

Interactions of adjuvants on adhesion and germination of *Isaria fumosorosea* on adults of *Diaphorina citri*

André Arnosti¹, Italo Delalibera Junior², Marcos Roberto Conceschi², Celeste Paola D'Alessandro², Raphael Vacchi Travaglini³, Maria Izabel Camargo-Mathias^{1*}

¹Universidade Estadual Paulista "Júlio de Mesquita Filho"/IB – Depto. de Biologia, Av. 24-A, 1515, C.P. 199 – 13506-900 – Rio Claro, SP – Brasil.

²Universidade de São Paulo/ESALQ – Depto. de Entomologia e Acarologia, Av. Pádua Dias, 11 – 13418-900 – Piracicaba, SP – Brasil.

³Universidade Estadual Paulista "Júlio de Mesquita Filho"/FCA – Depto. de Proteção Vegetal, Rua José Barbosa de Barros, 1780 C. P. 237 – 18610-307 – Botucatu, SP – Brasil.

*Corresponding Author <micm@rc.unesp.br>

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ABSTRACT: Asian citrus psyllid, *Diaphorina citri*, is considered the most important citrus pest worldwide, as it transmits Huanglongbing – serious citrus disease. New efficient and sustainable strategies to control this pest have been investigated and the use of entomopathogenic fungi has become a promising alternative. The objective of this study was to evaluate the effects of adjuvants a) Tween 80 at 0.01 % (v/v); b) Silwet L77 at 0.025 % (v/v) and c) KBRAJ at 0.075 % (v/v) on adhesion, germination and pathogenicity of *Isaria fumosorosea* ESALQ-1296 (5×10^6 conidia mL⁻¹). Female adults of *D. citri* used in this experiment were sprayed on *Citrus limonia* seedlings. The sprayed insects were analyzed through scanning electron microscopy (SEM) to identify the most susceptible integument regions for fungus attachment and the effect of adjuvants used. In the pathogenicity test, adjuvants Silwet L77 and KBRAJ presented a higher efficiency than Tween 80. Fungi adhered predominantly to the ventral posterior (abdomen) region in comparison with the dorsal anterior (thorax) region. In addition, adjuvants Silwet L77 and KBRAJ presented faster germination (< 48 h) of *I. fumosorosea* spores when compared to Tween 80 (> 72 h). Conidial germination in the dorsal part of the thorax of the insects was observed only with adjuvant KBRAJ 72 h post inoculation.

Keywords: biological control, Asian citrus psyllid, entomopathogenic fungi, formulation, integument

Introduction

Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is considered the most important citrus pest worldwide due to the transmission of bacteria '*Candidatus Liberibacter americanus*' and '*Candidatus Liberibacter asiaticus*', causal agents of citrus Greening disease or Huanglongbing (HLB) (Bové, 2006; Chu et al., 2016). HLB causes yield losses and poor fruit quality, resulting in a significant economic impact in citrus growing regions, especially in American and Asian continents (Kuchment, 2013; Teixeira et al., 2005; Tiwari et al., 2011; Spreen and Zansler, 2016). In Brazil, HLB has spread quickly throughout citrus groves in the states of São Paulo and Minas Gerais and there are currently 32 million trees with the disease symptoms (Fundecitrus, 2017).

To avoid economic damage due to HLB, farmers rely solely on chemical insecticides to control *D. citri* (Bové, 2006). However, the use of these products affects negatively the environment and induces resistance to insecticides (Tiwari et al., 2011). Entomopathogenic fungi have been reported as promising candidates to control *D. citri* (Meyer et al., 2007; Subandiyah et al., 2000; Stauderman et al., 2012). These fungi can be easily mass-reared *in vitro* and are capable of infecting all life stages of the insect pest by penetrating the host cuticle. The first step in fungi infection corresponds to the adhesion of reproductive structures (conidia) to insect cuticle, which occurs through physical and chemical mechanisms between the pathogen and the host insect (Hajek and Eastburn, 2003; Holder and Keyhani, 2005; Travaglini et al., 2016;

Vestergaard et al., 1999). In this sense, the use of chemical adjuvants in fungal formulations is an alternative to improve contact and adhesion of conidia to the external region of the insect. This strategy improves "spreading" and/or "wetting" in the target region, facilitating adhesion of entomopathogenic fungi conidia to the insect cuticle (Cowles et al., 2000; Tipping et al., 2003). Ausique et al. (2017) and Conceschi et al. (2016) have carried out several experiments to select fungal candidates against adults of *D. citri*. Fungal strains were screened in the laboratory and *I. fumosorosea* ESALQ-1296 was selected as the most virulent strain. These studies demonstrated that efficacy of the entomopathogenic fungi depended on the adjuvants used. Field trials were performed to test the efficacy of this strain in commercial citrus orchards during one year and the results showed that *I. fumosorosea* ESALQ-1296 was as effective as the chemical insecticides in most trials. These studies showed that the potential of *I. fumosorosea* ESALQ-1296 supported the development of a new biopesticide registered in Brazil by Koppert Sistemas Biológicos LTDA (Challenger®). In this study, we investigated how adjuvants can affect the fungus performance by improving adhesion, germination and penetration on adults of *D. citri*.

Materials and Methods

Isaria fumosorosea conidial suspensions

Entomopathogenic fungus *I. fumosorosea* ESALQ-1296 was obtained from adults of whitefly *Bemisia tabaci* biotype B (Hemiptera: Aleyrodidae) collected in

Jaboticabal, São Paulo, in 2001. This strain was identified using the elongation factor 1- α (EF1- α) and the internal transcribed spacer (ITS) regions ITS1 and ITS2 (ITS1-5.8S-ITS2) sequences, according to D'Alessandro et al. (2014). These sequences were compared to GenBank sequence data for genus *Isaria*, and ESALQ-1296 strains were identified as *I. fumosorosea* (unpublished data). Strain ESALQ-1296 was deposited in the entomopathogen collection of Laboratório de Patologia e Controle Microbiano de Insetos, Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Universidade de São Paulo (USP), Piracicaba, São Paulo, Brazil. *I. fumosorosea* ESALQ-1296 was cultivated in 9 cm diameter Petri dishes containing potato dextrose agar (PDA; Difco®) and kept in incubator ($26 \pm 1^\circ \text{C}$, 12 h photophase) for ten days. To prepare the fungal suspensions, conidia were harvested from the culture with a sterilized spatula. Three dilutions in distilled water were performed and the conidia concentration was adjusted to 5×10^6 conidia mL^{-1} , according to Ausique et al., 2017. Fungal suspensions were separately homogenized with the following adjuvants: Tween 80 (Composition: Polysorbate 80) (Oxiten®[®], Brazil) at 0.01 % (v/v), used as control because it is not toxic to the insect; Silwet L77 (Momentive Performance Materials Indústria de Silicones LTDA®, Brazil) at 0.025 % (v/v) and KBRAdj (unregistered composition) at 0.075 % (v/v). Concentrations of adjuvants were selected based on Mascarin et al., 2014, Ausique et al., 2017 and recommendations of the manufacturer.

Insect collection

Adults of *D. citri* were reared on *Murraya paniculata* (L.) Jack (Rutaceae) plants in steel cages (60 cm length \times 60 cm width \times 50 cm depth) and covered with voile fabric (sheer net-like fabric) to allow aeration, according to Nava et al., 2007. Ten to 15 day-old adults of *D. citri* were used for the bioassays and histological analyses.

Pathogenicity tests

Experiments on adults of *D. citri* were performed on 10-cm seedlings of *C. limonia* Osbeck cultivated in black plastic tubes (20 cm height \times 1.5 cm diameter and 50 mL volume) containing potting mix substrate (pine bark and peat). For each replicate, 20 adults per plant were confined in a transparent plastic cup (14 cm height \times 7 cm diameter and 500 mL volume) with lateral square openings (11 cm height \times 5 cm length) covered with voile fabric (sheer net-like fabric with size of 0.68 mm height \times 0.76 mm length) to allow aeration. Treatments were performed with distilled water (control), each adjuvant alone (Tween 80, KRAAdj and Silwet L77) and *I. fumosorosea* ESALQ-1296 conidia suspensions (*If*) with each adjuvant (*If* + Tween 80, *If* + KBRAdj and *If* + Silwet L77). Each seedling containing 20 adults of *D. citri* was typically sprayed with 400 μL of each treatment using a handheld airbrush sprayer (SW-168 model, Pneumatic SAGYMA Tools®). This volume provided a uniform coverage of seedlings without runoff. After spraying, plants were kept in a room with

conditions controlled at $25 \pm 2^\circ \text{C}$, 60-80 % relative humidity (RH) and 12 h of photophase. Adult mortality was assessed daily over a 10-day incubation period. Dead insects were transferred to a humid chamber with 24-well cell culture plates (EASYPATH, Brazil) containing cotton moistened with sterile distilled water to allow the development of the external mycelium and fungus sporulation. Fungal sporulation on the cadavers was confirmed after five days. The assay followed a randomized experimental design with three replicates per treatment, and the whole experiment was conducted independently three times at different dates. Data on daily adult mortality were analyzed with the nonparametric Kaplan-Meier estimator at 5 % level. Survival curves were compared through the Log-Rank test, considering 5 % probability level. These analyses were performed using the IBM SPSS Statistics 22 software (2013).

Scanning Electron Microscopy (SEM) Analysis

Adults of *D. citri* analyzed under SEM were anesthetized with CO_2 before spraying. Each adjuvant was sprayed alone as control to evaluate effects on the *D. citri* cuticle. Insects (30 females per treatment) were sprayed with fungal suspensions and each adjuvant in a Potter tower at 15 PSI (Pound-force per square inch). Later, the females of *D. citri* treated were placed on lime seedlings (*Citrus limonia*) and kept in room in controlled conditions at $25 \pm 2^\circ \text{C}$, 70 ± 10 % relative humidity and 12 h of photophase. At intervals of 24, 48 and 72 h, ten insects of each treatment were anesthetized at 4°C for 2 min in the fridge and fixed in paraformaldehyde 4 % at 4°C for 48 h. After, the females were dehydrated in crescent series of acetone (70, 80, 90, 95 and twice at 100 %, for 10 min each bath) and critical dried point. The insects were mounted on steel stubs with double-sided adhesive tape to be coated with gold in sputtering Balzers model SCD050. Only females were then analyzed and photographed using the Scanning Electron Microscope (SEM) Hitachi TM3000 (Hitachi High-Technologies Corporation/ Japan) operated at 15 kV in the Laboratório de Microscopia do Departamento de Biologia, Instituto de Biociências, UNESP, Rio Claro, SP, Brazil.

Deposition of *Isaria fumosorosea* conidia on *Diaphorina citri* adults

To estimate conidial deposition on the body of adults of *D. citri*, fungal suspensions with adjuvants were sprayed as described in section of Pathogenicity tests. Ten insects were sprayed per replicate ($n = 6$ replicates), totaling 60 insects per treatment. After drying, each replicate was transferred to 1.5 mL Eppendorf tubes containing 1 mL of sterile distilled water (0.01 % v/v Tween 80). The tubes were vortexed for 2 min to facilitate removal of conidia attached to insect bodies. Then, 100 μL from each tube was placed onto selective PDA medium (containing 0.5 g L^{-1} Cyclohexamide, 0.2 g L^{-1} Chloramphenicol, 0.5 g L^{-1} 65 % Diodine and 0.01 g L^{-1} Cristal Violet) and kept in growth chamber at $26 \pm$

1° C with 12 h of photophase for four days. The colony-forming unit (CFU) was quantified to estimate the number of conidia deposited on insect bodies. CFU data per insect were compared using the Tukey test at 5 % significance and Laercio package using statistical software "R" (R Development Core Team, 2013).

Results

Pathogenicity of *Isaria fumosorosea* ESALQ-1296 on adults of *Diaphorina citri*

The survival of *D. citri* varied between treatments ($\chi^2 = 271$; $df = 6$; $p < 0.001$) (Figure 1). Treatment of *I. fumosorosea* ESALQ-1296 + KBRAdj presented higher mortality than the other treatments (73.3 ± 6.9 % of adult mortality and 64.1 ± 7.4 % of fungal sporulation). *I. fumosorosea* ESALQ-1296 + Silwet L77 caused 59.0 ± 6.8 % of mortality and 54.8 ± 7.5 % of sporulation, followed by *I. fumosorosea* ESALQ-1296 + Tween 80 with 46.8 ± 12.2 % of adult mortality and 27.5 ± 10.3 % of fungal sporulation. Mortality caused by KBRAdj and Silwet L77 alone did not differ statistically with *I. fumosorosea* ESALQ-1296 + Tween 80 resulting in 37.0 ± 11.2 % and 37.0 ± 11.2 %, respectively. Mortality caused by Tween 80 alone and water (control) were lower than 16.0 ± 10.7 % and did not differ statistically.

Effect of adjuvants on *D. citri* cuticle

The ultramorphological analysis revealed that application of adjuvants KBRAdj and Silwet L77 after 72 h caused changes to the insect cuticle (Figure 2). This was evident by the presence of more electron-dense regions (arrows). Tween 80 did not affect the cuticle structure

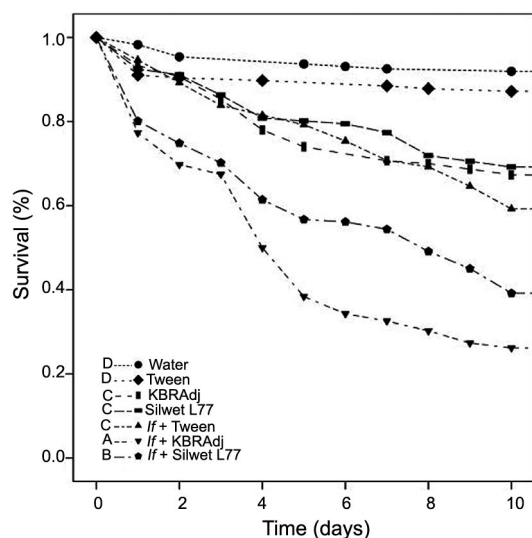


Figure 1 – Survival of adults of *Diaphorina citri* after spraying with adjuvants (Tween 80, KBRAdj and Silwet L77) alone or associated with entomopathogenic fungus *Isaria fumosorosea* (If). Water was used as control. Statistical differences in survival (Log-Rank test $p < 0.05$) are represented by uppercase letters.

(Figures 2 H-J; K-M). In the group exposed to adjuvant KBRAdj, the dorsal region remained intact (Figures 2 B-D); however, the ventral region was affected (Figures 2 Q-S). Similar effect was found for adjuvant Silwet L77 (Figures 2 N-P = ventral region and Figures 2 E-G = dorsal). Therefore, these findings clearly showed that the adjuvants evaluated in this study affected the ventral cuticle of the insects, modifying the structure, causing morphophysiological changes to this important physical barrier.

Adhesion and germination of *Isaria fumosorosea* on *D. citri* cuticle

The dorsal anterior and ventral posterior regions (Figure 2 A and Figure 3 A) displayed important differences in the infection process (adhesion and germination) of the fungus on the integument of adult females of *D. citri* with adjuvants Tween 80, Silwet L77 and KBRAdj (Figure 3).

After 24 h of exposure to fungal suspension with adjuvants Tween 80, Silwet L77 and KBRAdj, conidia adhered (black arrows) to both regions of the insect (dorsal anterior and ventral posterior); preferably next to the trichoid sensilla insertion and/or next to the wax droplets secreted by the exocrine glands (Figures 3 B, C, D, K, L and M). However, no conidial germination was observed in any region of the insect body. Regarding conidial suspension associated with KBRAdj, conidia adhesion was more significant in the ventral posterior region (Figure 3 K) than in the dorsal anterior (Figure 3B).

Observations after 24 h showed that conidia remained adhered after 48 h of exposure for the three adjuvants (Figures 3 E, F, G, N, O and P). Conidial germination was observed in the ventral posterior region for both Silwet L77 (Figure 3 O) and KBRAdj (Figure 3 N). In addition, germ tube formation was also observed for KBRAdj.

Adhered (persistent) conidia were found 72 h after conidial suspension had been applied in association with the three adjuvants. No conidia germination was observed on the dorsal anterior region for adjuvants Tween 80 (Figure 3 J) and Silwet L77 (Figure 3 I). However, this same region (dorsal anterior) showed conidial germination when the suspension was associated to KBRAdj (Figure 3 H). The ventral posterior region displayed persistent conidia; however, germination processes were not observed in association with Tween 80 (Figure 3 S). Adjuvants Silwet L77 (Figure 3 R) and KBRAdj (Figure 3 Q) favored germination; in addition, hyphae were observed penetrating the integument. These observations are summarized in Table 1.

Deposition of *Isaria fumosorosea* conidia on adults of *Diaphorina citri*

The number of conidia recovered from adults of *D. citri*, estimated by the number of colony-forming units (CFU) per insect, was higher in the treatments with KBRAdj and Tween 80 than with Silwet L77 ($F_{2,16} = 31.02$; $p < 0.0001$) (Table 2).

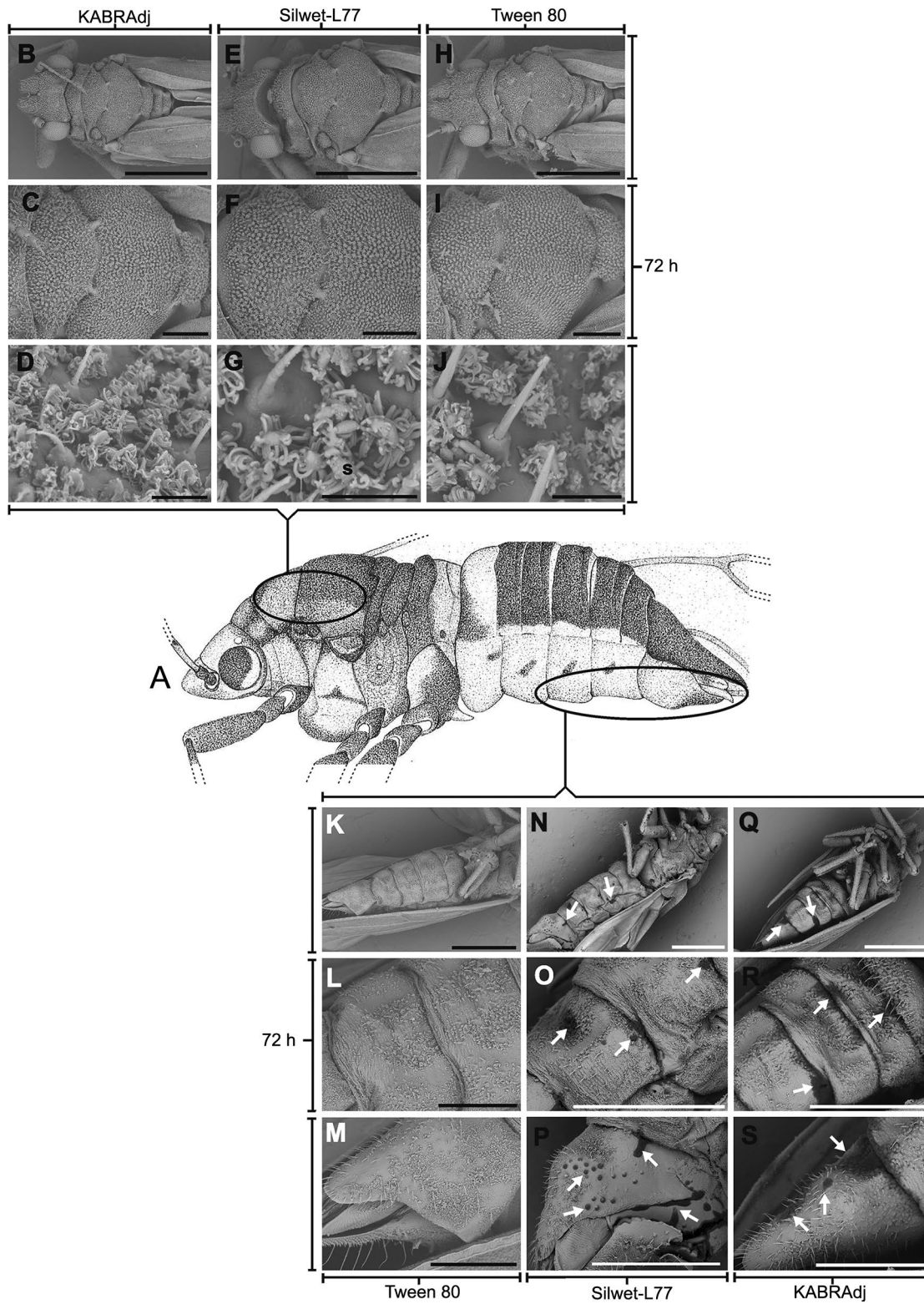


Figure 2 – A: Ultramorphology of the integument of female adults of *Diaphorina citri*. B-J: anterior dorsal and K-S: posterior ventral regions of adult females subjected to different adjuvants. The affected regions (areas of cuticle degradation), mainly the ventral region, are indicated by the white arrow. Scale Bars: B, E and H: 500 μ m; C, F and I: 250 μ m; D, G and J: 30 μ m; K, N and Q: 1000 μ m; L, O and R: 300 μ m; M, P and S: 200 μ m

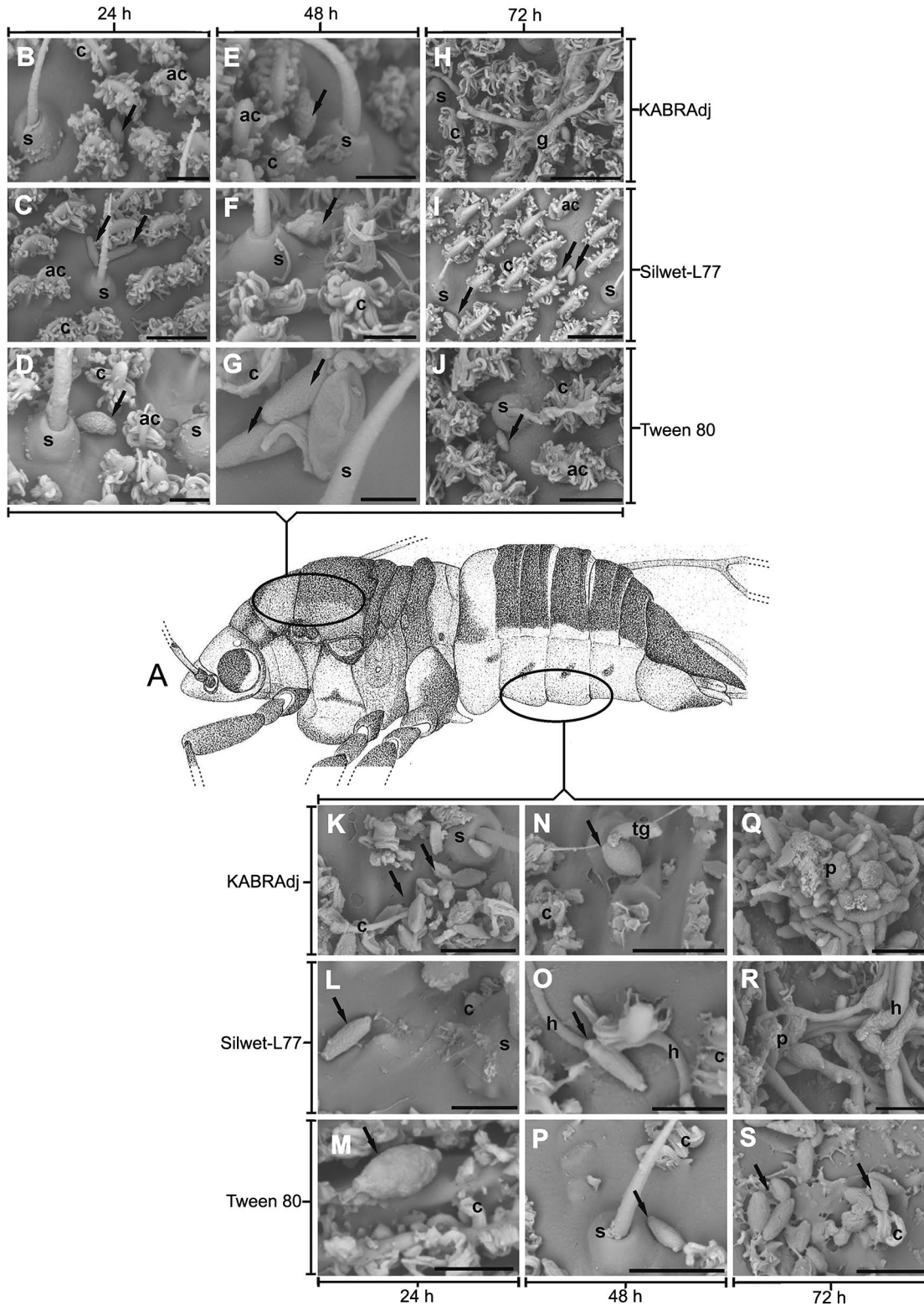


Figure 3 – Ultramorphology of the integument of female adults of *Diaphorina citri*. A: dorsal (anterior) and ventral (posterior) regions, sites of *Isaria fumosorosea* adhesion, germination. Ultramicroscopic images of the dorsal surface after 24 h (B, C and D), 48 h (E, F and G), and 72 h (H, I and J) and ventral region after 24h (K, L and M), 48 h (N, O and P) and 72h (Q, R and S) of exposure to *I. fumosorosea* conidial suspensions associated to adjuvants KABRAAdj, Silwet L77 and Tween 80. ac: acanthi; w: wax; h: hyphae; g: germination; tg: germ tube; s: trichoid sensilla; black arrows: conidia. Scale Bars: H, I and J =12.5 µm; C, K, N and P = 10 µm; Q, R and S = 7.5 µm; B, D, E, F, L, M and O = 5 µm; G = 2.5 µm.

Table 1 – Conidial adhesion and germination of *Isaria fumosorosea* ESALQ-1296 (If) influenced by body region of females of *Diaphorina citri* (dorsal anterior = DA and ventral posterior = VP) and adjuvants (Tween 80, Silwet L77 and KBRAdj).

	If + Tween 80			If + Silwet L77			If + KBRAdj		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
DA	a	a	a	a	a	a	a	a	g
VP	a	a	a	a	g	g	a	g	g

a = adhesion; g = germination.

Table 2 – Deposition of *Isaria fumosorosea* conidia on the body of adults of *Diaphorina citri* after spraying 5×10^6 conidia mL⁻¹ with adjuvants Tween 80, KBRAdj and Silwet L77.

Treatments	Mean CFU per insect* ± SE
<i>I. fumosorosea</i> + Tween 80	609.0 ± 39.0 a
<i>I. fumosorosea</i> + KBRAdj	648.0 ± 30.9 a
<i>I. fumosorosea</i> + Silwet L77	166.7 ± 13.9 b
Coefficient of variation (%)	30.9 %

*CFU = Colony-Forming Unit per insect; means followed by different letters present statistical differences by the Tukey test ($p < 0.05$).

Discussion

Our results showed that adjuvants play a major role in the control of adults of *D. citri* by fungus *I. fumosorosea*, as suggested by Ausique et al. (2017). The addition of adjuvants KBRAdj and Silwet L77 enhanced fungus efficacy. These adjuvants may interact with insect cuticle, favoring the fungal infection process. An understanding of insect integument is necessary to explain this interaction, the first barrier to fungal infection. Arnosti et al. (2016) observed the morphophysiology of *D. citri* integument to identify the most susceptible regions through which entomopathogenic fungi can penetrate the insect body. Lipid agents probably protected most integument, mainly wax, produced by the exocrine glands. Particular characteristics of the external and internal morphology, including the presence of integumentary and glandular structures, such as acanthi and trichoid sensilla (external) and cuticle thickness (internal) may affect the process of conidia adhesion and infection (Arnosti et al., 2016). The presence of these elements could hinder germination and penetration of hyphae produced through insect defense responses via integument (Holder and Keyhani, 2005; Ortiz-Urquiza and Keyhani, 2013).

Adjuvants are typically used as spreading or wetting agents to improve aqueous sprays of pesticides in agriculture (Holloway et al., 2000). Associations of adjuvants with hydrophobic conidia can enhance efficacy of entomopathogenic fungi against hemipteran insect pest (Mascarim et al., 2014; Santos et al., 2012). Different adjuvants used in formulations of entomopathogenic fungi are effective on the hydrophobic insect integument, allowing distribution and germination of conidia on the insect surface, which results in conidia penetration into the insect body (Cunha, 2009; Peng and Xia, 2011). Adjuvant Silwet L77 and KBRAdj are classified

as surfactants and are capable of increasing spreading and wetting of pesticide sprays; therefore, the addition of these adjuvants to the fungal suspension may have increased the distribution and adhesion of *I. fumosorosea* conidia to the body of adults of *D. citri*. The three adjuvants did not affect viability of conidia when mixed just before spraying (data not shown). Mascarin et al. (2014) demonstrated that Silwet L77 is a better wetting agent than Tween 80 on a paraffinic wax surface. However, the higher control efficacy of adults of *D. citri* by *I. fumosorosea* + KBRAdj and *I. fumosorosea* + Silwet L77 treatments is probably not related to the higher adhesion of conidia. In addition, when fungal suspension was associated with Tween 80, adhesion of conidia was similar to KBRAdj; however, conidia started to germinate only after 72 h.

The analysis of the external surface of insects showed that the preferred infection sites for fungus adhesion are the dorsal anterior and ventral posterior, considering that the wing protects the dorsal posterior, while the ventral anterior is highly sclerotized, as well as other parts, such as legs. However, only adjuvant KBRAdj allowed conidial germination on the dorsal anterior region of females. Adjuvants Silwet L77 and KBRAdj were more efficient when associated to the fungus *I. fumosorosea*, probably providing conditions for germination and penetration. These adjuvants affected the insect cuticle, as evidenced by the presence of more electron-dense regions, which indicates disruptions on the insect cuticle, probably by reducing lipid, wax or even substances capable of retarding or impairing fungal germinations (fungistatic properties) on the integument.

Thus, these results bring a promising perspective for the biological control of *D. citri* using entomopathogenic fungus *I. fumosorosea* associated to adjuvants, especially KBRAdj, which has been proven to optimize fungal action, probably by overcoming the physical barrier of the integument and/or creating a favorable micro-environment.

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Authors' Contributions

Conceptualization: Arnosti, A.; Camargo-Mathias, M.I.; Delalibera, Jr., I.; Data acquisition: Arnosti, A.; Travaglini, R.V.; Conceschi, M.R.; D'Alessandro, C.P.; Data Analysis: Arnosti, A.; Travaglini, R.V.; Conceschi,

M.R.; D'Alessandro, C.P.; Design of methodology: Arnosti, A.; Travaglini, R.V.; Conceschi, M.R.; D'Alessandro, C.P. Writing and editing: Arnosti, A.; D'Alessandro, C.P.; Camargo-Mathias, M.I.; Delalibera, Jr., I.

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