

Forage quality in cultivars of *Brachiaria* spp.: association of lignin and fibers with anatomical characteristics.

Janaína Mauri¹, Vânia Helena Techio¹, Lisete Chamma Davide*¹, Daniele Laís Pereira¹, Fausto Souza Sobrinho², Fabrício José Pereira¹

¹Federal University of Lavras – UFLA, Department of Biology, Lavras – Minas Gerais State. Zip Code 37.200-000, Brazil

²Embrapa Dairy Cattle, Juiz de Fora – Minas Gerais State. Zip Code 36.038-330, Brazil

*Corresponding author: lisete.ufla@gmail.com

Abstract

Studies to quantify lignin and fibers chemically and anatomically were conducted to evaluate the nutritional quality of forage. This study aimed to assess differences in the amount of lignin and fiber in the leaves of cultivars from three species of *Brachiaria*. These differences were correlated with structural characteristics of sclerenchyma and vessel elements. *Brachiaria brizantha* (Hochst.) Stapf. cv. Marandu, *Brachiaria decumbens* Stapf. Prain. cv. Basilisk, and *Brachiaria ruziziensis* Kennedy (R. Germ & Evrad) cv. Kennedy were chemically and anatomically analyzed using fluorescence techniques and plant microtechnique procedures to evaluate the amount of lignin and fibers in leaves. *B. brizantha* showed the lowest cell-wall lignin content in fibers from the mesophyll region, and the average gray level was 177.43 compared to 197.63 for *B. decumbens* and 195.77 for *B. ruziziensis*. In the midrib, the fiber cell-wall thickness was 3.27 μm in *B. brizantha*, 57.18% thinner than *B. ruziziensis* (5.14 μm) and 9.48% thinner than *B. decumbens* (3.58 μm). *B. brizantha* cv. Marandu showed the lowest amount of lignin based on cell-wall fiber characteristics.

Keywords: *Brachiaria brizantha*; *Brachiaria decumbens*; *Brachiaria ruziziensis*; Gray levels; Quantitative plant anatomy.

Abbreviations: ADF_acid detergent fiber; CEL_cellulose; CP_Content of crude protein; IVDMD_ *in vitro* dry matter digestibility; LIG_lignin; NDF_neutral detergent fiber.

Introduction

One of the most important economic activities in Brazil is pasture-based livestock. Tropical regions are characterized by large numbers of forage species with great potential for use in ruminant feed (Karia et al, 2006). Plants of the genus *Brachiaria* (Poaceae) are forage plants that are adapted to various environmental conditions and have high dry matter yield, high adaptability, acceptable nutritional value, and adequate growth during most of the year (Costa et al., 2005). Paciullo et al. (2007) estimated that 80 to 90% of pasture areas in the country are made up of forage species of this genus.

Forage quality is usually determined by chemical analysis of the stems and leaves to identify chemical groups that can interfere with plant digestibility by ruminants (Jerba et al., 2004; Bauer et al., 2008; Pariz et al, 2011; Santos et al., 2014). The quality of *Brachiaria* is given by its nutritive value relative to digestibility and its crude protein content in the cell wall. Higher content of lignin and fiber corresponds with lower quality (Jerba et al., 2004). A major factor for low digestibility is the advanced age of the forage available for grazing, which can increase the production of dry mass and decrease chemical quality (Pariz et al., 2010; Medeiros et al., 2011). Digestibility is influenced by microorganisms inhabiting the rumen of certain herbivorous animals that have the ability to digest cellulose but not lignin. The chemical characteristics of forage with high concentrations of lignin in the cell wall impair the digestibility of dry mass and thus limit consumption by ruminants (Clipes et al., 2010).

When associated with cell-wall components (cellulose, hemicelluloses, and pectins), lignin blocks the activity of enzymes or microorganisms in the rumen in forage tissues. However, the degradation of these polysaccharides in the cell wall composition is rarely complete and varies according to the lignin content, the species, and age (Jerba et al., 2004; Clipes et al., 2010). In recent years, quantitative plant anatomy has been used as a complementary tool to assess the quality of forage and define the amount of lignin in the plant tissue. Indicators generated by this technique enable comparison of species or cultivars. In Brazil, some studies have shown an association between anatomy and quality of forage based on the amount of lignin (Alves de Brito et al., 2004; Medeiros et al., 2011).

Forage quality evaluation requires precise knowledge of the content and quantification of the components that inhibit digestion of the cell wall structure. Therefore, the quantification of lignin is essential. Greater proportion of lignified tissues leads to poorer digestibility of the forage (Jerba et al., 2004). Various studies have assessed the degree of digestibility of *Brachiaria* according to the lignin content (Alves de Brito et al., 2004; Santos et al., 2004; Moraes et al., 2005; Bauer et al., 2008; Maranhão et al., 2009; Medeiros et al., 2011 and Santos et al., 2014). However, these studies were not conclusive and left gaps for improving the presentation of the results, reducing costs for assessing forage quality, and associating chemical analysis with plant anatomy. The present study aims to assess differences in the

content of lignin and fibers in the leaves of cultivars of three *Brachiaria* species and to analyze how these differences are correlated with structural characteristics of sclerenchyma and vessel elements.

Results

Chemical characterization and anatomical description

There were no significant differences among the cultivars analyzed regarding the percentage of cellulose content (CEL), *in vitro* dry matter digestibility (IVDMD), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin content (LIG), and crude protein content (CP) (Table 1). The cultivars also showed no major anatomical differences in the general arrangement of tissues in the leaves. In the leaf blade region, a uniseriate epidermis covers both sides of the leaves in the three species. The epidermis of the adaxial surface presents bulliform cells in groups of 3 to 5 cells (Figs. 1A, C, and E). In all species studied, there are stomata in the abaxial and adaxial surfaces, and there may be trichomes on the adaxial surface (Figs. 1 A, C, and E).

In the mesophyll, we observed 3 to 4 layers of homogeneous chlorophyll parenchyma with cells of various size and morphology (Figs. 1 A, C, and E). We also observed closed collateral vascular bundles with a well-developed layer of bundle sheath cells and an inner layer formed by a ring of fibers in all three studied species (Figs. 1 A, C, and E). In the midrib region, all species have similar uniseriate epidermis structure on both surfaces of the leaf (abaxial and adaxial). Adaxially, there is a region containing sclerenchyma fibers just below the epidermis and throughout the midrib (Figs. 1 B, D, and F). Vascular bundles of various sizes are located on the abaxial surface of the leaves. Bundles with large size have extensions of the bundle sheath consisting of sclerenchyma fibers (Figs. 1 B, D, and F). We observed two large metaxylem vessels on the adaxial surface and phloem on the abaxial surface (Figs. 1 B, D, and F). In the central region of the midrib, there is a large region of parenchyma (Figs. 1 B, D, and F). There are no significant differences in the arrangement of tissue of the midrib of the three species analyzed.

Quantitative anatomy

Table 2 lists the results of the quantitative evaluation of the structure of lignified tissues. The gray level in the cell walls of *B. brizantha* cv. Marandu fibers was lower than that observed for *B. ruziziensis* cv. Kennedy and *B. decumbens* cv. Basilisk in the leaf mesophyll region. In the midrib region, there were no differences in the gray level of the cell walls of fibers between the species. No difference was detected in the number of fibers among the three species in the mesophyll region (Table 2), but in the midrib region, *B. decumbens* cv. Basilisk had fewer fibers than *B. ruziziensis* cv. Kennedy and *B. brizantha* cv. Marandu.

The cell walls of *B. ruziziensis* cv. Kennedy fibers were thinner than those in *B. brizantha* cv. Marandu and *B. decumbens* cv. Basilisk in the mesophyll region. However, in the midrib region, *B. brizantha* cv. Marandu showed a lower cell wall thickness, while *B. decumbens* had intermediate thickness and *B. ruziziensis* cv. Kennedy had higher thickness (Table 2). There were no significant differences in the proportion of lignified cells between the species studied in both the mesophyll and midrib regions (Table 2).

Discussion

Structurally, the leaves of the three cultivars showed no significant differences in the arrangement of tissues. This is important given the large variability in leaves of plants of the family Poaceae (Dengler et al., 1994). The homogeneity of the leaf structure allows for comparisons because the quantitatively fibers analyzed are in the same locations (vascular bundles in the mesophyll region, on the abaxial surface of the midrib, and associated with the adaxial epidermis of the midrib).

There may be variability in the proportion of these tissues among cultivars, since it is common among different plants of *Brachiaria* (Alves de Brito and Rodella, 2002; Alves de Brito et al., 2004). However, the tissue proportions between cultivars do not limit individual analysis of sclerenchyma fibers (the target of this study) and should be the focus of future research. Ultimately, there are no major structural differences in the anatomical characterization of the three cultivars. The similarities allow for analysis of the sclerenchyma fibers without major barriers of variability in the arrangement of leaf tissues.

The chemical analysis did not show significant differences between species regarding the amount of important components of plants as forage. However, Sobrinho et al. (2009) analyzed the forage quality of *Brachiaria* species cultivars, including *B. brizantha* cv. Marandu, *B. decumbens* cv. Basilisk, and *B. ruziziensis* cv. Comum. The *B. ruziziensis* cv. Comum cultivar presented the best results. Porto et al. (2009) reported that *B. brizantha* cv. Marandu showed lower CP and NDF content and higher IVDMD in the leaf blade compared with other species of forage grass. Santos et al. (2014) worked with different genotypes of *B. ruziziensis* and demonstrated low lignin and fiber content in this species compared with other forage. Likewise, for *B. decumbens*, Bauer et al. (2008) found lower content of cellulose and lignin in the cell-wall composition, which impairs digestibility compared to other species of Poaceae.

Factors such as the number of fibers and the lignin content adversely affect the digestibility of forages (Clipes et al., 2010). However, studies have been inconclusive because there are descriptions indicating that the three species contain less lignin and fiber compared with other species. It is well known that the lignin content in fibers varies according to the environment, genetic factors, and plant age (Lev-Yadun, 2010). Therefore, assessing only the fiber content in the plant sample may not be sufficient, since fibers with less lignin may not be exactly detrimental to forage quality. A more detailed analysis of cells may further elucidate this issue.

Leaves are the main source of green matter in forage and can be used to determine forage quality (Santos et al., 2014). Studying the different tissues in forage leaves is essential for understanding the quality of forage and may lead to new actions in breeding these plants (Basso et al., 2014). The sclerenchyma and xylem are less digestible plant tissues (Paciullo, 2002), which is directly related to the high lignin deposition (Bauer et al., 2008).

Lignin is a phenolic substance that emits natural fluorescence due to the presence of phenyl-coumarin in its structure. Lignin fluorescence can be captured by spectroscopy, which is used in the analysis of fibers, wastewater, and other applications (Albinsson et al., 1999). Brundrett et al. (1988) described a method for staining lignin with blue aniline and assessment in fluorescence microscopy.

Table 1. Chemical analysis of leaves of *Brachiaria ruziziensis* cv. Kennedy, *B. brizantha* cv. Marandu, and *B. decumbens* cv. Basilisk.

Cultivars	CEL (g kg ⁻¹)	IVDMD (g kg ⁻¹)	ADF (g kg ⁻¹)	NDF (g kg ⁻¹)	LIG (g kg ⁻¹)	CP (g kg ⁻¹)
Kennedy	24.74a±1.55	71.55a±2.19	30.36a±1.74	60.11a±0.70	4.48a±0.38	15.27a±0.04
Marandu	26.14a±1.05	71.31a±4.04	31.18a±1.85	61.17a±1.14	4.01a±0.09	15.19a±0.60
Basilisk	24.73a±1.77	71.39a±1.67	30.30a±2.14	60.28a±3.15	4.35a±0.09	14.94a±2.60

Means followed by the same letter in rows are not significantly different by Scott-Knott test at 5% significance. CEL - Cellulose; IVDMD - *in vitro* dry matter digestibility; ADF - acid detergent fiber; NDF - neutral detergent fiber; LIG - lignin; CP - crude protein

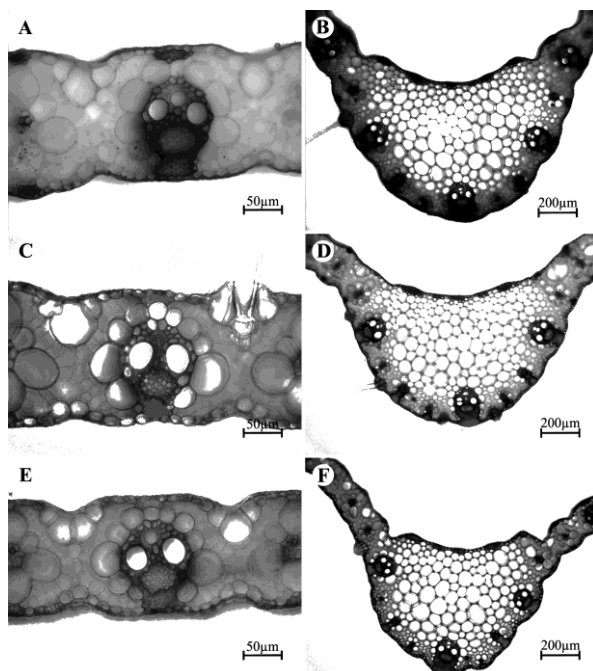


Fig 1. Anatomical structure of the mesophyll (A, C, and E) and the midrib (B, D, and F) of *B. ruziziensis* cv. Kennedy (A, B), *B. brizantha* cv. Marandu (C, D), and *B. decumbens* cv. Basilisk (E, F) in cross section.

Table 2. Mean values of the gray level, number of fibers and vessel elements, wall thickness, and proportion of cells with lignin for *Brachiaria ruziziensis* cv. Kennedy, *B. brizantha* cv. Marandu, and *B. decumbens* cv. Basilisk.

Mesophyll					
Cultivars	GL	NF	NVE	W.T. (µm)	P.C.L. (%)
Kennedy	195.77 a	80.70 a	3.46 a	3.03 b	11.03 a
Marandu	177.43 b	77.53 a	3.56 a	3.50 a	11.07 a
Basilisk	197.63 a	82.63 a	3.50 a	3.52 a	12.09 a
Midrib					
Kennedy	173.33 a	623.23 a	11.63 a	5.14 a	13.41 a
Marandu	172.03 a	652.50 a	10.10 b	3.27 c	13.57 a
Basilisk	166.63 a	566.23 b	10.53 b	3.58 b	14.17 a

Means followed by the same letter in the column are not significantly different by Scott-Knott test at 5% significance. GL - Gray level; NF - number of fibers; NVE - number of vessel elements; WT - wall thickness; PLC - proportion of lignified cells.

This direct relationship enabled quantitative evaluation of lignin in the cell walls of fibers and sclerenchyma in the leaves of the *Brachiaria* species studied. *B. brizantha* cv. Marandu showed a lower lignin content compared with *B. decumbens* cv. Basilisk and *B. ruziziensis* cv. Kennedy, despite the thicker cell wall in the leaf mesophyll. The thick cell wall cannot be directly correlated with lower digestibility of these cells, because cellulose may be one of the most important energy sources for ruminants (Bauer et al., 2008). The lower amount of lignin in fibers of *B. brizantha* cv. Marandu can be favorable for a better utilization of these leaves by ruminants, compared with *B. ruziziensis* cv. Kennedy and *B. decumbens* cv. Basilisk, since other quantitative characteristics of fibers were not significantly

different between the three species in the leaf mesophyll region. In the midrib region, there is a large amount of fibers in the adaxial and abaxial surfaces in all species studied. *B. decumbens* has a lower amount of fibers compared to *B. brizantha* and *B. ruziziensis*, but *B. brizantha* shows a lower thickness of the cell walls in these fibers. As the amount of lignin was the same in the fibers of the midrib region for the three species, fewer fibers and lower wall thickness probably increase the digestibility of that leaf area. These characteristics of *B. brizantha* and *B. decumbens* seem to be more favorable for the use of this leaf region. In Brazil, *Brachiaria* breeding for forage started in the 1970s, and in 1984, *B. brizantha* genotypes were commercially released. This species is therefore one of the first to be used in

breeding programs in Brazil (Jank et al., 2011). Due to intense improvement over at least 30 years, this species has the best conditions for use as forage, such as lower lignin content in the fibers of the leaf mesophyll and thinner walls in the midrib region compared to *B. ruziziensis* and *B. decumbens*. However, *B. decumbens* and *B. ruziziensis* are very promising species for new breeding programs because both species have high palatability and nutritional value (Jank et al., 2011). In particular, *B. ruziziensis* has a high potential for improvement by increasing forage quality, and there is an effort to improve this species for resistance to pests and to increase its nutritional value (Souza Sobrinho et al., 2010; Jank et al., 2011). To increase the quality of these species as forage, it is necessary to reduce the lignin content, cell wall thickness, and number of fibers in the midrib and the leaf mesophyll. The results can be easily applied to existing genotypes following the methodology of this study and guide future breeding to improve the quality of this species as forage.

Materials and Methods

Plant material

This study evaluated the plants *Brachiaria brizantha* (Hochst.) Stapf., cv. Marandu, *Brachiaria decumbens* Stapf. Prain., cv. Basilisk, and *Brachiaria ruziziensis* (R. Germ & Evrad), cv. Kennedy. These cultivars were grown in an experimental field in Embrapa in the municipality of Valença (Rio de Janeiro, Brazil). The experiment was conducted in the field in plots of two rows that were 3 m long. Only the three cultivars mentioned were grown in these plots. Rows were spaced 1 m apart, and plants were spaced 0.5 m apart within the rows. The soil was classified as Haplic Gleysol. The soil was amended with limestone and 300 kg of 08-28-16 NPK + Zn fertilizer. Topdressing was carried out subsequently with 50 kg N ha⁻¹. Irrigation was held when soil moisture reached 50% of field capacity. The water volume daily applied was sufficient to restore 100% of field capacity. The uniformity cut of plots was done within 60 days, and plants were harvested 29 days later.

Chemical characterization

At the 29th day after the uniformity cut, we collected the forage (cut at ground level) and separated the leaves and stems. Fully expanded leaves were dried in a forced ventilation oven for 72 hours at 55°C. After drying, leaves were ground in a sieve (1.0 mm) for chemical analysis at the forage analysis laboratory (Embrapa Dairy Cattle, Juiz de Fora, Minas Gerais). The crude protein content, neutral detergent fiber, acid detergent fiber, cellulose, lignin, and in vitro dry matter digestibility were determined by near infrared spectroscopy (NIRS) (FOSS, 5000, Denmark). For analysis, 20-g dry-weight samples were used. Chemical methods used for NIRS calibration were described from leaf samples (dried at 55°C) by the gravimetric method according to Silva and Queiroz (2006). The in vitro dry matter digestibility was assessed according to the methodology by Tilley and Terry (1963). Crude protein, cellulose, lignin, neutral detergent fiber, and acid detergent fiber were quantified according to the methodology described by Van Soest (1994).

Anatomical description

At the 29th day after regrowth, fully expanded leaves were collected from the three cultivars. The leaves were fixed in F.A.A._{70%} (formaldehyde: glacial acetic acid: ethanol) for 72 hours according to Johansen (1940). After fixation, the leaves were preserved in 70% ethanol at room temperature until analysis. Freehand cross sections of the leaves were obtained with a steel blade at the lower third of the leaf blade just after the end of the sheath. Analyses were performed on the lower third of the blade because the vascular system of leaves has acropetal development, and the sclerenchyma in the vascular bundles of most of the basal parts of the leaves mature more rapidly (Beck, 2010). Sections were cleaned in a solution of 50% sodium hypochlorite and washed in distilled water twice for 10 minutes. The sections were then stained with a mixture of safranin and astra blue (0.01% safranin and 0.99% astra blue) (Kraus and Arduin, 1997) and mounted on semi-permanent slides in 50% glycerol (Johansen, 1940). Images of the slides were captured with a capture microscope (Leica DMLS, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) coupled to a digital camera Nikon SIGHT DS-SII. For each cultivar, we captured an image of each mesophyll and midrib portion of the leaf.

Quantitative anatomical analysis

For quantitative anatomical analysis in fluorescence microscopy, cross sections were made using the same techniques described above for anatomical description in the same region of the leaf blade. Sections were cleaned in 50% sodium hypochlorite and washed in distilled water twice for 10 minutes. Slide staining and mounting of the sections were carried out according to the method by Brundrett et al. (1988). First, the cross sections were placed in a solution of distilled water with 0.1% (w/v) berberine hemi-sulphate for 1 hour, washed in distilled water, kept in 0.5% (w/v) aniline blue for 30 minutes, and washed again with distilled water. Sections were placed on slides containing 0.1% (w/v) FeCl₃ in 50% (v/v) glycerol (prepared by adding glycerin to the filtered aqueous FeCl₃). After 5 minutes, the slides were covered with coverslips. The entire procedure of slide staining and mounting was performed in the dark in a black gerbox. Slides were kept under refrigeration (\pm 6°C), and images were captured within 24 hours after the preparation. The observations were made with a fluorescence microscope (Olympus BX60) equipped with a cooled monochrome camera (Olympus Optical CO., LTD., Tokyo, Japan). Images were captured with ultraviolet excitation/emission wavelengths of 358 to 461 nm (Brundrett et al., 1988). We examined six slides per cultivar/species and five fields on each slide totaling 30 fields per cultivar/species for each characteristic assessed. The fields were captured in the mesophyll of the leaf blade and in the midrib region. In the leaf blade area, we selected the first bundle of larger caliber after the midrib to evaluate the lignified region by gray level and wall thickness of the fibers. We also assessed the proportion of the area containing lignified cells to calculate the percentage of lignified area relative to the total area in the mesophyll and midrib. We counted the number of fibers and vessel elements per vascular bundle in the leaf blade region. In the midrib, we selected the largest vascular bundle on the abaxial surface in the center of the rib for the assessment of gray levels and wall thickness of the fibers. The counting of the fibers and vessel elements in this region included the whole midrib region, as did the percentage of lignified cells and counting of all sclerenchyma cells, which emitted

fluorescence due to the presence of lignin. For the analysis of photomicrographs, UTHSCA Image Tool software was used for image analysis to count the number of fiber cells and vessel elements, and the gray level was assessed by histogram. Image J software was used to evaluate the proportion of lignified cells. The experimental design was a completely randomized with three treatments and 30 replicates for each feature. The experimental plot was one field evaluated for each feature.

Statistical analysis

Statistical analysis was done using the statistical software Sisvar 5.0 (Ferreira, 2011) for all data on the three cultivars studied and clone plants from *B. ruziziensis*. First, the data were tested for normality using the Shapiro-Wilk test, and data without a normal distribution were transformed by square root transformation. Then, the data were subjected to analysis of variance, and the means were compared by the Scott-Knott test at $p \leq 0.05$.

Conclusion

The species *B. brizantha* has fibers with lower lignin content and lower cell wall thickness compared with *B. decumbens* and *B. ruziziensis*. These characteristics indicate better performance of this species as forage. *B. decumbens* and *B. ruziziensis* can also be used in new breeding programs to reduce the number of fibers and lignin.

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