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Development of Nematophagous Fungi on Dead Adults of *Aedes aegypti* (Diptera: Culicidae) and Molt Inhibition Experimental

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A B S T R A C T

This study aimed to evaluate development of *Duddingtonia flagrans* on dead adults of *Aedes aegypti* (Diptera: Culicidae) and molt inhibition experimental. The mosquitos used in the experiment were killed previously, and they were used as a basis for chitinase production by the fungus. Two in vitro experimental assays, were performed in separate steps. The assay A, the development of the fungus *D. flagrans* (AC001) grown from 2% chitin agar on dead adult females of *A. aegypti* was evaluated. In the assay B, inhibition of the molting of larvae (from second to fourth stage) that were immersed in conidia from AC001 was experimentally evaluated. The development of *D. flagrans* on adult females of *A. aegypti* was observed experimentally and it was observed that only the larvae from the control group suffered molting of L_2 to adults, with the following percentages: 86% (L_4), 6.5% (pupa) and 7.5% (adults). On the other hand, the larvae treated with conidia of AC001 suffered molting until only the larval stage (L_4). In this study we observed the development of *D. flagrans* on dead adults of *Aedes aegypti* (Diptera: Culicidae) and molt inhibition experimental under laboratory conditions. However, much still needs to be researched before finally being employed as a strategic alternative control.

Introduction

Aedes aegypti (Diptera: Culicidae) is the principal urban vector of dengue, Chikungunya and Zika (Gibbons & Vaughan, 2002, Vasconcelos, 2015). The

absence of an effective vaccine against dengue, chikungunya and zika virus makes essential the realization of actions to control populations of the vector of this disease. Control of *A. aegypti* has been conducted over the years, by means of chemical control, based on the use of organophosphate insecticides (Furtado *et al.*, 2005) and insect growth regulators (Lucia *et al.*, 2009; Salokhe *et al.*, 2012; Silva & Mendes, 2007). However, there is a growing concern about the use of chemicals insecticides in relation to selection of *Aedes* spp. populations resistant to the same (Dusfour *et al.*, 2011; Lima *et al.*, 2011; Prophiro *et al.*, 2011; Tikar, Kumar, Prasad & Prakash, 2009). Several studies have been performed encompassing the biological control with entomopathogenic fungi in an attempt to develop alternative methods to chemical control of *A. aegypti* (Kay & Vu, 2005; Leles *et al.*, 2010; Leles & D`Alesandro, 2011; Luz *et al.*, 2007, 2008; Scholte, Takke & Knols, 2007; Silva *et al.*, 2004). However, the use of other groups of fungi, such as nematophagous fungi and their extracellular enzymes on arthropod vectors of diseases have been successfully tested (Bogum *et al.*, 2005; Braga & Araújo, 2014, Azevedo *et al.*, 2015).

Braga *et al.*, (2013) and Azevedo *et al.*, (2015) reported the development and the enzimatic activity of nematophagous fungi with entomopathogenic activity against ticks species, vector of diseases, starting from the principle that the exoskeleton of arthropods is composed of chitin, one polysaccharide that is recognized by the lectins from the most of nematophagous fungi.

Arthropods are good sources of chitin under laboratory conditions. In this sense, the activity of *D. flagrans* could be tested on other arthropods. However, this study aimed to evaluate the development of *D. flagrans* on dead adults of *A. aegypti* that were used as a basis for chitinase production by the fungus.

In Brazil the introduction and rapid dispersion process of two new arboviruses for the Americas, the Chikungunya virus, probably introduced in 2014 and the Zika virus, possibly introduced in the period during the World Cup held in 2014, emphasizes the need in improving the control in areas infested with *A. aegypti*. Therefore, it is increasing the enormous challenge of epidemiological surveillance in early detection of the new areas with transmission to minimize the impact of these diseases in the population (Vasconcelos, 2015). Thus, new control strategies may come to add other existing.

This study aimed to evaluate the development of *Duddingtonia flagrans* on dead adults of *Aedes aegypti* (Diptera: Culicidae) and molt inhibition experimental under laboratory conditions.

Materials and Methods

Fungus

An isolate of the nematophagous fungus *D. flagrans* (AC001) was used. This isolate is kept in test tubes at 4 °C containing 2% corn-meal-agar (2% CMA) and in the dark for 10 days. This isolated is derived from soil Viçosa, Minas Gerais, Brazil and comes from mycology collection of the Laboratory of Parasitology, Department of Veterinary Medicine of the Federal University of Viçosa, Minas Gerais, Brazil .It have continually been transferred to various media and among them Agar-chitin (2% CA) Braga *et al.*, (2013).

Conidia Production

To obtain conidia, 10 ml of water containing 0.1% Tween 80 was added to the plates

containing the fungus grown on 2% chitin-agar. The obtained suspension was stirred with glass beads in a vortex apparatus and then filtered on absorbent cotton, following the methodology used by Sousa *et al.*, (2006). The quantification of conidia was performed with a Neubauer chamber.

Obtaining Larvae and Adults of *Aedes aegypti*

The mosquitos used in the experiment were killed previously, and they were used as a basis for chitinase production by the fungus. *Aedes aegypti* from Rockefeller strain, obtained from a colony present at the Laboratory of Chemistry and Function of Proteins and Peptides (LQFPP) State University of North Fluminense (UENF) were kept in cages containing aqueous solution of 10% sucrose. Eggs were collected and kept in a container with water for two days. The larvae were feed with commercial food for mouse manually ground (approximately 5 g per 100 larvae/vial). From to 3-5 days after egg hatching and emergence of the larvae, pupae were removed with the aid of a Pasteur pipette reversed. The larvae were then transferred to a plate that was kept inside a cage, until emergence of adults. In the adult phase, mosquitoes were fed with sucrose solution (10%) for maintenance and females were fed with rabbit blood for egg laying and/or experimental procedures (Lelis, 2012).

Assays

Two *in vitro* assays, named A and B, were performed in separate steps. In the assay A, the development of the fungus *D. flagrans* (AC001) grown from 2% chitin agar on dead adult females of *A. aegypti* was evaluated under laboratory conditions. In the assay B, inhibition of the molting of larvae (from second to fourth stage) that were immersed in conidia from *D. flagrans* (AC001) was experimentally evaluated.

Assay A

Development of the Fungus on Dead Adult Females of *A. aegypti*

In this assay the methodology described by Braga *et al.*, (2013) has been followed, where dead adult females of *A. aegypti* were washed in distilled water, dry and placed on sterilized filter paper to remove excess of water. They were then separated and immersed for 5 seconds in fungal suspension with 10^6 conidia/ml of the fungus *D. flagrans* grown in 2% CA and placed in Petri dishes (6 cm diameter) and incubated in BOD (Biological Oxygen Demand) at 25 ± 1 º C and 80 ± 10% relative humidity, in the dark. Two groups were formed, one group treated with the dilution described above and one control group. Each Petri dish of treated and control groups contained 5 specimens, totaling 30 individuals for each treatment, with 6 repetitions. In the control group, females were immersed in distilled water for 5 seconds and incubated in BOD at 25 ± 1 ºC and 80 ± 10% relative humidity in the dark. The colonization of the fungus on the females of *A. aegypti* was monitored daily for 7 days (Lopes *et al.*, 2008). After colonization, the arthropods were disinfected in accordance with the methodology of Alves (1998). Colonization was assessed by visualization of microstructures characteristics (conidia and/or chlamydospores), according to the criteria of Duddington (1955) and Van Orschot (1985).

Assay B

The percentage inhibition of molting of larvae between stages (L_2) to pupa was

experimentally evaluated. The concentration of 10^6 conidia ml was used. Two groups, a control group and a treated group, with six repetions per group, were formed using plastic cups 3.5 x 4.2 cm that were covered with a transparent mesh. The treated group was formed by adding twenty second stage larvae (L_2) of *A. aegypti* and 18 ml of autoclaved distilled water, and then 2 ml of the spore suspension. The control group contained only 20 ml of autoclaved distilled water and 20 L_2. The larvae were incubated in a environmental chamber at 25 ºC and manually fed every two days with grinded ration for mouse. Every day, the groups were observed for changes in the larval to pupal stage (Sousa, Santos, Elias & Luz, 2006).

Results and Discussion

In the assay A, the colonization of conidia of the fungus *D. flagrans* (AC001) produced from 2% CA was evidenced on dead adult females of *A. aegypti*. The proof of this colonization could be observed after the visualization of growth of the conidia (AC001) (Fig 1. A-I). Then, the fungus was reisolated in Petri dishes containing medium 2% WA and the presence of simple adhesive hyphae and conidia with 25-50 µm in length and 10-15 µm in width was proven, as observed by Braga *et al.*, (2013) (Fig. 1. I).

In the assay B, it was observed that only the larvae from the control group suffered molting of L_2 to adults, with the following percentages: 86% (L_4), 6.5% (pupa) and 7.5% (adults), in the control group. On the other hand, the larvae treated with conidia of *D. flagrans* suffered molting until only the larval stage (L_4), at the end of seven days (Fig. 2A).

Arthropods are good sources of chitin under laboratory conditions and in the assay A, the development of *Duddingtonia flagrans* on dead adults of *Aedes aegypti* (Fig. IA) by the fungus grown in medium 2% CA was demonstrated. This information is particularly important in the biological point of view, since there is the abundant presence of chitin. However, the authors also point out that dead adults insects were used, in order to observe a possible colonization from the supplementation of the fungus in a rich chitin-based medium and through the end of the experiment were able to observe the internal colonization of insects through visualization of chlamydospores (Fig .1-I). However, as previously mentioned, the particularities of arthropods classes should be taken into account. Nematophagous fungi are known to "eaters of helminths" and have proven their worth over the years (Barron, 1977; Gronvold *et al.,* 1996; Larsen, 1999). However, some genera of these fungi have entomopathogenic potential as, for example, the genus *Paecilomyces*. In this regard, Fiedler & Sosnwska (2007) have demonstrated the pathogenicity of the nematophagous fungus *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) on a number of potentially harmful arthropods in agriculture. In the work of Braga *et al.,* (2013) was evidenced for the first time the production of a chitinase by the nematophagous fungus *D. flagrans* (AC001), which plays an important role in the fungus x arthropods infection process.

In the assay B, experimentally it was observed that the conidia of *D. flagrans* (AC001) obtained from growth in medium 2% CA somehow inhibited the change in larval stages to adults. These results are in agreement with the work of Braga *et al.*, (2013) who proposed that conidia from the medium 2% CA were essential for the interaction and subsequent destruction of engorged females of *A. cajenense*, however, the authors call attention to certain points: (a) in that study, conidia were produced from agar with *Amblyomma cajenense*

chitin; (b) There are no protocols for the use of chitinase derived from nematophagous fungi on arthropods, with only one report cited earlier; (c) the authors suggest that the interaction of the fungus *D. flagrans* with the arthropod was possible due to the action of a chitinase produced by this fungus, enzyme which has had its production induced by the growth of the fungus in culture medium rich in chitin (2% CA).

Figure.1 A-H. Development of Duddingtonia Flagrans on Dead Adults of *Aedes aegypti* (Diptera: Culicidae). I. Presence of D. Flagrans Chlamydospores on the Colonized Insect

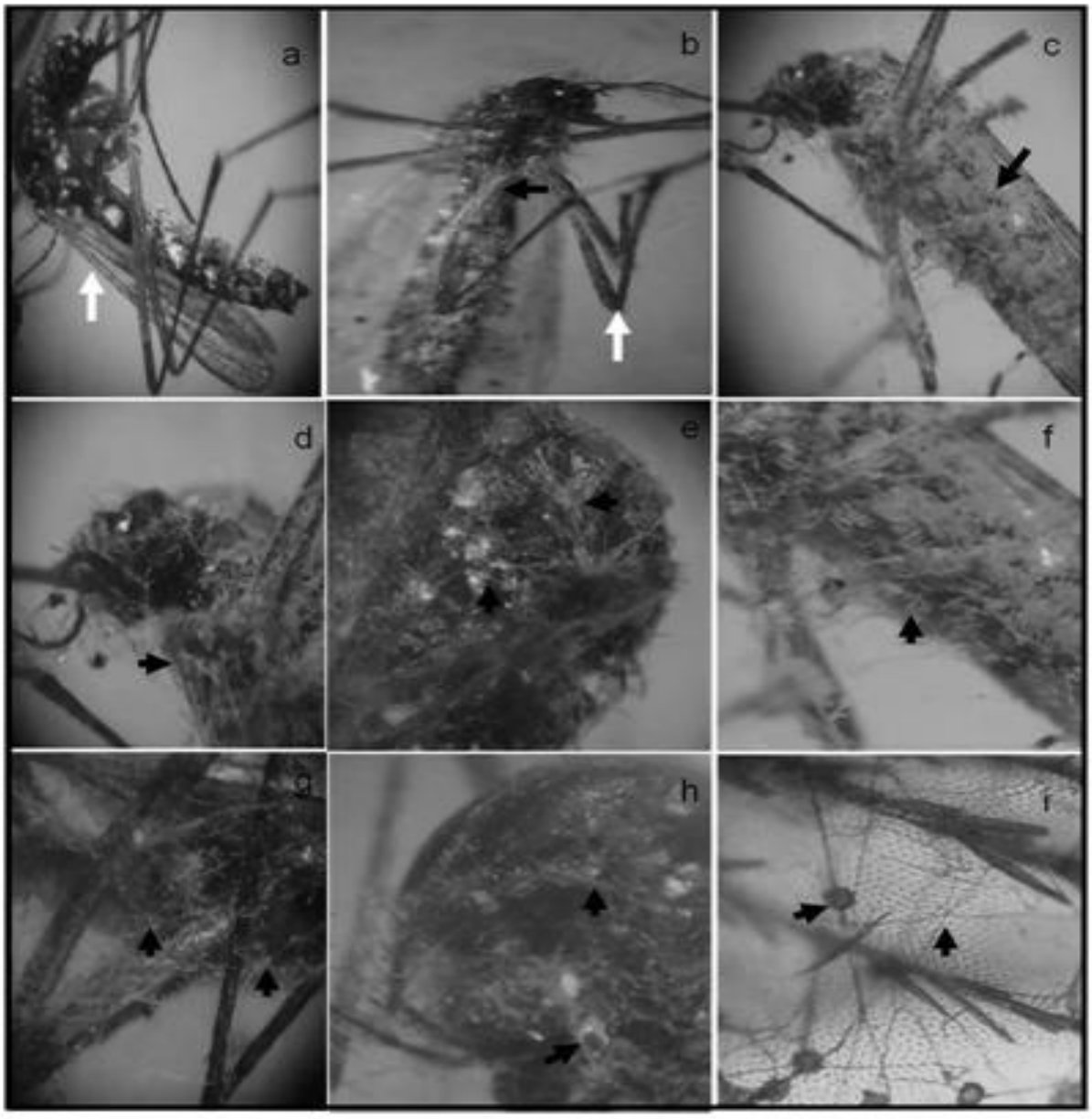
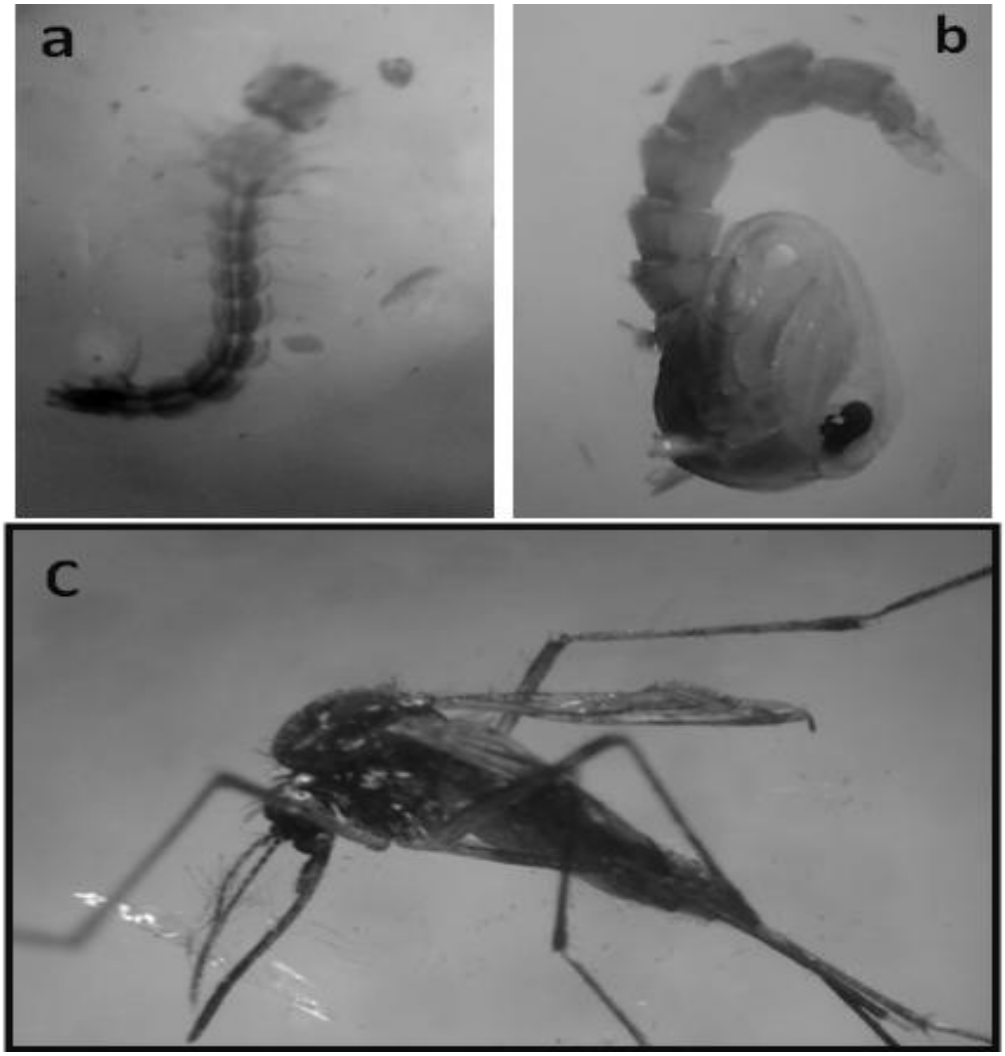


Figure.2A-C- A Larvae (L4); B (pupa) and C (adults) of *Aedes aegypti* in the Control Group without Treatment with *Duddingtonia Flagrans* Conidia, after Seven Days



Another important point that deserves to be discussed is the concentration of conidia used in the *in vitro* control of *A. aegypti*. Most papers mention that concentrations may vary 10^6-10^8 conidia/mL (Leles *et al.*, 2011; Santos *et al.*, 2009; Scholte *et al.*, 2007). However, these studies focus on the use of entomopathogenic fungi. In this paper, the authors suggest that the concentration of 10^6 conidia/mL was enough for the inhibition of larval stage and subsequent colonization of the adult insects and thus, the authors call attention to the first report of the interaction of spores from a nematophagous fungus (AC001) on larvae of *A. aegypti*, which in the future may prove to be an additional tool of research.

In this study we observed the development of *D. flagrans* on dead adults of *Aedes aegypti* (Diptera: Culicidae) and molt inhibition experimental under laboratory conditions. The authors mention that the mosquitoes used in the experiment were killed previously, serving only as a basis for chitinase production by the fungus.

However, much still needs to be researched before finally being employed as a strategic alternative control.

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