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

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Diferenças estruturais foliares entre biótipos resistente e suscetível ao glyphosate de *Digitaria insularis* (L.) Fedde

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Foliar differences of *Digitaria insularis*

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Abstract - We had, as an objective, characterizing, anatomically, the leaves of two populations of *D. insularis* (resistant biotype and susceptible biotype), which were collected in cropping areas in Paraná. In order to do this, a trial design in random sets was employed, which were repeated ten times. To obtain the data, it was evaluated the structural and quantitative variables of foliar blades of anatomical parts, which belonged to different leaves in the mature stage that were removed from distinct resistant and susceptible biotypes of *D. insularis*. The structural variables of the cross-sectional parts evaluated from the foliar blades were: the thickness of the foliar blade in the intercostal region (TBI); thickness of the keel (TK); stomata number (SN) and thickness of cuticle (TC) in the adaxial and abaxial faces. The resistant biotypes of *D. insularis* differ from the susceptible ones in several structural parameters, among them, there is the foliar blade in the interveinal region that was 7.3 ticker 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 the cuticle in the adaxial face (TCD), which was 53.8% thicker in the resistant biotype. In this way, we concluded that the anatomical foliar characteristics observed in the resistant biotypes, which differ from the susceptible ones, can be related to the reduction in the absorption and the herbicides translocation speed, constituting, therefore, possible mechanisms of resistance to glyphosate.

Key words: biology, leaf, sourgrass, thickness of cuticle, weed.

Resumo - Tivemos como objetivo caracterizar anatomicamente as folhas de duas populações de *D. insularis* (biótipo resistente e biótipo suscetível) coletadas em áreas agrícolas no Paraná. Para isso, utilizou-se delineamento experimental em blocos ao acaso, com dez repetições. Para a obtenção dos dados, foram avaliadas variáveis estruturais quantitativas de secções anatômicas da lâmina foliar, pertencentes a diferentes folhas no estágio maduro retiradas de distintos biótipos resistentes e suscetíveis de *D. insularis*. Entre as variáveis estruturais das secções transversais da lâmina foliar avaliadas foram: espessura da lâmina foliar na região intercostal (ELI); espessura da quilha (EQ); número de estômatos (NE); espessura da cutícula (EC) nas faces adaxial e abaxial. Os biótipos resistentes de *D. insularis* diferem dos susceptíveis em vários parâmetros estruturais, entre eles, quanto a lâmina foliar na região internervural que foi 7,3% mais espessa no biótipo resistente, o que também foi observado na espessura da quilha com 11,3% e na espessura da cutícula na face adaxial (ECD) que foi 53,8% mais espessa no resistente. Dessa forma, concluímos que as características anatômicas foliares observadas nos biótipos resistentes que diferem dos susceptíveis, podem estar relacionadas com a redução na absorção e na velocidade de translocação de herbicidas, constituindo assim possíveis mecanismos de resistência ao glyphosate.

Palavra-chave: biologia, folha, capim-amargoso, espessura da cutícula, planta daninha.

Introduction

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

It is a herbaceous and rhizomatous plant, perennial, present slow initial growth, grows in bunches and into the upright position, has fluted culms, with up to 150 centimetres of length, has leaves with acuminate and linear blades, long sheath, with little hairiness and present a membranous ligule and photosynthetic metabolism C_4 (Moreira & Bragança 2010). Regarding its inflorescences, in *D. insularis*, these are terminal with long stems, with branched panicles that may measure 30 centimeters, the spikelets have silky trichomes and can be oval to lanceolate shaped Carvalho *et al*. (2011).

Among the weed plants resistant to glyphosate, the *D. insularis* is one of the species that present 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 are reported, however, with many variations regarding the Resistance Factor (FR). Martins *et al*. (2016) mentioned that this species has FR = 3.1 and Reinert *et al*. (2013) FR > 16. In the West of Paraná, Ferreira *et al*. (2018) verified FRs between 2.7 and 7.7, while Licorini *et al*. (2015) observed the FR with a variation of 6.2 to 16.8. On the other hand, (Pavan 2018), described FR > 129 in biotypes collected in the municipality of Assis Chateaubriand, therefore, this place becomes the location to be considered with the biggest trouble in the matter of controlling plants.

The emergence of resistant biotypes arises from the alteration of different mechanisms, which can be related to the absorbance and herbicide translocation, target enzyme alteration and foliar anatomy (DNA) (Alcantara de La Cruz *et al*. 2016; Carvalho *et al*. 2012; González-Torralva *et al*. 2014; Kaundun e*t al*. 2011; Nandula *et al*. 2012; Sammons

& Gaines 2014; Salas *et al*. 2015; Yu *et al*. 2015). In the case of *D. insularis*, the mechanisms of resistance to glyphosate can be related to alterations in the foliar anatomy, in the absorbance and differential translocation and, still, in the 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 absorbance of the herbicide were verified (Barroso *et al*. 2015). The variation in the distance of vascular bundles, as much as the stomata and trichomes quantity are among the verified modifications.

The fact is that the characteristics related to the foliar structures can change in the same species, according to the plant age or having relation only with its resistance. In this regard, Lopez-Ovejero *et al*. (2017) revealed that the populations of *D. insularis* resistant to glyphosate occurrence in Brazil, and this result was a combination of biological characteristics with cropping practices that employed specifically the glyphosate as a herbicide in the areas of soy and corn plantations. Therefore, it is believed that the resistant biotype *D. insularis*, employed in the current study, can present biological characteristics that have been changed.

In face of the exposed facts, the current essay had as an objective the evaluation of leaves' anatomical characteristics, in two populations of *D. insularis* collected in cropping areas in Paraná.

Material And Methods

It was employed a trial design in random sets, with ten repetitions. The treatment was composed of the biotype of *D. insularis* resistant and susceptible to glyphosate.

It was used seeds of the F_1 generation, which were stored in the low-temperature chamber, with a temperature of 10°.C and relative humidity of 4.6%.

The seeding took place on November 19th, 2018. After 14 days from the seeding (DAS), the seedlings were transplanted in plastic vases with a capacity of 3 dm^{-3}, containing clay-textured soil. The two last leaves completely expanded of each plant were harvested when plants presented six actual leaves.

The harvested leaves were taken into the laboratory for washing and separation. 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%.

The data referring to the quantitative and structural variables were obtained from 10 anatomic parts, removed from 10 different leaves, from different resistant and susceptible to *D. insularis* biotypes. In order to have an anatomic analysis, the middle third of two leaves of each one of the repetitions was employed. In this region, transversal sections were performed, made through freehand cuts and with the help of a razor blade. The sections were clarified in sodium hypochlorite, in the concentration of 50%, washed with distilled water and colored with Alcian Blue and Basic Fuchsin (Kraus & Arduin 1998) and assembled in semi-permanent plates in glycerin 80% (kaiser 1880).

To observe the stomata through paradermal views, portions of the leaves' middle third were disassociated with hydrogen peroxide and acetic acid in a concentration of 1:1, according to the methodology adapted from Franklin, (1945) and colored with safranin. All the plates were assembled in a semi-permanent glycerin 70% environment. The anatomic images were taken through an optical microscope attached to a camera (CMOS – Bioptika model) and connected to the computer, considering that the evaluations were made through software, called TCapture 5.1.

The data referring to the structural variables were analyzed from ten anatomical sections, which were originated from ten different leaves, collected from different resistant and susceptible to *D. insularis* biotypes. With the help of the software Excel 2010, the following characteristics were measured: 1) the thickness of the foliar blade in the intercostal region (TBI); 2) thickness of the keel (TK); 3) thickness of chlorophyllin parenchyma (TCP); 4) the of stomata number (SN); 5) stomatal index (SI); 6) thickness of cuticle in the adaxial (TCB) and abaxial (TCD) faces; 7) the thickness of the outer tangential wall of the epidermic cells in the abaxial (EPB) and adaxial (EPD) faces; 8) length of bulliform cells (BC); 9) diameter of the central vascular bundle (DCB); 10) diameter of the secondary vascular bundle (DSB); 11) distance between the terciary vascular bundles (DTB); 12) diameter of the tertiary bundles vascular cells of the sheath (DBS); 13) size of the fibre strings associated to the keel (SS) in the adaxial e abaxial faces; 14) size of the fibre strings located in the internervural regions in the abaxial face (SSIB); 15) phloem area of the central vascular bundle (PAC); 16) phloem area of the secondary vascular bundles (PAS); 17) xylem size of the central vascular bundle (XSC) and 18) xylem size of the secondary vascular bundles (XSS).

The stomatal index were obtained through the formula calculation: SI = SN/(EC + SN)*100, in which SI is the stomatal index, SN is the stomata number and CE is the epidermal cells number.

The data obtained from the calculations were submitted to variance analysis, with the comparison of the averages through the Tukey test, with a probability percentage of 5%.

Results and Discussion

The occurrence and distribution of stomata on the foliar plate in paradermal views is similar among the biotypes with stomata organized into parallel lines, in which the ribs are apparent and the guard cells are shaped as dumbbells (Figure 1). This organization of stomata and guard cells characteristics are considered common in the plant family Poaceae (Machado *et al*. 2008; Nicolau *et al*. 2010). We also highlighted that, despite this kind of plant being amphihypostomatic (stomata in both epidermal faces, but with predominance in the abaxial face), in the current study, only the leaf's adaxial face was analyzed.

The thickness of the foliar blade in the internervural region (TBI) also varies among the biotypes, considering it to be 7.3% thicker in the resistant biotype (Table 1). Also, the keel (TK), in the central rib region is 11.3% thicker in the resistant biotype (Table 1), with a higher number of cells' layers (5-6) of aquifer parenchyma, concerning the susceptible one (3-4) (Figure 2C-D). As in bulliform cells, the investment in cells with wide vacuoles, as aquifer parenchyma, can act in the herbicide storage, guaranteeing a broader resistance to glyphosate.

The difference observed in the number stomata (NS) among the biotypes highlighted that the biotype of *D. insularis* that is resistant presents a number of stomata 7.3% higher in comparison to the susceptible. The stomatal index results (SI), in turn, did not differ statically. However, we must emphasize that the used plants presented only six actual leaves, being, therefore, considered young plants and can suffer changes in these variables when they get 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 are located in the abaxial foliar face. On the other hand, Procópio *et al*. (2003) reported that the fewer number of stomata in the adaxial face would be one of the main obstacles to the herbicides' penetration.

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

The thickness of the cuticles and outer tangential walls of the epidermic cells are larger in the resistant biotype (Table 1). The TCD values were 53.8% higher in the resistant biotype, however, there was no divergence regarding this aspect in the abaxial face. By contrast, regarding the values of the outer tangential walls of the epidermic cells, there was a difference in both faces, considering that they were thicker in the resistant biotypes, with a difference of 78.6% in the EPD and 15.4% in the EPB.

Higher values regarding the thickness of 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, the wax deposition was reported by Barroso *et al*. (2015), which contributes to the herbicide continuity on the foliar surface (Galon *et al*. 2013), with absorption in a slower way (Galvani *et al*. 2012) due to the hydrophobic nature of the epicuticular wax to the detriment of hydrophilic one of the glyphosate (Monquero *et al*. 2004).

The bulliform cells are higher in the resistant biotypes, with a difference of 18.6% in relation to the susceptible ones. This observed difference 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 *Conyza canadensis* (L.) Cronq. resistant biotypes, it happens higher glyphosate retention in the interior of the cells' vacuoles compared to the ones of susceptible plants. These authors comment that, possibly, this difference is generated by the presence of a higher concentration of a specific carrier to glyphosate in the vacuole in resistant biotypes.

The difference between the resistant and susceptible biotypes of *Echinochloa* spp. regarding this parameter, the length of the foliar blade, was also observed by Ferreira et al. (2012).

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

The thickness of the resistant biotype's chlorophyllin parenchyma (TCP) was also numerically higher, although it did not differ statistically from the susceptible one. In both biotypes, the chlorophyllin parenchyma is from the homogeneous type with reduced intercellular space and cells radially arranged around the vascular sheath, which characterizes the Kranz anatomy occurrence in the biotypes, aspects already indicated for the species and related to the C_4 photosynthetic metabolism (Jesus e*t al*. 2009; Paciullo *et al*. 2002).

The DCB and the DSB surpassed, in 65.9 and 51.9%, respectively, the susceptible biotype. The resistant biotype presents a larger diameter of the central vascular bundle (DCB) and the diameter of the secondary vascular bundles (DSB), with a difference of 738.8 and 218.6 µm, respectively (Table 1). However, the distance between the tertiary vascular bundles (DTB) and the diameter of the vascular sheath cells of the tertiary bundles (DBS) were 10.4 and 26.2%, respectively, smaller compared to the susceptible ones.

The size of the fibre string associated with the keel in the adaxial e abaxial faces and the internevural region in the abaxial face was smaller in the resistant biotype (Table 2). The differences are 14.2 and 10.4% in the length and the width of the TCD, and 8.2 and 34.8% in the length and width of the TCB. To the SSIB, the observed differences were 20.1% in the length and 26% in the width.

In relation to the phloem, the resistant biotype possesses a larger area than the susceptible one (Table 2). The phloem area of the central vascular bundle (PAC) and the secondary vascular bundles (PAS) surpass the susceptible biotype in 315.7 and 117.2 µm, respectively. In addition, the xylemetic tissue area of the central vascular bundle (XSC) and in the secondary ones (XSS), the resistant biotype presented the smaller measurements. The differences in length and width of the XSC were 4.2 e 7.5%, respectively, and the XSS differed in 7.7% in the length and 16.3% in the width.

Morphoanatomical alterations between the resistant and susceptible biotypes can have occurred in virtue of a biological change in the resistant plant. It is suggested that other studies must be performed to verify if the anatomical differences patterns will be kept between the resistant and susceptible biotypes. However, it must be highlighted that new researches must be carried out with plants that present an elevated factor of resistance, as it was tested in this essay, besides a susceptible biotype.

Conclusion

The resistant biotype of *D. insularis* presents differences in the anatomical characteristics of the leaves in relation to the susceptible ones, such as the larger thickness of the foliar blade in the region, the larger thickness of the keel, the larger thickness of the cuticle in the adaxial face, outer tangential walls of the epidermic cells are more thickened in the two faces and bigger bulliform cells that can function as the main resistance glyphosate mechanisms in this biotype.

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Interest conflict

The authors declare that they have no known competing interests in the work reported in this paper.

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