

Antimicrobial activity of *Stomoxys calcitrans* against *Beauveria bassiana sensu lato* isolates

Atividade antimicrobiana de *Stomoxys calcitrans* contra isolados de *Beauveria bassiana sensu lato*

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Abstract

This study had the aims of evaluating the antimicrobial characteristics of *Stomoxys calcitrans* (Diptera: Muscidae) larvae against the fungal isolates CG138, CG228 and ESALQ986 of *Beauveria bassiana sensu lato* (Balsamo-Crivelli) Vuillemin, 1912 (Hypocreales: Cordycipitaceae). *S. calcitrans* eggs, larvae and pupae were exposed to these same isolates. Statistical analysis showed that the immature stages of *S. calcitrans* were not susceptible to the fungal isolates used, regardless of the exposure method. Diffusion test on solid culture medium revealed that macerated *S. calcitrans* larvae exposed to isolate CG138 reduced CG138 fungal development. The analysis of the chromatographic profiles indicated that the macerate or mucus of larvae of the control group and the groups exposed to the isolate CG138 presented different profiles. Reduced development of the isolate CG138 on the larvae cuticle was observed by means of scanning electron microscopy.

Keywords: *Stomoxys calcitrans*, *Beauveria bassiana*, antimicrobial activity, biological control.

Resumo

Este estudo teve como objetivos avaliar as características antimicrobianas de larvas de *Stomoxys calcitrans* (Diptera: Muscidae) contra os isolados CG138, CG228 e ESALQ986 de *Beauveria bassiana sensu lato* (Balsamo-Crivelli) Vuillemin, 1912 (Hypocreales: Cordycipitaceae). Ovos, larvas e pupas de *S. calcitrans* foram expostos a estes mesmos isolados. Após análise estatística, foi verificado que os estágios imaturos de *S. calcitrans* não foram susceptíveis aos isolados utilizados, independentemente do método de exposição utilizado. O teste de difusão em meio sólido mostrou que quando o isolado CG138 foi exposto a macerado de larvas houve redução do desenvolvimento fúngico. A análise dos perfis cromatográficos mostrou que o macerado ou muco de larvas do grupo controle e dos grupos expostos ao isolado CG138 apresentaram diferenças nos perfis. Um desenvolvimento reduzido do isolado CG138 na cutícula de larvas foi observado pela microscopia eletrônica de varredura.

Palavras-Chave: *Stomoxys calcitrans*, *Beauveria bassiana*, atividade antimicrobiana, controle biológico.

Introduction

Stomoxys calcitrans (Linnaeus, 1758) is an important hematophagous fly that infests Brazilian livestock (GUIMARÃES, 1984), causing annual losses to breeders in this country estimated at US\$335.46 million (GRISI et al., 2014). This fly has been

described parasitizing many species of wild and domesticated animals, including pets, as well as humans (GUIMARÃES, 1984).

Beauveria bassiana sensu lato (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) is among the most widely studied entomopathogenic fungi (ALVES, 1998) and has been described parasitizing *S. calcitrans* adults with low prevalence (SKOVGARD & STEENBERG, 2002). Different isolates have been used to control arthropods that affect livestock (FERNANDES et al., 2006) and

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crops (ALVES, 1998). Other studies have assessed this species' genetic variability and geographic distribution (FERNANDES et al., 2006; FERNANDES et al., 2009), interaction with chemical insecticides (McCOY & TIGANO-MILANI, 1992), tolerance to stress conditions and performance (FERNANDES et al., 2007), enzymatic activity, production of toxins and action on beneficial arthropods, animals and humans (ZIMMERMANN, 2007).

A good deal of research is currently focused on pest control methods, in line with the principles of integrated pest management (CARLBERG, 1986). However, few studies have examined the use of entomopathogenic fungi for biological control (WATSON et al., 1995; SKOVGARD & STEENBERG, 2002; MORAES et al., 2008), or the antimicrobial mechanisms of the immature stages of *S. calcitrans*.

Methods such as inhibition zone tests have confirmed that the intestinal extracts of the stable fly *S. calcitrans* present antimicrobial activity (HAMILTON et al., 2002). Urbanek et al. (2012) demonstrated fungistatic activity against entomopathogenic fungi in secretions from *Forcipomyia nigra* larvae. The most widely used technique for antimicrobial peptide purification and characterization consists of their separation through reversed-phase high performance liquid chromatography (HPLC). HPLC has shown that the antimicrobial peptide identified as stomoxin, which is involved in epithelial immunity mechanisms in the gut of *S. calcitrans* adults, is expressed and secreted exclusively in this fly's foregut. Stomoxin presents a wide spectrum of activities that affect the growth of microorganisms (BOULANGER et al., 2002). Han et al. (1998) found that larvae of *Drosophila* sp. infected by entomopathogenic fungi produced peptides as a temporary adaptive immunological response. Scanning electron microscopy has been used to prove that certain fungi present inefficient parasitism on arthropods (MELO et al., 2007; URBANEK et al., 2012).

The present study used two exposures methods of immature stages of *S. calcitrans* to fungi (CG138, CG228 and ESALQ986 isolates of *B. bassiana* s.l.) in the laboratory for evaluated the antimicrobial activity. Macerates and mucus of *S. calcitrans* larvae sensitized with the fungal isolates and macerates and mucus of the larvae of the control group were used to assess the inhibition capacity of the three isolates, through the diffusion test on solid culture medium. Chromatographic profiles were obtained through HPLC to show possible differences between samples. *S. calcitrans* larvae exposed to the isolate CG138 were examined by means of scanning electron microscopy to investigate fungal adhesion and development on the cuticle and natural openings of the larvae.

Materials and Methods

Stomoxys calcitrans colony

The *S. calcitrans* adults were kept in plastic cages (60cm L × 30cm W × 30cm H) and supplied with citrated bovine blood (0.38%), while immature stages were maintained using an autoclaved rearing medium composed of a homogenized mixture of sugarcane pulp (66 g), wheat flour (25 g), sodium bicarbonate (1 g), meat meal (8 g) and distilled water (127 ml) (CHRISTMAS, 1970; MORAES, 2007).

Beauveria bassiana sensu lato

The fungal isolates were cultured on potato dextrose agar (PDA, 39 g/l) and 1% yeast extract (YE) in Petri dishes in the dark at 25 °C, 70-80% RH for 15 days (FERNANDES et al., 2009).

To prepare the fungal suspensions, isolates were removed from the Petri dishes with a scalpel, suspended and agitated (vortex, 2 min.) in sterile solution (distilled water and 0.01% Tween in distilled water). A Neubauer chamber was used to count conidia; suspensions were diluted to obtain concentrations of 2×10^8 , 10^7 , 10^6 and 10^5 conidia.ml⁻¹ (con.ml⁻¹). Conidial viability was assessed by inoculating the suspensions in Petri dishes containing PDA and YE and counting the germinated and ungerminated conidia (ALVES, 1998; FERNANDES et al., 2009).

Immature stages of *S. calcitrans* exposed to fungi

Two fungal exposure methods were used to assess mortality of *S. calcitrans* immature stages and the percentage adult emergence after larval exposure.

In the first method, 12 groups of 80 *S. calcitrans* eggs (less than 24 hours-old) were immersed for 2 minutes in suspensions of the three *B. bassiana* s.l. isolates (CG138, CG228 and ESALQ986) at concentrations of 2×10^8 , 10^7 , 10^6 or 10^5 con.ml⁻¹. The eggs were then transferred to Petri dishes lined with filter paper moistened with 3 ml of sterile distilled water (SENNA-NUNES et al., 2002) and 3 grams of autoclaved rearing medium (MORAES, 2007). The same exposure method was carried out for 12 groups of 30 *S. calcitrans* larvae, used after 10 days of development. Finally, 8 groups of 32 *S. calcitrans* pupae, used 14 days after the eggs had been laid, were immersed for 2 minutes in suspensions of the *B. bassiana* s.l. isolates CG138 and CG228 at the same fungal concentrations and placed in Petri dishes. All the dishes were sealed with Parafilm®, which was perforated with a hypodermic needle (13 mm × 0.45 mm). The same procedure was used for the control groups, for which the eggs, larvae and pupae were immersed only in a diluent (solution of distilled water and 0.01% Tween 80) (MORAES et al., 2008).

In the second method, three groups of 80 eggs were immersed for 2 minutes in 0.5 ml of the suspensions of the isolates CG138, CG228 and ESALQ986 at a concentration of 2×10^8 con.ml⁻¹. Then, the eggs and suspension were transferred to Petri dishes with the same quantity of rearing medium, but the filter paper was previously moistened with 2.5 ml of the respective fungal suspension. The same method was used for 3 groups of 30 larvae with 10 days of development. The eggs and larvae of the control group were immersed in 0.5 ml of the same diluent and transferred to Petri dishes containing filter paper moistened with 2.5 ml of sterile distilled water and rearing medium. The dishes were sealed and the Parafilm® was perforated as described for the first method (MORAES et al., 2008).

The percentage mortalities of eggs, larvae and pupae were calculated, respectively, 5, 10 and 10 days after the biological assays. The percentage adult emergence was obtained 20 days after exposure of the larvae. The mortality relating to the effect of the fungal isolates was determined by adjusting the natural

mortality of the controls using Abbott's formula (ABBOTT, 1925). The percentages of mortality and emergence were evaluated using the chi-square test (Bioestat 4.0) with 95% significance (SAMPAIO, 2002). The assays were carried out twice to obtain more reliable results.

Antimicrobial activity of Stomoxys calcitrans

Mucus and macerate of *Stomoxys calcitrans* larvae

To collect the mucus and macerate of the *S. calcitrans* larvae exposed to the *B. bassiana* s.l. isolates, groups comprising nine 9-day-old larvae were exposed to the three fungal isolates as in the second method. After three days of incubation, the larvae were washed in sterile distilled water and placed in sterile cryogenic tubes (1.2 ml) for production of mucus (1 hour). The larvae were then removed and 900 µl of chilled sterile phosphate buffer solution (PBS) was added (0.1 M; NaCl 1.5 M; pH 7.4). To obtain the macerate, the larvae were placed in sterile microcentrifuge tubes (1.5 ml), macerated with a conical stick, homogenized with 900 µl of the buffer solution and stored in sterile cryogenic tubes (1.2 ml). The macerate and mucus from the larvae of the control groups were obtained in the same way, except that the larvae were only exposed to the diluent (sterile distilled water and 0.01% Tween). The entire procedure was performed in a laminar flow cabinet. The samples were manipulated on ice and stored in an ultrafreezer (−80 °C).

Diffusion test on solid culture medium

Ten microliters of fungal suspension of the three isolates (CG138, CG228 and ESALQ986) at a concentration of 1×10^8 con.ml^{−1} were spread evenly with a sterile swab, in Petri dishes containing PDA (39 g/l), YE (1%) and chloramphenicol (500 mg/l). Similarly to the method used by Hunt (1986) and Urbanek et al. (2012), two filter paper disks (6 mm in diameter) were impregnated with chloramphenicol (500 mg/l), autoclaved (120 °C for 20 min) and dried in a Pasteur oven (100 °C) and then were immersed in the macerate of larvae that had been previously exposed to the fungal isolates or to the macerate from the control larvae. After the procedure, the disks were placed in dishes containing the fungal suspension. The same procedure was carried out with the mucus samples. These dishes were incubated for three days at 25 ± 1 °C and 70 - 80% RH. The fungal inhibition zone was ascertained on the second and third days after the start of the experiment.

High performance liquid chromatography (HPLC)

The mucus and macerate samples from the larvae of the controls and groups exposed to the fungal suspension (CG138 of *B. bassiana*) were centrifuged (using at refrigerated centrifuge at 10,000 rpm, for 10 min at 4 °C) to obtain the supernatant. The diluent and sodium phosphate buffer were also evaluated. Ten microliters of each sample were applied to a C-18 reversed-phase column (Microsorb, 250 × 4.6 mm). An isocratic system (0.1% trifluoroacetic

acid in distilled water) with a flow of 0.6 ml/min was used. The peaks were detected at 220 nm. For each sample, a maximum period of 30 minutes was needed in order to view all the peaks (BOULANGER et al., 2002).

Scanning electron microscopy

Groups comprising eight 9-day-old *S. calcitrans* larvae were exposed to a conidial suspension of *B. bassiana* s.l. isolate CG138 at 2×10^8 con.ml^{−1}, as described in the second method of exposure. The fungal viability was appraised as described by Alves (1998). The larvae were removed from the Petri dishes at 0, 2, 15, 24, 48, 72 or 96 hours after fungal exposure. They were then fixed in super skipper solution [kerosene (17 pt), glacial acetic acid (11 pt), 95% ethanol (50 pt), isobutyl alcohol (17 pt) and dioxane (5 pt)] for 30 seconds, washed by means of immersion in Carl's solution [95% ethanol (28 pt), 45% formaldehyde (11 pt), glacial acetic acid (4 pt) and H₂O (57 pt)] and maintained in this same solution for 24 hours (GRODOWITZ et al., 1982). Two larvae were used at each of the above mentioned times, to observe whether the fixation process had removed the conidia. For this, the larvae were stained with lactophenol blue cotton, before and after the fixation process, and were viewed under an optical microscope between slides (40 ×) (ALVES, 1998). Six larvae of each group were dehydrated in a graded ethanol series (15 minutes in each of 30, 50, 70, 80, 85, 90 and 95% and 3 periods in 100%), dried in a CO₂ critical-point drier and then covered with gold particles (10 milliamperes/90 seconds). Electron micrographs were produced using a JEOL JSM-JEM 1011 scanning electron microscope, with the aim of viewing the conidia, fixation and fungal penetration structures (GRODOWITZ et al., 1982; DEDAVID et al., 2007).

Untreated larvae with or without sonication were compared with larvae that had been exposed to fungus. The larvae were washed in sterile distilled water and sonicated for 3 periods of 30 seconds at 20 kHz (Ultrasonic cell disruptor - Unique R2D091109) in sterile phosphate buffer solution (PBS) (0.1 M; NaCl 1.5 M; pH 7.4) at 4 °C. Scanning electron microscopy was performed, following the same steps as for the treated larvae.

Results

Immature stages of S. calcitrans exposed to fungi

The mortality rates in the treated groups were similar to those of the control groups, for both egg exposure methods (Tables 1 and 2). In some cases, such as the isolate CG138 in the first exposure method, lower percentage mortality was observed for the treated groups in comparison with the control, irrespective of the fungal concentration used (Table 1). The same result was observed in the second exposure method, when the eggs were exposed to the three isolates at a concentration of 2×10^8 con.ml^{−1}.

In the second exposure method, when the eggs and larvae remained in contact with the fungal isolates (2×10^8 con.ml^{−1}) until the end of the experiment, the larvae ingested conidia (Figure 1a), but they did not present behavioral changes, mummification or fungal proliferation on the cuticle or through natural openings.

Table 1. Mortality of immature stages of *Stomoxys calcitrans* immersed in aqueous suspensions of three isolates of *Beauveria bassiana* s.l. using the first method of exposure.

Isolates/Concentrations		First Assay			Second Assay		
		Eggs*	Larvae*	Pupae*	Eggs*	Larvae*	Pupae*
		CM (%)	CM (%)	CM (%)	CM (%)	CM (%)	CM (%)
CG 138	Control	0	0	0	0	0	0
	2x5 ⁵ con.ml ⁻¹	-28.2	0	11.1	-18,5	12.0	4.0
	2x5 ⁶ con.ml ⁻¹	-5.3	4.5	25.9	0	31.0	-3.9
	2x5 ⁷ con.ml ⁻¹	-14.2	18.1	-7.4	3.7	32.0	0
CG 228	2x5 ⁸ con.ml ⁻¹	-3.6	4.5	11.1	7.4	28.0	4.0
	2x5 ⁵ con.ml ⁻¹	10.7	-4.5	3.7	7.4	0	20.0
	2x5 ⁶ con.ml ⁻¹	-8.9	-27.2	0	9.2	-12.0	12.0
	2x5 ⁷ con.ml ⁻¹	7.1	-13.6	3.7	-33.3	8.0	-8.0
ESALQ 986	2x5 ⁸ con.ml ⁻¹	8.9	4.5	11.1	0	20.0	-11.9
	2x5 ⁵ con.ml ⁻¹	-14.9	-3.6	-	17.6	6.7	-
	2x5 ⁶ con.ml ⁻¹	8.9	-3.6	-	7.3	3.3	-
	2x5 ⁷ con.ml ⁻¹	6.0	7.1	-	-4.4	13.3	-
	2x5 ⁸ con.ml ⁻¹	1.5	7.1	-	16.2	13.3	-

CM = Corrected mortality by Abbot's formula. - = Not evaluated. * = No statistical difference between the mortality percentages in the treated groups and control group in each immature stage of *Stomoxys calcitrans*.

Table 2. Mortality of immature stages of *Stomoxys calcitrans* immersed and maintained in aqueous suspensions of three isolates of *Beauveria bassiana* s.l. using the second method of exposure.

Isolates/Concentrations		First Assay		Second Assay	
		Eggs*	Larvae*	Eggs*	Larvae*
		CM (%)	CM (%)	CM (%)	CM (%)
Control		0	0	0	0
CG 138	2x5 ⁸ con.ml ⁻¹	-16.3	0	-21.8	-12.5
CG 228	2x5 ⁸ con.ml ⁻¹	-5.4	-11.5	-5.4	-16.7
ESALQ 986	2x5 ⁸ con.ml ⁻¹	-5.4	-7.7	0	-16.7

CM = Corrected mortality by Abbot's formula. * = No statistical difference between the mortality percentages in the treated groups and control group in each immature stage of *Stomoxys calcitrans*.

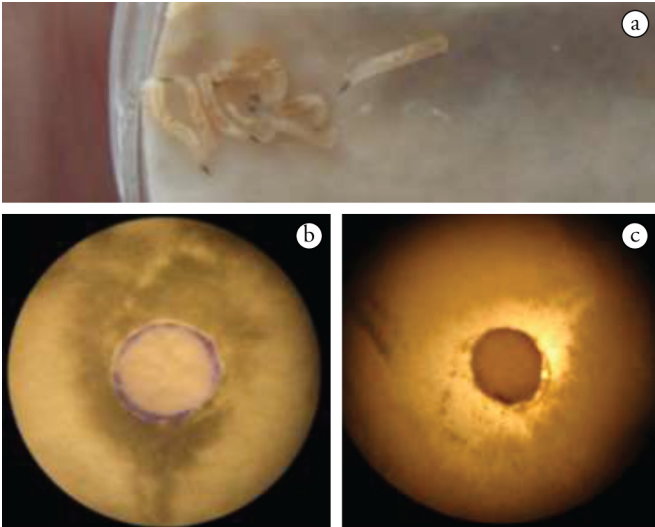


Figure 1. (a) *Stomoxys calcitrans* larvae ingesting conidia of *Beauveria bassiana* s.l. (b) and (c) Petri dish in which an inhibition zone of *B. bassiana* s.l. isolate CG138 was observed by using a macerate from larvae previously sensitized by the same fungus.

Fly emergence rates from the *S. calcitrans* larvae exposed to the two fungal exposure methods were not affected at the concentrations tested (Table 3). The three fungal isolates did not cause any deleterious effect or have any delayed action on the larvae, in either of the methods used.

Antimicrobial activity of Stomoxys calcitrans

Diffusion test on solid culture medium

The macerate and mucus from larvae of the control group did not cause the formation of an inhibition zone for the three *B. bassiana* s.l. isolates utilized (CG138, CG228 and CG986). However, the macerate of larvae previously sensitized with the isolate CG138 reduced the fungal development, leading to formation of an inhibition zone (Figures 1b, c). This was not observed with the other two isolates, where there was good fungal development around the disks moistened with mucus or macerate of larvae that had been exposed to those isolates. Moreover, no inhibition zone

was observed when the isolate CG138 was challenged with mucus from larvae previously exposed to that isolate.

High performance liquid chromatography (HPLC)

Alterations to the chromatographic profiles of the macerate and mucus of *S. calcitrans* larvae were observed after exposure to *B. bassiana* isolate CG138, thereby suggesting that the larvae had an

active response against fungal infection (Figures 2a-e). New peaks in the chromatographic patterns of *S. calcitrans* macerate were seen to have appeared, in comparison with the control group. However, the peaks observed in the chromatographic pattern of the mucus of the control group did not remain after larval exposure to the fungus, thereby indicating, respectively, production and mobilization of molecules such as antimicrobial peptide defense molecules.

Table 3. Fly emergence from *Stomoxys calcitrans* larvae exposed to aqueous suspensions of three isolates of *Beauveria bassiana* s.l. using the first and the second method of exposure.

Larval Exposure Method	Isolate	Concentrations	Emergence	
			First Assay	Second Assay
First*	CG 138	Control	63.3	60.0
		2x5 ⁵ con.ml ⁻¹	46.6	53.3
		2x5 ⁶ con.ml ⁻¹	56.6	36.6
		2x5 ⁷ con.ml ⁻¹	46.6	43.3
		2x5 ⁸ con.ml ⁻¹	63.3	43.3
	CG 228	Control	63.3	60.0
		2x5 ⁵ con.ml ⁻¹	66.6	66.6
		2x5 ⁶ con.ml ⁻¹	63.3	73.3
		2x5 ⁷ con.ml ⁻¹	63.3	60.0
		2x5 ⁸ con.ml ⁻¹	60.0	53.3
	ESALQ 986	Control	80.0	90.0
		2x5 ⁵ con.ml ⁻¹	90.0	86.6
		2x5 ⁶ con.ml ⁻¹	90.0	73.3
		2x5 ⁷ con.ml ⁻¹	83.3	73.3
		2x5 ⁸ con.ml ⁻¹	86.6	86.6
Second*	C	Control	76.6	80.0
	CG 138	2x5 ⁸ con.ml ⁻¹	83.3	76.6
	CG 228	2x5 ⁸ con.ml ⁻¹	86.6	86.6
	ESALQ 986	2x5 ⁸ con.ml ⁻¹	86.6	86.6

* = No statistical difference between the emergence percentages in the treated groups and control group of *Stomoxys calcitrans*.

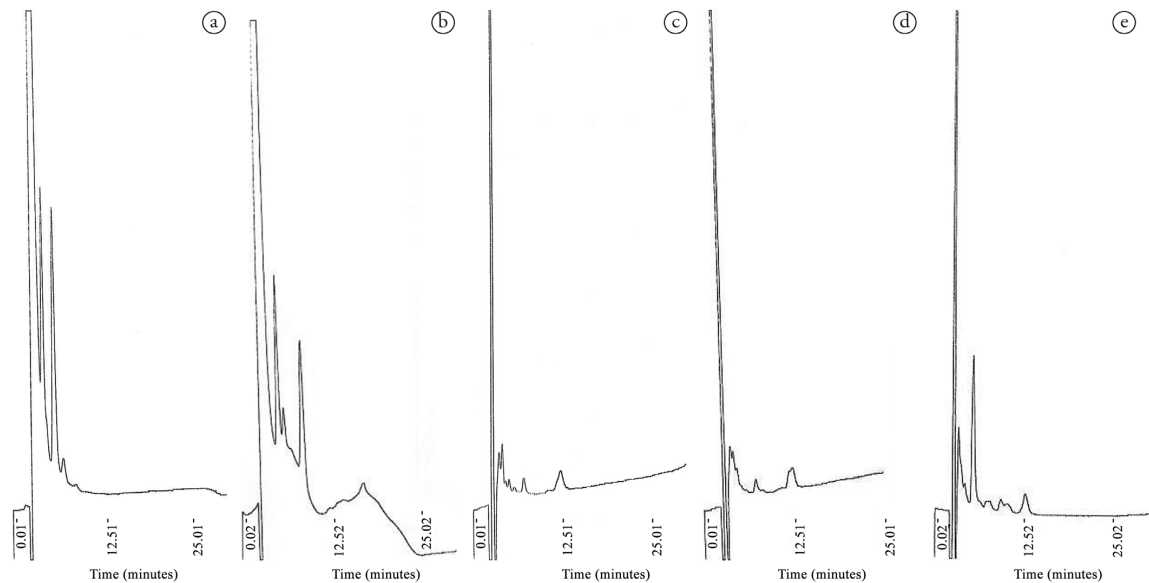


Figure 2. Chromatographic profile (HPLC) of (a) macerate of *Stomoxys calcitrans* larvae from control group. (b) macerate of *S. calcitrans* larvae sensitized with the isolate CG138 of *Beauveria bassiana*. (c) mucus from *S. calcitrans* larvae of the control group. (d) mucus from *S. calcitrans* larvae sensitized with the isolate CG138 of *B. bassiana* and (e) isolate CG138 of *B. bassiana*.

Scanning electron microscopy

The larvae were healthy and presented midgut content (consisting of rearing medium and conidia) when they were fixed with super skipper and Carl's solution (Figure 1a). These solutions did not remove conidia from the body surface of the larvae, as could be seen through optical microscopy.

No germinated conidia were observed in the natural orifices. Structures similar to germ tubes (Figure 3a), enzymatic action (Figure 3b) and appressoria were seen 24, 48, 72 and 96 hours after fungal exposure, but the colonization process was not homogenous. More than one germ tube per conidium was observed (Figure 3c) and some were very elongated, possessing appressorium (Figure 3d). Reduced hyphal development was observed on the body surface of *S. calcitrans* larvae, mainly after 72 and 96 hours.

Discussion

The results from this study demonstrated that the immature stages of *S. calcitrans* were tolerant to the isolates CG138 and CG228 of *B. bassiana* s.l. and the low mortality found with the isolate ESALQ986 confirmed what was observed with those isolates. Although this is considered to be an isolate that yields high pathogenicity among arthropods (FERNANDES et al., 2007), it was unable to cause satisfactory mortality rates. In a study on controlling the fly population that develops in bird droppings, Carlberg (1986) reported that *S. calcitrans* and *Fannia canicularis* flies were less susceptible to *Bacillus thuringiensis* than *Musca domestica* was. The tolerance of the immature stages of *S. calcitrans* to the *B. bassiana* s.l. isolates observed in the present study demonstrated that natural differences exist among fly species regarding their tolerance to infection by microorganisms. For this reason,

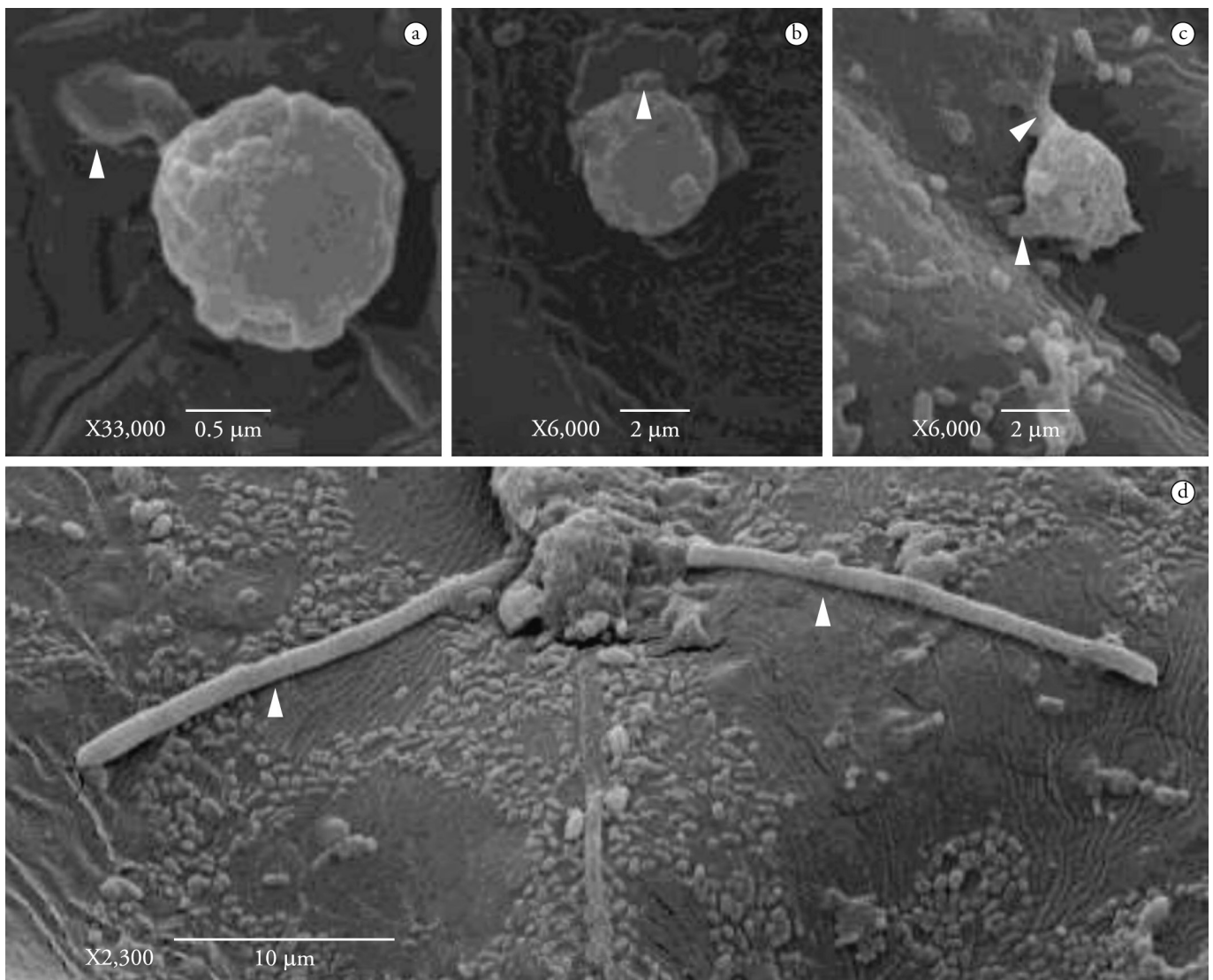


Figure 3. Scanning electron microscopy of *Stomoxys calcitrans* larvae exposed to *Beauveria bassiana* s.l. isolate CG138: (a and b) Germ tube and integument penetration (24 hours after fungal exposure) (arrow). (c) Development of more than one germ tube in one conidium (48 hours after fungal exposure) (arrows). (d) Development of more than one germ tube in one conidium (96 hours after fungal exposure) (arrows).

Watson et al. (1995) suggested that new control agents and the evolutionary mechanisms of these insects should be investigated, in order to develop effective biological control methods.

We used eggs and larvae in the second method because they are considered to be more susceptible to environmental conditions and to parasites and predators, in comparison with pupae (NEVES & FARIA, 1988). The aim of this method was to promote high fungal infestation, but no significant mortality was observed in comparison with the control groups. Instead, the percentage mortality of the eggs and larvae exposed to the isolates was actually lower. It should be pointed out that the high infestation of this method would not be found in a natural environment or in cases of fungal application in places where the immature stages of *S. calcitrans* develop, which should cause high mortality. This method was used to ascertain whether the first exposure method was influencing the results, mainly because of the use of larvae in the tests, because the intense movement and mucus production might favor removal of the conidia from the surface. Immersion was also utilized to promote intense contact and favor ingestion of the conidia.

The results from the second egg exposure method differed from those reported by Moraes et al. (2008), using *Metarhizium anisopliae* with a similar exposure method. They found 100% non-viability of eggs exposed to concentrations of 2×10^8 and 10^7 con.ml⁻¹. The fact that *M. anisopliae* caused high mortality is suggested to be related to the presence of certain enzymes produced by this fungus (GABRIEL, 1968; ALVES, 1998); another possibility could be the time of infection that occurred faster for *M. anisopliae* than for *B. bassiana* s.l. as observed when subterranean termite were exposed (MOINO et al., 2002).

Unlike the results obtained using the eggs, the mortality rates of the larvae and pupae were similar to those found by Moraes et al. (2008), because no significant difference was observed when *S. calcitrans* larvae and pupae were treated with conidial suspensions of *M. anisopliae*. According to Watson et al. (1995), *S. calcitrans* may have developed mechanisms to tolerate the presence and parasitism of certain microorganisms, possibly related to the highly contaminated environment in which its immature stages develop.

Another interesting observation was the absence of conidial non-viability of the three *B. bassiana* s.l. isolates, because 100% germination was obtained upon re-isolation of the suspensions used, along with good development of the fungal isolates in the Petri dishes with the immature stages. Fungal colonization of the rearing medium contributed towards development in a medium that was more favorable to the immature stages, which may have been due to the degradation of organic matter or to the availability of the microorganisms themselves as a food source (SCHMIDTMANN & MARTIN, 1992; PEROTTI et al., 2001; ROMERO et al., 2006). This can be confirmed by the higher mortality rates (Tables 1 and 2) and lower emergence rates (Table 3) observed in the majority of the control groups in the experiments, using both exposure methods. Further confirmation is provided by the good morphological appearance of the immature stages and the intense ingestion of conidia and fungal content in the gut of the larvae (Figure 1a), without causing mobility or behavioral changes or pupal malformation, in contrast to the observations of

Moraes (2007). That author described liquefied pre-emerged larvae, larval paralysis, mummification and pupal malformation when *S. calcitrans* eggs and larvae were exposed to high concentrations of *M. anisopliae* (MORAES, 2007). Moreover, in the present study, no behavioral change were noted among the flies that emerged from the pupae treated with fungal suspensions or in either of the two larval exposure methods, unlike what was reported by Alves (1998), who described infected insects suffering from lack of coordination, paralysis or fungal growth in orifices or more fragile sites of the cuticle.

The test on solid culture medium, through formation of the inhibition zone, showed whether the macerate or mucus of the larvae had an inhibitory effect on the three *B. bassiana* s.l. isolates and whether this response was related to the previous exposure of the larvae. The fungal growth observed around the disks moistened with the macerate or mucus from larvae of the control groups suggests that the antimicrobial mechanisms were not previously active to fend off a fungal infection (SILVA, 2002).

The formation of a fungal inhibition zone on disks impregnated with the macerate from larvae sensitized with the isolates CG228 or ESALQ986 may be related to the conditions needed for fungal germination on the solid medium used, in which the ideal nutrient, temperature and humidity conditions are provided for development. These factors may have overcome the inhibitory capacity of the macerate and the mucus from the larvae previously sensitized to these isolates, or the minimum concentration of macerate or mucus necessary to impede fungal development may not have been reached. According to Hunt (1986), the caprylic acid present in *Dendroctonus ponderosae* and *Hyphantria cunea* can inhibit the development of *B. bassiana* s.l. Furthermore, according to that author, the low concentration found in *D. ponderosae* was insufficient to cause fungal inhibition and form an inhibition zone, while that of *H. cunea* showed moderate inhibition under the experimental conditions used. Urbanek et al. (2012) reported on antimicrobial assays performed with mixtures of fatty and pyroglutamic acids. Interestingly, *B. bassiana* strain Dv-107 was more susceptible to mixtures than *B. bassiana* strain Tve-N39.

The different chromatographic profiles found for the control group and for the infected larvae demonstrated that the larvae responded to the fungal infection. In general, the observation of new chromatographic peaks in the macerate and mucus, not associated with the peaks of the fungal isolates, suggests that antifungal activity fighting the infection was present. According to Dangl & Jones (2001), peptides are considered to be primitive elements of the immune response of living beings, and the routes for induction are relatively well conserved in insects. HPLC has shown that the peptide identified as stomoxin is expressed and secreted exclusively in the stable fly's foregut and presents a wide spectrum of activities that affect the growth of microorganisms (BOULANGER et al., 2002). Urbanek et al. (2012) showed that the *E. nigra* secretion contained 12 free fatty acids and that the most effective acids against bacterial and entomopathogenic fungal growth were pelargonic (9:0), capric (10:0) and palmitoleic (16:1).

The colony-forming process of the isolate CG138 (Figures 3a-d) that was observed through scanning electron microscopy was not enough to kill *S. calcitrans* larvae, since there was no diffuse colonization on the larval integument even with 100% germination

on PDA medium. Garcia et al. (2005) reported that *M. anisopliae* s.l. conidia took 18 hours post-infection to germinate on the cuticle of *Rhipicephalus sanguineus*, and thus established that a period of 48 to 72 hours was required for colonization to be disseminated. Urbanek et al. (2012) suggested that the secretion of *F. nigra* larvae serves as a physical barrier against various possibly pathogenic microorganisms.

Scanning electron microscopy demonstrated that conidia on the larval surface would be affected by a defense mechanism of mucus or integument, which would delay fungal development, similar to what was described for *Bradysia hygida* larvae with bacteria and yeast (CANDIDO-SILVA et al., 2007), for *S. calcitrans* adults with bacteria (BOULANGER et al., 2002) and for *F. nigra* larval secretions (URBANEK et al., 2012).

According to Hunt et al. (1984), the development of more than one germ tube in one conidium (Figures 3c, d) is related to the excess amount of nutrients required to maintain larval viability in the current experiment. These nutrients contributed to the development of errant-type hyphae, which delayed the parasitism on the integument.

The fungal inhibition in the diffusion test on solid culture medium caused by the macerate of larvae previously exposed to the isolate CG138, in association with the five retention times observed in the chromatographic profile of the same macerate (Figure 2a) and the results from the scanning electron microscopy, indicates that an antimicrobial response was triggered against the fungal infection, as also noted in studies on entomopathogenic fungi by Munks et al. (2001), using adult forms of *S. calcitrans*, and by Han et al. (1998), investigating *Drosophila* sp. larvae. According to Gillespie et al. (2000), the fatty acids of insects have a profound effect on fungal spore germination: they are toxic and fungistatic.

These results obtained from HPLC are preliminary; however, these could indicate that similar peptides or molecules with protective characteristics are produced after exposure to the fungus. More studies are needed to elucidate these molecules and their participation in *S. calcitrans* protection against external agents, in order to assess the existence of factors relating to the antimicrobial activity observed with immature stages of *S. calcitrans* in fungal exposure tests. In addition, the unsatisfactory results regarding mortality and adult emergence that were reported in the different exposure bioassays were related to the ability of the stable fly's immature stages to restrain the entomopathogenic action of *B. bassiana* s.l. The difficulty in specifying the substances involved in this response lies in the absence of studies reporting on mucus production by larvae of this species and on its composition and protective activity, since the few studies in the literature cover adult forms of the fly (BOULANGER et al., 2002).

Daffre et al. (2001) reported that studies aiming towards finding natural or synthetic substances with antimicrobial activity and alternative mechanisms not used by conventional antibiotics should be conducted. Several researchers have also mentioned the importance of researching, purifying and chemically, biologically and structurally characterizing efficient new antimicrobial substances that can be used against infections (DAFFRE et al., 2001).

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