AGRONOMY SCIENCE AND BIOTECHNOLOGY

RESEARCH ARTICLE

# Leaf anatomy of *Urochloa brizantha* and *Urochloa ruziziensis* (Poaceae) plants subjected to different fertilization management practices

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## ABSTRACT

Brazilian agribusiness is heavily influenced by beef export. Thus, it is important to evaluate the quality and growth rate of forage species used as cattle food. Given that the structural organization of leaf cells and tissues is directly related to forage-plant growth, we aimed to analyze the foliar anatomy of *Urochloa brizantha* and *U. ruziziensis* subjected to different fertilization management practices. Experimental design was completely randomized split-split-plots. Plots were allocated to fertilization strategies, namely single and split fertilizer application. Subplots were allocated to two fertilizer doses for the carrying capacities of 7 and 10 head of cattle per hectare (AU ha$^{-1}$). Sub-subplots were distributed to the forage plant species *U. brizantha* and *U. ruziziensis*. Three leaves were collected from each treatment for the anatomical processing of cross-sections, epidermal imprints and diaphanizations. Based on a parsimonious model, *U. brizantha* showed thicker leaf tissues than *U. ruziziensis*. Split fertilizer application increased thickness of the adaxial and abaxial cuticle and of bulliform cells. Fertilizer dose of the higher carrying capacity determined increased thickness of the leaf blade, bulliform cells, and regular homogeneous parenchyma on both studied species. We concluded that increased fertilizer dose and split application interfere with the leaf anatomy of *U. brizantha* and *U. ruziziensis*, providing both species with enhanced growth and yield.

**Keywords**: *Brachiaria*, compost, histotechnique, pastures, plant development, sustainability.

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## INTRODUCTION

Having 217 million cattle head, which accounts for 14.3% of all cattle in the world, Brazil is currently one the world's largest beef producers (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA], 2018; EMBRAPA, 2021). One of the features of Brazilian beef production is the use of forage species as cattle food. Pastures are the main factors responsible for the uptake of nutrients and fibers by cattle (Tonello et al., 2011). Therefore, improving forage management aimed at increasing yield, carrying capacity and nutritional quality is essential for achieving a more efficient and sustainable use of pastures.

Assessing plant mineral nutrition is pivotal to achieve optimal forage yield. There is a general consensus over the fact that split application of fertilizers, especially nitrogen and phosphorous, leads to enhanced nutrient use efficiency by forage species. As forage growth and development are associated with split fertilizer application, alterations in plant physiology and anatomy are to be expected, especially on leaves (Mateus et al., 2011). The anatomical structure and cellular arrangement of leaves influence both growth and development of forage species, and consequently they affect cattle nutrition. Cattle nutritional value is directly related to the low digestibility of some internal leaf structures by rumen microorganisms (Valente et al., 2011).

The leaf blade of *Urochloa* (syn. *Brachiaria) brizantha* and *U. ruziziensis*, both of which have high yield in pastures, may have low digestibility depending on the proportion of their foliar tissues. For instance, the sclerenchyma, which typically has high amounts of fibers, and the xylem, which has some cellular elements with lignin-impregnated walls, are more resistant to colonies of rumen microbes that promote digestion (Cabral, Bauer, & Cabral, 2011). In addition, compact cell arrangement of some tissues and increased cell wall thickness may also negatively affect plant nutritional value (Sobral, Fries, & Silva, 2011). Leaf maturation is also relevant for the study of forage quality, as leaf and stem growth are associated with increased amounts of lignified tissues, owing to the higher proportion of sclerenchyma that is usually found on these organs at later stages of plant development (Basso & Barbero, 2015).

The internal structure of plant organs is directly associated with nutrient availability, i.e., with the amount of macro and micronutrients available for plant uptake in the environment, which interferes with the internal cellular organization of the plant body (Somavilla & Ribeiro, 2011). Hence, studies on the relationship between fertilizer dose and plant anatomical structure are of great importance, insofar as they allow for an evaluation of the efficiency of fertilization management practices, by means of a comparative analysis of the proportion of leaf tissues between different species. In that sense, we aimed to assess the effects of different management practices on the foliar structure of *U. brizantha* and *U. ruziziensis*, in order to test which fertilizer dose and application strategy is best for each species in terms of providing higher yield and quality for use in animal nutrition.

## MATERIAL AND METHODS

The assay was conducted at the experimental field of Federal University of Viçosa – Rio Paranaíba Campus. Climate in the region is type Cwa according to Köppen's classification, with well-defined winter and summer seasons. Temperatures in the three coldest months average between -3 and 18.5 ºC. Soils are red-yellow dystrophic oxisols with clayey texture (EMBRAPA, 2018). The 20-m$^2$

experimental area was initially desiccated with 4 L $ha^{-1}$ glyphosate. Soil was prepared in 0.3-m spaced lines, over which 10 kg $ha^{-1}$ of *Urochloa brizantha* and *U. ruziziensis* seeds were sown in January 2020. For weed control, we manually weeded the area and then applied 1.5 L $ha^{-1}$ 2,4-D, to enable crop development. The area was sprinkler irrigated. Water depth was applied aiming to restore 100% of potential evapotranspiration, which was measured by a Davis Vantage Pro2 automated weather station.

Experimental design was completely randomized split-split-plots with three factors and two replicates, totalizing 16 experimental units. Plots were fertilized with two application strategies, namely single and split. Subplots had fertilizer applied at two doses. Sub-subplots were cultivated with two species, *U. ruziziensis* and *U. brizantha*. From each experimental unit, ten random samples were selected as replicates. Plots were established over a 10 x 10 m area, whereas subplots and sub-subplots were fit over a same 4.5 x 4.5 m area, with a 1-m border among them.

Topdressing fertilization was performed two months after sowing in single and split applications. For single application, the total fertilizer dose was applied in the first month of the experiment. For split application, the total fertilizer dose was split six times over six months, i.e., with one application per month.

We adopted the Mass Balance Method (Novais et al., 2007) to calculate the fertilizer doses for two carrying capacities: 7 and 10 head of cattle per hectare (AU $ha^{-1}$). The method yielded a 920 kg $ha^{-1}$ urea, 95 kg $ha^{-1}$ MAP and 450 kg $ha^{-1}$ KCl dose for the 7 AU $ha^{-1}$ carrying capacity and a 1450 kg $ha^{-1}$ urea, 250 kg $ha^{-1}$ MAP and 880 kg $ha^{-1}$ KCl dose for the 10 AU $ha^{-1}$ carrying capacity. These were the calculated doses for single application. They were split in six for the split application, as previously described.

In the last month of fertilizer application, i.e., 284 d after sowing, we collected three expanded leaves (n = 3) from the internodes of plants at each experimental unit, totalizing 48 samples for anatomical analyses. Fragments from the leaf mid region were fixed in $FAA_{50\%}$ (Johansen, 1940) and stored in 70% ethanol. For structural and micromorphometric evaluations, samples were dehydrated in an increasing ethyl series and embedded in histological resin (Historesin, Leica®, Heildelberg, Germany). Cross-sections (8-µm thick) were obtained in an automated rotary microtome (Leica RM 2255) and stained with toluidine blue (O'Brien & McCully, 1981). Glass slides were mounted with Permount® (Thermo Fisher Scientific).

For the micromorphometric analysis, images were captured in a BX 41 photomicroscope coupled with a digital camera and analyzed in an Olympus CX 41 microscope. Images were also obtained in an Olympus TH4-100 inverted photomicroscope coupled with a digital camera. Three fields were photographed from each leaf cross-section, and from each field three measurements were taken using ImageJ software. We measured the thickness of the cuticle, adaxial and abaxial epidermis, bulliform cells, mesophyll, and leaf blade (in micrometers). To analyze the epidermis in frontal view, leaf samples were diaphanized through a modified protocol, in which clarification was performed using undiluted sodium hypochlorite, in contrast with the usual method, which preconizes the use of 20% and 50% solutions. Leaf fragments were kept at 35 ºC in an oven for four days (Kraus & Arduim, 1997). After clarification, samples were stained with three droplets of 1% safranin in 50% ethanol (Kraus & Arduim, 1997) and semi-permanent glass-slides were mounted in glycerin jelly (Kaiser, 1880 *apud* Kraus & Arduim, 1997) and photographed in an Olympus BX 41 photomicroscope.

For stomatal index assessment, we obtained epidermal imprints following usual methods (Kraus & Arduim, 1997), i.e., by applying instant adhesive glue over a glass slide and pressing leaf fragments against it. We photographed three fields (n = 3) from both leaf surfaces. Stomatal index was calculated using the equation proposed by Cutter (1978): (SI) = [NS/(CC + NS)] x 100, where SI is the stomatal index, NS is the number of stomata and CC is the number of epidermal common cells. Data was obtained using AnatiQuanti software (Aguiar et al., 2007).

Data was subjected to exploratory analysis and outliers were removed. Replicated values were reduced to the mean value for experimental units. We used mixed linear models, whereby plots, which were the randomization unit, were considered to be subjected to random effects. Model significance was subjected to likelihood-ratio test, which sufficed to detect differences between means, as factors had only two levels. Data was analyzed using R software (R Core Team, 2021). The nlme package was used to adjust mixed linear models, following R Package Documentation (2021).

## RESULTS AND DISCUSSION

In cross-section, leaves of *Urochloa brizantha* and *U. ruziziensis* are amphistomatic. Epidermis is uniseriate on both leaf surfaces and bulliform cells are present on the adaxial leaf surface. The cuticle is overall thick, but its thickness differs between the two species. Mesophyll is homogenous and radiated, with collateral bundles being encircled by a conspicuous sheath (Kranz anatomy) (Figures 1 and 2).

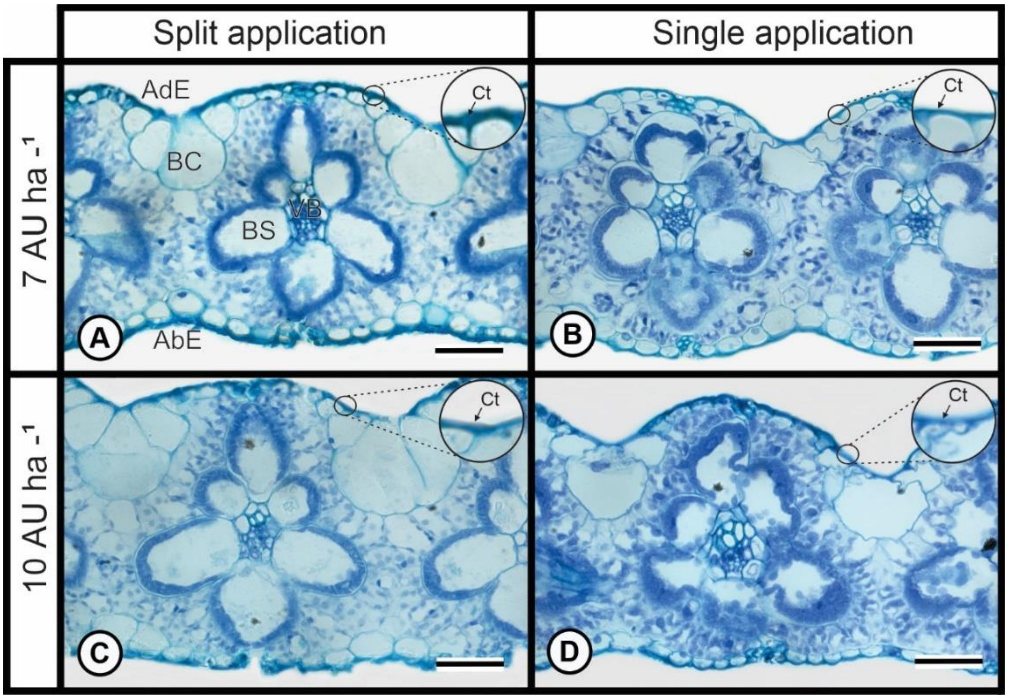

**Figure 1.** Leaf anatomy of *Urochloa brizantha* (cross-sections) subjected to the lower fertilizer dose (7 AU ha$^{-1}$) split-applied (A), lower fertilizer dose (7 AU ha$^{-1}$) in a single application (B), higher fertilizer dose (10 AU ha$^{-1}$) split-applied (C), and higher fertilizer dose (10 AU ha$^{-1}$) in a single application (D). BS = bundle sheath; BC = bulliform cells; Ct = cuticle; AbE = abaxial epidermis; AdE = adaxial epidermis; VB = vascular bundle. Bars = 50 µm.

Qualitative analyses revealed only lipid impregnations on epidermal cells

(Figures 1 and 2). According to Paciullo (2002) and Carneiro, Bordignon, Scheffer-Basso and Agnol (2008), the nutritional value of forage grasses can be inferred from traits of foliar cells and tissues. For instance, epidermal cells may show thick secondary cell walls impregnated with lignin and lipid substances, which thereby negatively affects digestibility; this, however, was not found in our study. In both studied species we observed several bulliform cells on the adaxial epidermis of the leaf blade. Carneiro et al. (2008) reported that an average three to five of these specialized cells per group of cells provides the plant withincreased resistance against water stress.

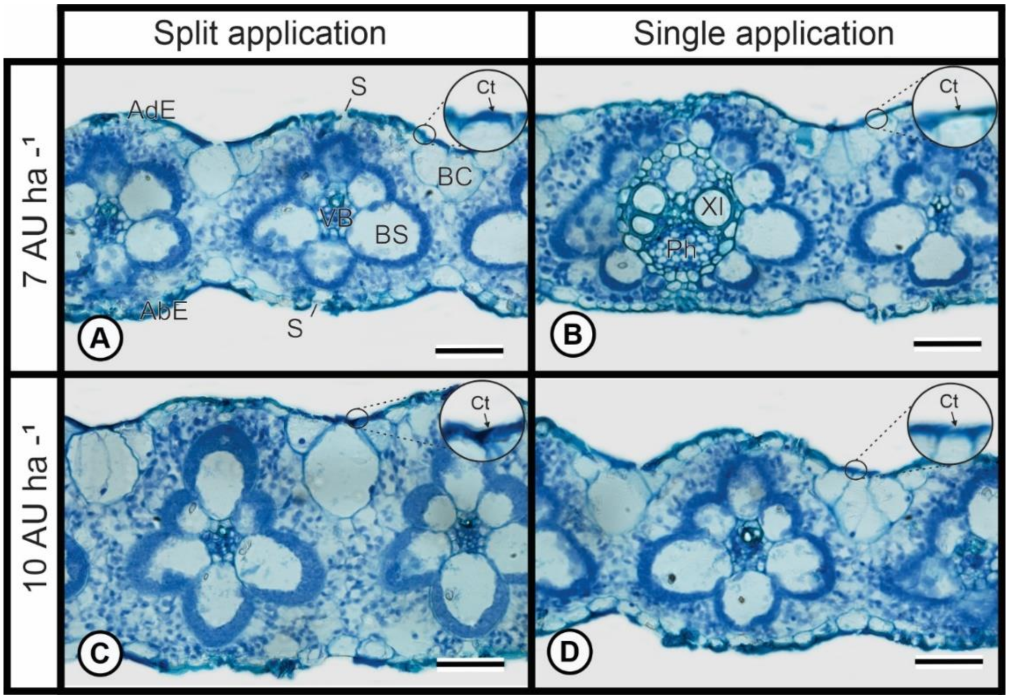

**Figure 2.** Leaf anatomy of *Urochloa ruziziensis* (cross-sections) subjected to the lower fertilizer dose (7 AU ha$^{-1}$) split-applied (A), lower fertilizer dose (7 AU ha$^{-1}$) in a single application (B), higher fertilizer dose (10 AU ha$^{-1}$) split-applied (C), and higher fertilizer dose (10 AU ha$^{-1}$) in a single application (D). BS = bundle sheath; BC = bulliform cells; Ct = cuticle; AbE = abaxial epidermis; AdE = adaxial epidermis; S = stomata; Ph = phloem; Xl = xylem; VB = vascular bundle. Bars = 20 µm.

Forage species with C4-metabolism, Kranz-anatomy bundle sheaths and homogenous chlorenchyma are less recommended as cattle food than grasses with the C3-metabolism anatomy, as the former have reduced intercellular spaces and more compactly arranged cells, which thus hampers leaf degradation during digestion (Carneiro et al., 2008). Although the cell arrangement in C3 forage species leaves provide them with higher digestibility, the species studied herein, which have Kranz anatomy, show higher growth, higher foraging potential and higher resistance against elevated temperatures, while also showing increased yield with lower amounts of water (Valente et al., 2011).

In frontal view, the epidermis of both species showed common cells with sinuous walls, hairs, silica bodies, calcium oxalate crystals and paracytic dumbbell-shaped stomata (Figure 3). According to Wang and Chen (2020), stomata morphology may determine an enhanced physiological efficiency, in the form of an increased resistance against water stress and unfavorable climate conditions. In that sense, the dumbbell shape allows for rapid regulation of the opening and closing of the stomatal pore, thereby promoting an increased water use efficiency (Wang & Chen, 2020).

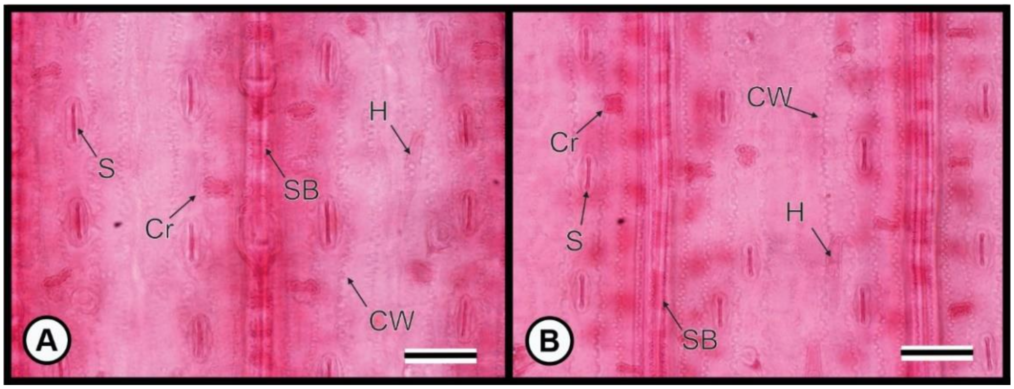

**Figure 3.** Leaf epidermis (frontal view – diaphanization) of *Urochloa brizantha* (A) and *U. ruziziensis* (B). SB = silica bodies; Cr = calcium oxalate crystals; S = paracytic stomata; CW = sinuous cell walls;

H = hairs. Bars = 50 µm.

In a preliminary analysis we found no significant interaction ($p > 0.05$) in the combination of the factors application strategy, dose, and species, for any of the evaluated variables. Therefore, based on the most parsimonious model, and without including interactions, the factors application strategy, dose, and species were analyzed independently (Table 1).

**Table 1.** Mean thickness (µm) of the adaxial and abaxial cuticle, adaxial (AdE) and abaxial (AbE) epidermis, mesophyll, leaf blade, and bulliform cells; and stomatal index (%) of the adaxial and abaxial epidermis of *Urochloa brizantha* and *U. ruziziensis* plants subjected to different fertilizer doses and application strategies according to carrying capacities.

| Response variable | Fertilizer application strategy | | Fertilizer dose (kg ha$^{-1}$) | | Species | |
|---|---|---|---|---|---|---|
| | Split | Single | Lower | Higher | *U. brizantha* | *U. ruziziensis* |
| **Thickness (µm)** | | | | | | |
| AdE cuticle | 4.62 a[1] | 4.04 b | 4.28 a | 4.38 a | 4.49 a | 4.16 b |
| AbE cuticle | 4.94 a | 4.48 b | 4.65 a | 4.77 a | 4.96 a | 4.46 b |
| Adaxial epidermis | 11.3 a | 13.4 a | 12.1 a | 12.6 a | 11.9 a | 12.8 a |
| Abaxial epidermis | 11.9 a | 12.1 a | 12.0 a | 12.0 a | 12.3 a | 11.7 a |
| Mesophyll | 218 a | 209 a | 204 a | 222 b | 223 a | 204 b |
| Leaf blade | 251 a | 239 a | 236 a | 254 b | 255 a | 235 b |
| Bulliform cells | 74.7 a | 67.7 b | 67.7 a | 74.8 b | 70.8 a | 71.6 a |
| **Stomatal index (%)** | | | | | | |
| Adaxial epidermis | 14.4 a | 14.4 a | 14.8 a | 14.0 a | 14.5 a | 14.2 a |
| Abaxial epidermis | 18.6 a | 17.4 a | 18.3 a | 17.7 a | 18.8 a | 17.3 a |

[1] In a same row for each factor, means followed by the same letter do not differ significantly by the likelihood-ratio test ($p > 0.05$).

Split application led to increased thickness of the adaxial and abaxial cuticle as well as of bulliform cells in both species (Table 1; Figures 1 and 2). It is well known that split application can significantly interfere with crop growth and development, determining several morphophysiological and anatomical alterations (Cabral et al., 2016). Such an influence of split fertilizer application on plant morphology has been reported by Cabral et al. (2016) to *U. brizantha* cv. Marandu. In the first stage of crop establishment, the total amounts of nitrogen and potassium were equally split into four applications. That, along with the replacement of urea by ammonium sulfate during the first stage of plant growth, determined a dry-matter yield proportional to the increase in amount of those fertilizers. Split nitrogen application allows for decreased losses by lixiviation, volatilization and denitrification in the soil, which thereafter leads to increased yield (Albuquerque, Pegoraro, Vieira, Amorim, & Kondo 2012).

As for the higher carrying capacity (10 AU ha$^{-1}$) dose, we found increased thickness of the leaf blade, bulliform cells and homogenous parenchyma (mesophyll) in both species (Table 1; Figures 1 and 2). Studying the leaf anatomy of *U. brizantha*, Guimarães et al. (2021) also found an influence of nutrient amount on internal leaf structure. The authors reported that increasing phosphate doses positively correlate with the increase in thickness of bulliform cells, mesophyll, and consequently of the leaf blade, therefore obtaining results similar to our own findings. The authors also stated that bulliform cells are strongly correlated with increased digestibility of dry matter and higher amounts of crude protein in the forage, due to the easier degradation of such cell type, which is characterized by having thin cell walls and a thin cuticle. On the other hand, increased mesophyll thickness allows for optimized photosynthesis, as $CO_2$ fixation is thereat more efficient, which consequently determines higher plant yield (Gama, Sales Junior, Castanheira, Silveira, & Azevedo, 2017). In addition to these modifications, the presence of dumbbell-shaped stomata is also advantageous for plant yield, as they enable higher water use efficiency by the plant (Wang & Chen, 2020).

The factors application strategy and dose showed no influence in relation to either species. Yet, some tissues showed thickness differences between the two species. *U. brizantha* showed higher thickness of the leaf blade, interveinal chlorenchyma, and adaxial and abaxial cuticle than *U. ruziziensis* (Table 1). Mauri et al. (2019) reported distinct foliar anatomical structures to those two species, with thicker ground parenchyma occurring in the leaf blade mid region of *U. brizantha* than in that of *U. ruziziensis*. Furthermore, we found a thicker abaxial epidermis on leaves of *U. brizantha*; it is possible that the cuticle, a lipid-impregnated epidermal structure, is also thicker in that species.

## Influence of application strategies and doses on the species leaf anatomy

Since the factors application strategy and dose showed no significant effect on the species, we found no anatomical difference between the two species in relation to fertilization treatments. Thus, in both species the alterations found on the internal leaf structure were due to factors acting individually (Figures 1 and 2). However, when we compare tissue structural organization between the two species without considering the fertilization treatment, *U. brizantha* and *U. ruziziensis* show distinct structures, which are due to the natural anatomical variation found among different species, without the interference of external environmental factors (Mauri et al., 2019). In that sense, *U. brizantha* showed higher thickness of the leaf blade, regular homogenous parenchyma, and adaxial and abaxial cuticle than *U. ruziziensis*, as revealed by morphometric analysis (Figures 1A and 2A).

### Relationship between alteration in structural organization, split fertilizer application and amount of nutrients

Split fertilizer application, without the interference of different doses, determined an increase in cuticle thickness on both species (Table 1; Figures 1A and 2A). Split application also led to an increased thickness of bulliform cells on the two species, but the higher dose contributed the most (Figure 1C). Split fertilizer application enhances the efficiency of nutrient absorption/adsorption by roots. Since in split application the fertilizer is applied at lower doses over a longer period, the loss of highly volatile nutrients such as phosphorous and urea through lixiviation and volatilization are thereat prevented, hence promoting a more efficient use of those minerals by the plant (Teixeira, Spironello, Furlani, & Sigrist, 2002).

In *U. brizantha*, the cuticle, which covers the outer periclinal wall of epidermal cells from the adaxial and abaxial leaf surfaces, showed higher thickness on plants subjected to split application (Figures 1A and 1B). Such difference in cuticle thickness according to the application strategy was also found in *U. ruziziensis* (Figures 2A and 2B), but it was less noticeable than in *U. brizantha*, which has a naturally thicker cuticle. Fertilizer dose showed no influence on cuticle thickness, as revealed by a comparative visual analysis between leaf cross-sections of plants subjected to the same dose in single (Figures 1D and 2D) and split (Figures 1C and 2C) applications. The cuticle is formed by cutin and waxes, which are waterproofing lipids produced by epidermal cells (Azevedo et al., 2018). Thicker cuticles enable decreased water loss, lower transpirations rates and increased water use efficiency, while also preventing overheating of the leaf surface by ultraviolet radiation (Silva, Dias, & Arruda, 2016). As it covers the epidermis, the cuticle is the first barrier against pathogens, and thus its increased thickness represents an optimization of such protection. Pita-Barbosa, Sant'Anna-Santos, Silva, Azevedo, & Rocha (2009) evaluated the effects of fluoride phytotoxicity on *U. decumbens* and *U. brizantha* whereas Gama et al. (2017) assessed the effects of fertilizer levels on *Coffea arabica*, and the authors of both studies reported a decreased cuticle thickness. The authors also concluded that rupture or decreased thickness of the cuticle render leaves more exposed to water loss and water stress, as well as to fungal infection due to increased wetness of the leaf surface.

We found increased thickness of bulliform cells in *U. brizantha* plants subjected to split application of the lower fertilizer dose (Figure 1A) in comparison with plants subjected to the same dose in a single application (Figure 1B). Such alteration resulting from split application without interaction with fertilizer dose was also found in *U. ruziziensis* plants (Figures 2C and 2D). Bulliform cells are voluminous, specialized cell types that occur on the adaxial epidermis, having as main function the regulation of mechanisms responsible for the curling of leaves when they are less turgid. Single, immediate application of fertilizers restricts macro and micronutrient availability, as it does not prevent the occurrence of losses by lixiviation and/or volatilization. Adjustments of tissue thickness may be a major metabolic regulation mechanism for plant survival under stress conditions (Boeger & Poulson, 2006). This is a possible explanation for the occurrence of variations in thickness of bulliform cells according to the fertilizer application strategy.

The amount of nutrients in the fertilizer dose without interference of the application strategy also caused alteration in the thickness of bulliform cells of *U. brizantha*, increasing such variable on leaves of plants subjected to the higher fertilizer dose (Figure 1D). Contrastingly, we found lower bulliform cell thickness on leaves of plants subjected to the lower dose yet without split application (Figure 1B). We also found such difference in bulliform cell thickness due to the higher fertilizer dose on leaves of *U. ruziziensis* plants (Figures 2B and 2D). There are few studies correlating modifications in the leaf internal structure of brachiaria species with the amount of nutrients, yet a relationship between those two factors has been proven. Rosolem and Leite (2007), for instance, reported decreased mesophyll thickness in plants of *Coffea arabica* var. Catuaí and Mundo Novo due to boron deficiency, while Corrêa et al. (2009) found increased thickness of the leaf blade, palisade parenchyma and spongy parenchyma in oregano plants (*Origanum vulgare L.*) subjected to increasing doses of organic fertilizer.

Therefore, the lower nutrient dose, i.e., the one for the lower carrying capacity (7 AU ha$^{-1}$), led to decreased thickness of specialized epidermal cells, which can be explained by the phenotypic plasticity of the studied plants to different environmental conditions and nutrient availabilities. Lower nutrient dose propitiates a different environment for the plant, inducing adjustments in leaf architecture in response to that factor (Corrêa et al., 2009). Thicker, turgid bulliform cells enable the leaf to remain extended, thereby maintaining the plant physiological status more stable. Moreover, the vacuole of bulliform cells is able to store large amounts of water, and ergo with increasing cell thickness higher water contents can be stored (Gobbi, Rasmo, Ventrella, Garcez Neto, & Rocha, 2011).

We found thicker and more voluminous bulliform cells on leaves of *U. brizantha* and *U. ruziziensis* compared with the other factors (Figures 1C and 2C). Thus, split application of the higher nutrient dose yielded taller bulliform cells, in contrast with those factors analyzed individually. This is because split application increases the time that nutrients remain in the soil, and the higher dose provides a higher amount of minerals to the plant, thereby creating a more favorable environment for plant development (Teixeira et al., 2002). According to Gobbi et al. (2011) and Matschi et al. (2020), bulliform cells are advantageous to plant metabolism and development, as they allow for higher water storage due to their larger vacuole. Bulliform cells also have high water permeability, which enables leaf shrinkage under stress conditions, thereby preventing plant dehydration.

Fertilizer dose also significantly altered mesophyll and leaf blade thickness in both species, yet without interference of the application strategy. Leaves of plants subjected to the higher fertilizer dose (10 AU ha$^{-1}$) showed thicker mesophyll (Figures 1 and 2). Such increase was conspicuous in both species, considering the higher fertilizer dose and the same application strategy (Figures 1C and 2C). The phenotypic plasticity of the two studied species enabled them to show increased thickness of leaf ground tissue due to the overlapping of the homogenous regular parenchyma. Such increased mesophyll thickness consequently leads to an increased thickness of the leaf blade, which in turn propitiates enhanced plant resistance against water stress and high sunlight incidence, both of which could otherwise cause leaf overheating and consequent metabolic alterations (Boeger & Poulson, 2006).

Stomatal index of the adaxial and abaxial epidermis of neither species differed significantly in relation to fertilizer dose or application strategy (Table 1). Stomatal index is little correlated with plant mineral nutrition, being chiefly influenced by factors such as water availability, as demonstrated by Santos, Freitas, Techio and Sobrinho (2013), who found increased values of stomatal density and stomatal index on leaves of *U. ruziziensis* plants subjected to higher water levels. Other factors that may affect this variable are high sunlight incidence, which promotes cell division and differentiation in stomata (Santiago et al., 2001), and high salinity, which is associated with decreased stomatal index (Parés, Arizaleta, Sanabria, & Garcia, 2008). Leaves of the studied species are amphistomatic (Figures 1A and 2D). This trait represents and adaptation of species that are kept in sunny environments, with the number of stomata being proportional to transpiration rates, as amphistomatic leaves show more efficient transpiration (Medeiros, Pinto, Castro, Rezende, & Lima 2011). Additionally, dumbbell-shaped stomata also represent an adaptation of grasses to warmer regions, insofar as they allow for increased water use efficiency by means of a more agile mechanism of opening and closing of the stomatal pore (Wang & Chen, 2020).

Epidermal thickness varied in neither species with the evaluated factors (Table 1). Similarly, we found no visual evidence of lignin deposition on the walls of epidermal cells. Lignification enhances the mechanical resistance and waterproofing of the secondary cell wall, thereby providing it with higher protection against pathogens. However, increased lignin amounts on leaves of forage species are not an advantageous trait. As rumen microorganisms are unable to degrade lignin, ruminants cannot digest it. Therefore, higher lignin contents are a hindrance for plant yield, as they lead to decreased forage potential (Silva, Alquini, & Cavallet, 2005).

## CONCLUSIONS

The higher nutrient dose increased mesophyll and leaf blade thickness, whereas split fertilizer application increased adaxial and abaxial cuticle thickness in both studied species. None of the tested management practices increased lignification of secondary cell walls. Height of bulliform cells was the only variable that increased in response to split fertilizer application and higher fertilizer dose.

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