Anatomy of the saltgrass (*Distichlis spicata* L.) leaves with irrigation and without irrigation

Anatomía de hojas de pasto salado (*Distichlis spicata* L.) con riego y sin riego

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ABSTRACT. *Distichlis spicata* is a gramineous halophyte species that grow around the coastal areas. The objective of the study was to identify the modifications in the anatomical structure of stomates of the *D. spicata* leaf epidermis, cultivated with irrigation and without irrigation conditions. Two populations were selected, 1) plants of *D. spicata* with irrigation and 2) plants of *D. spicata* without irrigation. Ten plants per replication were selected from each population, and from each selected plant, the fourth leaf was taken. Each leaf was cut longitudinally into four segments which were dried and analyzed using an Electronic Scanning Microscope. The variables measured were the stomatal density, length, and width of stomates and pores, the stomatal area, the proportion of the pore area concerning the size of the stomata, and the stomata proportion per surface. The results showed that *D. spicata* populations with irrigation and without irrigation showed anatomical modifications in the structure of stomates in the adaxial epidermis but not in the abaxial epidermis. In the adaxial epidermis, the plants with irrigation showed higher values of stoma length, stoma width, stoma area and pore length, while plants without irrigation showed higher values of proportion pore/stoma and stomatal density. The *D. spicata* plants without irrigation showed small stomates than plants with irrigation, also, the plants without irrigation, increased the accumulation of salt crystals in the abaxial epidermis.
Keywords: Halophyte grass, stomates structures, gramineous, arid zones.

RESUMEN. *Distichlis spicata* es una especie de gramínea halófita que crece en las zonas costeras. El objetivo del estudio fue identificar las modificaciones en la estructura anatómica de estomas de la epidermis foliar de *D. spicata*, cultivadas con riego y sin riego. Se seleccionaron dos poblaciones, 1) plantas de *D. spicata* con riego y 2) plantas de *D. spicata* sin riego. De cada población se seleccionaron 10 plantas por repetición y de cada planta seleccionada se tomó la cuarta hoja. Cada hoja se cortó longitudinalmente en cuatro segmentos que se secaron y analizaron utilizando un microscopio electrónico de barrido. Las variables medidas fueron la densidad estomática, largo y ancho de estomas y poros, el área estomática, la proporción del área de poros con respecto al tamaño de los estomas y la proporción de estomas por superficie. Los resultados mostraron que la población de *D. spicata* con riego y sin riego mostraron modificaciones anatómicas en la estructura de los estomas en la epidermis adaxial pero no en la epidermis abaxial. En la epidermis adaxial, las plantas con riego presentaron valores más altos de longitud de estoma, ancho de estoma, área de estoma y longitud de poro, mientras que las plantas sin riego mostraron valores más altos de proporción poro/estoma y densidad estomática. Las plantas de *D. spicata* sin riego presentaron estomas más pequeños respecto a las plantas con riego; además, las plantas sin riego incrementaron la acumulación de cristales de sal en la epidermis abaxial.
Palabras clave: Halófitas, estructuras de estomas, gramíneas, zonas áridas.

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INTRODUCTION

Distichlis spicata is a halophyte grass that grows and develops in extreme environments characterized by saline and flooding, its presence has been reported in Saskatchewan, Canada to Patagonia, Argentina, also in the tidal marshes east, south, and west of North America (Lazarus *et al*. 2011). In Mexico, this species is reported in the northwest, north-northwest, southwest, and gulf (Soreng *et al*. 2003, Frías-Ureña *et al*. 2022). In the coastlines of Baja California Sur (the Gulf of California and the Ocean Pacific), its presence has also been indicated (León de la Luz *et al*. 2018).

This is a plant with the potential for remediation of the soils affected by salinity (Rojas-Oropeza *et al*. 2022) and has also been treated for its capacity to produce forage in desert-like conditions (Norman *et al*. 2013), where the water is a scarce resource, and for the irrigation of the crops and animal feeding only salinized water is used, since the “sweet water” is destined for population and industry (INEGI 2017). There are some studies that refer the use of water for irrigation in traditional crops; however, the halophytes can dissolve oxalate grains (calcium or magnesium) (Mata-González *et al*. 2021), from the osmotic adjustment and potassium preference over sodium within the membrane potential. This ability provides to the halophytes a better physiological and morphometric response under saline and extreme ecosystems (Al-Shamsi *et al*. 2020).

According to the criteria of Céccoli *et al*. (2015), when the plant is under dry conditions, appear some anatomical xeromorphic such as the increase of the number of fasciculate hairs in the hairless indumentum, that avoid the loss of water by transpiration. These anatomic changes respond to specific and regional climatic conditions, and even to geospatial variations within the same ecosystem (Madewell *et al*. 2022, Rojas-Oropeza *et al*. 2022). In that context, we hypothesized that *D. spicata* plants with irrigation and without irrigation (rainfed) conditions can modify their morphometric characteristics such as the stomates that are structures that define the maintenance of the boundary layer, breathing, and the efficient use of water. The objective of the study was to identify modifications in stomatal morphology in leaf blades of *D. spicata*, grown with irrigation and without irrigation conditions in an arid area of Mexico.

MATERIALS AND METHODS

Study area

The study was developed in the Center for Biological Research of the Northwest, S.C. (CIBNOR, Acronym in Spanish) situated in the coastal lands of El Comitán, in the southern portion of the Baja California Peninsula, located to the northwest of La Paz city between 24° 08’ 10.03 N and 110° 25’ 35.31 W. According with Aguilera and Martínez (1996) this area is situated in the region around the world with high aridity. The site has a type Bw (h’) hw (e) climate considered as semi-arid with xerophilous vegetation (García 2004). The average annual temperature varies between 22 and 23 °C and precipitation is scarce, between 100 y 250 mm annually, with annual evaporation between 1.758-2.472 mm (Agüero-Fernández *et al*. 2018).

Distichlis spicata L. populations

The *D. spicata* plants were sampled from two populations. One population consisted of plants with irrigation and agronomic management. These plants were planted on 16 plastic boxes (57×38×7 cm, L, W, D) using sand of dunes as substrate. The plastic boxes with *D. spicata* plants were placed inside a greenhouse with roof covered with white anti-aphid mesh model 55, with 30% of shade mesh. Under this mesh, another black mesh model 20 with 35% of shade was placed for a total shading of 65%. One replication was represented by four plastic boxes and the experiment consisted of four replications.

The second population consisted of wild *D. spicata* plants, without irrigation and agronomic management, which were growing close to a *Jatropha* spp. population; both species subjected and exposed to environmental conditions. The plot of *D. spicata* was composed of many scattered plants; however, four plots of similar dimensions to those of the first population were selected and each plot was considered as one replication.

Selection and analysis of _D. spicata_ plants

Ten plants per replication with similar characteristics were selected from each population and from each selected plant, the fourth leaf was taken. Then, each leaf was cut longitudinally into four segments of 3 cm from the base to the apex, which were carried to the CIBNOR microscopy laboratory and washed with running tap water for 3 min to eliminate the excess of salt that could be contained the surfaces of the leaves. Afterwards, each segment was dried at critical point CPD SAMDRI PVT-3D. From the four segments, two segments were used to analyze the adaxial epidermis (80 segments per population) and the other two to analyze the abaxial epidermis (80 segments per population). Each segment was analyzed using an Electronic Scanning Microscope (Hitachi, S-300N, Hitachi Science System Ltd., Japan), and previously four quadrants were located to 500x within a field of 1171.1 × 254.6 µm.

Morphometric characteristics of stomates

In the readings of each segment, the stomatal density (mm^{-2}), length and width of stomates (µ), and the stomata area from the connection (guards' cells + pore) (León de la Luz and Fanjul 1983) were determined. The length and width of the pores (µ), the proportion of the pore area concerning the size of the stoma (%), as well as the stomata proportion per surface (%) were also determined. A total of 160 segments were observed for each population, 80 observations from abaxial epidermis and 80 from adaxial epidermis, with four counts per segment with a total 640 fields observed per population.

Statistical analysis

Kolmogorov-Smirnov test (Massey 1951) was performed on the data to test normality. Data were analyzed using univariate analysis of variance (ANOVA) according to a completely randomized design of one-way of classification, with two populations of *D. spicata* (with irrigation and without irrigation) modeled as fixed factor. The differences between the means were determined by Tukey's HSD multiple range test at $p \leq 0.05$. The data were analyzed using Statistica® v. 13.5. In order to meet the assumptions of homogeneity of normality and homoscedasticity, data transformations were performed when it was necessary. The stomata density for the adaxial and abaxial epidermis was transformed according $\boldsymbol{x} = \sqrt{\boldsymbol{x} + \mathbf{2.0}}$ and $\boldsymbol{x} = \sqrt{\boldsymbol{x} + \mathbf{2.5}}$.

RESULTS

Morphometric characteristics of stomates in the adaxial epidermis

Some morphometric characteristics of stomates in the adaxial epidermis showed significant differences among *D. spicata* populations (Table 1). The plants without irrigation showed a greater proportion of pore with respect to the stoma ($p \leq 0.001$) and showed a higher stomatal density ($p < 0.001$) in response to the water stress.

Table 1. Morphometric characteristics of the stomates in the adaxial epidermis in *D. spicata* plants with and without irrigation.

Variables	***Distichlis spicata*** **populations**			**Standard error**
	With irrigation	**Without irrigation**	***P value***	
Stoma length (μ)	27.53 ± 2.8a	23.04 ± 1.43b	0.001	5.04
Stoma width (μ)	0.35 ± 0.10a	0.15 ± 0.06b	0.001	0.010
Stoma area (μ^2)	9.91 ± 3.04a	3.65 ± 1.54b	0.001	9.78
Pore length (μ)	12.7 ± 2.15a	10.42 ± 2.47b	0.01	1.35
Pore width (μ)	0.022 ± 0.01a	0.028 ± 0.01a	0.93	0.0009
Pore area (μ^2)	0.28 ± 0.15a	0.28 ± 0.09a	0.17	0.0005
Proportion pore/stoma (%)	0.02 ± 0.04b	0.08 ± 0.05a	0.001	0.0008
Stomatal density (mm^2)	6.8 ± 1.2b (2.95)	33.5 ± 6.5a (5.91)	0.0001	0.08

Values in parentheses correspond to transformed data according to $\sqrt{x+2}$. The data correspond to the means ± standard deviation. Rows with different letters differ significantly (Tukey HSD $p \leq 0.05$).

Morphometric characteristics of stomates in the abaxial epidermis

The morphometric characteristics of stomates in the abaxial epidermis did not show significant differences among *D. spicata* populations (Table 2). The figure 1 shows the stomates of the abaxial epidermis of leaves of two *D. spicata* populations growing with and without irrigation. The leaves of *D. spicata* plants without irrigation accumulated more lumps of salt on the surface. The accumulation of salt crystals increased in the abaxial epidermis of the leaves of *D. spicata* without irrigation (Figure 1B) than those with irrigation (Figure 1A). The figure 2 (A-1 & A-2) shows the different anatomical structures of the abaxial epidermis of a *D. spicata* leaf that grow with irrigation, while the figure 2 (B1 and B2) shows a *D. spicata* leaf that grow without irrigation. The figure 2A (population with irrigation) shows prominent marginal stingers, developed trichomes with hair shapes and papillae. The figure 2A-1 and A-2 shows absence of crystals, while the population without irrigation shows crystals (Figure 2B-1) without hairs but trichomes predominated (Figures 2 B-1 & B-2) in the costal and intercostal region. The distribution of stomates in the plants of both populations was disperse (Figure 2).

Table 2. Morphometric characteristics of the stomates in the abaxial epidermis in *D. spicata* plants with and without irrigation.

Variables	*Distichlis spicata* populations		*P value*	Standard error
	With irrigation	Without irrigation		
Stoma length (μ)	22.62 ± 1.49a	23.10 ± 8.18a	0.50	0.24
Stoma width (μ)	0.21 ± 0.06a	0.17 ± 0.07a	0.12	0.02
Stoma area (μ^2)	4.79 ± 1.56a	3.86 ± 4.72a	0.12	0.46
Pore length (μ)	14.60 ± 0.01a	14.23 ± 0.01a	0.18	0.19
Pore width (μ)	0.023 ± 0.01a	0.019 ± 0.01a	0.59	0.002
Pore area (μ^2)	0.35 ± 0.19a	0.27 ± 0.09a	0.36	0.043
Proportion pore/stoma (%)	0.08 ± 0.04a	0.09 ± 0.06a	0.73	0.004
Stomatal density (mm^2)	4.4 ± 0.02(0.35)a	4.6 ± 0.02 (0.35)a	0.54	0.012

Values in parentheses correspond to transformed data according to $x = \sqrt{x + 2.5}$. The data correspond to the means ± standard deviation. Rows with same letters do not differ significantly (Tukey HSD $p \leq 0.05$).

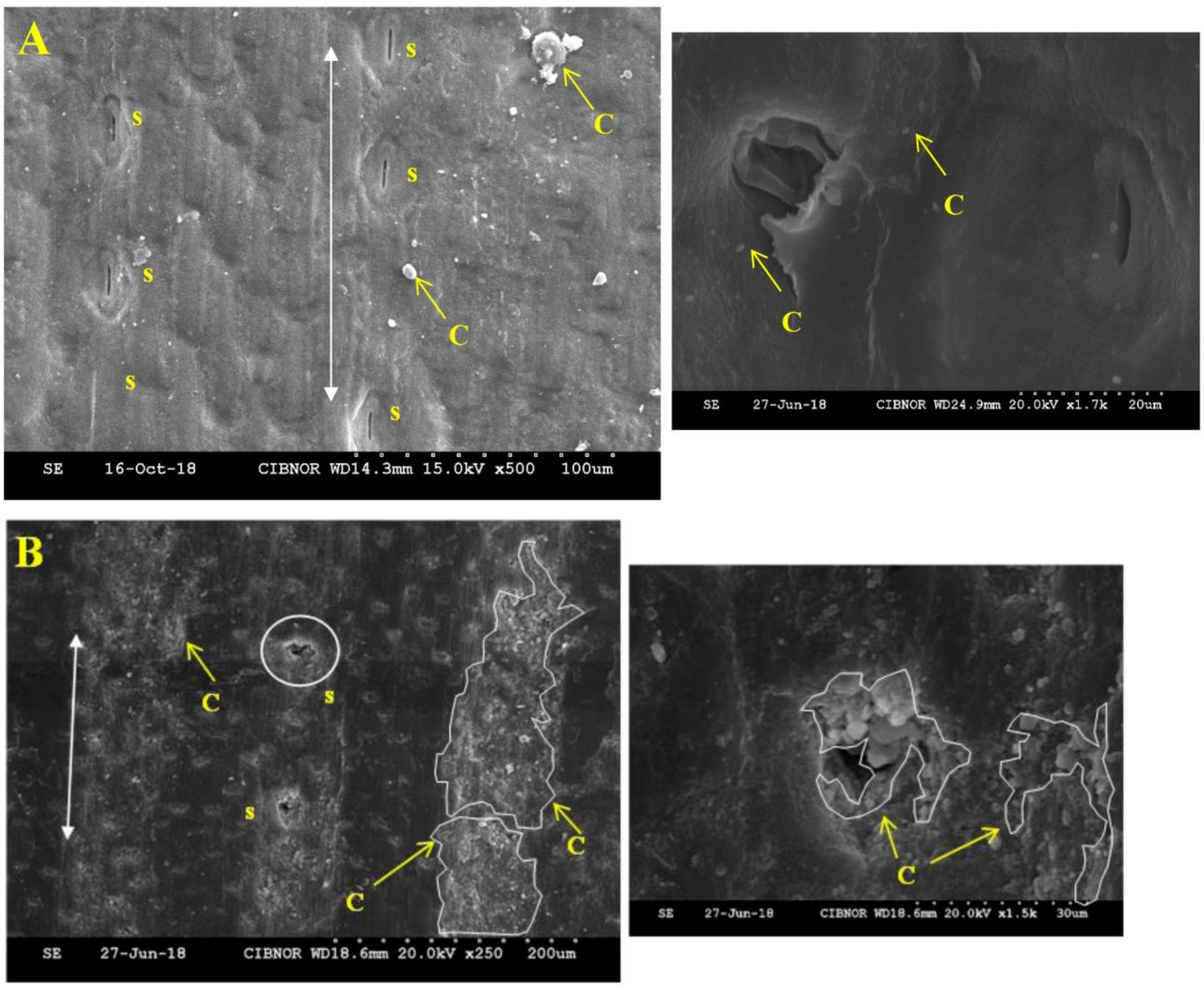

Figure 1. Stomates in the abaxial epidermis in *D. spicata* leaves of two populations (A) with irrigation and (B) without irrigation. s = Stomates; C = crystals; *. Arrows indicate the arrangement of the stomata.

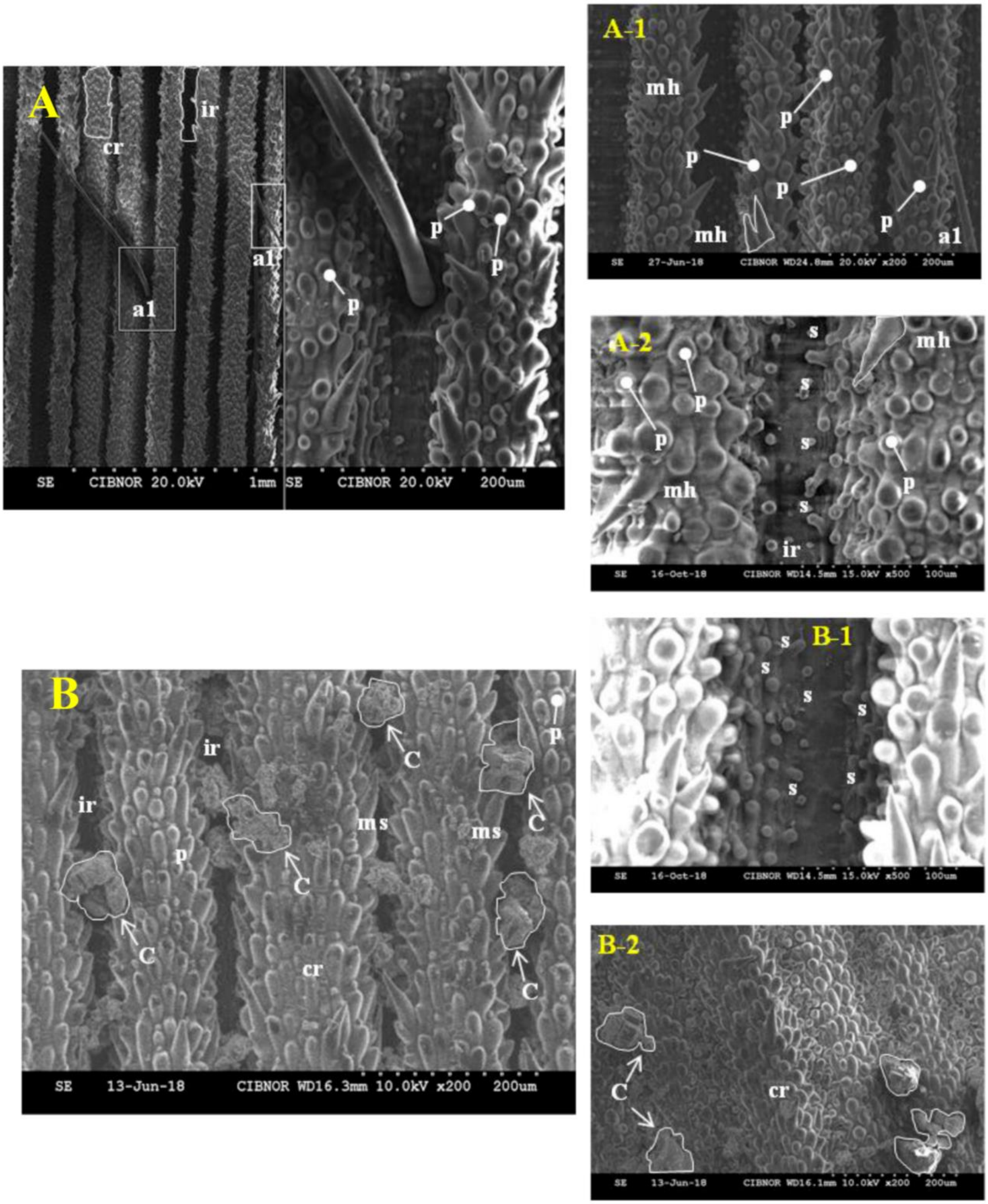

Figure 2. View of the abaxial epidermis (A1-B1 and A2-B2) of *D. spicata* from two populations (A) with irrigation and (B) without irrigation. ms = Marginal stinger; ir = Intercostal region; cr = Costal region; p = Papillaris; mh = Micro hair; s = Stomates; oc = Oxalate crystals.

DISCUSSION

The size range (between 15.1-37.9 µm) of the stomata of *D. spicata* in the present study coincided of the stomates defined by Wilkinson (1979) and those indicated (21-24 um length) by Soreng *et al*. (2003) in the characterization of grasses for the new world, the size of stomates for both *D. spicata* populations is considered as medium; however, the size of the stomata of the plants grown without irrigation was smaller. This response was reported by Maricle and Maricle (2018) who expressed that plants growing under water deficit conditions promote the presence of small stomates that allow the control in opening and closing and avoiding the escape of water. The large stomates difficult the control of opening and closing when water availability is limiting (Drake *et al*. 2013).

The differences between the ratio of pore size respect that of the stoma and the high stomatal density in plants with water deficit is associated to the reduction of the potential canopy conductance which increases the use water efficiency since stomatal density is directly related to this potential (Fraser *et al*. 2009). The values of all morphometric characteristics of the stomates of both *D. spicata* populations are lower than those reported by García *et al*. (2008) in 15 halophyte species and by those reported in *Pseudoroegneria spicata* ssp. inermis evaluated under different dose of water supply and range of temperatures; however, the reduction in the number of stomates in the plants with irrigation are in agreement with those reported by Fraser *et al*. (2009) showing the same pattern of reduction associated to the moisture available in the soil, doing an economic or rational use of the water (Paradiso *et al*. 2017).

Referring to the morphometric characteristics of stomas of the abaxial epidermis, similar results were reported by Maricle *et al*. (2007) when evaluated the effects of stress by salinity and drought in halophyte grasses, observed in the treatments of induction of drought stress, an increase in the concentration of crystals in the leaves to compensate the efficiency of the photosynthesis by the reflectance of light, without effects on the stomatal conductance. Although in the present study this effect was not observed, Fraser *et al*. (2009) reported a similar criterion in *Pseudoroegneria spicata* showing a decrease in stomatal distribution on the abaxial surface due to an increase in temperature, where the residual soil moisture ceased to be a conditioning factor. In this case, the radiant energy reflected in the soil was the factor that determined the stomatal morphometry of the abaxial epidermis. This effect could be present in this study; however, the information was not registered. According to Da-Silva *et al*. (2020) the dispersion of the stomates is related to the order of the parallel veins of the leaf epidermis, which can be located parallel or without a specific orientation as observed in the Figure 1. The stomates were located at the same level of the epidermal cells, like the results reported by Kuster *et al*. (2020), who concluded that halophytes growing under beach environments shows this stomates orientation, while halophytes growing on rocks, the stomates are sunk below the epidermis as a compensatory mechanism for water loss, and as well as other adaptations for different growth conditions (Da Silva *et al*. 2020).

According with the criterion of He *et al*. (2014), the presence of oxalate crystals in the epidermis of the plant is associated with different defense and compensation mechanisms, for example, keep calcium levels inside the epidermis, protection from herbivory, and detoxification of heavy metals because of the epidermis can accumulate toxic metals such as Zn, Cd, Sr, Pb and Al. The oxalate crystals also contributing to provide rigidity to the tissues giving structural support

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to the organs, and intervention in the dispersion and concentration of the light rays, increasing in this way, the photosynthetic efficiency. The accumulation of oxalate crystals may be related to the dilution effect of water, reducing its availability by controlling the amount of water in the soil. The salt is concentrated at the stomatal and epidermis level, since the functioning of the salt excretory gland does not discriminate from water availability to eliminate excess Na, K, and Cl ions when it works actively (in a diurnal rhythm) (Kobayashi 2008). According to the criteria of He *et al*. (2014), this response constitutes a defense-compensation mechanisms, that could be a compensation for water deficit and extreme edaphoclimatic conditions, typical of arid climates, while the abundance of crystals is related to a phenotypic response (Semenova *et al*. 2010).

The halophyte plants show variability in the characteristics of the stomatal apparatus (Kuster *et al*. 2020, Da Silva *et al*. 2020). In this study, the amphistomatic (leaves with stomates on both surfaces) character of the leaves of *D. spicata* plants was evidenced (Figures 1 & 2) regardless of the growth condition of the plants (with or without irrigation). Other species exhibit different stomatal apparatus, for example, *Blutaparon portulacoides* show leaves in which stomata are present on the upper epidermis (called epistomatic) (Arruda *et al*. 2009), *Jacquinia armillaris* and *Remiria maritima* show leaves with stomata restricted to the lower surface (hypostomatic) (Kuster *et al*. 2016). Other species associated to the sand dunes or ecosystems near the coastal, show an amphistomatic character such as *Ipomoea pes-caprae* (Da Silva *et al*. 2020), *Acicarpha spathulata*, *Alternanthera maritima*, *Canavalia rosea*, *Hydrocotyle bonariensis*, and *Sporobolus virginicus* (Boeger and Gluzezak 2006, Arruda *et al*. 2009, Kuster *et al*. 2016).

The absence of crystals in the population with irrigation and without hairs but trichomes predominated could be related to the cooling of the leaf, one of the main functions developed by the trichomes (Zucol *et al*. 2019). Similar micromorphology was observed by Bell (2010) in a new species of *Distichlis* (Poaceae, Chloridoideae) from Baja California, Mexico. The results showed in this study suggests that *D. spicata* population with irrigation stablishes the anatomy of the abaxial epidermis to reduce the losses through the evapotranspiration, developing anatomical xeromorphic characters such as marginal stingers or bicellular hairs; these structures maintain the surface tension of water to avoid excessive loss (Morris *et al*. 2019). This phenomenon is known as border layer formation, which maintain water availability and modify the Na exudation rate, response that coincide with those reported by Faraday and Thomson (1986) and Apóstolo (2005). Jáuregui *et al.* (2014) stated that the form of mineral suspension exudation is from the union with the products of the photorespiration, such as glycolates and glyoxylates; consequently, when the plant through the boundary layer controls the evapotranspiration and the photorespiration, reduces the synthesis of their respective products and the presence in the abaxial epidermis (Boer *et al*. 2021). The plants under drought conditions (without irrigation) invests in the proliferation of structures, mainly trichomes, to minimize the effect of the heat and achieve a thermoregulation that is reflected at the level of the cell membrane reducing the loss of electrolytes and the possible use for the salt excretion (Faraday and Thomson 1986, Semenova *et al*. 2010, Maricle and Maricle 2018, Zucol *et al*. 2019). This response needs to be studied and was not addressed in this study. The accumulation of salt at the level of stomas and epidermis that cause a rough appearance of the leaf, could limit the use of these halophytes grass in animal feed, which should be considered for further studies.

The distribution of stomates in the plants of both *D. spicata* populations was disperse and according to the criterion of stomatal density referred by Evert (2008), this study confirmed the low density of stomates in the abaxial epidermis of both populations (with irrigation and without irrigation); however, the organization of these structures in the abaxial epidermis of the leaves does not follow a specific pattern which sometimes can be explained.

CONCLUSIONS

The plants of the *D. spicata* populations with irrigation and without irrigation showed anatomical modifications in the structure of stomates in the adaxial epidermis but not in the abaxial epidermis. In the adaxial epidermis, the plants of *D. spicata* with irrigation showed higher values of stoma length, stoma width, stoma area and pore length, while plants without irrigation showed higher values of proportion pore/stoma and stomatal density. The pore width and pore area did not showed differences between *D. spicata* populations. The *D. spicata* plants without irrigation showed small stomates than plants with irrigation. The plants without irrigation, also increased the accumulation of salt crystals in the abaxial epidermis.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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