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Characterization of stomata and glandular trichomes of *Aloysia citriodora* submitted to water stress and seasonal variation[1]

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ABSTRACT – The objective of this study was to evaluate the stomatal density and glandular trichomes as well as the morphometry of stomata of *Aloysia citriodora* leaves submitted to different water availability throughout the seasons. The experiment was carried out in a greenhouse under a randomized block design, composed of four water availability (25%, 50%, 75%, and 100% of the field capacity) throughout the four seasons (autumn, winter, spring, and summer). We evaluated stomatal density and glandular trichomes, and stomatal morphometry (stomata length and width and stomata area). Stomatal density and stomatal morphometry were affected by the seasons, while water availability affected the glandular trichomes density throughout the seasons. There is a higher stomatal density in the spring, and lower in the winter. Winter and spring presented greater stomata length and stomata area. Glandular trichomes density increases in winter and summer under low water availability, with a higher density in the abaxial epidermis.

Keywords: epidermis, seasons, stomata, water availability

RESUMO – Caracterização estomática e de tricomas glandulares de *Aloysia citriodora* submetidas a condições de estresse hídrico e variações sazonais. O objetivo deste estudo foi avaliar a densidade estomática e de tricomas glandulares, bem como a morfometria estomática em folhas de *Aloysia citriodora*, submetidas a diferentes disponibilidades hídricas ao longo das estações do ano. O experimento foi conduzido em casa de vegetação em delineamento de blocos casualizados, composto por quatro disponibilidades hídricas (25%, 50%, 75% e 100% da capacidade de campo) ao longo de quatro estações do ano (outono, inverno, primavera e verão). Foram avaliadas densidade estomática e de tricomas glandulares e morfometria estomática (comprimento e largura estomática e área estomática). As estações do ano modificaram a densidade estomática e morfometria estomática, enquanto a disponibilidade hídrica afetou a densidade de tricomas glandulares ao longo das estações. Na primavera ocorre maior densidade estomática, enquanto no inverno ocorrem as menores densidades. As estações de inverno e primavera promovem maior comprimento de estômatos e área estomática. A densidade dos tricomas glandulares aumenta no inverno e verão sob baixa disponibilidade de água, sendo que maior densidade ocorre na epiderme abaxial.

Palavras-chave: disponibilidade hídrica, epiderme, estações do ano, estômatos

INTRODUCTION

Aloysia citriodora (Palau) is a plant of the Verbenaceae family, popularly known as "lemon verbena", "cidró" or "luisa herb". It has medicinal and aromatic properties with antimicrobial, neuropsychological, gastrointestinal, and antioxidant activities, being cultivated in several countries due to its high economic value (Bahramsoltani *et al.* 2018). It is an essential oil-producing plant, which is stored in liquid form in leaf glandular trichomes and has a characteristic aroma (Bakkali *et al*. 2008). However, the content and composition of volatile compounds, as well as the structures of glandular trichomes can be influenced by phytotechnical, environmental and physiological factors (Brant *et al*. 2009, Meira *et al.* 2013). Feijó *et al.* (2014) confirmed that the essential oil content in *Varronia curassavica* (Jacq.) is causally related to the glandular trichomes density.

Stomata are specialized epidermal cells responsible for regulating CO_2 absorption, fundamental for the photosynthesis and the control of water loss through transpiration, once these processes occur at the same time and are directly interconnected (Lee *et al*. 2016, Pociecha *et al*. 2016, Spinelli *et al*. 2016). The stomata development is controlled by environmental factors such as light and water supply (Bucher *et al*. 2017). Therefore, morphological characteristics and stomatal density are important for the production and survival of the species (Vatén & Bergmann 2012).

Water stress is one of the most impacting environmental factors that result in changes in plant function such as stomatal closure and reduction of gas exchange, which limits the photosynthetic process and consequently affects all plant metabolism (Campbell *et al*. 2010, Hu *et al*. 2010). Therefore, plants have developed acclimation mechanisms through anatomical and physiological modifications to ensure their survival (Hamed *et al*. 2016, Li *et al*. 2016, Santana-Vieira *et al*. 2016, Zaharah & Razi 2009, Zhang *et al*. 2016). Stomatal density and size are some of the indicators of species acclimation, when kept under water stress conditions (Makbul *et al.* 2011). The different seasons tend to modify stomatal density and index as a function of plant phenotypic plasticity. Besides, a higher stomatal density increases the plant's ability to perform gas exchange.

Thus, we have proposed that water deficit that can occur throughout the seasons, affect stomatal characteristics and glandular trichomes of *Aloysia citriodora*. Then, the objective of this study was to evaluate the stomatal density and glandular trichomes, as well as the stomatal morphometry of *Aloysia citriodora* leaves submitted to different water availability throughout the seasons.

MATERIAL AND METHODS

Study Location and Plant Material

The work was carried out in a greenhouse in the experimental area of the Federal University of Santa Maria, *campus* Frederico Westphalen, located at 27º 23' S, 53º 25' W, and 493 m altitude from August 2015 to March 2017. According to Köppen's classification, the region's climate is Cfa – humid temperate with hot summer and maximum air temperatures in warmer months above 22 °C (Alvares *et al*. 2013).

Aloysia citriodora plants were vegetatively propagated by the mini-cutting technique, with cuttings of approximately 10 cm and three mother-plant buds, disinfected in sodium hypochlorite solution (with 1% active chlorine) for one minute, and washed in distilled water. The mini-cuttings were planted in properly washed 2 × 2 × 5 cm phenolic foam, using a phenolic foam cell for each mini-cutting, inserting a knot in the substrate and two out of the foam. Phenolic foam plates were kept on a bench with sub-irrigation, and irrigation shifts controlled by a timer, with 15 minutes on and 60 minutes off. At night, only two periods of 15 minutes were performed.

After 13 days, a nutrient solution was supplied at a concentration of 25%. The electrical conductivity and the pH of the solution were maintained at approximately 300 µS and 6.0, respectively. After 68 days, the mini-cuttings were transplanted to five-liter pots containing commercial substrate Carolina® to complete seedling formation.

Experimental Treatments and Procedures

Plants with 152 days were transplanted to 14.3-liter pots filled with soil. The pots were painted with white paint and 100 g sugarcane bagasse was added to each pot to form the ground cover to avoid excessive water loss through the evapotranspiration process.

The experiment was performed in a randomized block design, 4 × 4 bifactorial, with four water availability levels (25%, 50%, 75%, and 100% of field capacity) with four replicates throughout the four seasons (autumn, winter, spring, and summer). The experimental unit consisted of four plants for stomatal density and glandular trichomes. For stomatal morphometry (length, width and area), we used four replicates of each of the three stomata, totaling 12 experimental units (stomata) per treatment.

All plants were irrigated with 100% of field capacity until the beginning of water restriction at 45 days before leaf collection (recuperation period after evaluation), that is, from the period that corresponds to half of each season. Water management was based on the humidity of the pot with soil, determined by weighing the pot daily with the aid of a digital scale (maximum capacity of 40 kg). The replacement of evapotranspired water was performed whenever the variation between the initial pot weight and the weight obtained on the day of evaluation was greater than or equal to 2%. Thus, the difference between the weights corresponded to the amount of water to be completed. The temperature inside the greenhouse was monitored using thermometers of maximum and minimum temperature of the day. The average temperature was determined by the following equation:

$$\text{Average temperature (ºC)} = \text{(maximum temperature + minimum temperature)}/2 \quad \text{(Eq. 1)}$$

Measurements

At the end of each season, stomata and glandular trichomes density of the abaxial and adaxial surfaces of the epidermis of the leaves, as well as stomatal morphometry (stomata length, stomata width and stomata area) were evaluated. For these analyses, we removed the leaves present in the fifth node from the apex towards the base of the plant. Cyanoacrylate ester adhesives were used for the impression of the abaxial and adaxial epidermis (Weyers & Johansen 1985, Campos *et al*. 2009). The blades were visualized with an optical microscope (Model LEICA DM 1000) with an attached camera (Model LEICA DFC 295), and the images were micrographed with the software Leica Application Suite (Version 3.0). The glandular stomata and trichomes were counted using Anati Quanti, a quantitative analysis software for studies of plant anatomy (Aguiar *et al*., 2007). Stomatal and glandular trichomes density were calculated per mm^2 of the leaf as a function of the photo area provided by the software, being 298 × 223 µm for a 40-fold increase.

To perform the scanning of electronic microscopy, the leaf samples were fixed in modified Karnovsky's solution (2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2) (Karnovsky 1965). Then, they were dehydrated in acetone 100%. The fragments were critical-point dried using CO_2 (CPD 030, BalTec, Balzers, Germany) and subsequently metallized with gold (FDU 010 Bal-Tec, Balzers, Liechtenstein). The observations and photographic documentation were performed using the scanning electron microscope (Leo 1430 VP, Zeiss, Cambridge, United Kingdom), and all the images were processed digitally.

The same images for stomatal and glandular trichome counts were used for stomatal morphometry. Measurements were performed with the software ImageJ and expressed in µm. Regarding morphometry, we evaluated the following variables: stomata length and width (stomatal pore + guard cell), and stomata area. The stomata area was estimated by multiplying the length and width of the stomata, as described by Lima *et al*. (2010) e Pinheiro *et al*. (2020):

$$\text{Stomata area } (\mu m^2) = \text{length } (\mu m) \times \text{width } (\mu m) \qquad \text{(Eq. 2)}$$

Statistical Analysis

Data were subjected to analysis of variance to assess the effect of seasonality and different levels of water availability. When significant, a regression analysis was performed for quantitative data and comparison of means for qualitative data by the Scott-Knott's test at 5% significance, using the statistical program Genes (Cruz 2013).

RESULTS

Temperature conditions inside the greenhouse showed high thermal amplitude. The effect of seasonality showed periods with high temperatures, reaching values close to 50 ºC in the spring and others with low temperatures, reaching negative values during autumn (-0.2, -1.8, and -2.1 ºC, respectively). In autumn, maximum and minimum temperatures were observed, ranging from 41.5 and -2.1 ºC, and the average temperature for the period was 19.9 ºC (Fig. 1A). For the winter season, the average temperature was higher than in the autumn (23.2 °C), but this period presented a higher thermal amplitude with maximum and minimum temperatures of 46.6 and 2 °C, respectively (Fig. 1B). In spring, temperatures ranged from 47.9 to 10.8 °C with an average temperature of 28.7 °C (Fig. 1C). The average temperature for summer was 30 °C, with maximum and minimum temperatures of 45.8 and 11.6 °C, respectively (Fig. 1D).

Aloysia citriodora presents stomata in only one of the leaf epidermis surfaces, being classified as hypostomatic, that is, the stomata are present only in the abaxial epidermis of the leaf. These characteristics were confirmed in the present study by scanning electron microscopy micrographs of the adaxial and abaxial epidermis of *Aloysia citriodora* leaf (Fig. 2).

Through the analysis of variance, the stomatal density of the abaxial epidermis and the stomatal morphometry (stomata width and stomata area) presented significant differences only for the seasonality factor ($p < 0.05$). Stomata length did not present differences for any factor (Tab. 1). Glandular trichomes density in two faces of the epidermis was significant for the interaction between seasonality and water availability (Tab. 1).

The highest stomatal density of the abaxial leaf epidermis was observed in spring (397 stomata mm^{-2}). The lowest stomatal density was observed during winter, with approximately 178 stomata mm^{-2} (Fig. 3).

Stomata width variable was significantly higher in the winter and spring seasons when compared to the others (13.2 and 12.7 µm, respectively) (Fig. 4A). The stomata area presented similar results, being significantly higher in the winter and spring seasons, with 346 and 340 μm^2, respectively (Fig. 4B).

The abaxial epidermis of the leaf presented high glandular trichomes density, with some differences depending on the season (Fig. 5A). The highest glandular trichomes densities were observed in the winter and summer (366 and 471 glandular trichomes mm^{-2}, respectively). In the autumn, we verified the lowest density of trichomes (109 glandular trichomes mm^{-2}), adjusting linearly as the water availability increased. In the winter, the best-fitted curve was quadratic and the condition of 100% of field capacity presented lower glandular trichomes density (366 glandular trichomes mm^{-2}). Spring also adjusted quadratically, with higher glandular trichomes density in extreme water availability, i.e., 25% and 100%. In the summer, the curve that best fitted was the cubic due to water availability, with higher glandular trichomes density for water availability of 25% and 75% of the field capacity (Fig. 5A).

The adaxial epidermis presented lower glandular trichomes density when compared to the abaxial epidermis since the highest average observed in this surface was 59 trichomes mm^{-2} in the spring with water availability of 25% of the field capacity (Fig. 5B). The autumn season showed a quadratic tendency, with higher glandular trichomes density at 50% of field capacity. For the summer season, the best fit curve was quadratic, and the lowest densities were found in the water availability of 50% of field capacity. In winter, the quadratic curve was the best fit curve, with higher glandular trichomes density at water availability of 75% of field capacity (55 glandular trichomes mm^{-2}). Spring also showed a quadratic response, but the glandular trichomes density was significantly higher at 25% of field capacity (Fig. 5B).

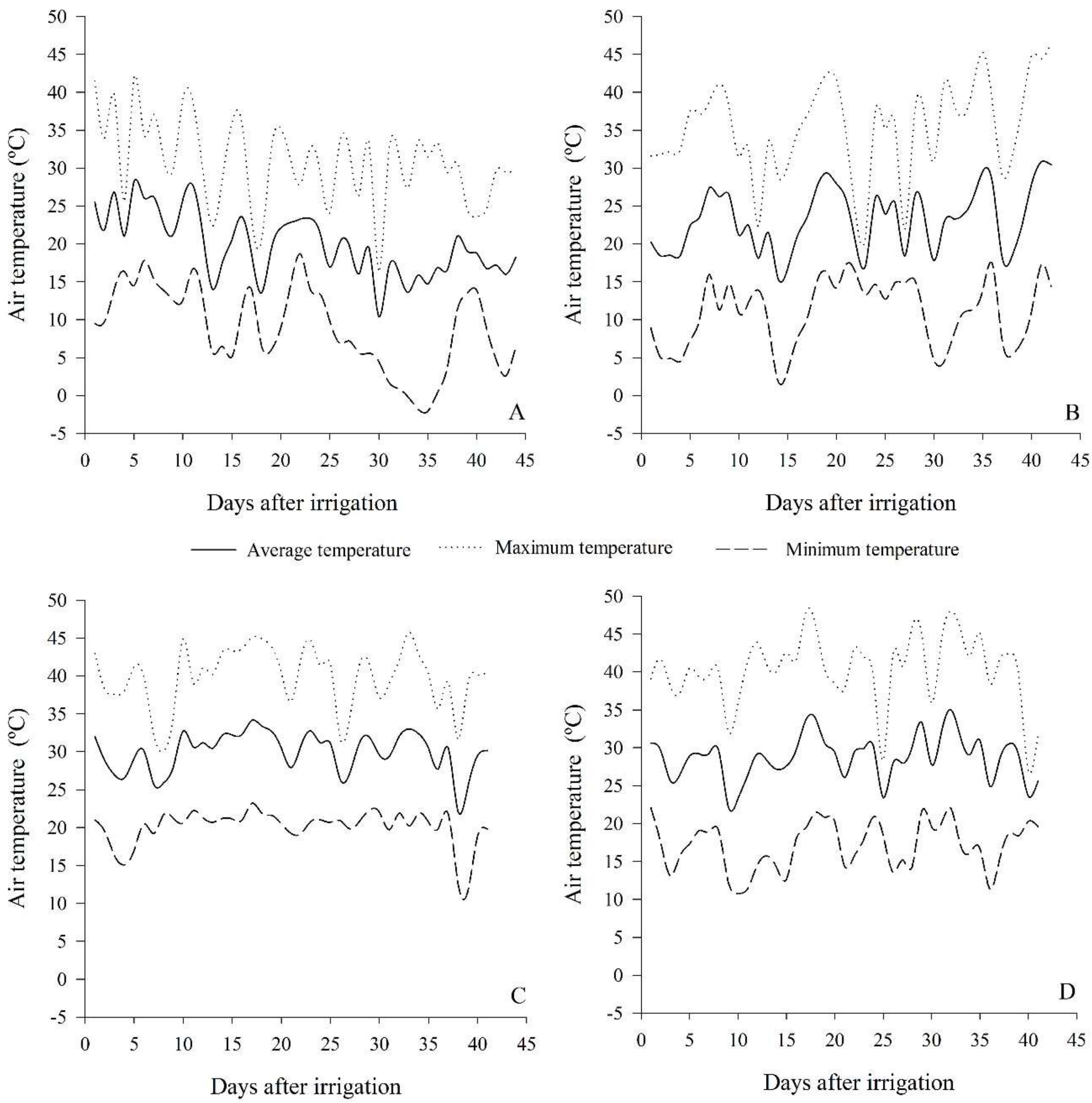

Figure 1. Maximum, minimum and average air temperatures were recorded inside the protected environment when *Aloysia citriodora* was under different irrigation levels. **A.** Autumn 2016; **B.** Winter 2016; **C.** Spring 2016; **D.** Summer 2016/2017.

DISCUSSION

The results of the work showed that the seasons affect the stomatal density and stomata size, and spring provided higher stomatal density of *Aloysia citriodora*, as well as the formation of larger stomata in the leaves. The glandular trichomes of the abaxial and adaxial epidermis of this species were influenced by seasonality and water availability, with different responses to these conditions.

The presence of stomata only in the abaxial epidermis is related to the adaptation factor of the species to environmental factors such as high radiation and temperature and low water availability, since the genus *Aloysia* is also cultivated in North Africa (Carnat *et al*. 1999), an environment with a characteristic subtropical climate with hot summer. In addition, *Aloysia citriodora* stomata have a reniform shape, surrounded by a variable number of epidermal cells that do not differ in shape and size, characterizing it as anomocytic and with striae around the stomata (Gattuso *et al*. 2008), which are characteristics observed in this study (Fig. 2).

During the spring, there were more favorable environmental conditions to increase stomatal density, allowing greater gas exchange with the environment and the production of photoassimilates. In the winter, the stomatal

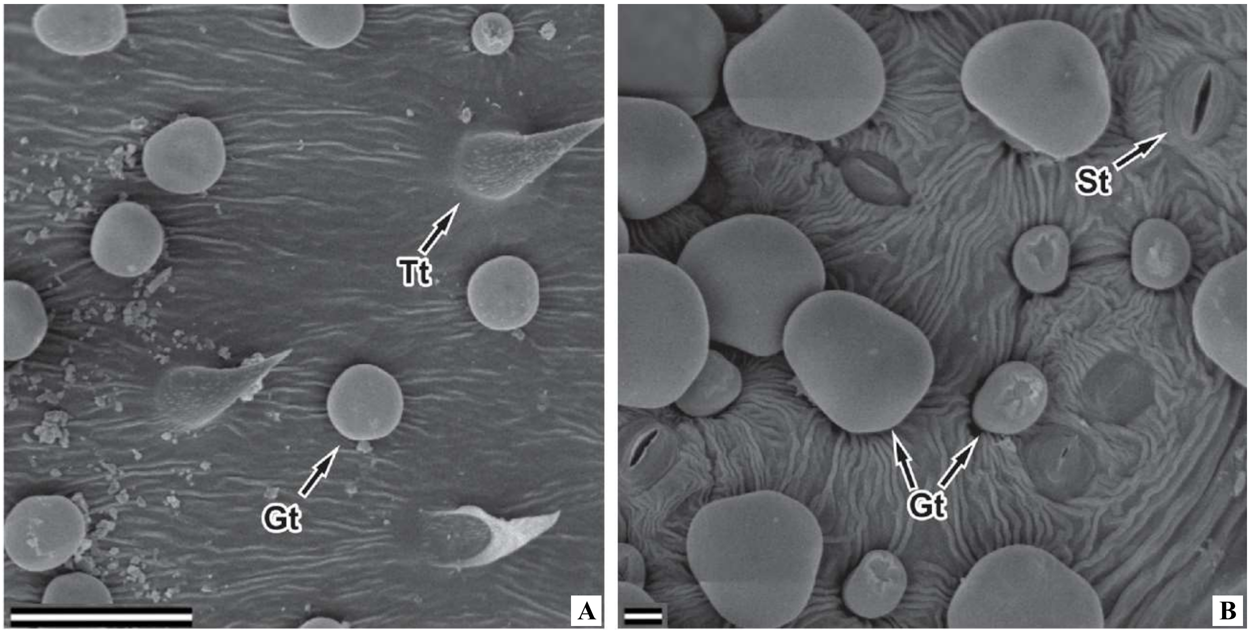

Figure 2. Details of the stomata and trichomes of the epidermis of *Aloysia citriodora* leaves from scanning electron microscopy. **A.** Stomata and trichomes of the adaxial epidermis; **B.** Stomata and trichomes of the abaxial epidermis; Tt = Tector trichome. Gt = Glandular trichome; St = Stomata. Bars: A = 100 µm; B = 10 µm.

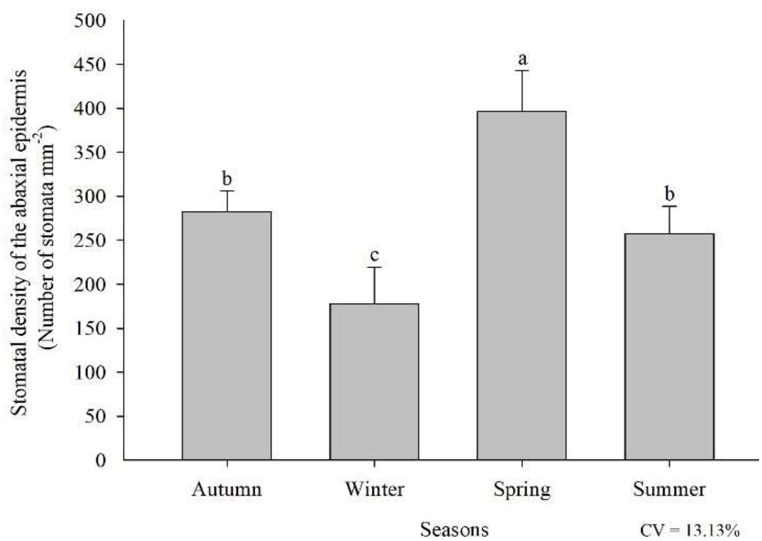

Figure 3. Stomatal density of the abaxial epidermis of *Aloysia citriodora* leaves throughout different seasons. *Means followed by the same lower-case letter do not differ from each other by the Scott-Knott's test ($p < 0.05$).

density was lower due to the lower temperatures and less transpiration, with larger cells. The increase in stomatal density is related to the greater ability of plants to absorb CO_2 from the atmosphere, to increase the photosynthetic capacity through a better volume absorption of this gas.

The higher stomatal density is due to the fact that *Aloysia citriodora* shows higher growth in October (spring) and reached maximum growth during the summer (Brant *et al*. 2008). Generally, stomatal density is positively correlated with photosynthetic rate, as the number of stomata directly influences the process of transpiration and photosynthesis, making it an important parameter to evaluate the acclimation capacity of plants under different environmental conditions (Vráblová *et al*. 2018). Therefore, the relationship between the photosynthetic and transpiration rate makes it easier to determine the water use efficiency by plants, which is causally related to the stomatal density of the leaves. *Aloysia citriodora* is a species of tropical and subtropical climates, then leaf loss may occur during the colder periods of the year (Paulus *et al*. 2013). Therefore, the lower stomatal density in winter may be related to the unfavorable environmental conditions for growth since

Table 1. Summary of the analysis of variance for stomatal density of the abaxial epidermis (SDAB), stomata length (SL), stomata width (SW), stomata area (SA), glandular trichomes density of the abaxial epidermis (GTAB), and glandular trichomes density of the adaxial epidermis (GTAD) in leaves of *Aloysia citriodora*. Degrees of freedom (DF), Sources of variation (SV); Coefficient of variation (CV). *Significant at 5% by the F-test.

SV	DF	Medium square					
		SDAB	SL	SW	SA	GTAB	GTAD
Blocks	3	909.59	1.95	3.83	4599.56	1935.80	533.83*
Water availability	3	2131.57	7.74	1.57	4577.40	3832.78	4096.30*
Season	3	130663.26*	10.45	15.58*	18103.42*	376932.64*	172.83
Water availability × Season	9	1868.50*	3.07	0.94	1843.92	4147.74*	570.70*
Residue	36	624.12	1.46	0.99	1435.59	1089.39	128.15
CV(%)		8.96	4.69	8.21	12.06	11.05	16.69

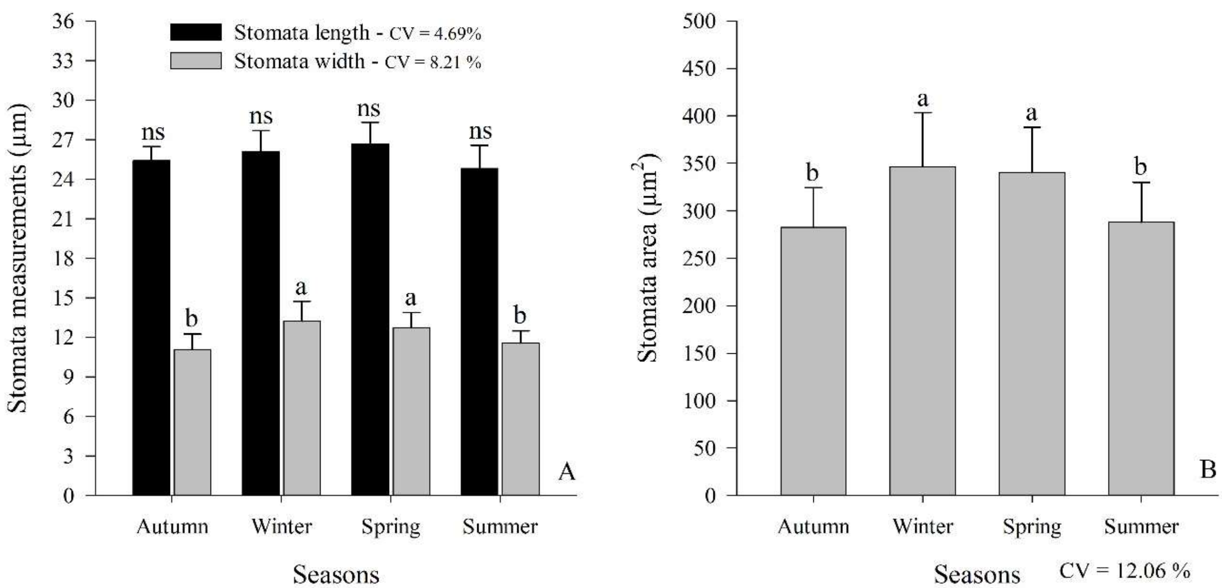

Figure 4. Stomatal morphometry of *Aloysia citriodora* leaves throughout different seasons. *Means followed by the same lower-case letter do not differ from each other by the Scott-Knott's test ($p < 0.05$). **A.** Stomata length and stomata width; **B.** Stomata area.

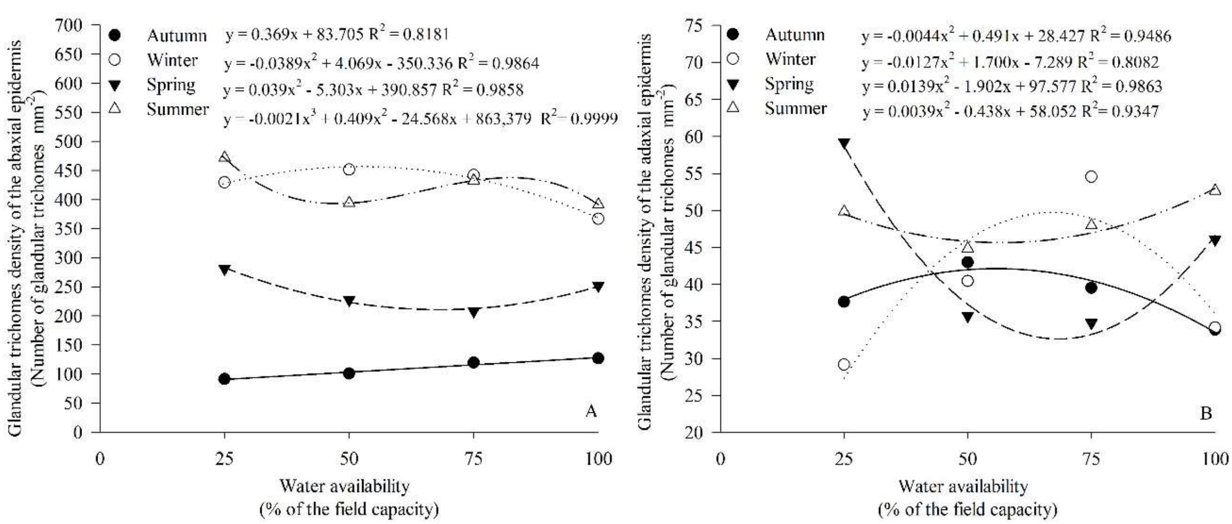

Figure 5. Glandular trichomes density of *Aloysia citriodora* leaves epidermis under different water availability throughout the seasons. **A.** Glandular trichomes density of the abaxial epidermis; **B.** Glandular trichomes density of the adaxial epidermis.

the plants reduce their metabolism and go into dormancy in colder periods. Generally, the higher stomatal density is related to the occurrence of smaller stomata, allowing gas exchange, and improving the stomatal conductance of the leaf, besides promoting greater resistance to the passage of water molecules during leaf transpiration, while the CO_2 molecule is normally captured due to its smaller size and diffusion resistance (Laza *et al*. 2010, Fermino Jr. & Fockink 2017). The high density of small stomata is related to plant acclimation to minimize transpiration water loss and promote carbon input for photosynthetic activity, because the smaller the size of the stomata, the faster the process of opening and closing the stomatal pore (Doheny-Adams *et al*. 2012, Drake *et al*. 2013, Schmidt *et al*. 2017). In this work, the largest number of smaller stomata found in the fall and summer seasons may be related to the acclimation mechanism of the plants, mainly to the temperature factor.

In contrast, winter presented the lowest stomatal density and the largest stomata width in *Aloysia citriodora*. Spring was similar to winter for the variable stomata width, standing out as a favorable season for plant growth and development, since it presented high stomatal density, and also characterized by larger stomata, proving that the plants acclimatize according to the changes in the environment.

Seasonality changes the environmental conditions throughout the year (i.e. temperature, solar radiation, photoperiod, etc.), and in this study, this effect was the factor that influenced the characteristics of density and stomatal morphometry. Therefore, plants can adjust their morphometry and stomatal densit as a function of environmental conditions (Pearce *et al*. 2006). Cell differentiation allows modifications of morphological characteristics, acting as a defense mechanism of plants against biotic or abiotic stress (Potters et al. 2007). In the present study, the different water availability did not influence the stomatal density and morphometry, probably due to the water restriction that started in the middle of each season, that is, the stomata were already differentiated in the leaves and did not influence the phenotypic plasticity.

Glandular trichomes are epidermal appendages that perform important functions in plants as they store secondary metabolites that are related to plant defense mechanisms against biotic or abiotic factors (Cui *et al*. 2011, Stratmann & Bequette 2016). In this study, glandular trichomes were observed on both surfaces of the leaf epidermis (abaxial and adaxial), however, with a greater amount in the abaxial epidermis.We observed variations in the density of glandular trichomes with different water availability throughout the seasons. The abiotic factors, such as the effect of different water availability throughout the seasons, influence the quantity and quality of metabolite components, as well as the morphophysiological characteristics and modifications of plants in which epidermal cells are induced to differentiate into stomata or trichomes, under different conditions (Bartoli *et al*. 2014). Thus, the plasticity that the plants of *Aloysia citriodora* presented was able to facilitate acclimatization according to the environmental changes.

The higher glandular trichomes density may indicate abiotic or biotic stress conditions in which the plants remain subjected. The higher occurrence of glandular trichomes is also related to the higher production of essential oil that usually occurs under stressful conditions, as this secondary metabolite needs to be stored in glandular trichomes (Lange & Srividya 2019). The production of secondary metabolites of aromatic plants, such as terpenoids, are stored in glandular trichomes and act as a plant defense strategy (Maffei 2010). Thus, a higher glandular trichomes density in *Aloysia citriodora* indicates greater production and storage capacity for essential oil, since it is the main metabolite produced by the species.

The highest glandular trichomes densities were observed during winter and summer and under lower water availability. The function of trichomes is to protect against environmental stress (Cui *et al*. 2011), so the higher glandular trichomes densities suggest that the cultivation of *Aloysia citriodora* during winter and summer seasons with low water availability are unfavorable for these plants. In the adaxial epidermis of leaves, summer provided higher glandular trichomes density under water availability corresponding to 25% and 100% of the field capacity. However, the highest glandular trichomes densities were observed in spring with 25% and winter with 50% and 75% of the field capacity. These seasons have extreme temperature and radiation conditions, so the presence of more glandular trichomes in the leaves protects the plant against both heat and cold. Under conditions of high temperature or low water availability, trichomes reduce leaf temperature, favoring the maintenance of CO_2 assimilation and consequently the adaptation of plants to those conditions (Ehleringer & Mooney 1978). Likewise, under conditions of low water availability, more trichomes act to protect plants against water loss (Karabourniotis *et al*. 2020).

Therefore, *Aloysia citriodora* cultivated in the winter and summer, associated with low water availability, increases the density of glandular trichomes as an acclimation mechanism to tolerate these unfavorable environmental conditions.

The results are essential to understand some of the anatomical responses of *Aloysia citriodora* when subjected to water stress, as well as to present which conditions tend to favor glandular trichome production, which may result in higher production of essential oil produced by the secondary metabolism of this species. However, further studies are needed to generate information about the production and chemical composition of the essential oil for this species and to determine the season and water availability that promotes greater productivity.

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