Foliar structural differences between glyphosate-resistant and glyphosate-susceptible biotypes of Digitaria insularis (L.) Fedde[1]

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ABSTRACT

Digitaria insularis (L.) Fedde stands out for its resistance to glyphosate and this characteristic may have a relationship with structural alterations. In such context, this research aimed at the characterization of the foliar anatomical structure of two populations of *D. insularis* (glyphosate-resistant and glyphosate-susceptible biotypes) collected in agricultural areas of Paraná, a Brazilian state. The experiment was conducted at the Laboratory of Botany of the Universidade Estadual do Oeste do Paraná – Unioeste, Brazil. The resistant biotypes of *D. insularis* differ from the susceptible ones in several structural parameters. Among them, the Mesophyll Thickness in the interveinal region was 7.3% thicker in the resistant biotype, which was also observed in the thickness of the keel, in the percentage of 11.3%, and in the thickness of cuticles in the adaxial surface (TC_{ad}), which was 53.8% thicker in the resistant biotype. In this way, we concluded that the resistant biotypes of *D. insularis* differ from the susceptible ones in several anatomical foliar characteristics, therefore, they present possible mechanisms of resistance to glyphosate.

Keywords: biology; leaf; sourgrass; thickness of cuticles; weed.

INTRODUCTION

The genus *Digitaria* involves various plant species distributed in every region of the world. In Brazil, there are 26 native species and 12 exotic ones. Among them, *Digitaria insularis* (L.) Fedde, popularly known as sourgrass (Gemelli *et al.*, 2012) can be mentioned.

D. insularis is a perennial, herbaceous and rhizomatous plant. It also has a C_4 photosynthetic metabolism, is about 150 cm tall, has slow initial growth, has erect culms, leaves with long sheaths, membranous ligules, and blades that are acuminate and linear (Moreira & Bragança, 2010). The inflorescences of *D. insularis* plants are terminal, with long stems and branched panicles that may measure 30 cm in length. The spikelets, on the other hand, have silky trichomes and can be oval to lanceolate-shaped (Carvalho *et al.*, 2011).

Among the weed plants that are resistant to glyphosate, the *D. insularis* is one of the species that presents the higher amount of cases reported in Brazil (Brunharo *et al.*, 2014; Gazola *et al.*, 2016). In literature, many cases of resistance in populations of *D. insularis* in Brazilian cropping areas were reported. However, they portrayed several variations regarding the Resistance Factor (RF). Martins *et al.* (2016) mentioned that this species has the RF = 3.1 and Reinert *et al.* (2013)and it is important to characterize this resistance levels for reasons of rational recommendations for management measures. Thus, an assay was conducted to elaborate comparative dose-response curves between two biotypes

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sourgrass weed, one resistant (R RF > 16. In the West of Paraná, Ferreira *et al.* (2018) verified that the RF was between 2.7 and 7.7, while Licorini *et al.* (2015) observed that the RF varied between 6.2 to 16.8. Pavan (2018), in turn, described the RF > 129 in biotypes that were collected in the municipality of Assis Chateaubriand - PR, therefore, this place stands out for the difficulty in controlling plants.

The emergence of glyphosate-resistant biotypes arises from the alteration of different mechanisms, which can be related to the absorption and herbicide translocation, target enzyme alteration (DNA) and foliar anatomy (Carvalho *et al.*, 2012; Sammons & Gaines, 2014; Salas *et al.*, 2015; Yu *et al.*, 2015). In the case of *D. insularis,* it is no different. The mechanisms of resistance to glyphosate can be related to alterations in the foliar anatomy, in different translocations, and absorption. Additionally, it can also be related to genetic mutations (Carvalho *et al.*, 2011; Gomes *et al.*, 2017; Takano *et al.*, 2017).

In the leaves of resistant plants, alterations that impose several impediments to the absorption of the herbicide were verified (Barroso *et al.*, 2015). Among the verified changes, it is possible to mention the distance of vascular bundles, stomata and trichomes quantity.

The fact is that the characteristics related to the foliar structures can change in the same species, according to the age of the plant, or even nurture a relationship only with its resistance. In this sense, Lopez-Ovejero *et al.* (2017) reported that the populations of *D. insularis* glyphosate-resistant occurrence in Brazil, as a result of a combination of biological characteristics with cropping practices, which specifically employed glyphosate as a herbicide in the areas of *Zea mays* (corn) and *Glycine max* (soybeans) plantations. Therefore, it is believed that the glyphosate-resistant *D. insularis* biotype, which was analyzed in the current study, presents biological characteristics that could be changed.

In light of the exposed facts, the current research considered, as the main objective, the evaluation of the anatomical characteristics of leaves in two populations of *D. insularis* (glyphosate-resistant and glyphosate-susceptible biotypes). The samples were collected in agricultural areas of the Paraná state.

MATERIAL AND METHODS

The experiment was conducted at the Laboratory of Botany of the Universidade Estadual do Oeste do Paraná – Unioeste, Brazil. The first-stage seeds (F0) of *D. insularis* that were used in the research were collected from geographically distinct agricultural areas, in the municipality of Toledo, which is located at 24°43'32'' 'S' (latitude) and 54°46'58,9'' 'W' (longitude), and in the municipality of Engenheiro Beltrão, which is located at 23°09' 'S' (latitude) and 52°26' 'W' (longitude). Moreover, both municipalities are located in the Paraná, a Brazilian state. The collected seeds were stored in the cold chamber, at a temperature of 10 °C and relative humidity of 4.6%.

Seeds of the first generation (F1) were used (such seeds were harvested from the adult plants that were produced in a greenhouse, at the same time when the F0 seeds were collected). The seeding took place on November 19th, 2018, in plastic trays containing the plant Max ® organic substrate. The germination started seven days after sowing. Fourteen days after seeding (DAS), the seedlings were transplanted in plastic pots with a capacity of 3 dm^3, containing clay-textured soil.

It was employed a completely randomized design, with ten replications. The treatment was composed of glyphosate-resistant and glyphosate-susceptible *D. insularis* biotypes.

When the plants presented six leaves, two true leaves, which were completely expanded, were harvested. The collected leaves were taken to the laboratory for washing and separation of the sections. After the washing, the leaves were immediately fixed on FAA 50 (formaldehyde 37% glacial acetic acid and alcohol 50% in the proportion 1:1:18) (Johansen, 1940) and stored in ethanol 70%.

For the anatomical analysis, the intermediate region of the leaves was employed. In this region, transverse sections were performed, made through freehand cuts with the help of a razor blade. The sections were clarified in sodium hypochlorite, in a concentration of 50%, washed with distilled water and colored with Alcian Blue 1% and Basic Fuchsin 0,05% (Kraus & Arduin, 1997) and mounted in semipermanent slides in glycerin 80%.

For the stomata quantification through paradermal views, portions of the middle-third of the leaves were disassociated with hydrogen peroxide 30% and P.A. glacial acetic acid in a concentration of 1:1, according to the methodology adapted from Franklin (1945), and then colored with safranin 0,5%. All the plates were prepared with semi-permanent slides with glycerin 70%. The anatomic images were taken through an optical microscope (Olympus CX31 RBSFA model, Shinjuku-ku, Tokyo, Japan), using a 40X objective, which was attached to a digital camera (Olympus EP 50 model, Shinjuku-ku, Tokyo, Japan).

The quantification was performed visually, with the aid of the TCapture 5.1.1 image program (Tucsen Camera Quick Start), and the calculation of the Stomatal Index was obtained by the formula: , where SI is the Stomatal Index, SN is the number of stomata in a known area and EC is the number of epidermal cells, according to the methodology from Cutter (1986).

The data referring to the quantitative structural variables were collected from different *D. insularis* glyphosate-resistant and glyphosate-susceptible biotypes. The structural variables were: 1) the Mesophyll Thickness in the interveinal region (MT); 2) keel thickness (KT); 3) chlorophyll parenchyma thickness (CPT); 4) the Stomata Number (SN); 5) Stomatal Index (SI); 6) thickness of the cuticle adaxial (CT_{ad}) and abaxial (CT_{ab}) surfaces; 7) the thickness of the outer tangential wall of the epidermic cells in the adaxial (TW_{ad}) and abaxial (TW_{ab}) surfaces; 8) length of bulliform cells (BC); 9) central vascular bundle diameter (CVBD); 10) secondary vascular bundle diameter (SVBD); 11) the distance of vascular bundles (BD); 12) diameter of the sheath cells of the tertiary bundles (SCT); 13) the size of the fiber bundle associated to the keel in the adaxial (FBk_{ad}) and abaxial (FBk_{ab}) surfaces; 14) size of the fiber bundle that is located in the interveinal regions in the abaxial (FBi_{ab}) surface; 15) phloem area of the central vascular bundle (PHc); 16) phloem area of the secondary vascular bundles (PHs); 17) xylem size of the central vascular bundle (XYc) and 18) xylem size of the secondary vascular bundles (XYs). The mensuration was made through software, which is called TCapture 5.1.1 image (Tucsen Camera Quick Start).

The data obtained was submitted to the F-test for analysis of variance and, when significant, they were compared by the Tukey-test at 5% probability.

RESULTS AND DISCUSSION

The occurrence and distribution of the stomata on the leaf blade, in paradermal view, is similar among the biotypes with the stomata organized into parallel rows in the ribbing, presenting guard cells shaped as dumbbells (Figure 1). Such an organization of the stomata and characteristics of the guard cells are considered common in the family Poaceae (Machado *et al.*, 2008; Nicolau *et al.*, 2010). We also highlighted that, despite this kind of plant being amphi-hypostomatic (stomata in both epidermal surfaces, but with predominance in the abaxial surface), in the current study, only the adaxial surface of the leaf was analyzed.

The Mesophyll Thickness (MT) in the interveinal region also varies among the biotypes. The variation is 7.3% thicker in the resistant biotype (Table 1). Also, the thickness of the keel, in the central rib region, is 11.3% thicker in the resistant biotype (Table 1), presenting more layers of aquifer parenchyma cells (5-6), in comparison with the susceptible one (3-4) (Figure 2C-D). As in species with bulliform cells, the investment in cells with wide vacuoles, such as aquifer parenchyma, can act in herbicide storage, which presents a greater resistance level to glyphosate.

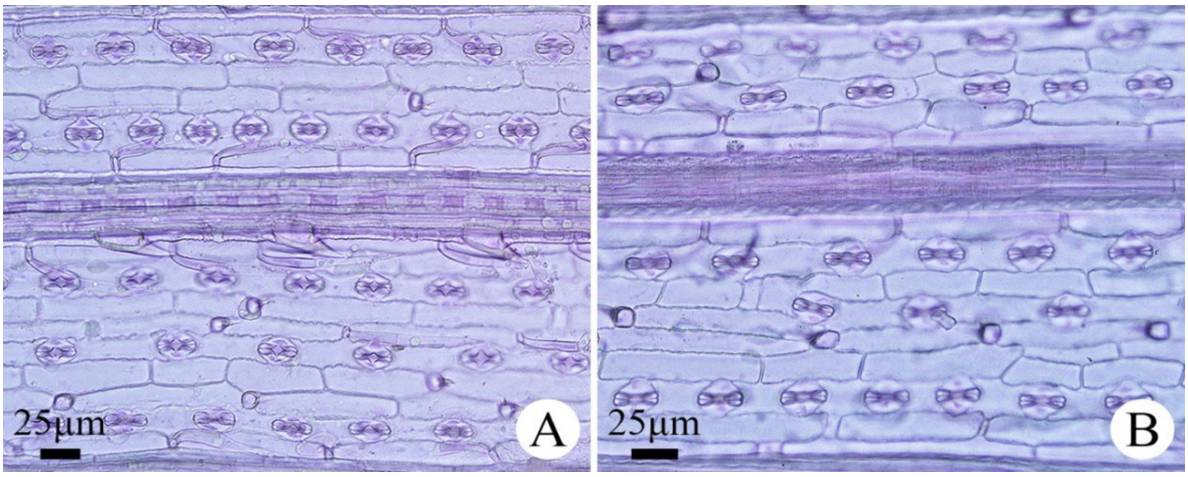

Figure 1: Detail of Paradermal view, which highlights the abaxial surface stomata of the leaves of glyphosate-resistant (A) and susceptible (B) *Digitaria insularis* biotypes, in the V_6 stage.

Table 1: Averages of anatomic variables analyzed in the glyphosate-resistant and susceptible *Digitaria insularis* biotypes, in the V_6 stage

Variable	Resistant	Susceptible	CV%
	(µm)		
MT	93.9 A	87.5 B	4.81
KT	188.7 A	169.6 B	7.82
CPT	47.7 A	46.1 A	4.11
CT_{ad}	1.63 A	1.1 B	6.78
CT_{ab}	0.88 A	0.9 A	4.49
TW_{ab}	2.5 A	1.4 B	10.26
TW_{ad}	1.5 A	1.3 B	15.79
BC	32.5 A	27.4 A	5.53
CVBD	3130.2 A	2391.4 B	9.70
SVBD	1699.8 A	1481.2 B	13.06
BD	16.4 B	18.3 A	2.05
SCT	3.1 B	4.2 A	5.69
	(mm^2)		
SN	88.0 A	82.0 B	5.48
	(%)		
SI	77.0 A	76.4 A	2.25

Means followed by the same uppercase letter in the column do not differ statistically by t-test, presenting a significant probability of 5%. Foliar Mesophyll Thickness in the interveinal region (MT), kell thickness (KT), chlorophyll parenchyma thickness (CPT), cuticle thickness adaxial (CT_{ad}) and abaxial (CT_{ab}) surfaces, the thickness of the outer tangential wall of the epidermic cells in the abaxial (TW_{ab}) and adaxial (TW_{ad}) surfaces, length of bulliform cells (BC), central vascular bundle diameter (CVBD), secondary vascular bundle diameter (SVBD), vascular bundles distance (BD) and diameter of the sheath cells of the tertiary bundles (SCT), Stomata Number (SN), Stomatal Index SI); CV% = coefficient of variation.

Regarding the difference observed in the Stomata Number (SN), we can mention that the biotype of *D. insularis* that is resistant presents a number of stomata 7.3% higher in comparison to the susceptible one. The Stomatal Index (SI) results, in turn, did not differ statically. However, we must emphasize that the used plants presented only six true leaves, therefore, they are considered to be young plants and can suffer changes in the variables that were previously mentioned when they grow older.

Moreover, the relationship of the stoma variable with herbicide resistance is considered controversial. To Tuffi-Santos *et al.* (2009), the stomata would be an unlikely path to glyphosate absorption, once the higher number of stomata is located in the foliar abaxial surface. On the other hand, Procópio *et al.* (2003) reported that the inferior quantity of stomata on the adaxial surface would be one of the main obstacles to herbicide penetration.

Independent from the obstacles presented by the leaves, the surfactant employment can diminish their natural impediments and ensure the success in the chemical control of the weeds (Shonherr, 2006), a fact also reported by Ferreira *et al.* (2002).

The thickness of the cuticles and outer tangential walls of the epidermic cells is larger in the resistant biotype (Table 1). Moreover, the thickness on the adaxial surface part of the cuticles was 53.8% higher in the resistant biotype. However, the result did not differ in the abaxial surface, regarding the analyzed aspect. By contrast, mentioning the values of the outer tangential walls of the epidermic cells, there was a difference in both surfaces, and they were thicker in the resistant biotypes, with a difference of 78.6% in the tangential wall of the epidermic cells in the adaxial part (TW_{ad}) surface, and 15.4% in the tangential wall of the epidermic cells in the abaxial surface part (TW_{ab}).

Higher values regarding the thickness of the cuticles and epidermic walls of cells stand out for constituting the first obstacles to the herbicide, making its entrance into the foliar blade difficult (Machado *et al.*, 2008; Marques *et al.*, 2012). Furthermore, about the cuticles of *D. insularis* leaves, wax deposition was reported by Barroso *et al.* (2015), as well as the herbicide's difficulty to be in contact with the leaf surface (Galon *et al.*, 2013), resulting in a slower absorption (Galvani *et al.*, 2012) due to the hydrophobic nature of the epicuticular wax to the detriment of hydrophilia of the glyphosate (Monquero *et al.*, 2004).

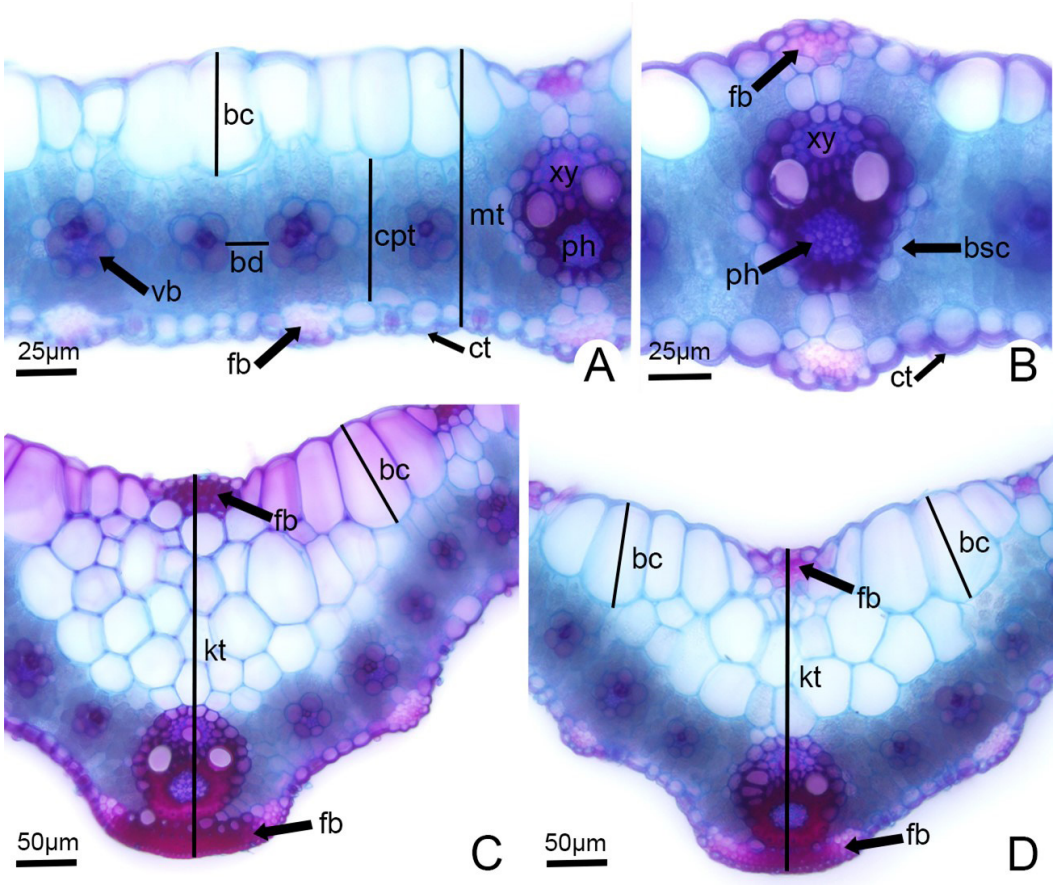

Figure 2: Photomicrograph of the transversal sections of foliar blades of glyphosate-resistant (A-C) and susceptible (B-D) *Digitaria insularis* biotipes. A. General view of the intercostal region, highlighting the analyzed variables: bulliform cell (bc), thickness of the foliar blade Mesophyll Thickness (mt), the distance of the secondary vascular bundle (bd), vascular bundle (vb), fiber bundle (fb), xylem (xy), phloem (ph), cuticle (ct). B. Detail from the secondary vascular bundle region, reinforcing the variables: fiber bundle (fb), xylem (xy), phloem (ph), cuticle (ct), bundle sheath cells (bsc). C-D. General view of the keel bulliform cell (bc), fiber bundle (fb) and thickness (kt) in the keel region of the glyphosate-resistant and susceptible *D. insularis* biotypes, respectively, in the V_6 stage.

The bulliform cells are higher in the resistant biotypes, with a difference of 18.6% in relation to the susceptible ones. The difference of variables also was observed by Ferreira *et al.* (2002), who noticed the distinctions between the resistant and susceptible biotypes of *Echinochloa* spp. These observed differences can represent a higher capacity of storing the herbicide in the interior of the vacuole, reducing, this way, their translocation. According to Ge *et al.* (2010), in resistant biotypes of *Conyza canadensis* (L.) Cronq., higher glyphosate retention in the interior part of the vacuoles of the cells is observed, when we compare this aspect with other types of susceptible plants. These authors commented that, possibly, this difference is generated by the presence of a higher concentration of a specific transporter for glyphosate in the vacuole in resistant biotypes.

Contrasting with the pieces of data that were presented in this current study, Gomes *et al.* (2017), by evaluating 12 biotypes of *D. insularis* with several levels of resistance (glyphosate-susceptible, mildly glyphosate-susceptible and glyphosate-resistant), did not verify any difference between the biotypes regarding the foliar thickness. Possibly, the difference in the resistance degree in such weeds might not have been sufficient to cause alterations.

The chlorophyll parenchyma thickness (CPT) of the resistant biotype was also numerically higher. In both biotypes, the chlorophyll parenchyma belongs to the homogeneous type, which has reduced intercellular space and cells radially arranged around the vascular sheath, which characterizes the occurrence of the Kranz anatomy in the biotypes, aspects that were already indicated for the species and related to the C_4 photosynthetic metabolism (Paciullo *et al.*, 2002; Jesus *et al.*, 2009).

Table 2: Averages of the anatomic variables analyzed in the resistant and susceptibles biotypes of *Digitaria insularis*, in the V_6 stage

Variable	Measurement	Resistant	Susceptible	CV%
		(µm)		
FBk_{ad}	Length	24.3 B	28.3 A	8.20
	Width	16.4 B	18.3 A	22.32
FBk_{ab}	Length	77.9 B	84.9 A	6.28
	Width	9.0 B	13.8 A	7.64
FBi_{ab}	Length	21.8 B	27.3 A	9.89
	Width	10.8 B	14.6 A	12.84
XYc	Length	41.2 B	43.0 A	4.41
	Width	29.4 B	31.8 A	6.56
XYs	Length	30.1 B	32.6 A	8.25
	Width	23.6 B	28.2 A	15.11
		(µm²)		
PHc		595.6 A	279.9 B	9.89
PHs		255.1 A	137.9 B	20.77

Means followed by the same uppercase letter in the column do not differ statistically by t-test, presenting a significant probability of 5%. Size of the fiber bundle associated with the keel in the adaxial (FBk_{ad}) and abaxial (FBk_{ab}) surfaces, size of the fiber bundle located in the interveinal region in the abaxial (FBi_{ab}) surface, xylem size of the central vascular bundle (XYc) and xylem size of the secondary vascular bundles (XYs) phloem area of the central vascular bundle (PHc), phloem area of the secondary vascular bundles (PHs). CV% = Coefficient of variation.

The central vascular bundle diameter (CVBD) and the secondary vascular bundle diameter (SVBD) of the resistant biotype overcame the susceptible one by 65.9 and 51.9%, respectively. The resistant biotype presents a larger CVBD and SVBD, with a difference of 738.8 and 218.6 µm, respectively, in comparison to the susceptible biotype (Table 1). However, the distance between vascular bundles (BD) and the diameter of the sheath cells of the tertiary bundles (SCT) was, respectively, 10.4 and 26.2%, smaller when compared to the susceptible ones.

The size of the fiber bundles associated with the keel in the adaxial and abaxial surfaces and the interveinal region in the abaxial surface was smaller in the resistant biotype (Table 2). The differences were 14.2 and 10.4% in the length and the width of the CT_{ad}, and 8.2 and 34.8% in the length and width of the CT_{ab}. To the FBi_{ab}, the observed differences were 20.1% in the length and 26% in the width.

Regarding the phloem, the resistant biotype possesses a larger area than the susceptible one (Table 2). The phloem area of the central vascular bundle (PHc) and the secondary vascular bundles (PHs) surpass the susceptible biotype in 315.7 and 117.2 µm, respectively. Concerning the xylem tissue area in the central vascular bundle (XYc) and the secondary ones (XYs), the vascular area was smaller. The differences in length and width of the XYc were 4.2 and 7.5%, respectively, and the XYs differed by 7.7% in the length and 16.3% in the width.

In light of these observations, leaf structural differences were verified between the glyphosate-resistant and glyphosate-susceptible *D. insularis* biotypes. These alterations can have occurred due to a biological change in the resistant plant. Therefore, it is suggested that other studies must be performed to verify if the anatomical patterns and differences between the resistant and susceptible biotypes will remain the same.

In practice, the weeds must be controlled when they are still small, in order to avoid problems, especially with plants that are resistant to herbicides, because increasing the use of complex chemical-control strategies will be necessary, such as the need for larger doses, the use of herbicide mixtures with different action mechanisms or sequence applications.

CONCLUSION

The resistant biotype of *D. insularis* presents differences in the anatomical characteristics of the leaves in relation to the susceptible one. Among the differences, we can mention greater Mesophyll Thickness in the interveinal region, greater keel thickness, and greater cuticle thickness in the adaxial surface. Besides that, the outer tangential walls of the epidermic cells are denser on the two surfaces and have larger bulliform cells. This aspect can be seen as the main resistance to glyphosate mechanisms in this biotype.

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