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# Morphological mapping of the integument of adult females of *Diaphorina citri* Kuwayama, targeting the development of control strategies

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**ABSTRACT**

***Diaphorina citri* Kuwayama is currently the most important agriculture pest for citriculture worldwide, being reported in the American and Asian continents, causing economic losses of great impact and transmitting the bacterial pathogen "Huanglongbing" (HLB, Yellow Dragon Disease), also known as "Citrus Greening". The objective of this study was to characterize morphologically the external and internal integument of the insect regions that have a greater contact area for the entomopathogenic agents: anterior dorsal region: prescutum (psc2) and scutum (sc2), and the ventral posterior segmental (Segments III and IV). Ultramorphological tools [Scanning electron microscopy (SEM)] were used for the external characterization and histological techniques to analyze the internal integument (hematoxylin/aqueous eosin) of both regions. The ultra morphological results showed the presence of wax and sensilla in the external integument, more frequently found in the anterior dorsal region in comparison with the ventral segment. The histology showed that the cuticle is thinner in the ventral segment region when compared with the anterior dorsal, being more susceptible to infections by entomopathogens.**



## INTRODUCTION

*Diaphorina citri* Kuwayama, 1908 is a pest insect of great importance for the world citriculture, once it is a vector of several pathogens. This species has deserved special attention in research for transmitting the bacteria '*Candidatus* Liberibacter americanus' and '*C.* Liberibacter asiaticus', agents of "Huanglongbing" (HLB, Yellow Dragon Disease), also known as "Citrus Greening" (Beloti et al., 2013; Pinto et al., 2012; Gottwald et al., 2007; Bové, 2006). For transmitting these diseases, *D. citri* is currently the most important citrus pest in the American and Asian continents (Burckhardt and Ouvrard, 2012; León et al., 2011; Tiwari et al., 2011; Bonani et al., 2009).

The integument of the insects is a very important mechanical barrier, protecting against desiccation and many times against predators (Gullan and Cranston, 2012; Chapman, 1998).

The integument is formed by the epidermis and the cuticle, one of the main structures for the biological success of the Order Insecta (Gullan and Cranston, 2012; Chapman, 1998) and whose main function is to form the rigid exoskeleton that separates the internal tissues of the insects from the environment in which they live. It is known that the insect cuticle is secreted by the epidermis and is constituted by: a) epicuticle, the thinner layer and b) procuticle, the thicker

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layer (Gullan and Cranston, 2012; Chapman, 1998; Binnington and Retnakaran, 1991; Hadley et al., 1986; Hepburn, 1985).

The outermost surface of an insect is covered by the epicuticle, which is subdivided into internal and external epicuticle.

The presence of wax is observed covering the external surfaces of the insects, preventing them from suffering dehydration (desiccation), once the wax contains lipid substances with hydrophobic behavior (Gullan and Cranston, 2012).

The procuticle is mainly constituted by chitin, and is also subdivided into two layers: the exocuticle (outermost and thinner) and the endocuticle (thicker and innermost) (Gullan and Cranston, 2012; Chapman, 1998; Binnington and Retnakaran, 1991; Hadley et al, 1986; Hepburn, 1985).

Ongoing research has been carried out to find new methods to control the pest insects. In addition to controlling the pests, the methods must be efficient and harmless to non-target organisms and the environment. Thus, further studies approaching the morphological characterization of insects' integument, still scarce in the literature, are needed to understand the function of this important barrier for the protection and isolation of the insect from the environment, aiming to develop new strategies to control this pest. These data will be useful in the development of biological products, mainly using entomopathogenic fungi, which act penetrating the integument (Alves, 1998).

Considering all the exposed, this study had the objective to map the morphology of the integument in *D. citri* adult females, currently considered the most important citrus pest, aiming to identify the regions with greater susceptibility to the adhesion and penetration of entomopathogenic fungi, once this information can support other studies on the biological control of this pest.

## MATERIALS AND METHODS

### Collection of the adult females

Male and female *D. citri* adults were collected from the Laboratory of Pathology and Insect Microbial Control of ESALQ/USP/ Piracicaba SP, Brazil, and maintained in climatized room at 25±2°C; 65-80% HR and 12 h photophase. Murta *Murraya paniculata* (L.) JACK (Rutaceae) plants were kept in 60 × 60 × 50 cm steel cages covered with anti-aphid screen. Ten plants were infested with 30 adult *D. citri* couples for the oviposition of the females. After 6 days, all the insects were collected from the plants and to perform sexing and the separation of 30 adult females with 1 day of age to be used in this study.

### Scanning electron microscopy (SEM)

Ten *D. citri* female adults were anesthetized through thermal shock (2 min in fridge at 4°C**)** and fixed in paraformaldehyde 4% for 48 h, at 4°C. After, the females were dehydrated in crescent series of acetone (70, 80, 90, 95 and twice in 100%, for 10 min each bath). After drying in critical point, they were mounted in steel stubs with double-sided adhesive tape to be coated with gold in sputtering. The specimens were then analyzed and photographed using scanning electron microscope Hitachi TM3000 (Hitachi Higt-Technologies Corporation/ Japan) in the Laboratory of Microscopy of the Biology Department, Biosciences Institute, UNESP Rio Claro, SP, Brazil.

### Histology

Ten adult *D. citri* females were collected and anesthetized through thermal shock in refrigerator to be posteriorly fixed in paraformaldehyde 4% for 48 h at 4$^{o}$C. Then, the material was dehydrated in crescent series of alcohol (70, 80, 90 and 95%), for 1 h each bath, transferred to Leica embedding resin (Leica Micro systems/Germany), included and sectioned with a microtome Leica RM2255 in 3 µm-thick sections.

The sections were collected on glass slides and processed according to the Harris hematoxylin/aqueous eosin staining technique (Junqueira and Junqueira, 1983). For this, the sections were rehydrated in distilled water for 1 min, stained with hematoxylin for 10 minutes and washed in tap water. The sections were then stained with eosin for 10 minutes, washed, and dried at room temperature. The final slide mounting was performed in Canada balsam. After dried in incubator at 37°C the sections were analyzed and photographed with photomicroscope Leica DM4000 (Leica Microsystems/ Germany).

### Morphology

Schemes were elaborated from the median histological sections obtained to better illustrate the position and the composition of each integument layer.

All the results obtained here were described based on the terminology proposed by Ouvrard et al. (2002, 2008) and, Gullan and Cranston (2012), once the former had previously analyzed the external morphology of several species, including insects of the same superfamily (Psylloidae) studied here, and the latter performed the characterization of the insect's integument. Studies by Chapman, (1998), who has also studied and characterized the integument of several insects, were used for this description.

## RESULTS

### Ultramorphology

#### *Dorsal region*

The present study used scanning electron microscopy technique to focus on the mesothorax, more precisely the prescutum (psc2) and scutum (sc2), regions (Figures 1 A e B), once , these regions have the largest area of contact with the environment and are consequently more susceptible to the adhesion of conidia fungi. They present external surfaces with very similar morphologies; that is., the presence of sculptures on the surface and specialized structures, such as achanti and sensilla (especially trichoid).

The sensilla, with sensitive function, are approximately 15 µm long and are observed at intervals of 16 µm (Figure 1B 1). It is important to highlight that these regions show large amounts of wax, produced by specialized epidermal glands and secreted to the surface of the insect via integument pores (channels) distributed by the insect body (Figure 1 B1).

#### *Ventral region*

In the ventral region (abdominal), mainly in the segments III and IV, achanti are not observed; however, the external surface is heterogeneous, with wax deposition, and trichoid sensilla. The latter are 50% less frequently/µm$^2$ observed and are distributed throughout the surface at approximately 30 µm intervals, with similar length (15 µm) to those found in the dorsal region (Figures 2A-C-C1).

### Histology

The histological analysis of the dorsal region (Figure 1C-E-E1) showed that the integument, specifically the prescutum (psc2) (Figure 1C), is constituted by: a) epicuticle (outermost); b) procuticle (only the exocuticle was observed); c) region of cuticle formation and d) epidermis (innermost). In the region where the scutum (sc2) (Figure 1E-E1) is found, and in the ventral (abdominal) region the following layers are observed: a) epicuticle (outermost); b) procuticle (subdivided in exo and endocuticle) and c) the epidermis (innermost).

For a better understanding of the histology, the layers are described from the outermost to the innermost region of the integument, and the descriptions correspond to the layers that show morphologic differences. These differences are mainly observed in the sclerotized layer (exocuticle) and epidermal cell profile (epidermis) of the regions studied here.

The dorsal region of the prescutum (psc2) has the thickest sclerotized layer (Figure 1 C), followed by the scutum (sc2) (Figsure 1 E and E1) and the ventral region (segments III and IV) (Figure 2 D). Regarding the epidermal cell profile (epidermis), there is similarity between the prescutum (psc2) layers (Figure 1 C) with the segmental ventral region (Figure 2 D), where the cell morphology is cubic, while the dorsal region of the scutum (sc2) displays cells with pavementous shape (Figsure 1 E-E1).

#### *Epicuticle (ep)*

The outermost and thinnest layer, observed as a thin and dark line (black arrow in the Figures 1C, and D and 2 D and E).

#### *Procuticle*

This layer is subdivided into two other layers, named exo (exo-outermost) and endocuticle (endo-innermost) (Figure 1 E-E1 and F-F1). The exocuticle observed here displays brownish color and is approximately 5 µm thick in the region of the (sc2); 3 µm thick in the region of the (psc2) and 1 µm thick in the ventral region (Figures 1 E-E1 and F; Figures 2 D and E).

The sclerotization in the ventral region is less intense in comparison with the dorsal region. It is important to highlight that, in the (sc2) region, it was not possible to distinguish the exo layers from the endocuticle ones, once the insects were completely sclerotized (Figure 1C and D).

Innermost layer of the procuticle, weakly stained by hematoxylin and eosin in comparison with the exocuticle (Figure 1 E-E1 and F-F1; Figure 2 D and E). This layer of the (psc2) dorsal region was not easily observed, probably due to the previous sclerotization, which did not allow it to be distinguished from the exocuticle (Figure 1 C and D).

#### *Epidermis*

This layer is formed or by a cube-shaped or by a pavementous one which constituting a monolayed cubic or pavementous epithelia, respectively. The cell limits were not observed. These cells constitute a simple epithelium, named pavementous and cubic respectively. The cubic epithelium was observed in the prescutum (psc2) dorsal region, where the cells present rounded nuclei (n) with decondensed chromatin, located in the central region of the cell (Figure 1C and D). This morphology was also found in the ventral region (Figure 2 D and E). It was also observed that the epidermis

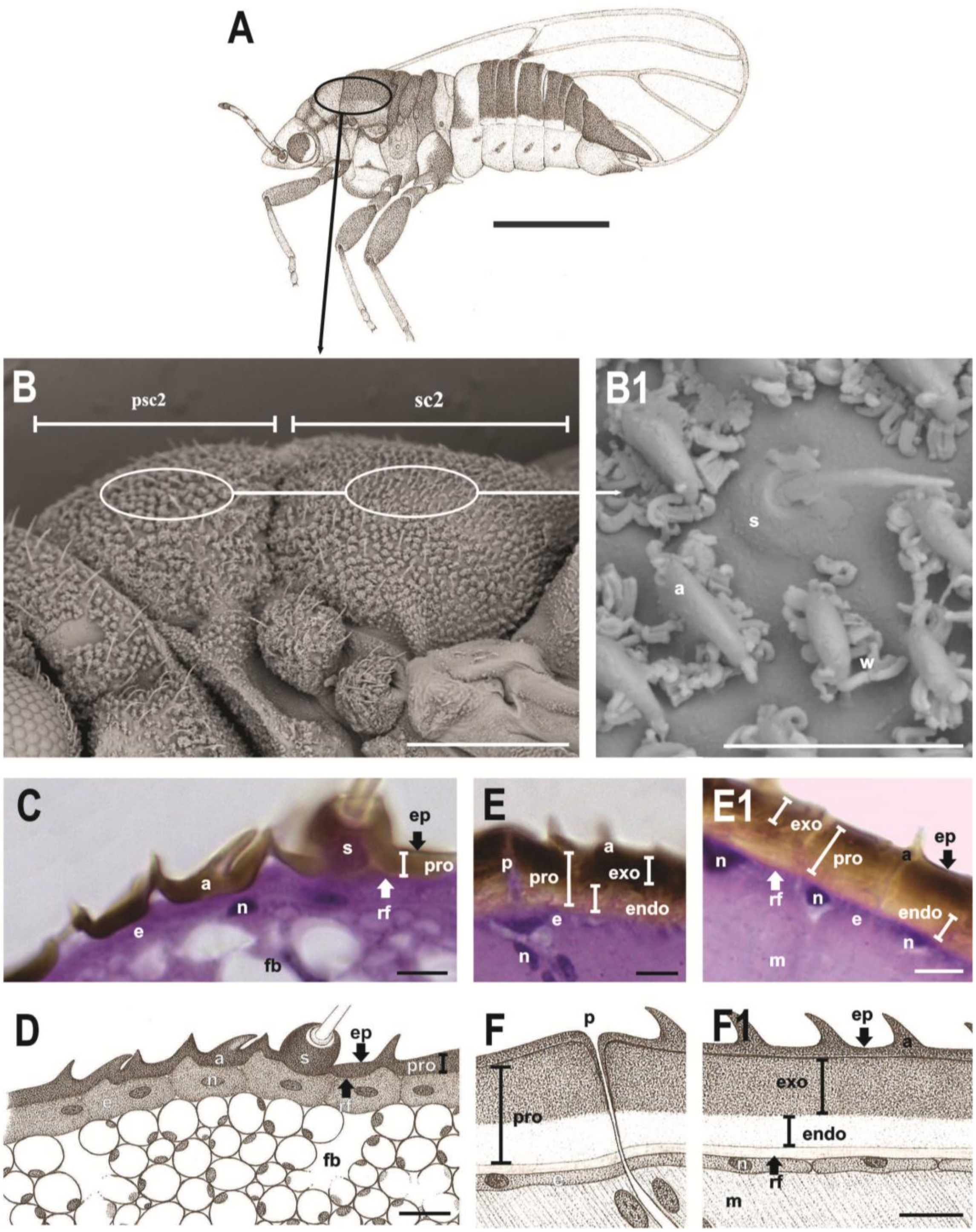


**Figure 1.** A, Schematic representation *Diaphorina citri* female adults; B-B1, Photomicrographs (SEM) showing details of the external surface of the anterior dorsal region. Specifically in B1, details of the trichoid sensilla, acanthi (a) and wax droplets (w) deposited on the cuticle can be observed; C-E-E1, Median histological sections of the anterior dorsal region stained with HE, showing the cell and tissue organization of the prescutum (psc2) (C) and scutum (sc2) (E-E1); D and F-F1, Schematic representation of the prescutum (psc2) (D) and scutum (sc2), (F-F1) integument layers, obtained from the observation of median histological sections of the respective regions. a: cuticle specialization (acanthus).

ep: Epicuticle; pro: procuticle; exo: exocuticle; endo: endocuticle; e: epidermis; w: wax droplets; n: nucleus; fb: fat body; m: muscle; p: pore; s: sensilla.

Scale: A, 1 mm; B, 500 µm; B1, 15 µm; C-F-F1, 5 µm.

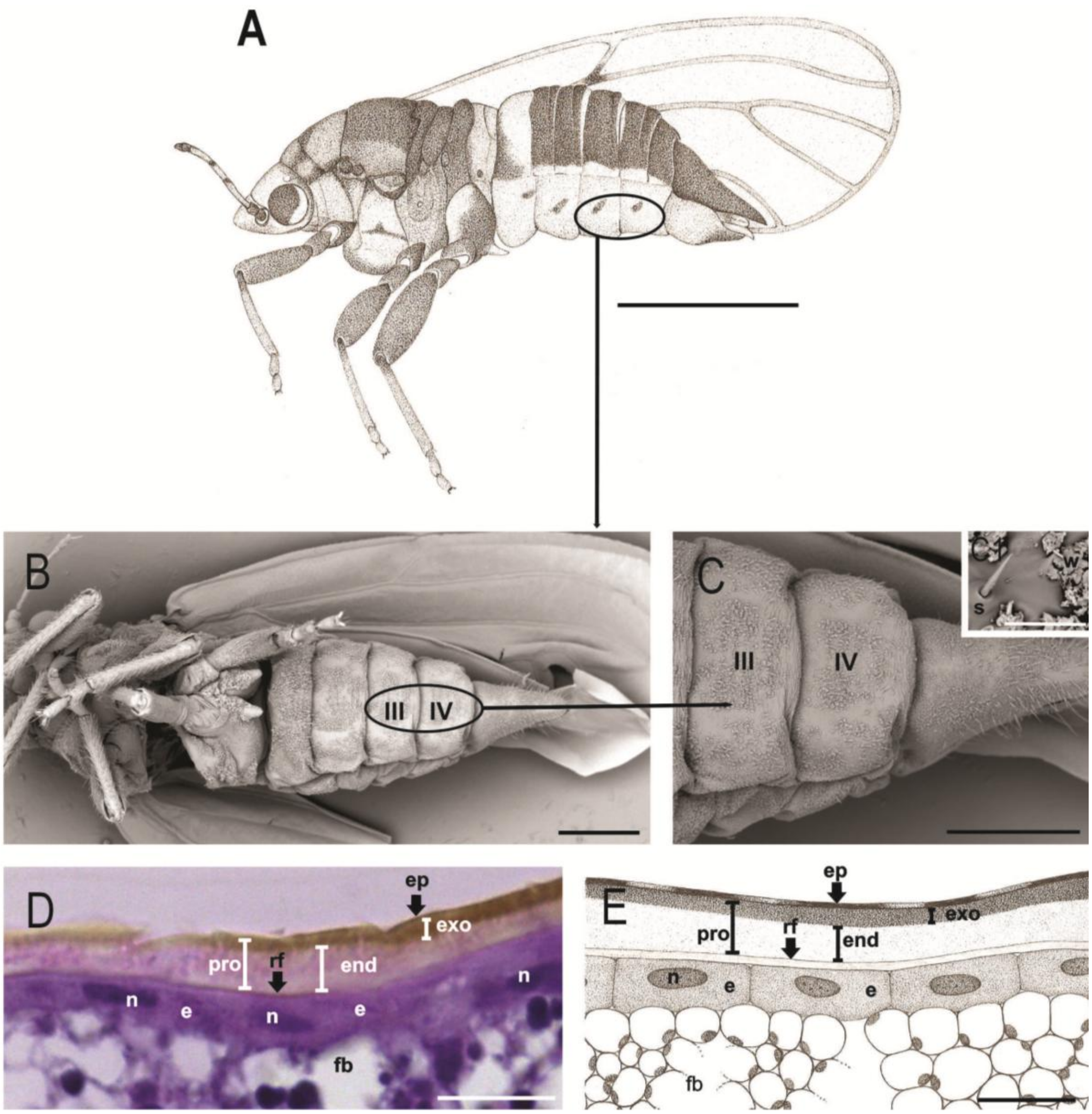


**Figure 2.** A, Schematic representation of *Diaphorina citri* female adults; B-C, photomicrographs (SEM) of the posterior ventral region of the insect. Specifically in C-C1, note the details of the integument surface showing the irregular distribution of the trichoid sensilla, as well as the rare presence of wax droplets; D, median histological sections of the posterior ventral region of the insect, stained with HE, showing the cell and tissue organization of the integument in the segments (III-IV); E: schematic representation of the integument layers (III-IV) obtained from the observation of the median histological sections.
ep: Epicuticle; pro: procuticle; exo: exocuticle; endo: endocuticle; e: epidermis; w: wax droplets; n: nucleus; fb: fat body; m: muscle; s: sensilla.
Scale: A: 1 mm; B-C:250 µm;C1:15 µm D and E: 5 µm.

described previously is supported by a thick layer of adipocytes (fb) (Figures 1 C e D; Figures 2 D and E).

Epidermis with pavementous cells was observed in the scutum (sc2) dorsal region (Figures 1 E-E1 and F-F1). Specifically in this region, this layer is immediately subjacent to the cuticle formation region (rf) and the latter is supported by a muscle layer (m) of the thorax (Figures 1E-E1 and F-F1). Still in this region, the epidermis is very thin, with cells presenting flat nuclei (n). In addition, the cuticle shows interruptions in the scutum (sc2), corresponding to openings (channels) of dermal exocrine glands, where the secretion produced (wax) is released to the exterior of the body. For a better understanding of the results, data are summarized in Table 1.

**Table 1.** Summary of the morphological and histological results obtained from the analysis of the integument in *D. citri* female adults.

| | **Dorsal region** | **Ventral region (III and IV segments)** |
|---|---|---|
| Sculptures and cuticular specializations | Acanthi and Trichoid Sensilla | Trichoid Sensilla (fewer) |
| Presence of wax | Large amount | Small amount |
| Epicuticle | psc2 / sc2: Present | Present |
| Exocuticle | sc2: distinct endo and exo | Present |
| Endocuticle | psc2: fused exo and endo | Present |
| Cuticle formation region | psc2 / sc2: Present | Present |
| Epidermis | psc2: simple cubic epithelium and sc2 simple pavementous epithelium | Simple cubic epithelium |
| Pore channels | | |
| psc2 | Absent | Absent |
| sc2 | Present | Absent |

## DISCUSSION

The insect *D. citri* belongs to the superfamily Sternorrhyncha, which comprises approximately 3890 species already described (Burckhardt and Ouvrard, 2012; Li, 2011). Several studies focusing on this insect have been carried out, especially concerning the development of control strategies (Conceschi et al., 2016; Orduño-Cruz et al., 2016; Hoy et al., 2010). However, little is known about the integument of the insects, their primary defense barrier. In this sense, morphological studies aiming to better understand the internal and external organization and the biology of these insects have been little developed, mainly using adult individuals, the phase of dispersion of the insect, and consequently the phase of disease dissemination. In general, the adult individuals are more resistant to chemical and biological control agents. The recognition of the morphological constitution of the organisms provides indispensable data, bringing new perspectives in the search of strategies to control pest insects. Studies developed on *D. citri* have usually focused on the body regions considered the most important for the survival of the insect: the anterior region, comprising the head, and the abdomen, where the reproductive systems, responsible for the generation of new individuals, are located (Dossi and Cônsoli, 2014, 2010; Garzo et al., 2012). In this species, despite being relatively large when compared with the rest of the body, the thoracic and abdominal regions have been neglected in terms of morphology (Drohojowska et al., 2013).

Regarding the insects considered economic pests, it is known that their integument is an important barrier against the action of entomopathogens, whose infections can lead the insect to death (Small and Bidochka, 2005; Alves, 1998; St. Leger et al., 1986a, 1986b). Therefore, considering the great economic importance of this insect, a citriculture pest, understanding and morphohistologically characterizing its integument, the first barrier to allow its survival, will provide relevant information for the comprehension of its biology and consequently allow the identification of the most susceptible regions concerning the control of these individuals. With specific regard to biological control, this knowledge will help to map the regions of the insect's body that are more vulnerable to infections by entomopathogenic agents.

This study showed that the integument of *D. citri* present dorsal scutum (sc2), and prescutum (psc2), with less susceptibility to these infections when compared with the ventral region due to the heavy sclerotization and thickening of the cuticle in the dorsal regions in comparison with the ventral one (segments). The latter is potentially more susceptible to infections, once its external surface is less sculptured, with fewer sensilla and consequently a greater surface for contamination and penetration of microorganisms. Despite showing intense sclerotization, the scutum (sc2), presents several pore channels, structures that correspond to natural glandular openings, which could be an entrance door for pathogens, via conidia. On the other hand, the production and accumulation of wax could prevent the adhesion of conidia due to the presence of fungistatic and fungicide compounds in its composition, decreasing germination. Thus, the first information obtained here demonstrated that the *D. citri* female integument region was less susceptible to infection in comparison with the prescutum (psc2) (dorsal), probably due to the intense sclerotization, corroborating Hajek and St Leger (1994), Hassan and Charnley (1989) who stablished that the level of sclerotization and consequent rigidity of the integument would directly affect the virulence of the entomopathogenic fungus.

According to information available in the literature, the external surface of the insects in general displays some cuticular specializations, such as: hairs, sensilla, achanti, multicellular thorn bristles and microtrichia. Moreover, the cuticle surface itself shows irregularities (drawings), named sculptures (Richards and Richards, 1979) with the most varied morphologies, which consequently leads each insect to have a more or a less specialized exoskeleton. In addition to these structures and the sculpturation, deposition of wax covering the external surface of many insects is observed, which occurs via wax release through channels and/or pores, from the epidermal gland that produces this substance towards the surface of the insect (Gullan and Cranston, 2012).

With specific regard to *D. citri,* this morphohistological study also allowed the observation of integument specializations such as trichoid sensilla and achanti, both more frequently found in the prescutum (psc2) and in the scutum (sc2), (dorsal region), and less frequently found in the segments of ventral region. Regarding the sensilla physiology, according to Kristoffersen et al. (2006) and Chapman (1998), these would be mechanosensory structures. Therefore, in addition to the epidermal cells, the sensilla would also be composed by cells with nervous activity (neurons) which would allow the insect to detect variations, such as temperature and humidity (Onagbola et al., 2008); while the acanthi would provide physical protection to the insect when exposed to environmental variations (Richards and Richards,1979).

It was observed that the surface covering of adult *D. citri* is constituted by the deposition of wax both on the dorsal and ventral regions. This wax of lipid nature is mainly constituted by hydrocarbonates, and has the function to reduce the cuticle permeability, protecting the insect against desiccation and avoid the entrance of toxins and pathogens, an efficient physical barrier according to Gibbs and Rajpurohit (2010). The hydrophobicity of the waxy layer would certainly reduce the adhesion of chemical and biological products via aqueous application. Further investigation on tensoactives to increase the wettability of these regions is needed to increase the efficiency of pesticides.

Other ultramorphologic studies have been carried out on insects belonging to the superfamily Psylloidae*,* and among them is the study by Drohojowska et al. (2013). However, these authors, contrarily to this study, did not report the presence of cuticular specializations; therefore, these structures are being described and characterized here for the first time in the *D. citri* species.

In a broad sense, the results obtained in this study indicate that the different integument regions of the psyllid *D. citri* females body are more vulnerable to contamination and infection by entomopathogenic agents. Therefore, the mesoscutum dorsal (m) and the ventral region (segments) were considered regions of easy penetration of external elements, due to the larger available surface in comparison with the rest of the insect body, offering a larger contact area for the adhesion of conidia. Furthermore, the presence of cuticular specializations (sensilla, sculpture and wax), mainly in the dorsal region, could facilitate the anchoring of conidia. Regarding the ventral region, the morphohistological characteristics of the integument showed that it is thinner and less rigid in comparison with the dorsal region, an organization that would certainly facilitate the penetration of entomopathogens.

In conclusion, this study brought the first information on the ultramorphology and histology of the dorsal and ventral integument of *D. citri*, an important agriculture pest, providing relevant data for further studies aiming at the development of more efficient biological control strategies.

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