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Article

Structural Evaluation of Damage Caused by Herbicide Clomazone in Leaves of Arborescent Species Native to Brazil

Avaliação Estrutural de Danos Causados pelo Herbicida Clomazone em Folhas de espécies Arbóreas Nativas do Brasil

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ABSTRACT - Herbicide clomazone is highly soluble in water, which increases the risk of leaching, resulting in contamination of sources of groundwater and waterways, affecting non-target organisms, with possible degradation of riparian areas. The objective of this work was to investigate the possible anatomical modifications of the leaf tissue of twelve arborescent species native to Brazil grown in soil contaminated by clomazone, in order to microscopically characterize leaf damage caused by the herbicide, and investigate whether the structural damage precedes visible damage. Through micro morphometrics, the thickness and the area occupied by the tissues were measured in a cross section of the leaves of the forest species: adaxial and abaxial epidermis, palisade and spongy parenchyma. It has been found that the species evaluated were affected by the presence of the pesticide in the soil, and the variable with the largest negative changes was the spongy parenchyma. Among the species, *Inga marginata*, S*chizolobium parahyba* and *Handroantus. serratifolius* had higher tolerance to the herbicide, demonstrating potential for studies on the mechanism by which these plants exert such tolerance. Overall, the results revealed that the forest species studied demonstrate significant alterations in leaf anatomy in response to the presence of residues of herbicide clomazone in the soil, even in leaves with no visible lesions.

Keywords: *Inga marginata*, *Handroanthus serratifolius*, forest species, phytoremediation, leaf anatomy.

RESUMO - O herbicida clomazone é altamente solúvel em água, fato que aumenta os riscos de lixiviação, acarretando contaminação de mananciais de água subterrâneas e cursos d'água, atingindo organismos não alvos, com possível degradação de matas ciliares. Objetivou-se neste trabalho investigar as possíveis modificações anatômicas do tecido foliar de mudas de 12 espécies arbóreas nativas do Brasil, cultivadas em solo contaminado por clomazone, com o intuito de caracterizar microscopicamente danos foliares causados pelo herbicida, bem como investigar se os danos estruturais precedem os danos visuais. Por meio de avaliações micromorfométricas, foram medidas, em secção transversal das folhas das espécies florestais, a espessura e a área ocupada pelos tecidos: epiderme adaxial e abaxial, parênquima paliçádico e parênquima lacunoso. Constatou-se que as espécies avaliadas foram afetadas pela presença do pesticida no solo, sendo a variável com maiores alterações negativas o parênquima lacunoso. Entre as espécies, ***Inga marginata****,* ***Schizolobium parahyba*** *e* ***Handroantus serratifolius*** *apresentaram maior tolerância ao herbicida, demonstrando potencial para estudos sobre o mecanismo pelo qual estas plantas exercem essa tolerância. De modo geral, os resultados obtidos permitem afirmar que as espécies florestais estudadas apresentam*

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alterações relevantes na anatomia foliar em resposta à presença de resíduos do herbicida clomazone no solo, mesmo em folhas sem injúrias visíveis.

Palavra-chave: *Inga marginata*, *Handroanthus serratifolius*, espécies florestais, fitorremediação, anatomia foliar.

INTRODUCTION

Environmental contamination due to the use of herbicides in agricultural practices is a global problem (Steffen et al., 2011). Clomazone, an active ingredient of Gamit®, is an important herbicide employed in crops as paddy field rice, soybean, maize, tobacco, cotton, sugarcane and cassava (Brasil, 2015). This herbicide is applied in preemergence in the control of various weed species, presenting moderate persistence in the soil, but it is soluble in water (1,100 mg L^{-1} at 25 ^{o}C), which gives it greater toxic power when in the presence of abundant water (Zanella et al., 2008). These properties of the clomazone molecule indicate a high potential for displacement in the environment with the water depth for irrigation and drainage, which may cause poisoning to sensitive plants downstream of the application sites, besides contamination of groundwater and watercourses.

Clomazone belongs to the chemical group of isoxazolidinones and works by inhibiting the biosynthesis of carotenoids (Kruse, 2001). It is absorbed by the apical meristem of the plant (preferably the root), making a transpirational flow (Senseman, 2007). Symptoms of its presence in the plant are mainly expressed by the formation of albino tissue in younger organs (Rodrigues and Almeida, 2011).

The observation of symptoms in plant species surrounding the crop areas may indicate the occurrence of species tolerant both to the presence of pollutants and bioindicators, i.e., very sensitive ones (De Temmerman et al., 2004). However, although there are morphological symptoms indicating the presence of clomazone in the ground, studies about herbicide presence monitoring methods are limited, including the selection of bioindicators.

Also, in view of plants that are organisms extremely sensitive and may react similarly to the effects of different pollutants, having visible injuries, such as necrosis and chlorosis, as well as visible physiological changes (De Temmerman et al., 2004), data based only on visual analysis can misidentify the presence of the pollutant and underestimate the potential of each species for biomonitoring (Sant'Anna-Santos and Azevedo, 2007).

In this context, micro symptoms have been used as biomarkers of atmospheric pollution and their detection by microscopy techniques is critical (Sant'Anna-Santos et al., 2006). Leaf micromorphology analyses have stood out as a tool in the pre-diagnosis of structural changes in response to different pollutants. Gupta and Ghouse (1986) have already emphasized that structural changes in the leaf epidermis of certain plants would have potential in monitoring the extent and degree of environmental pollution. In studies on the effect of glyphosate drift on eucalyptus, microscopic characters were used as a tool for detecting injuries caused to the tissues in the presence of the pesticide (Tuffi Santos et al., 2006). Sant'Anna-Santos and Azevedo (2007) have shown that microscopic changes have preceded visible symptoms in *Spondias dulcis* in response to fluoride. Thus, it is clear that microscopic analyses are essential to aid the early diagnosis of injuries, helping to clarify the mechanisms of action of molecules (Sant'Anna-Santos et al., 2006, 2007).

Given the above, this study has aimed to investigate the anatomical changes in the leaf tissue of 12 native tree species in Brazil grown in soil contaminated by clomazone, in order to microscopically characterize foliar damage caused by this herbicide and investigate whether the structural damage has taken place prior to visible damage.

MATERIALS AND METHODS

The experiment was installed and conducted in a greenhouse. The experimental design consisted of randomized blocks with four replications, in a factorial arrangement (2 x 12). The first factor consisted of plants with the application of clomazone and plants without herbicide application (control). The second factor consisted of seedlings (plants for eight months in a nursery) of 12 forest species: *Inga marginata*, *Handroanthus serratifolius*, *Jacaranda puberula*, *Cedrela fissilis*, *Calophyllum brasiliense*, *Psidium myrsinoides*, *Tibouchina granulosa*, *Caesalpinia ferrea*, *Caesalpinia pluviosa*, *Terminalia argentea*, *Schinopsis brasiliensis* and *Schizolobium parahyba*, acquired at Brazilian horticultural garden at Instituto Estadual de Florestas (IEF; State Institute of Forests) in the city of Diamantina, MG, and grown in pots measuring 8 dm^3 with an area of 0.0314 m^2, filled with a properly fertilized substrate.

The substrate used consisted of a sample of Red-Yellow Latosol (Embrapa, 2006) collected at the 0-20 cm layer in an uncultivated area. Therefore, without herbicide application history and the physicochemical characteristics are shown in Table 1.

Table 1 - Physical and chemical composition (prior to fertilization) of a Red-Yellow Latosol at the 0-20 cm layer used in the experiment

Granulometry analysis (dag kg^{-1})		
Sand	Silt	Clay
38	6	56

Chemical analysis												
pH	P	k	Ca	Mg	Al	H+Al	SB	t	T	m	V	MO
(H_2O)	(mg dm^{-3})		($cmol_c$ dm^{-3})							(%)		(dag kg^{-1})
6.1	0.7	25	1.7	0.5	0	3.7	2.3	2.3	6	2	38	1

Two applications of herbicide clomazone were provided at 20 day intervals (at 60 and 80 days after planting), each one corresponding to half of the commercial dose of technical product Gamit® of 2 L ha^{-1} (Brasil, 2015) in order to simulate leaching of the herbicide downstream of the application area, since it does not reach the groundwater in its entirety. The applications were made with a precision pipette directly in water containment plates placed under the pots in order to simulate water absorption by roots from a groundwater contaminated by herbicides. For irrigation, throughout the experiment water was placed in the containment plates, being provided to the plant by capillarity.

Anatomical analyses were made on fully expanded leaves (third node from the stem apex) due to having high metabolic activity and a defined anatomical structure. Two collections were carried out, always at seven days after herbicide application. This material was fixed in FAA 70 (formalin-aceto-alcohol) (5% of formaldehyde at 40%, 5% of glacial acetic acid and 90% of ethyl alcohol at 70%) and then transferred to ethanol 70 GL (Gay Lussac). Two leaves were taken from each repetition, of which fragments of 0.5 cm^2 were extracted from the middle region of an apparently healthy leaf, which were serially dehydrated in ethyl alcohol and embedded in glycol methacrylate (Historesin, Leica Instruments, Heidelberg, Germany). Anatomical sections were obtained in a rotary microtome with an automatic motorized coarse feed (model RM2155, Leica Microsystems Inc., Deerfield, USA). From each sample, three histological slides were taken, each containing 15 sections, which were stained with toluidine blue/pH = 4.0 (O'Brien and Mccully, 1981) and mounted on (toluene-based synthetic resin mounting medium) Permount™. Five sections of each slide were photomicrographed and analyzed with the aid of image processing and advanced quantitative analysis Anati Quanti 2.0 software. Thickness and the proportional area of tissues were measured: leaf blade, palisade and spongy parenchyma, adaxial e abaxial epidermis.

The data obtained were turned into percentage relative to the control, since the leaves of the species studied presented data with considerable amplitude of mean values. Later, they were subjected to analysis of variance and the means, when significant, were: among the species grouped according to the Scott-Knott test at 5% probability of error and in the means comparisons within each species the Tukey's test was used at 5% probability of error.

RESULTS AND DISCUSSION

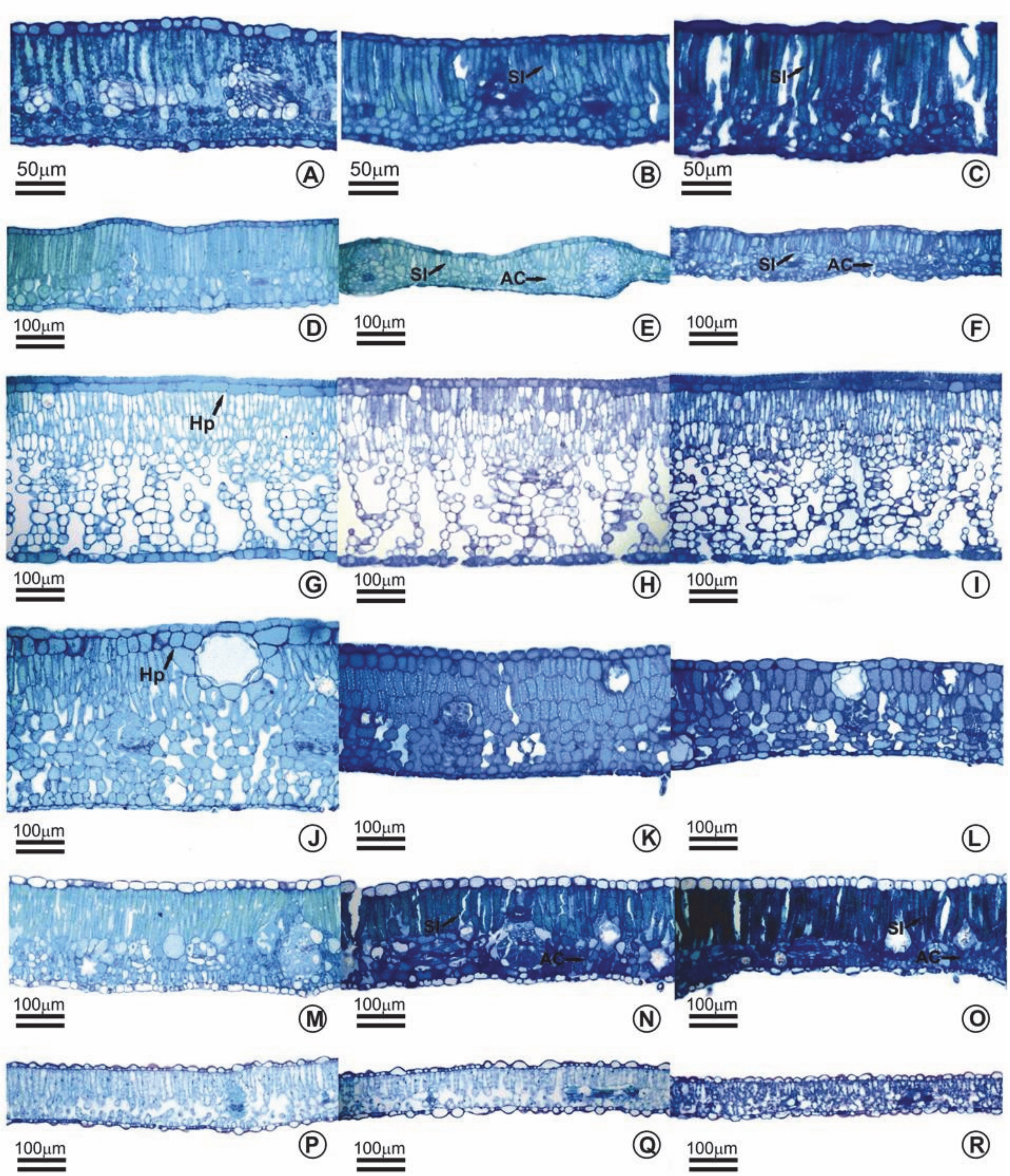
The anatomical analysis of the cross section of the leaf blade of forest species revealed a predominance of leaf blade consisting of uniseriate epidermis, mesophyll dorsiventral formed by the one to three layers of cells in the palisade parenchyma, ranging from slightly elongated to very long, and four to nine layers of spongy parenchyma, with amplitude variation of intercellular spaces. Most species have large intercellular spaces, except for *S. brasiliensis* and *J. puberula*, which present a compact mesophyll (Figures 1A and D). Uniseriate hypodermis toward the adaxial side was observed in *C. brasiliense* and *P. myrsinoides* (Figures 1G and J).

Plants treated with herbicide clomazone showed reduction in the ratio of mesophyll tissues in most species tested (Figures 1 and 2). Among the variables analyzed, thickness of the spongy parenchyma (SP) had the highest reduction percentage: on average 16.3 and 17.9% for the first and second applications, respectively (Figures 1 and 2), followed by palisade parenchyma (PP), adaxial epidermis (AET) and abaxial epidermis (ABE), with an average reduction in both applications of 13.1, 8.22 and 7.73%, respectively (Table 2).

S. brasiliensis decreased in thickness of AET in 18.9% (Figures 1A-C) when comparing the herbicide applications, totaling 27.26% compared to the control (Table 2). As for the species, the data obtained in samples collected after the second application of the herbicide revealed that *S. brasiliensis* and *T. argentea* were negatively affected, with an average reduction of 22.6%, followed by *J. puberula*, *C. fissilis*, *P. myrsinoides* and *C. ferrea* with an average reduction in AET of 8.59%. However, *C. fissilis* tended to recovery from the injury, since among the applications there was increased thickness of AET. The decrease of AET, even moderately, may interfere with the protection and prevention of tissue water loss (Bukovac et al., 1990). However, its increase determines prevention from sweating, being an important factor to mitigate the penetration of radiation inside the leaf, protecting the layers of the photosynthetically active mesophyll (Cen and Bornman, 1993). Môro et al. (1999) have noticed anatomical changes such as significant increase in number, size and shape of bulliform cells, besides the almost complete disappearance of key epidermal cells of *Zea mays* submitted to herbicide nicosulfuron.

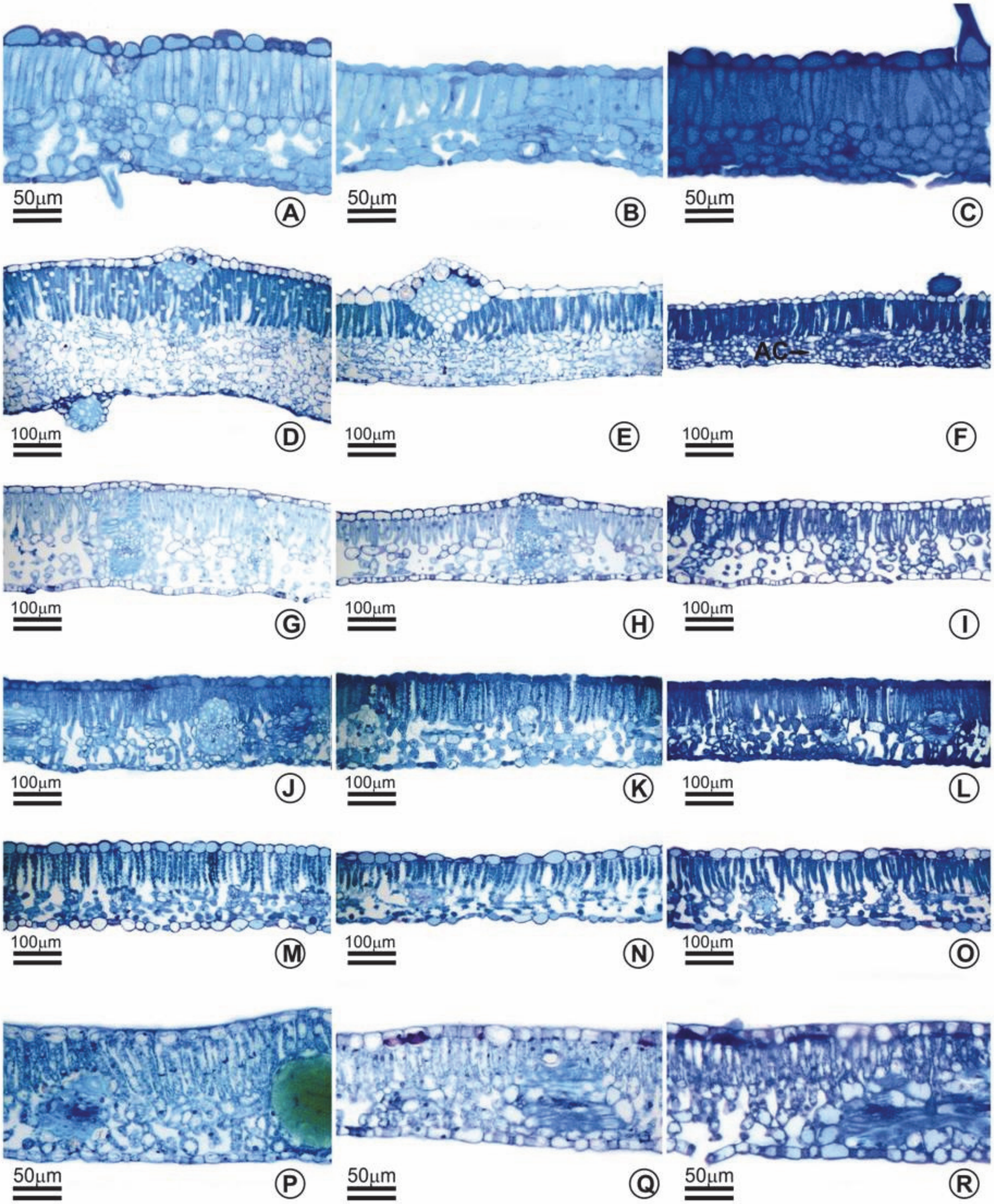
For the variable palisade parenchyma (PP), *T. argentea* showed significant decrease compared to both applications of 21.8% (Figures 1M-O) (Table 2). Among the species, there was a reduction of 30.7, 29.9, 28.1 and 17.2% in *P. myrsinoides*, *J. puberula*, *C. ferrea* and *T. granulosa*, respectively (Figures 1J-L, D-F, P-R and Figure 2D-F), in samples collected after the first application of herbicide clomazone. The same was observed for *P. myrsinoides*, *J. puberula*, with a reduction of about 39%, followed by *C. ferrea* and *T. granulosa*, with an average reduction of 22.2% in samples collected after the second application (Table 2). Variable PP, an important characteristic linked to leaf protection against high intensity of light (Tuffi Santos et al., 2008), was negatively affected by the presence of the pesticide. This reduction may be due to a decrease in the growth rate of plants, since clomazone indirectly affects the photosynthetic process, causing a decrease in the availability of metabolites and hence plant growth (Pasqualini et al., 2002). However, the increase of this tissue seen in *I. marginata* may be linked to the deposition of the contaminant and may be an evidence of phytovolatization or phytodegradation in leaf level. In this case, the plant could be releasing the pollutant transformed or in the original form.

The spongy parenchyma (SP) is adjacent to PP and facing the leaf abaxial side. In that portion of the leaf tissue the largest thickness reduction ratios were observed. The species submitted to treatment had in general a decrease of 12.4% and 13.05% for the first and second applications, respectively (Table 2). There was an SP reduction of species *J. puberula* (Figures 1D-F), *P.myrsinoides* (Figures 1J-L), *T. argentea* (Figures 1M-O), *C. ferrea* (Figures 1P-R), *C. fissilis* (Figures 2A-C) and *T. granulosa* (Figures 2D-F) in samples collected after the first application of the herbicide (Table 2). As for leaf samples collected after the second application of the herbicide, *J. puberula* (Figure 1F), *T. argentea* (Figure 1O) and *T. granulosa* (Figure 2F), they showed the highest reduction ratios. SP was the most negatively affected variable analyzed, resulting in a



In the first column, cross sections of leaves of forest species without herbicide application. In the second, a leaf cross section corresponding to the first application of clomazone. And in the third, a leaf cross section corresponding to the second herbicide application.

Figure 1 - Cross section of leaves of forest species: *Schinopsis brasiliensis* (A-C), *Jacaranda puberula* (D-F), *Calophyllum braziliensis* (G-I), *Psidium myrsinoides* (J-L), *Terminalia argentea* (M-O), *Caesalpinia ferrea* (P-R). Sinuosity (SI) of the cell wall, cell flattening (CF) and hypodermis (Hp).



In the first column, cross sections of leaves of forest species without herbicide application. In the second, a leaf cross section corresponding to the first application of clomazone. And in the third, a leaf cross section corresponding to the second herbicide application.

Figure 2 - Cross section of leaves of forest species: *Cedrela fissilis* (A-C), *Tibouchina granulosa* (D-F), *Handroanthus serratifolius* (G-I), *Inga marginata* (J-L), *Schizolobium parahyba* (M-O), *Caesalpinia pluviosa* (P-R). Cell flattening (CF).

Table 2 - Adaxial epidermal thickness (AET), palisade parenchyma (PP), spongy parenchyma (SP), abaxial epidermis (ABE) e leaf blade (LB) of leaves of arborescent species treated with clomazone. The values represent the percentage to relative the control (100%)

Species	AET (%)		PP (%)		SP (%)		ABE (%)		LB (%)	
	1st Appl	2nd Appl	1st Appl	2nd Appl	1st Appl	2nd Appl	1st Appl	2nd Appl	1st Appl	2nd Appl
Inga marginata	97.33 A a	98.99 A a	98.39 A a	100.00 A a	100.00 A a	100.00 A a	98.10 A a	100.00 A a	99.08 A a	100.00 A a
Handroanthus serratifolius	93.52 A a	100.00 A a	87.81 A a	88.16 A a	91.78 A a	91.168 A a	94.14 A a	87.48 A a	91.55 A a	95.05 A a
Jacaranda puberula	89.55 A a	88.43 A b	70.03 A b	64.36 A c	71.66 A b	69.45 A b	84.47 A a	79.47 A b	73.43 A b	69.27 A b
Cedrela fissilis	80.31 A a	88.37 A b	95.36 A a	93.68 A a	75.46 A b	80.55 A a	94.84 A a	91.58 A a	86.16 A a	88.20 A a
Calophyllum brasiliensis	96.22 A a	97.99 A a	98.92 A a	97.29 A a	100.00 A a	99.37 A a	91.94 A a	96.61 A a	99.80 A a	99.33 A a
Psidium myrsinoides	87.99 A a	87.08 A b	69.28 A b	57.64 A c	66.39 A b	48.89 B c	99.13 A a	98.13 A a	70.13 A b	56.75 A c
Tibouchina granulosa	97.93 A a	97.06 A a	82.79 A b	78.64 A b	76.07 A b	68.89 A b	78.51 A a	90.5 A a	80.59 A b	75.78 A b
Caesalpinia ferrea	95.95 A a	90.21 A b	71.87 A b	76.87 A b	72.35 A b	79.52 A a	93.45 A a	89.14 A a	76.21 A b	80.35 A b
Caesalpinia pluviosa	95.44 A a	95.62 A a	89.41 A a	92.29 A a	88.11 A a	89.29 A a	96.56 A a	95.11 A a	90.95 A a	91.75 A a
Terminalia argentea	89.15 A a	79.9 A c	100.00 A a	83.69 B a	78.68 A b	74.24 A b	90.35 A a	70.32 B b	92.71 A a	80.89 A b
Schinopsis brasiliensis	91.69 A a	72.74 B c	100.00 A a	100.00 A a	90.37 A a	85.08 A a	98.38 A a	96.88 A a	99.65 A a	96.19 A a
Schizolobium parahyba	94.09 A a	97.03 A a	93.31 A a	95.61 A a	94.19 A a	98.17 A a	99.17 A a	100.00 A a	94.61 A a	98.47 A a
VC (%)	9.94		13.26		14.9		10.28		11.21	

Means followed by the same lowercase letter in the column do not differ by the Scott-Knott test at 5% probability. Means followed by the same uppercase letter in the line do not differ by the Tukey's test at 5% probability or rror.

decrease in the size of the leaf blade. The frequently observed morphological change was the decrease in intercellular spaces, with greater density of parenchymal cells, providing tissue compression (Figures 2D, F). A similar result was found in fluoride toxicity studies in vegetables (Sant'Anna-Santos and Azevedo, 2010), highlighting the importance of microscopy as an important element to compare the sensitivity of species in response to pollution (Pita-Barbosa et al., 2009). Its reduction decreases the light absorption by the leaf due to impairment of gas diffusion in the leaf mesophyll (Vogelman et al., 1996), since in dorsiventral leaves SP, with its irregular architecture and wide intercellular space, increases the distance that light reaches in the leaf through reflection in the gas/liquid interfaces (Lambers et al., 1998). In this sense, the species affected by clomazone can, in the field, reduce competitiveness by underusing available light. In the species tested, in general SP had the highest negative changes, which would hinder its establishment in open areas, as this tissue is more required in such a situation. Besides the fact that clomazone interferes with protection against photo-oxidation, promoting destruction of chlorophyll, induced by the absence of carotenoids (Vidal, 1997).

The evaluation of the epidermis thickness of the abaxial side (ABE) of the species tested showed that only *T. argentea* had a decrease ($p<0.05$) in the tissue thickness between the first and second applications of the herbicide. *T. argentea* and *S. brasiliensis* showed the highest reduction ratios (Table 2).

In addition to the changes already mentioned for the mesophyll, it is possible to observe small meanders in the cell wall in *S. brasiliensis* (Figures 1A, C), *J. puberula* (Figures 1D, F) and *T. argentea* (Figures 1M, O), flattening of the spongy cells in *J. puberula* and *T. argentea*, decrease in intercellular spaces in *T. argentea*, and a certain disorganization of the mesophyll in *C. ferrea* (Figures 2D, F).

The anatomical changes mentioned above have contributed to reducing the total thickness of the leaf blade (LB), with *P. myrsinoides* standing out with a reduction of 43.2% (Figures 1J, L and 2).

Anatomical analyses taking into account the ratio that each tissue occupies in the leaf blade through area measurements in the cross section of the leaf have shown that, overall, the tissue ratios were not significantly affected when compared with samples from the different applications of clomazone, with some exceptions. *T. argentea* and *S. brasiliensis* were negatively affected in 17.5 and 26.95%, respectively, in relation to the area of the adaxial epidermis (AET), followed by *P. myrsinoides*, with an average reduction of 49.5% in the ratio of the palisade parenchyma (PP), and *T. argentea*, *S. brasiliensis* and *T. granulosa*, with a decrease of 17.4, 12.2 and 14.9, respectively, regarding the area of abaxial epidermis (ABE). For the latter, tissue ratio integrity recovery was observed (Table 3).

Among the species, it was noticed that *T. argentea* and *S. brasiliensis* had the lowest reduction rates ($p<0.05$) in the ratio of AET. However, *H. serratifolius*, *J. puberula*, *C. brasiliensis*e and

Table 3 - Measurements of the adaxial epidermal thickness (AET) area, palisade parenchyma (PP), spongy parenchyma (SP), abaxial epidermis (ABE) and leaf blade (LB) of leaves of arborescent species treated with clomazone. The values represent the percentage to relative the control (100%)

Species	AET (%)		PP (%)		SP (%)		ABE (%)		LB (%)	
	1st Appl	2nd Appl	1st Appl	2nd Appl	1st Appl	2nd Appl	1st Appl	2nd Appl	1st Appl	2nd Appl
Inga marginata	95.67 A a	96.19 A a	97.99 A a	100.00 A a	97.61 A a	100.00 A a	92.41 A a	96.25 A a	97.62 A a	100.00 A a
Handroanthus serratifolius	92.98 A a	99.98 A a	83.21 A b	87.95 A a	85.35 A a	81.84 A a	93.94 A a	90.81 A b	86.42 A a	92.48 A a
Jacaranda puberula	85.62 A a	91.04 A a	69.96 A b	65.09 A b	63.58 A b	66.66 A b	87.35 A a	83.87 A b	69.88 A b	69.47 A b
Cedrela fissilis	92.51 A a	91.14 A a	85.64 A b	88.25 A a	70.18 A b	82.33 A a	87.49 A a	93.29 A a	80.19 A b	86.49 A a
Calophyllum brasiliense	99.73 A a	100.00 A a	88.54 A a	95.81 A a	95.37 A a	96.35 A a	92.01 A a	99.82 A a	93.89 A a	96.95 A a
Psidium myrsinoides	90.39 A a	97.09 A a	69.78 A b	50.41 B c	64.78 A b	53.90 A b	100.00 A a	100.00 A a	68.85 A b	58.55 A b
Tibouchina granulosa	98.30 A a	92.76 A a	76.090 A b	74.85 A b	69.39 A b	67.96 A b	85.05 B a	97.05 A a	74.97 A b	73.92 A b
Caesalpinia ferrea	97.54 A a	96.20 A a	75.39 A b	83.88 A a	67.74 A b	77.43 A a	93.97 A a	95.46 A a	75.70 A b	83.65 A a
Caesalpinia pluviosa	94.29 A a	95.89 A a	96.68 A a	96.42 A a	96.33 A a	91.52 A a	97.46 A a	97.36 A a	98.28 A a	94.53 A a
Terminalia argentea	93.44 A a	82.47 B b	100.00 A a	83.86 A a	81.57 A a	80.40 A a	93.65 A a	82.59 B b	94.77 A a	83.45 A a
Schinopsis brasiliensis	90.49 A a	73.05 B b	100.00 A a	100.00 A a	93.22 A a	86.71 A a	99.03 A a	87.77 B b	98.66 A a	95.13 A a
Schizolobium parahyba	94.85 A a	97.82 A a	96.04 A a	98.70 A a	89.78 A a	97.29 A a	100.00 A a	100.00 A a	94.10 A a	98.73 A a
VC (%)	7.97		13.37		16.1		7.69		11.9	

Means followed by the same lowercase letter in the column do not differ by the Scott-Knott test at 5% probability. Means followed by the same uppercase letter in the line do not differ by the Tukey's test at 5% probability or error.

P. myrsinoides have positively stood out, since the possible recovery of this tissue ratio can be seen in these species (Table 3). The ratio of PP was negatively affected in samples from the first application of herbicide in *H. serratifolius*, *J. puberula*, *C. fissilis*, *P. myrsinoides*, *T. granulosa* and *C. ferrea*, aggravating to *P. myrsinoides*, with decrease of 49.5% in the second application. SP remained as the most affected variable analyzed, corroborating the results obtained with the linear measurements. However, in the analysis of species, *J. puberula*, *C. fissilis*, *P. myrsinoides*, *T. granulosa* and *C. ferrea* had the lowest values: on average, 32.8% of decrease in the ratio of such tissue in the leaf (Table 3) already in the first application of the herbicide. In AET it was possible to see differences ($p<0.05$) among the applications, highlighted by species *T. argentea*, *S. brasiliensis* and *T. granulosa*. However, for the letter an increase of the AET thickness was noticed when comparing the applications. *H. serratifolius*, *J. puberula*, *T. argentea* and *S. brasiliensis* had a significant reduction of ABE thickness from the second application of clomazone in the analyses among the species (Table 3).

The reduction in the ratio of leaf tissue can be attributed to the application of clomazone, since this one results in photo-oxidation and destruction of chlorophyll induced by the absence of carotenoids (Vidal, 1997), directly interfering with photosynthesis and causing less plant dry matter production (Takahashi et al., 2009). However, in some species, decline was observed in tissue integrity in the first application of the herbicide, demonstrating an "alarm stage" at the beginning of the disorder characterized by loss of stability of the structures (biomembrane proteins) and the functions that maintain the vital activities (energy-producing metabolism and biochemical processes) (Larcher, 2006). These plants showed recovery in samples collected after the second application of clomazone, indicating restitution in the form of repair processes such as protein synthesis or de novo synthesis of protective substances (Larcher, 2006).

Even if the leaves were already fully expanded, the negative effect of the product is justified due to the possible application of the commercial formulation additives (Malkomes, 2000), which is a direct effect of clomazone on the de novo synthesis of pigments eventually destroyed by photodamage or other means, or even the combined effect of these phenomena.

The results allow us to state that the forest species studied show significant changes in leaf anatomy in response to the presence of clomazone herbicide residues in the soil, even in leaves with no visible injuries.

Plants treated with herbicide clomazone have presented in greater or lesser extent changes in micromorphometric measurements of the leaf tissues. It should be noted that the samples were taken prior to the onset of symptoms in the external morphology. Among the species analyzed, *T. argentea*, *S. brasiliensis*, *J. puberula* and *P. myrsinoides* were more sensitive to the presence of the herbicide in the soil, demonstrating their potential as bioindicators. The results show the poisoning caused by herbicide clomazone to forest species, but *I. marginata*, *S. parahyba* and *H. serratifolius* have stood out for maintaining the integrity of tissues close to the control even under stress conditions. This reinforces the need for more studies on the mechanism exerted by these plants to present this tolerance.

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