

Ultrastructural and Histochemical Changes in Glyphosate-Tolerant Soybean Leaves Exposed to Glyphosate

Nayane C. P. Bomfim¹, Márcio S. Silva¹, Liliane S. Camargos¹ & Aline R. Martins¹

¹ Faculdade de Engenharia de Ilha Solteira, Universidade Estadual Paulista, São Paulo, Brazil

Correspondence: Aline R. Martins, Faculdade de Engenharia de Ilha Solteira, Universidade Estadual Paulista, São Paulo, Brazil. Tel: 55-(18)-3743-1969. E-mail: aline.botanica@gmail.com

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Abstract

Is it transgenic soy, resistant to glyphosate, does not suffer any injury or stress in contact with this herbicide? Anatomic studies of plant tissue are necessary to answer this question. This study investigated the influence of glyphosate in glyphosate-resistant soybean plants by analysis of leaf ultrastructure and histochemistry in a morphophysiological context. The experiment was carried out in a greenhouse, using RR soybean seeds (*Glycine max* (L.) Merrill, cultivar BRS Valiosa) in pots containing vermiculite and washed sand (1:1). Between the phenological stages V₂ and V₄, two treatments with glyphosate [N-(fosfonometil) glicina] were sprayed once a week: recommended dose (5.0 mg ae plant⁻¹) and control (0.0 mg ae plant⁻¹), with four repetitions each. Samples of midrib and internervural area of the leaves were fixed, dehydrated in ethyl series and blocks were sectioned at a 5-10 μm thickness. The material was stained with toluidine blue 0.05% and blades mounted on "Entellan". Glyphosate decreased the thickness of the adaxial epidermis, palisade parenchyma, spongy parenchyma and total thickness of the leaf. Although, the diameter of companion cell was decreased with herbicide treatment, the diameter of the vase element increased, also increasing the size of the vascular bundle. Ultrastructural and histochemical changes caused by glyphosate can extend dysfunctions in the metabolic apparatus and plant relationship with the environment, given the inter-relation between tissue structure and its functions.

Keywords: epidermis, foliar injury, parenchyma, plant morphophysiology, vascular bundle

1. Introduction

Brazil is the second largest producer of soybeans (*Glycine max*) in the world accounting for more than 30% of world grain production. Soybean is an essential component for animal feeding with increasing use in food for humans according to National Supply Company (CONAB, 2016) and United States Department of Agriculture (USDA, 2017).

In 2016, 185.1 million ha were cultivated with genetically modified plants (GM) crops worldwide (De Vos & Swanenburg, 2018). Brazil takes the 2nd place in the world ranking with 49.1 million ha of GM crops planted, behind the United States (72.92 million ha) with 49.1 million ha of planted GM crops accounting for 27% of the world's arable land (International Service for the Acquisition of Agri-biotech Applications [ISAAA], 2017). There are 32.69 million hectare of transgenic soybeans, with 12.43 million hectares being cultivated with herbicide resistant cultivars, 36.7% (ISAAA, 2017).

The weeds limit soybean growth and development potential, decreasing plant productivity and grain quality (Hock, Knezevic, Martin, & Lindquist, 2005; Knezevic, Evans, & Mainz, 2003). The flagship for weed management is the chemical control with glyphosate, a post-emergence, systemic, broad-spectrum action herbicide used for annual and perennial weeds (Castle et al., 2004; Franz, Mao, & Sikorski, 1997). Glyphosate acts specifically by inhibiting enzyme 5-enolpiruvilchiquimate 3-phosphate synthase (EPSPs), which blocks the synthesis of the essential aromatic amino acids, namely phenylalanine, tyrosine and tryptophan (Duke, Rimando, Pace, Reddy, & Smeda, 2003).

Glyphosate is an isopropylamine salt of N-(phosphonomethyl)-glycine, an aminophosphonate analogue to the natural amino acid glycine, which therefore occupies its place in the protein synthesis. This herbicide is non-selective with low toxicity to animals, allowing deployment in rotation crops, such as corn, soybean, bean, enabling its use in large-scale in Brazilian agricultural system (Figueiredo, Silva, Boaretto, & Ribeirinho, 2011).

Advances in biotechnology have included the development of GM soybean cultivars, such as Glyphosate-resistant soybean (GR soybean), obtained by insertion of a gene (*aroA:CP4*) from bacterium *Agrobacterium* sp. strain CP4, which confers to these plants tolerance to glyphosate, allowing the use with recognized efficacy and broad spectrum of weed control in the soybean culture (Padgett et al., 1995; Dvoranen, Oliveira, Constantin, Cavalieri, & Blainski, 2008). Under treatment with glyphosate, GR soybean is not affected, because of a route deviation of the shikimic acid pathway by an alternative EPSPs enzyme not-inhibited by glyphosate. Thus, this technology optimizes the integrated-weed management (Santos et al., 2007).

In a review study by Martinez and Graham (2018), it identifies some deleterious effects of glyphosate in relation to GR cultures, such as: the glyphosate compromises the shikimic acid pathway, strongly decreasing the physiological defenses GR some cultivars, despite the glyphosate tolerance, making it weaker and more vulnerable to pathogenic attack; glyphosate-based herbicide may interfere with local microbial ecology: increase the population and/or virulence of some phytopathogenic microbial species; and reducing nutrient uptake by crops has the potential to further impair disease resistance. Martinez and Graham (2018), also points out that the above deleterious effects can occur in synergism, increasing and intensifying each other's negative consequences.

Despite its great economic importance, studies are scarce on morphophysiological and histochemical aspects of the species. Metcalfe and Chalk (1950) investigated several anatomical characteristics of the family and genus, referring to *Glycine max* by describing the disposition of vascular bundles of petiole and Fisher (1967) described the paranervular parenchyma in the mesophyll. Anatomical studies were conducted regarding variations in leaf thickness and stomatal frequency with factors such as gas exchange rate (Dornhoff & Shibles, 1976), water stress (Vidal & Pognonec, 1984), different nitrogen concentrations and irradiance levels (Sims, Seemann, & Luo, 1998).

Injury to plants may not present macroscopic symptoms, which justifies the microscopic analysis of plant tissue as a tool for early diagnosis of biotic or abiotic stresses (Tuffi Santos et al., 2008). Therefore, research on anatomical changes of the vascular bundle of leaf midrib of GR soybean subjected to glyphosate treatment is important since the use of this herbicide is common to the culture. It is also important to check whether these potential changes occur or not in the vascular tissue and how the plant responds to the changes. Thus, this study aims to understand the morphophysiological responses of GR soybean to glyphosate.

This study evaluated the influence of glyphosate in GR soybean through the analysis of ultrastructure and histochemistry in a foliar morphophysiological context.

2. Materials and Methods

The experiment was carried out in a greenhouse, with temperature control at 27 °C, without photoperiod control, at the *Laboratório de Fisiologia e Metabolismo Vegetal* of UNESP, Campus Ilha Solteira, SP. The experiment was carried out in a completely randomized design, with two treatments, using commercially available RR soybean seeds, cultivar BRS Valiosa. For each treatment, it was prepared 40 pots containing 2 plants per pot. Soybean seeds, after germination, were transferred to 4 L pots containing vermiculite and washed sand (1:1), where they were inoculated with *Bradyrhizobium* sp. obtained from maceration of nodules of pre-existing soybean plants. After effective nodulation, the plants received 100 mL of nutrient solution without nitrogen, according to Hoagland and Arnon (1950).

The two treatments were: with 5.0 mg ae plant⁻¹ glyphosate, (recommended dose: 720 g ha⁻¹, acid equivalent, according to Ludwig et al., 2011), (Roundup Ready[®] Monsanto Company, St Louis, MO, www.monsanto.com) and, without glyphosate, 0.0 mg ae plant⁻¹. The herbicide treatment was sprayed by means of a manual water sprayer, once a week between the vegetative stages V₂ (with second node and with fully developed trifoliate first leaf) and V₄ (with fourth node and with fully developed third trifoliate leaf) (Fehr, Caviness, Burmood, & Pennington, 1971).

The source of the herbicide used was Roundup[®], whose formulation corresponds to the following composition: N-(phosphonomethyl) glycine isopropylamine salt 480 g L⁻¹ (48% m v⁻¹), N-(phosphonomethyl) glycine (glyphosate) 360 g L⁻¹ (36% m v⁻¹), inert ingredients of 684 g L⁻¹ (68% m⁻¹).

After the period of 15-21 days, sufficient for the metabolization of the herbicide, completely expanded leaves were sampled and fixed in FAA 50 (Johansen, 1940) for anatomical analysis.

After 48 hours the material was stored in 70% alcohol. In the completely expanded leaf, the middle region of the leaf was analyzed at the midrib and internervular areas. The samples were dehydrated in ethylic series, included in hydroxy-ethyl-methacrylate (Leica Historessin) and the blocks were cut at 5-10 µm thick. The material was

stained with toluidine blue 0.05% in phosphate buffer and citric acid with pH between 4.5 and 6.0 (Sakai, 1973). The slides were mounted with synthetic resin “Entellan”.

For the study of single cells composing the xylem and phloem, we used tissue maceration technique in which the samples were treated with “Jeffrey solution”, a mixture of acids (10% chromic acid and 10% nitric acid, 1:1), to allow the dissolution of the middle lamella and isolation of the structures (Johansen, 1940), then the samples were stained, dehydrated and assembled in glycerin gelatin.

With the obtained lamina set, photomicrographs were performed in a trinocular microscope, with cross section. These images were submitted to image analysis using the program Image Tool 3.0, which allowed the measurement in micrometers (μm) of the epidermis thickness (abaxial and adaxial); parenchyma (palisade and spongy), vascular bundle and total leaf thickness. The diameter (μm) of the companion cells, sieve tube elements, vessel elements and fibers were measured. To measure the diameter of the vessel elements (xylem) of the central rib region, two metaxylem vessel elements were chosen, the two largest and most centralized. From tissue maceration, we can measure the length (μm) of vessel elements (short and elongated), fibers (short and elongated) and vascular bundle.

Histochemical tests were performed in fresh material, hydrated, fixed, and included in histological resin. To detect lipid substances, we used Sudan IV (Jensen, 1962); for starch, iodized zinc chloride (Strasburger, 1913); for phenolic compounds, ferric chloride (Johansen, 1940) and for pectic substances, Ruthenium red (Johansen, 1940). To check the natural aspect of the organ, we assembled the cuts of the material only in water, that is, without treatment and observed them under a light microscope.

All data were submitted to analysis of variance. The means of the treatments were compared by the Tukey test at 5% probability. Statistical analysis was performed using the software packages commercially available SISVAR® (Ferreira, 2011).

3. Results

Studies on anatomical characterization of plants subjected to glyphosate are scarce. However, the anatomical analysis in GR soybean allows characterizing and observing some changes. Regarding characterization of leaves of GR soybean, the mesophyll is dorsiventral with two layers of palisade parenchyma cells and four to five layers of spongy parenchyma cells (Figure 1) and the latter has sizes that vary significantly with intercellular spaces.

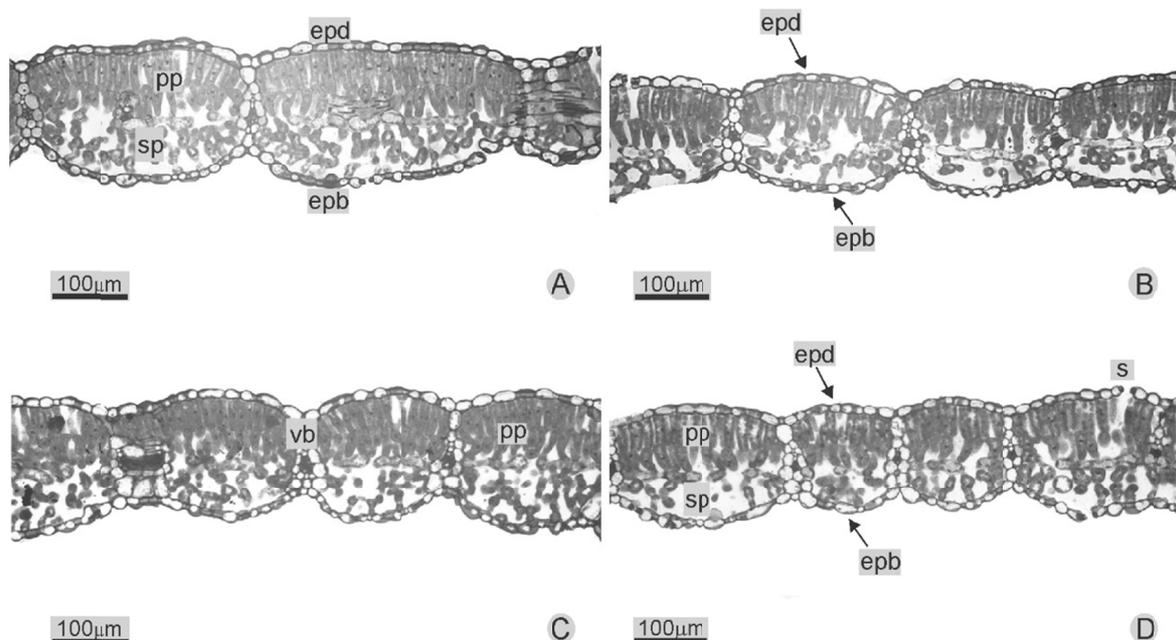


Figure 1. Photomicrographs of glyphosate-resistant soybean (*Glycine max*) leaves treated with glyphosate (C, D) and control samples (A, B) in cross-section. Midrib (A, C) and internervural area (B, D); pp: palisade parenchyma; sp: spongy parenchyma; epd: adaxial epidermis; epb: abaxial epidermis; vb: vascular bundle; s: stomata

The epidermis is uniseriate with elliptical circular contour on both sides, displaying fine leaf cuticle (Figure 1A, 1B and 1D). Stomata are present on both leaf sides (Figure 1D); therefore, the leaf receives the classification of amphistomatic.

In this experiment, in both foliar portions studied, it was identified decrease in structure thickness (Figures 1 and 2). In the internervural area, glyphosate decreased the thickness of palisade parenchyma (Figure 2D). In the midrib portion, thickness was decreased in adaxial epidermis, palisade parenchyma, spongy parenchyma (Figure 2A, 2C and 2D) and total leaf due to the treatment used (Figure 3).

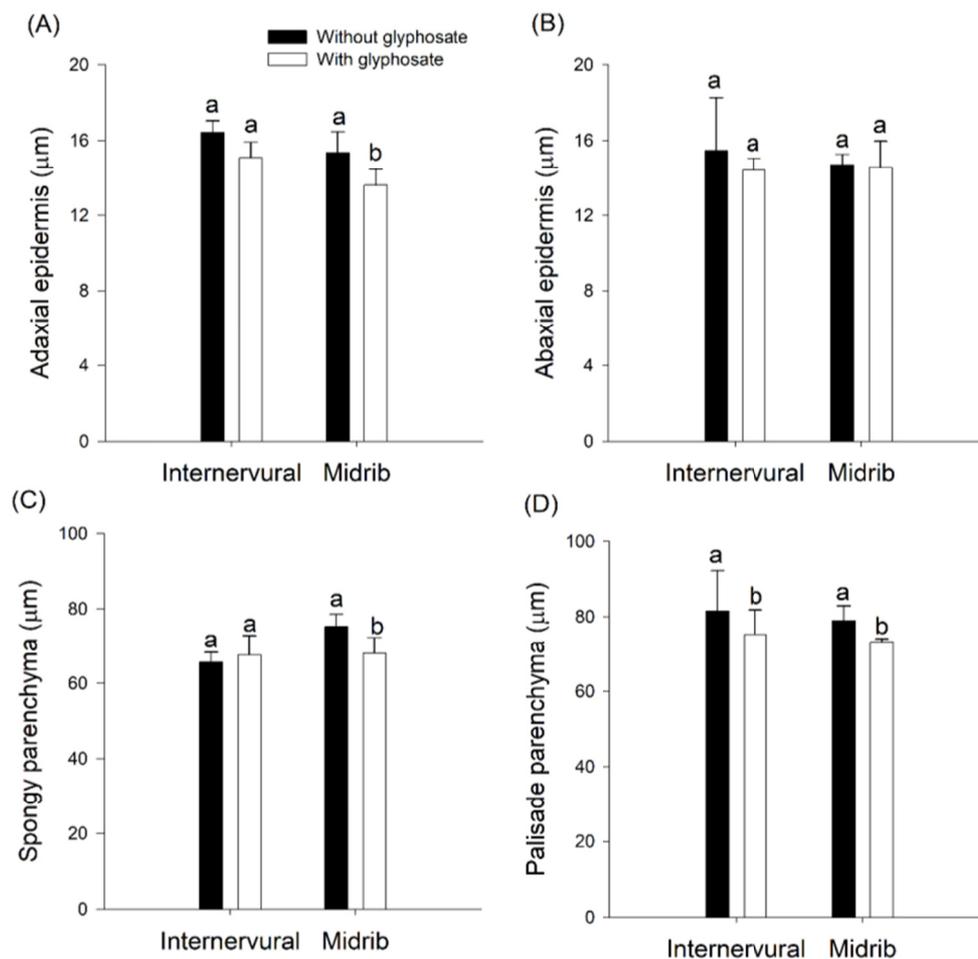


Figure 2. Abaxial epidermis thickness (A), adaxial epidermis thickness (B), spongy parenchyma thickness (C) and palisade parenchyma thickness (D) of internervural area and midrib of glyphosate-resistant soybean (*Glycine max*) leaves treated with glyphosate. Values followed by the same letter in the column do not differ from each other by the Tukey test at 5% probability; n = 48

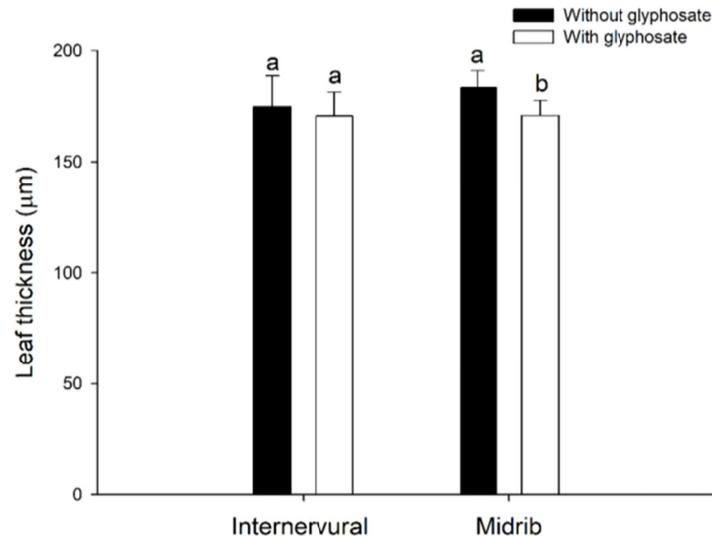


Figure 3. Leaf thickness of internervural area and midrib of glyphosate-resistant soybean (*Glycine max*) leaves treated with glyphosate. Values followed by the same letter in the column do not differ from each other by the Tukey test at 5% probability; n = 48

There was no change in thickness of the midrib vascular bundle between the treatments; however, the height of the vascular bundle increased when treated with glyphosate (Figures 4, 6A and 6B). There was a significant decrease in diameter of the companion cells when treated with glyphosate; nevertheless, there was no change in diameter of sieve tube elements and fibers in the presence of glyphosate in GR soybean leaves (Figures 4D, 4C, 4E, 5C and 5D). In this study, there was an increase in the diameter of the vessel elements (Figure 4F, 5C and 5D).

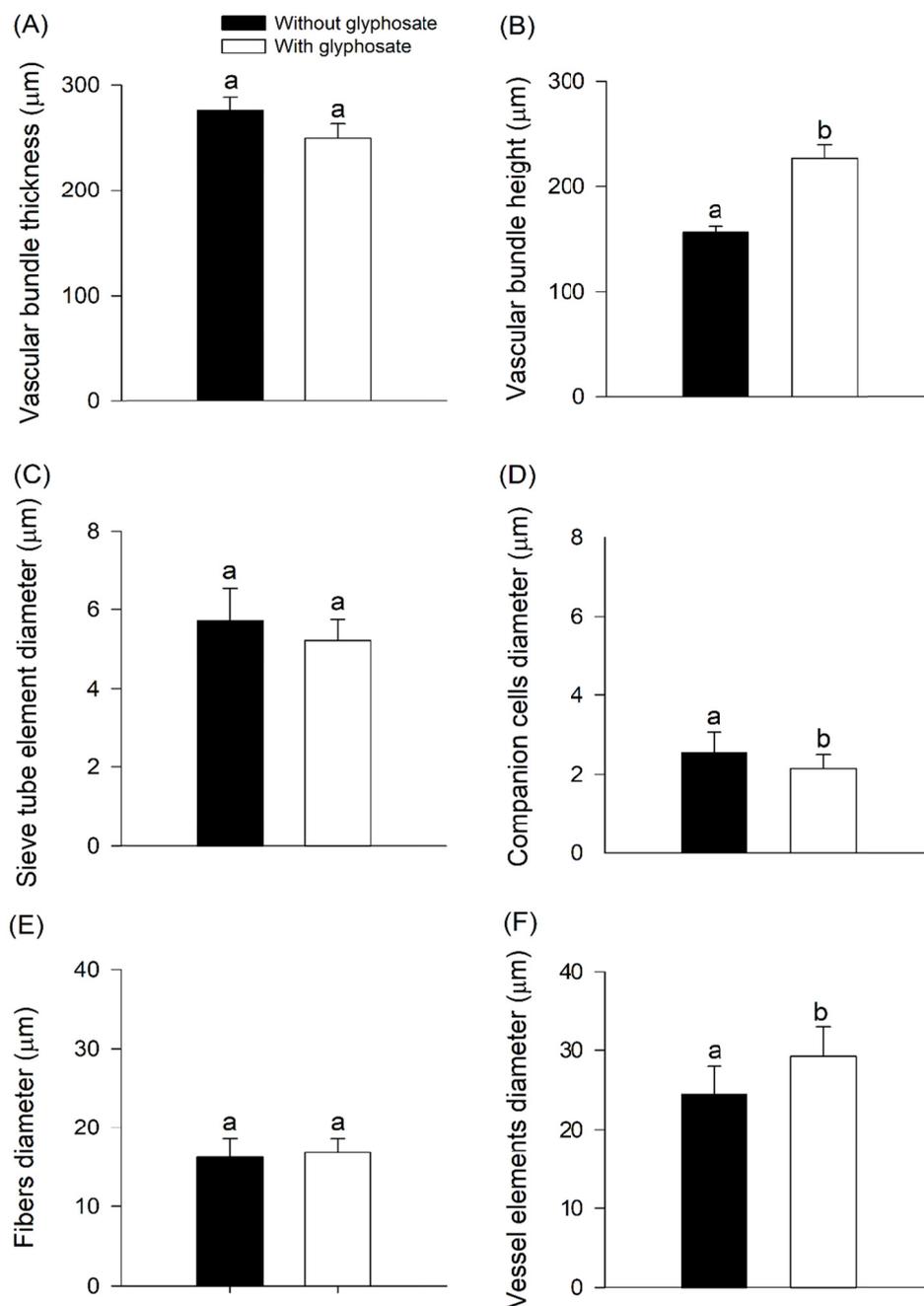


Figure 4. Vascular bundle thickness (A), vascular bundle height (B), sieve tube element diameter (C), companion cells diameter (D) fibers diameter (E) and vessel elements diameter (F) of internervural and nervural regions of glyphosate-resistant soybean (*Glycine max*) leaves treated with glyphosate. Values followed by the same letter in the column do not differ from each other by the Tukey test at 5% probability; n = 16

Figure 5 shows elongate fibers (E and F) and short fibers (G-H) in the coupled material of the midrib of GR soybeans. It was possible to identify and measure the length (µm) of some structures that compose the vascular bundle of midrib: two types of cells support, short (sclereids) and elongated (libriforms), and two types of the xylem vessel elements: shorter and elongated elements (Figure 5I, 5J, 5K and 5L). It was measured 24 cells of each type (fibers and vessel elements) for the control and treatment with glyphosate and we verified no statistical significant variation in the length of these cells, between the treatments (Figure 6).

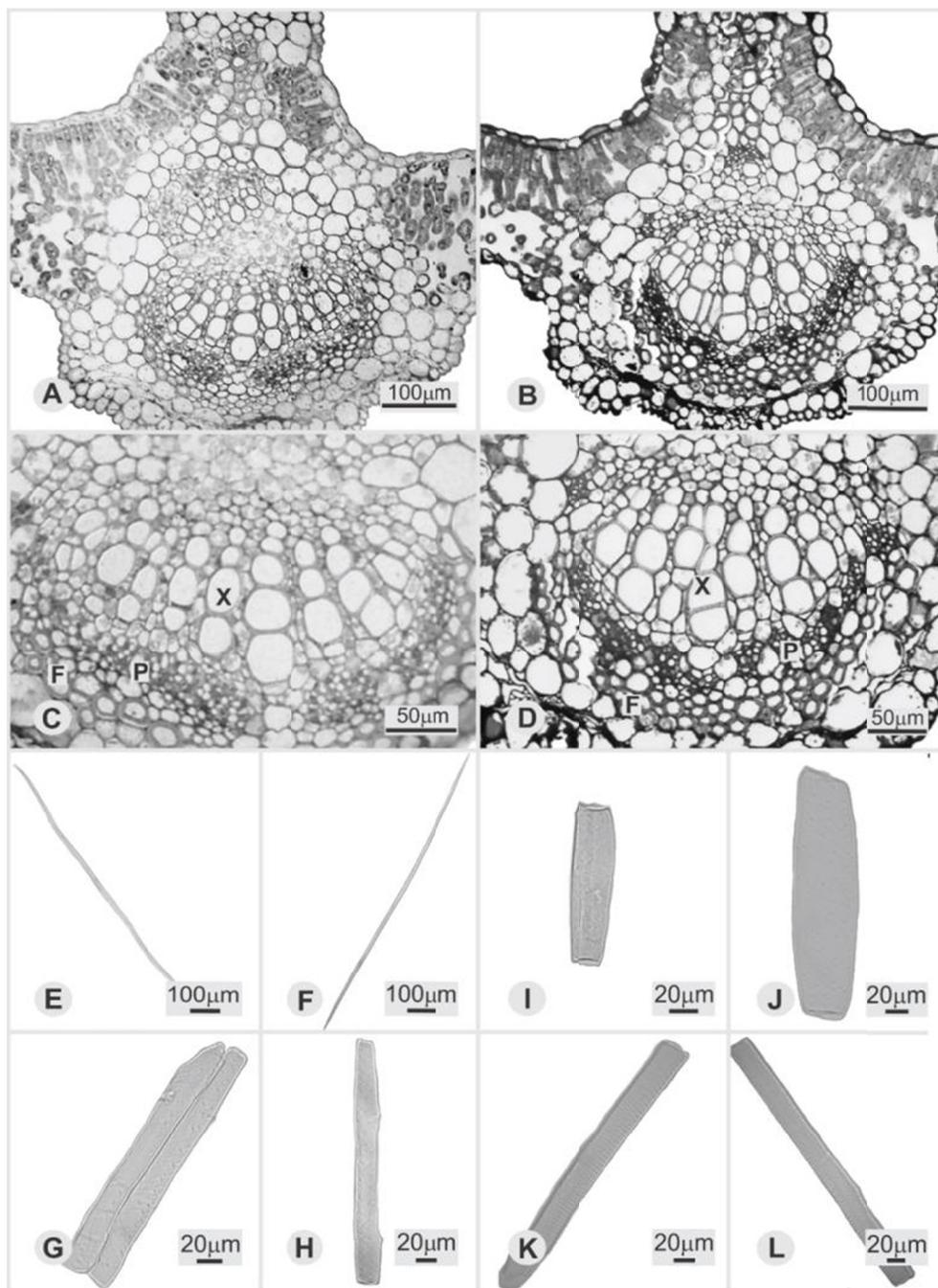


Figure 5. Cross-section of soybean leaves (*Glycine max*). (A, E, G, I, K) control samples and (B, D, F, H, J, L) glyphosate-resistant treated with glyphosate. (C, D) Vascular tissue of midrib leaf. Elongate fibers (E, F), short fibers (G, H), short vessel elements (I, J), elongate vessel elements (K, L). F: phloem; P: companion cells; X: metaxylem

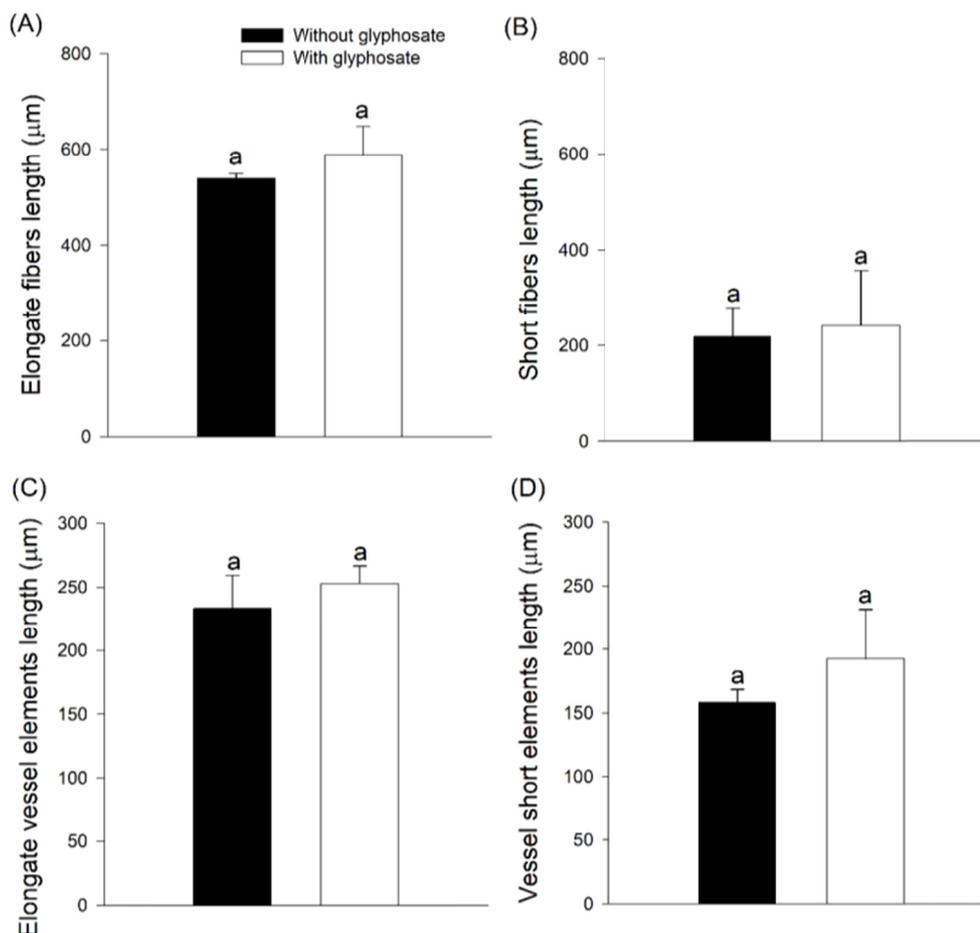


Figure 6. Average length of vascular tissue cells of internervural and midrib of glyphosate-resistant soybean (*Glycine max*) leaves treated with glyphosate. Elongate fibers length (A), short fibers length (B), elongate vessel elements length (C) and vessel short elements length (D). Values followed by the same letter in the column do not differ from each other by the Tukey test at 5% probability; n = 12

There is evidence of phenolic compounds (test with ferric chloride) in different regions of the vascular bundle as well as in the palisade parenchyma, in both treatments (Table 1). The test with iodized zinc chloride showed great starch grains in leaves of GR soybean, found in the palisade and spongy parenchyma and in the vascular bundle, for both treatments (Table 1). In the palisade parenchyma, there is greater occurrence of starch grains with the presence of phenolic compounds in the mesophyll.

Table 1. Histochemical test of soybean (*Glycine max* (L.) Merrill) leaves treated with glyphosate

Compounds	Treatments	
	Without glyphosate	With glyphosate
Phenols	+	+
Starch	+	+
Lipids	+	+
Pectin	+	-

Note. (+): Positive reaction; (-): Negative reaction.

The cuticle, that covers the entire surface of the leaf epidermis, showed positive response to lipid (Sudan IV test) in both treatments (Table 1). Pectin substances, between polysaccharides of plant cell wall, are the ones with greater importance in the water retention process. However, after testing with ruthenium red, pectin substances

were not observed in plants treated with glyphosate. These substances have been found only in control plants, in the epidermis, collenchyma, midrib and phloem (Table 1).

In this study, we observed that GR soybean features some level of sensitivity to glyphosate, due to microbiometric changes of most structures evaluated (Figures 2, 3 and 4).

4. Discussion

The tolerance mechanism conferred to the plant is not so efficient, since some ultrastructural changes have been found in GM soybeans, although the tolerance gene (*aroA*) does not affect its final production. The pathway deviation caused by transgene aims to keep the shikimic acid pathway, which is inhibited by specific action of glyphosate on enzyme EPSPS (Jaworski, 1972; Zablotowicz & Reddy, 2004). The shikimic acid pathway is directly responsible for the synthesis of amino acids, but there is no change in the amino acid concentration in GR soybean exposed to glyphosate (Bomfim et al., 2017).

According to Zobiolo, Oliveira Junior, Constantin, and Biffe (2011), the action mechanism of glyphosate is well defined by the literature; however, some direct and indirect effects on plants that can affect important processes such as plant growth are poorly studied.

The epidermis thickness decrease in the leaf implies greater vulnerability of the plant to the attack of plant pathogens (Tuffi Santos et al., 2005 and 2007). The epidermis is one of the first barriers against phytopathogens, according to Durbin (1988), which, along with wax quantity and quality, have a structural defense mechanism that occurs even before the phytopathogenic fungus infection.

Tuffi Santos et al. (2009) also reported changes in thickness of spongy parenchyma, palisade parenchyma and foliar blade, in addition to the death of epidermal cells in eucalyptus subjected to simulated drift with glyphosate. Although the case cited concerns non-GM plants, and glyphosate cause ultrastructural damages in leaves of GR soybean as observed this work. Still, it seems that the inserted gene in plants provides resilient mechanisms due glyphosate does not affect growth, productivity and yield of GR soybeans (Bomfim et al., 2017; Silva et al., 2018).

The decrease of cell wall thickness, palisade and spongy parenchyma, may negatively affect the photosynthetic efficiency of plants (Zobiolo et al., 2010), since chloroplast is aplenty in these cells, which may justify the decrease in chlorophyll concentration in GR soybean leaves exposed to glyphosate observed by Bomfim et al. (2017). In addition, parenchyma cells may show some characteristics that reflect the performance of some key activities in the plant as reserve, transportation, secretion, excretion of substances (Apezato-da-Glória & Carmello-Guerreiro, 2006).

According to Tuffi Santos et al. (2008), the palisade parenchyma is coupled to leaf protection against high luminous intensity. Plants suffer light saturation under excessive amount of solar radiation, decreasing efficiency of radiation use (D. Adams & W. Adams, 1992) and compromising productivity (Casaroli et al., 2007).

Little is known about the influence of glyphosate on the vascular tissue of GR soybean leaves. Despite glyphosate resistant, under certain conditions, GR soybean presented injuries to herbicide applications associated with the formulation used (Cerqueira, Gazziero, Duke, Matallo, & Spadotto, 2007). Several authors, such as Baas (1982), Baas and Schweingruber (1987), Carlquist (2001), Alves and Angyalossy-Alfonso (2000), report that environmental factors affect dimensions and even the arrangement of vascular elements.

Melo et al. (2007) state that in *Paspalum paniculatum*, under water deficiency conditions, there is no variation in the vascular bundle thickness both in leaves and roots; however, there is a decrease in the diameter of the elements of metaxylem. Stoyanova et al. (2002) observed no significant differences in water deficit effect and soil flooding that influence thickness of vascular bundles in maize leaves.

Castro et al. (2005) stated that when the plant is subjected to stress, the decrease of vessels can ensure increased transportation. Therefore, diameter reduction of metaxylem is a common response in plants subjected to stress conditions, especially considering water deficiency and can encourage water flow in the plant, according to Passioura (1982).

Apezato-da-Glória and Carmello-Guerreiro (2006) reported that fibers are support cells, responsible for rigidity of vascular tissues. They have elongated shapes and tapered ends, with higher dimension in the longitudinal axis. The fiber walls vary in thickness; however, they are generally thicker than walls of the other cells in the secondary xylem. Shorter fibers can also be called sclereids.

According to Digby and Wareing (1966), indole acetic acid (IAA) is an important factor in establishing the diameter of the vessel elements in *Robinia pseudacacia*, a tree from the Fabaceae family. IAA promotes

elongation of cambial derivatives on the formation of xylem vessels and fiber elements, and the application of gibberellins (GA) promotes fiber elongation. According to these authors, glyphosate may cause a fast decrease in IAA and GA content in plants by changing the xylem or phloematic tissue formation as evidenced in this study. On the other hand, GR soybean responds differently to the herbicide, not necessarily characterizing a negative effect to plant, but perhaps by modulating the response of plant hormone. Bomfim et al. (2017) observed the same for other metabolism aspects, in which glyphosate does not influence negatively growth and metabolism of GR soybean.

Histochemical tests play an important role in the identification and characterization of plant anatomy (Martins & Appezzato-da-Glória, 2006), which have long been used to identify the compounds present and their occurrence in plant tissues (Sant'Ana-Santos et al., 2006; M. Santos, Freitas, Aroucha, & A. Santos, 2009).

According to Santos et al. (2009), phenolic compounds are heterogeneous groups of substances in almost all plants, in vacuoles, cytoplasm or impregnated to cell wall. Such compounds are related to plant protection in terms of draining, animal attack, among others, despite questions regarding its functions. Souto and Oliveira (2005) stated that phenolic compounds are considered a type of chemical defense against herbivory.

Starch is one of the main reserve compounds in plants. Throughout evolution, starch has been used not only as a reserve for the plant itself, but also, as one of the most important energy sources to subsequent levels of the food chain in the ecosystems (Zeeman, S. Smith, & A. Smith, 2004). Therefore, various organs acquired the ability to produce enzymes that degrade starch with subsequent release of glucose to be used in energetic metabolism. In plant cells, starch is stored in the form of water-insoluble granules located in special organelles (Amaral, Gaspar, Costa, Aidar, & Buckeridge, 2007).

In analysis of the histochemical tests, relative differences were observed only for the presence of pectic substances, which may be related to the increase of the ethylene synthesis. Abu-Irmaileh et al. (1979) found that in bean, there was an increase in ethylene concentration in glyphosate-treated plants (Yamada & Castro, 2007). Although the role of ethylene in defense responses to pathogens is widely recognized, recent studies on *Arabidopsis* and crop species highlight an emerging role for ethylene in regulating growth and yield of organs under abiotic stress (Dubois, Van Den Broeck, & Inzé, 2018).

Abiotic stress conditions, which trigger the ethylene synthesis, include submersion, heat, shadow, exposure to heavy metals and high salt content, low nutrient availability and water deficiency (Skirycz et al., 2011; Thao et al., 2015; Zhang, Smith, Harberd, & Jiang, 2016; Dubois, Claeys, Van Den Broeck, & Inzé, 2017). In young leaves, plants exposed to environmental stress, ethylene regulates cell growth inhibition, influencing both cell division as well as its expansion (Skirycz et al., 2011; Dubois et al., 2018).

Ethylene increase, possibly caused by stress caused by herbicide in GR soybean, can weaken cell wall of leaves due to the action of enzymes that degrade cell wall, such as polygalacturonase and cellulase, the latter refers to a hydrolytic enzyme that acts in pectin degradation (Uenojo & Pastore, 2007) may be responsible for the change, confirmed by the test with ruthenium red.

5. Conclusion

The treatment with glyphosate in GR soybean decreased thickness of palisade parenchyma (internervural region) and thickness of the adaxial epidermis, palisade parenchyma, spongy parenchyma and total leaf (midrib region). Although the diameter of the companion cells was reduced with the treatment with the herbicide, the diameter of the metaxylem increased substantially, and this may have led to an increase in the size of the vascular bundle. However, such evidence is indicative of structural changes occurring in response to herbicide.

It is not possible to attribute the answer observed to a stressful condition, since stress not causes permanent damage on growth and grain yield. Therefore, the tolerance gene in fact contributes to the plant not to show indication of stress or herbicide toxicity, restricting the effects to a morphophysiological disorder, that is, a transitory change in some metabolic and/or structural aspect, leading to a response to overcome this adverse condition.

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