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Leaf-surface characterization and the effects of the herbicide saflufenacil on the leaves of weeds

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ABSTRACT

Effects of saflufenacil in *Merremia aegyptia* (Convolvulaceae), *Luffa aegyptiaca* (Cucurbitaceae) and *Mucuna aterrima* (Fabaceae - Leguminosae) were analyzed by scanning electron microscopy one day before and after application. Stomatal density, stomatal index and length of the stomatal aperture were determined by imprinting the leaf epidermis. These species showed stomata on the adaxial and abaxial epidermis. *M. aegyptia* showed paracytic stomata, unicellular tector trichomes on the abaxial surfaces, especially around leaf veins, and absence of epicuticular wax crystals. *L. aegyptiaca* was characterized by having anomocytic stomata, glandular trichomes and multicellular trichomes on both sides of the leaf, and did not have epicuticular wax crystals. *M. aterrima* showed anisocitic stomata, glandular and unicellular trichomes on both sides as well as a large amount of epicuticular wax crystals. The herbicide saflufenacil promoted dispersion of waxes, epidermal surface roughness and depletion of trichomes on *L. aegyptiaca* and *M. aterrima* plants. There were significant differences in the stomatal index, density and length of stomatal aperture among the studied species.

Key words: retention, stomatal density, trichomes, wax

Caracterização da superfície foliar e efeito de saflufenacil sobre as folhas de plantas daninhas

RESUMO

As características foliares de *Merremia aegyptia* (Convolvulaceae), *Luffa aegyptiaca* (Cucurbitaceae) and *Mucuna aterrima* (Fabaceae - Leguminosae) foram analisadas por microscopia eletrônica de varredura, antes e um dia após a aplicação do herbicida saflufenacil. A densidade, o índice e a abertura estomática, foram determinados através da impressão da epiderme foliar. As espécies apresentaram estômatos presentes na epiderme adaxial e abaxial. *M. aegyptia* mostrou estômatos paracíticos, tricomas tectores unicelulares na superfície abaxial, especialmente ao redor das nervura das folhas e ausência de cristais de ceras epicuticulares. *L. aegyptiaca* foi caracterizada por possuir estômatos anomocíticos, tricomas glandulares e tricomas multicelulares, em ambos os lados da folha e sem a presença de cristais de ceras. *M. aterrima* mostrou estômatos anisocíticos, tricomas glandulares e unicelulares, em ambos os lados, tal como grande quantidade de cristais de cera epicuticulares. O saflufenacil promoveu dispersão de ceras, rugosidade na superfície epidérmica das plantas de *L. aegyptiaca* e *M. aterrima*. Houve diferenças significativas no índice estomático, densidade e comprimento da abertura estomática entre as espécies estudadas.

Palavras-chave: retenção, densidade estomática, tricomas, ceras

Introduction

The species *Luffa aegyptiaca*, popularly known as loofah, is a weed in sugarcane plantations in Brazil. Although its occurrence is limited to isolated areas, the species causes serious damage to sugarcane due to its climbing behavior. The species intertwines on the stalk and leaves and vegetates at the apex of the culture, which promotes shading and reduction of photosynthesis (Kissmann & Groth 1999). Similarly, the velvet bean (*Mucuna aterrima*) and species of the *Ipomoea* and *Merremia* genera (Christoffoleti et al. 2007; Kuva et al. 2007) have also interfered with the production of mechanically harvested sugarcane crops.

Increased infestation of these weeds in areas of mechanically harvested sugarcane crops can be explained by some aspects of the biology of these plants, such as their large seed-reserve structures, capable of growing through a layer of straw on the soil, in addition to their negative photoblastic behavior (Monquero et al., 2009). Weed control using chemical methods is increasingly significant in sugar cane crop. Knowledge of weed anatomy has a determining role in this type of control because it can influence the absorption and translocation of herbicides.

Currently there are 24 active ingredients registered for weed control in sugarcane crops in Brazil, and recently, saflufenacil, a new herbicide developed by BASF, targeted for contact and residual broadleaf weed control was registered. Saflufenacil belongs to the pyrimidinedione family and inhibits protoporphyrinogen oxidase (Protox). Saflufenacil has a mildly acidic character, a pKa of 4.3, water solubility of 30 mg L^{-1} at pH 5.0 and 2,100 mg L^{-1} at pH 7.0 and a vapor pressure of 2.0 x 10^{-14} Pa at 25 ºC (BASF agricultural products, 2008).

Penetration of herbicides into plants can occur through aerial organs (leaves, stems, flowers and fruits), underground organs (roots, rhizomes and tubers) and seeds during germination and emergence as well as through the radicle and hypocotyl (Silva et al*.,* 2007). However, leaves are the main organ of weeds involved in the penetration of herbicides applied post-emergence (Procópio et al., 2003). The area of the leaf blade and the angle or orientation of leaves in relation to the spray pattern influences the amount of intercepted herbicide. However, the anatomical characteristics of leaves, such as the presence and quantity of trichomes and stomata, the thickness and composition of the cuticle layer and the presence and nature of waxes, will practically determine the ease with which herbicides will be absorbed (Hess & Falk, 1990). Thus, knowledge of leaf characteristics of target weeds and the characteristics of the spray solution is fundamental to the success of agricultural spraying.

The aim of this study was to characterize the leaf-surface anatomy (the layout, classification and density of stomata, stomatal index, length of the stomatal aperture and the presence of trichomes and epicuticular waxes) of *Merremia aegyptia*, *Luffa aegyptiaca* and *Mucuna aterrima* and to study the effects of the herbicide saflufenacil on some of these characteristics.

Material and Methods

Analysis of leaf surface - scanning electron microscopy

Merremia aegyptia, *Luffa aegyptiaca* and *Mucuna aterrima* were sown in 1000 mL capacity pots filled with soil classified as a dystrophic red oxisol and kept in a greenhouse. Ten days after seedling emergence, the seedlings were thinned to three plants per pot. The herbicide saflufenacil (50 g ha^{-1}) was applied to the plants when they had the 2nd pair of true leaves. Concurrently, blank solutions were sprayed on control plants. The herbicide was applied with a research backpack sprayer pressurized with CO_2 at a flow rate of 200 L ha^{-1}.

For analysis of the surfaces of the leaves by scanning electron microscopy, two segments of approximately 50 mm² from adaxial and abaxial surface were taken from the fully expanded median region of young leaves of *M. aegyptia, L. aegyptiaca* and *M. aterrima*. The leaves for analysis were collected before and one day after the herbicide application and eight plants for each species and each treatment were used.

Samples were prepared according to the Tanaka & Kitajima protocols (2009) used by the laboratory of the Electron Microscopy Applied to Agriculture Research Support Nucleus (Núcleo de Apoio a Pesquisa em Microscopia Eletrônica aplicada a Pesquisa Agropecuária – NAP/MEPA) of the Luiz de Queiroz College of Agriculture (Escola Superior de Agricultura "Luiz de Queiroz" – ESALQ/USP), where the image processing was also performed. Plant segments were fixed with modified Karnovsky's fixative (2.5% glutaraldehyde and 2.5% formaldehyde in 0.05 M sodium cacodylate buffer, pH 7.2, with 0.001 M $CaCl_2$). Samples were placed in 1.5 mL Eppendorf tubes containing this solution and kept in the refrigerator for 12 hours. Subsequently, samples were dehydrated by subjecting them to solutions of increasing concentrations of acetone (30, 50, 70, 90 and 100%), where each sample remained for approximately 10 minutes, and processing in the 100% acetone solution was repeated 3 times. After preparation, samples were placed in individual chambers and taken to the critical point-method drying apparatus. The apparatus consisted of a sealed chamber in which samples were immersed in liquid CO_2. The chamber was then heated to 40 °C, whereby CO_2 gas was slowly released, drying the samples.Dried samples were then assembled into stubs and sputter-coated with a thin layer of gold to a thickness of 0.05 microns to avoid rehydration (Bozzola & Russell 1992). Prepared samples were analyzed using a JEOL scanning electron microscope.

Leaf Surface Characterization

The stomatal density, stomatal index and length of stomatal aperture were determined on the adaxial and abaxial surfaces of the leaves, using 8 plants of each of the species studied.

The epidermis-imprinting technique with a cyanoacrylate ester-based adhesive was used to evaluate the epidermis of the species (Mendonça 2000). In this technique, a drop of adhesive is placed on a glass microscope slide, with the leaf surface to be analyzed facing the adhesive and pressed against the slide. After the adhesive has dried, the plant material was removed, leaving an impression of the epidermis on the slide. The

slide was observed on a light microscope (Olympus BX51) coupled to a computer with DP Controller and DP Manager software. Fields were chosen at random from different leaves and regions for each type of assessment, avoiding margins and proximity to the veins. There was no discrepancy among the values obtained; thus, averages among these values were calculated to determine the parameters for the stomatal index, length of stomatal aperture and stomatal density. The images were analyzed using the Anati Quanti software to determine the stomatal index and density. The lengths of the stomatal aperture in digitized images were measured using the ImagePro Plus 4.1 software from Media Cybernetics.

Results and Discussion

All species were classified with leaves as amphistomatic, i.e., having stomata on both sides (Appezzato-da-Glória & Guerreiro, 2003). The penetration of herbicides by stomata is considered low because most amphistomatic species have fewer stomata on the adaxial surfaces, which was confirmed in the present study (Table 1). Moreover, in agricultural spraying, it is challenging for the droplets to reach the abaxial surface; consequently, the importance of stomatal uptake by this surface is considered low. Another factor leading researchers to believe in the lack of importance of herbicide absorption through the stomata is the fact that these stomata are closed during various times of the day, including during night applications (Ferreira et al. 2002). However, the cuticular membrane present on the guard cells is thinner and more permeable, which would facilitate the entry of herbicides. According to Procópio et al. (2003), the use of silicone surfactants contributes to breaking the surface tension of a spray solution on the leaves, which then promotes greater spreading of the product and allows the stomata to play an important role in the penetration of herbicides; however, this type of surfactant is not compatible with all active ingredients.

Trichomes were not found on the adaxial surface of *M. aegyptia;* however, they are present on the abaxial surfaces, especially around the veins, and are thus unicellular tector trichomes (Figure 1). The stomata are of the paracytic type, meaning that they are flanked by two subsidiary cells whose longitudinal axes are parallel to the guard cells. The leaf epidermis is rough and did not show the presence of dispersed epicuticular wax crystals. The presence of amorphous waxes is normally associated with a low amount of wax on the leaf surfaces or the predominance of a primary alcohol in the chemical composition of the waxes (Baker & Bukovac, 1971). According to Monquero et al. (2004), the adaxial surface of *I. grandifolia,* another species belonging to the Convolvulaceae family, is also rough but without the presence of trichomes or wax crystals.

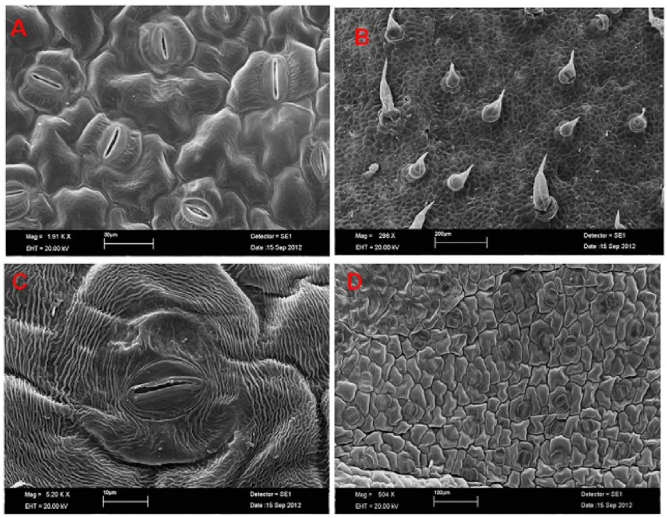

Figure 1. Abaxial (A-B) and adaxial (C-D) leaf surfaces of *M. aegyptia* with stomata and trichomes

Figure 2 shows that the herbicide saflufenacil caused no changes on the leaf surfaces of *M. aegyptia*. According to Hess & Falk (1990), all features of the leaf surface influence the deposition of the herbicide on the leaf surface, such as the presence of trichomes and glands that can leave the cells of the epidermis completely hidden. Trichomes on the leaf surfaces can intercept droplets, preventing them from reaching the epidermis. The adhesion of the drops on the trichomes occurs even when the trichomes are simple and occur at low density. Although the effectiveness of herbicide absorption by the trichomes is still partly unknown, there is a negative correlation between the adherence of herbicides to the trichomes and the effectiveness of these products.

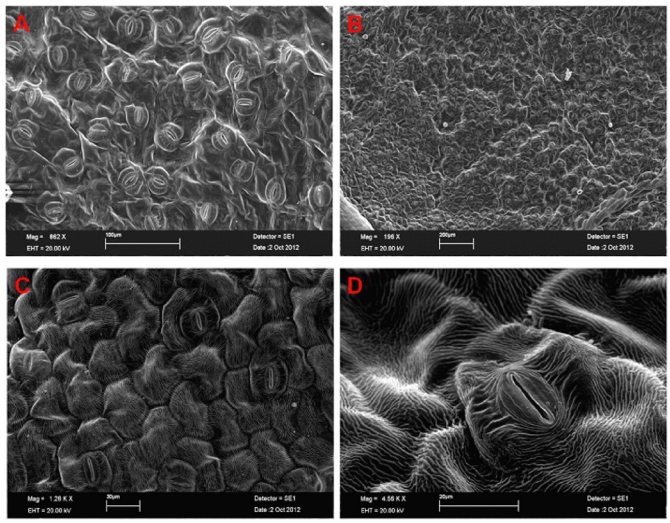

Figure 2. Abaxial (A-B) and adaxial (C-D) leaf surfaces of *M. aegyptia* after the application of the herbicide saflufenacil

Table 1. Characteristics of leaf surfaces of *L. aegyptiaca*, *M. aegyptia* and *M. aterrima*

Plant Species	Stomatal Distribution	Type of Stoma	Stomatal index (%)		Stomatal density (Stomata mm^{-2})		Length of stomatal aperture (µm)	
			Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
L. aegyptiaca	Amphistomatic	Anomocytic	7.03	32.54	37.79	74.21	13.98	18.24
M. aegyptia	Amphistomatic	Paracytic	23.55	52.00	22.76	62.37	15.95	18.57
M. aterrima	Amphistomatic	Anisocitic	6.04	21.01	6.83	29.14	12.86	12.54

In a study designed to assess the tolerance of 14 tomato genotypes to the herbicide acifluorfen, an inverse correlation was found between the activity of the herbicide and the frequency of trichomes, i.e., with more trichomes, the herbicide activity was lower (Ricotta & Masiunas, 1992). According to Mcwhorter & Ouzts (1993), the glandular trichomes often collapse and discharge a mucilage that can increase the oiliness of the leaf and inhibit the spreading of the herbicide droplets.

The abaxial and adaxial surfaces of *L. aegyptiaca* showed multicellular and glandular tector trichomes on both sides, an absence of epicuticular wax and anomocytic stomata, that is, stomata that lack differentiated subsidiary cells (Figure 3). It is important to mention that the cuticular membrane on the guard cells is thinner and permeable, which facilitates the entry of water. The herbicide saflufenacil made the abaxial and adaxial surfaces of this species rough, and there was depletion of trichomes (Figure 4). The epidermal cells and trichomes of plants submitted to herbicide were plasmolysed, this phenomenon occurs when the cell is placed in hypertonic medium, which is, when the external environment is more concentrated than the cytoplasm and the cell loses water through osmosis. Malpassi (2006) observed when lactofen is applied in *Eleusine indica*, some cuticular changes. Seven days after application, the cuticle was more undulated and folded than in the control due to plasmolysed subjacent epidermic cells. Moreover, some fractures or cracks can be observed in it, regardless the analyzed plant category, but are bigger and more frequent in the youngest plants. Stomatal cells were also plasmolysed, as the rest of typical epidermic cells and, consequently, the cuticle that covers them reflects the situation.

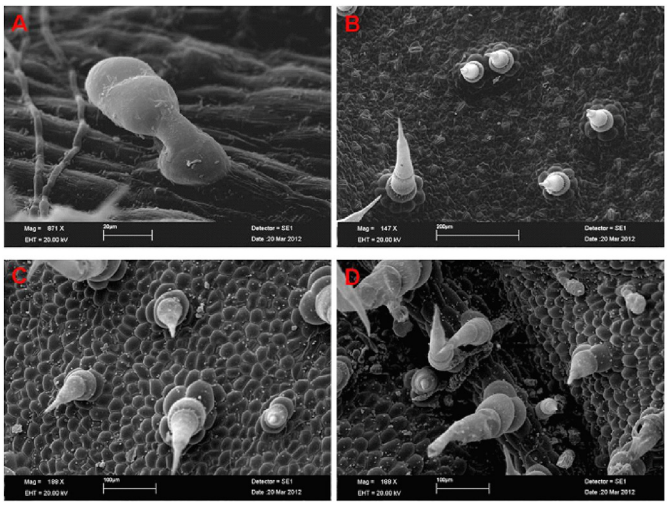

Figure 3. Abaxial (A-B) and adaxial (C-D) surfaces of *L. aegyptiaca* with stomata and pluricellular and glandular trichomes

Figure 5 shows the abaxial and adaxial surfaces of *M. aterrima*. The two surfaces showed many epicuticular wax crystals, glandular trichomes, unicellular tector trichomes and anisocitic stomata. After applying saflufenacil (Figure 6), there was corrosion of the epicuticular waxes on both of the leaf surfaces and the epidermic cells were plasmolysed. Herbicide retention on the leaf surfaces of plants is affected by wax, physical structural characteristics of the cuticle and the pilosity. Plants that have large amounts of epicuticular

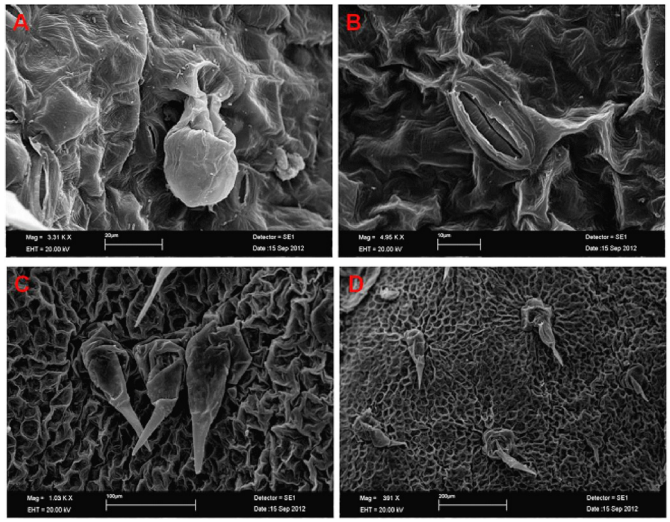

Figure 4. Abaxial (A-B) and adaxial (C-D) surfaces of *L. aegyptiaca* after the application of the herbicide saflufenacil

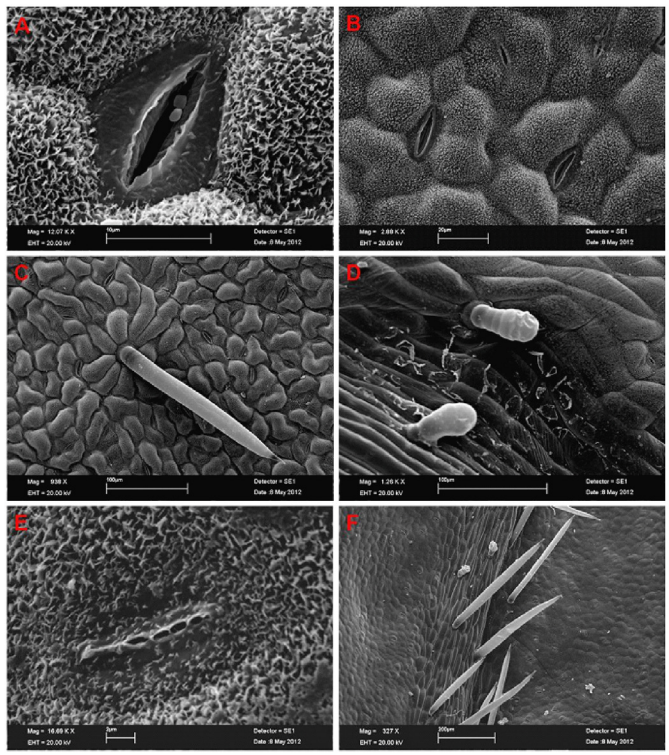

Figure 5. Abaxial (A-D) and abaxial (E-F) surfaces of *M. aterrima* with wax, stomata trichomes

waxes on the adaxial leaf surfaces may have lower retention of herbicide droplets, which have hydrophilic characteristics, and therefore lower absorption (Monquero et al., 2004). The leaves of *Populus balsamifera,* after being dipped for 40 seconds in chloroform, showed increased uptake of picloram (from 7 to 20%) and 2,4-D (from 8 to 29%) due to the removal of wax, which acted as a barrier to the entry of these herbicides (Sharma & Vanden Born, 1970).

Herbicides differ in structure and polarity, thereby crossing the cuticle with greater or lesser difficulty (Procópio et al., 2003). According to Silva et al. (2000), the exact mechanism of penetration is not yet known for all products, but one can say that the non-polar compounds follow the lipophilic route, and polar

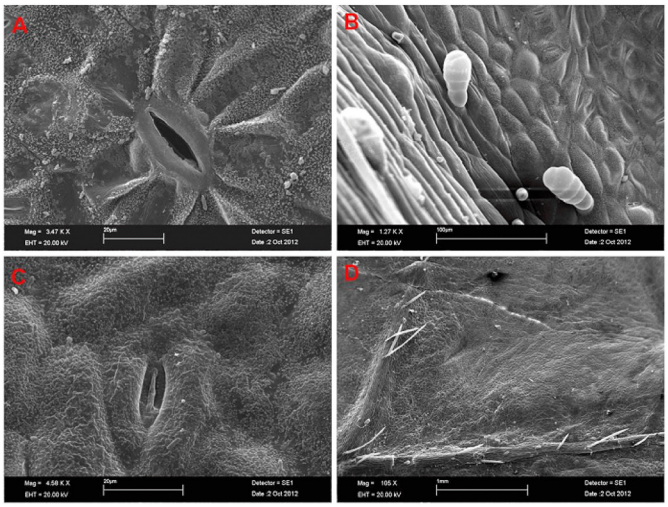

Figure 6. Abaxial (A-B) and adaxial (C-D) surfaces of *M. aterrima* after the application of the herbicide saflufenacil

compounds follow the hydrophilic route. Polar or hydrophilic herbicides (Kow <10) use pectin filaments, which can cross the cuticle if they are hydrated, as an absorption route. Marques et al. (2012) noted that the leaf anatomical structures can be related to a greater or lesser sensitivity of plants to herbicides and are important for chemical management. However, little is known about the contribution of each structural characteristic of the leaf to the process of absorption and translocation of herbicides.

The stomatal density was greater on the abaxial surfaces of the species studied (Table 1), and among the three species, *L. aegyptiaca* showed greater stomatal density on both the adaxial and abaxial surfaces. Procópio et al. (2003) observed that the main barrier to penetration of foliar herbicides in *Galinsoga parviflora* was the low stomatal density on the plant's adaxial surfaces. The stomatal index is a relatively constant parameter for each species (Cutter, 1986), although the *M. aterrima* species had the lowest values, followed by *L. aegyptiaca*, and the highest value was observed for *M. aegyptia*.

The length of stomatal aperture showed little variation among species, being higher on the abaxial surfaces. Ferreira et al. (2002) noted that the longer length of the stomatal aperture of *N. physaloides* could facilitate the breaking of the surface tension of the herbicide solution with the aid of an adjuvant.

Conclusion

The herbicide saflufenacil promoted wax dispersion, roughness and plasmolysis of epidermic cell and trichomes in *L. aegyptiaca* and *M. aterrima* but not in *M. aegyptia*.

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