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In vitro Control of *Aedes aegypti* Larvae Using *Beauveria bassiana*

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Abstract*—Aedes aegypti* larval survival rate was assessed after exposure to blastopores or conidia (mineral oil-in-water formulation or aqueous suspension) of *Beauveria bassiana* CG 479 propagules (blastospores or conidia). Here, mineral oil was used in the fungal formulation to control *Aedes aegypti* larvae. 1%, 0.5% or 0.1% mineral oil-in-water solutions were used to evaluate mineral oil toxicity for mosquito larvae. In the oil toxicity test, 0.1% mineral oil solution reduced only 4.5% larval survival; accordingly, this concentration was chosen for fungal oil-in-water formulations. Aqueous suspensions were prepared using 0.01% Tween 80® in sterile dechlorinated water. *A. aegypti* larvae (L_2) were exposed in aqueous suspensions or mineral oil-in-water fungal formulations at 1×107 propagules mL^{-1}; the survival rate (assessed daily, for 7 days) and the median survival time (S_{50}) were calculated. Seven days after the treatment, mosquito larvae survival rates were 8.56%, 16.22%, 58%, and 42.56% after exposure to oil-in-water blastospores, oil-in-water conidia, blastospores aqueous suspension and conidia aqueous suspension (respectively). Larvae exposed to 0.01% Tween 80® had 100% survival rate and the ones treated with 0.1% mineral oil-in-water had 95.11% survival rate. Larvae treated with conidia (regardless the presence of oil) or treated with blastospores formulation had survival median time (S_{50}) ranging from one to two days. S_{50} was not determined (ND) when larvae were exposed to blastospores aqueous suspension, 0.01% Tween 80® (aqueous control) or 0.1% mineral oil-in-water formulation (oil control). *B. bassiana* conidia and blastospores (mineral oil-in-water formulated or suspended in water) had potential to control *A. aegypti* mosquito larvae, despite mineral oil-in-water formulation yielded better results in comparison to aqueous suspensions. Here, *B. bassiana* CG 479 isolate is suggested as a potential biocontrol agent of *A. aegypti* mosquito larvae.

***Keywords*—**Blastospores, formulation, mosquitoes, conidia.

I. Introduction

AEDES aegypti (Diptera: Culicidae, Linnaeus 1762) is vector of arboviruses, such as dengue fever, yellow fever, zika and chikungunya that have become major public health concerns around the world [1]-[3]. The predominant methods for *A. aegypti* control are mosquito capture, removal of water collections that are potential spots for immature stages development or chemical control using insecticides [4], despite several studies have already proved the resistance of insect vectors to several chemical compounds besides its toxicity effects to the environment [5]-[8].

Studies with entomopathogenic fungi, such as *Beauveria bassiana*, have been developed seeking new approaches to control *A. aegypti* [9]-[11]. *B. bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) is an entomopathogenic fungi widely studied to control vectors of public health concern [9], [10]. The fungal infection starts with propagule adhesion, attachment and development of the germ tube and appressoria on the insect cuticle facilitating the fungal penetration into the hemocoel [12]-[14]. Into the hemocoel, fungal produce hyphal bodies (blastospores) for scape of the immune response host and continue the parasitism [15].

Blastospores have morphological characteristics that differentiate them from aerial conidia. This propagule is a hydrophilic structure with a thin cell wall. Blastospores are less hydrophobic than conidia, and not necessarily less resistant to adverse environmental conditions than conidia [16]. Blastospores need short time to be produced (2-4 days) in comparison to conidia that need approximately 14 days [17], [18]. Current research with *A. aegypti* larvae reported that blastospores are more virulent than conidia [19]-[21].

Formulations based on oils optimize fungal effect towards the insect host, protecting propagules from negative abiotic factors and favoring propagules adhesion on insect cuticle [22]. Therefore, the aim of this study was to evaluate and compare the virulence of blastospores and conidia (formulated or not) from *B. bassiana* CG 479 isolate against *A. aegypti* larvae.

II. Material and Methods

A. Mosquito Larvae

A. aegypti (rockefeller lineage) eggs were obtained from the Laboratory of Biochemistry and Molecular Biology of Arthropods (LBBMA)/Chemistry Department at the Exact Sciences Institute (Federal Rural University of Rio de Janeiro, Brazil). Larvae hatched in a container with sterile dechlorinated tap water (1.5 L). Larvae (L_2) were feed with sterile fish food (Alcon alevinos®) and kept at room temperature (27±2 °C). UFRRJ's Ethics Committee

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(CEUA/ICBS/UFRRJ) approved the mosquito colony establishment under the number 23083007342/2016-59.

B. Toxicity of Mineral Oil against Aedes aegypti Larvae

Mineral oil (Vetec Química Fina Ltda., Rio de Janeiro, Brazil) in different concentrations (1%, 0.5%, or 0.1%) was used to treat three cohorts of ten larvae (L_2) each (n=30 per treatment). Larvae were immersed in 10 mL mineral oil emulsions (1%, 0.5%, or 0.1%) in plastic tubes for seven days. Each test was carried out three different times. 0.01% polyoxyethylene sorbitan monooleate (Tween 80®, Sigma Chemical Co., St. Louis, MO, USA) was used to stabilize the oil-in-water emulsions. Killed larvae were removed for seven days, and the survivors were recorded daily.

C. Fungal Strain and Aqueous Suspensions

Beauveria bassiana CG 479 isolate was obtained from the National Center for Genetic Resources-CENARGEN, EMBRAPA, Brazil. The isolate was inoculated on potato dextrose agar medium (PDA) for 15 days under controlled temperature and humidity conditions (25 ± 1 °C; RH ≥ 80%). Conidia were harvested and suspended in 33 mL 0.01% Tween 80® (v/v) sterile dechlorinated tap water. The conidial suspension was homogenized for 1 min, quantified in a hemocytometer and adjusted to 1.0×10^7 conidia mL^{-1}.

For blastopores production, 3 mL of fungal suspension at 1.0×10^8 conidia mL^{-1} were inoculated into 42 mL Adamek's modified liquid medium [23] and incubated at 27 ºC and 150 rpm (TE-424, Tecnal®) for 72 h. The liquid culture medium was filtered with sterile gases and centrifuged at 3410 g for 5 min (Rotina 380R Hettich®). The supernatant was discarded, and 10 mL 0.01% Tween 80® sterile dechlorinated water solution was added. Centrifugation was repeated one more time. Blastospores suspension was adjusted to 1×10^7 blastospores mL^{-1}, using a hemocytometer [24].

D. Blastospores and Conidia Mineral Oil-in-Water Formulation

Formulations were prepared as follows: 10 microliters of sterilized mineral oil was added in 9.99 mL conidial suspension or 9.99 mL blastospores suspension at 1×10^7 propagules mL^{-1}.

E. Fungal Viability

Each conidial aqueous suspension or oil-in-water formulation was transferred to Petri dishes containing PDA supplemented with 0.05% chloramphenicol and incubated at 25 ± 1 °C and RH ≥ 80%. After 24 h, conidial viability was determined by direct optical microscopic observation at 400× magnification. 200 conidia were counted and the percentage of viability was accessed [24]. Blastospore (aqueous suspension or oil-in-water formulation) viability was assessed as described in conidia viability. However, after 24 h was observed the hyphal development using optical microscopic at 400× magnification.

F. Beauveria Bassiana Virulence against Aedes aegypti Larvae

10 larvae (L2) were placed in each plastic tube with 10 mL fungal suspension or 10 mL oil-in-water formulation at 1.0×10^7 propagules mL^{-1}. Three tubes were used per group (N= 30). Larvae in the aqueous control group were exposed to 0.01% Tween 80® (v/v) sterile dechlorinated tap water; and larvae in the oil-in-water control group were treated with 0.1% mineral oil. The larval survival rate was evaluated daily for seven days and the dead larvae were removed daily. The biological assay was performed under ideal conditions of temperature, humidity and photoperiod (25 °C, RH ≥ 80%, 8 hours of light) [25]. The biological assay was repeated three different times with new batches of conidia/blastospores and new larvae.

G. Re-Isolation of Entomopathogenic Fungi from Dead Aedes aegypti Larvae

Dead larvae were surface-sterilized by immersion in 70% alcohol (EtOH) for two minutes and then rinsed in sterile distilled water for one minute. Larvae were gently dried with sterile paper towel and transferred to petri dishes containing PDA supplemented with 0.05% chloramphenicol under controlled conditions (25 °C, RH ≥ 80%) to stimulate fungal development [26]. Seven days after incubation, fungal macromorphological characteristics were observed [27].

After fungal development, the microculture chamber was set up. The isolate was inoculated on PDA into the humidity chamber under controlled temperature and humidity conditions (25 ± 1 °C; RH ≥ 80%); after five days [28], micromorphological characteristics of the fungal isolate were observed using technique between slide and coverslip and staining with lactophenol and cotton blue [27] and viewed under light microscope at 400× magnification [29].

H. Statistical Analysis

Mean larval survival rates were expressed by mean ± standard error. The one-way analysis of variance (ANOVA) test was used to compare means with Tukey´s post hoc test to assess pairwise comparisons ($P \leq 0.05$) [25]. The log-rank test was used to calculate the larvae median survival time (S_{50}) [25]. The tests were performed using Prism, GraphPad, v.7.00, Inc (GraphPad Software, USA).

III. Results

A. Toxicity of Mineral oil Against Aedes aegypti Larvae

A. aegypti larvae exposed to 0.1% mineral oil-in-water formulation had the lowest survival rate (i.e., 4.5%) with no statistical difference (P=0.5619) in comparison to the aqueous control group (larvae exposed to 0.01% Tween 80) (Fig. 1). Nevertheless, larvae exposed to 0.5% or 1% mineral oil-in-water had statistically different ($P < 0.0001$) survival rates in comparison to aqueous control group. Accordingly, 0.1% mineral oil-in-water concentration was chosen to prepare the fungal mineral oil-in-water formulations. The results are expressed in Table I.

TABLE I
MEAN LARVAL SURVIVAL (%) ± STANDARD ERROR AND MEDIAN SURVIVAL TIME (S_{50}) OF *AEDES AEGYPTI* LARVAE EXPOSED TO DIFFERENT CONCENTRATIONS OF MINERAL OIL EMULSIONS

Oil concentration	Mean larval survival (%) ± standard error	S_{50} (days)
1%	63.67 ± 2.57 b	5
0.5%	70.33 ± 3.16 b	6
0.1%	95.5 ± 2.60 a	ND
Aqueous control	100 ± 0.00 a	ND

Means followed by the same letter did not differ significantly ($P \geq 0.05$). ND= Not determined. Larvae survival was evaluated daily for seven days. (Aqueous control) mosquito larvae exposed to 0.01% Tween 80®; (1%) larvae exposed to 1% mineral oil; (0.5%) larvae exposed to 0.5% mineral oil; (0.1%) larvae exposed to 0.1% mineral oil.

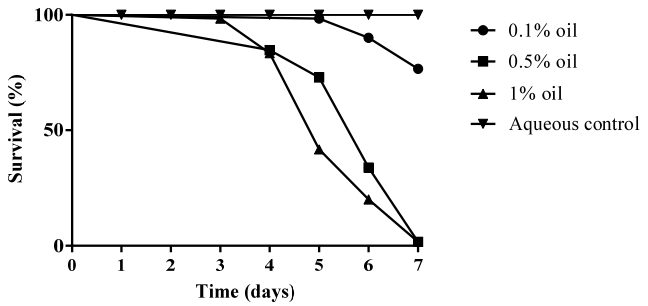

Fig. 1 Oil toxicity test. Survival rate of *Aedes aegypti* larvae exposed to mineral oil at 1%, 0.5% or 0.1%, daily for seven days. Aqueous control was immersed in 0.01% Tween 80® sterile dechlorinated H_2O solution

B. Fungal Viability

Conidia propagules from aqueous suspensions were 98% viable and fungal propagules from oil-in-water formulation had 95% viability 24 h after incubation on PDA. Blastospores aqueous suspension or oil-in-water formulation had 100% hyphal developed.

C. Beauveria bassiana Virulence against Aedes aegypti Larvae

Regardless the propagule (conidia or blastospores), *B. bassiana* isolate CG 479 (formulated or not) was effective to control *A. aegypti* larvae (Table II). Oil-in-water fungal propagules formulations yielded the lowest larval survival rates. Despite there was no difference when larval survival rates of conidia (formulated or not) were compared; blastospores oil-in-water formulation yielded better results than blastospores aqueous suspension (Table II).

Although the addition of 0.1% mineral oil increased the fungal virulence (Table II), there was no difference between the propagule type, i.e., blastospores aqueous suspension yielded the same larval survival rate than conidia aqueous suspension and blastospores oil-in-water formulation yielded the same larval survival rate than conidia oil-in-water formulation. In the present study it was not possible to determine (ND) the mean survival time (S_{50}) of mosquito larvae exposed to blastospores aqueous suspension (Table II). Therefore, conidial suspension was more efficient than blastospores suspensions.

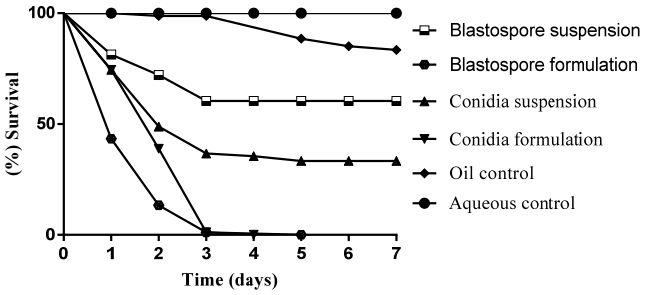

Fig. 2 Survival rate of *Aedes aegypti* larvae exposed to blastospores or conidia suspensions and oil-based formulations of *Beauveria bassiana* strain CG 479. Aqueous control was immersed in 0.01% Tween 80® sterile dechlorinated H_2O solution; (oil control) larvae was exposed to 0.1% mineral oil

TABLE II
GROUPS TREATED WITH FORMULATION OR SUSPENSION OF BLASTOSPORES AND CONIDIA

Group	Mean larval survival (%) ± standard error	S_{50} (days)
Blastospores suspension	58.00 ± 13.86 b	ND
Blastospores formulation	8.56 ± 2.73 c	1
Conidia suspension	42.56 ± 14.39 c b	2
Conidia formulation	16.22 ± 3.08 c	2
Oil control	95.11 ± 2.94 a	ND
Aqueous control	100 ± 0.00 a	ND

Means followed by the same letter did not differ significantly ($P \geq 0.05$). (Aqueous control) mosquito larvae exposed to 0.01% Tween 80®; (Oil control) mosquito larvae exposed to 0.1% mineral oil. ND = Not determined.

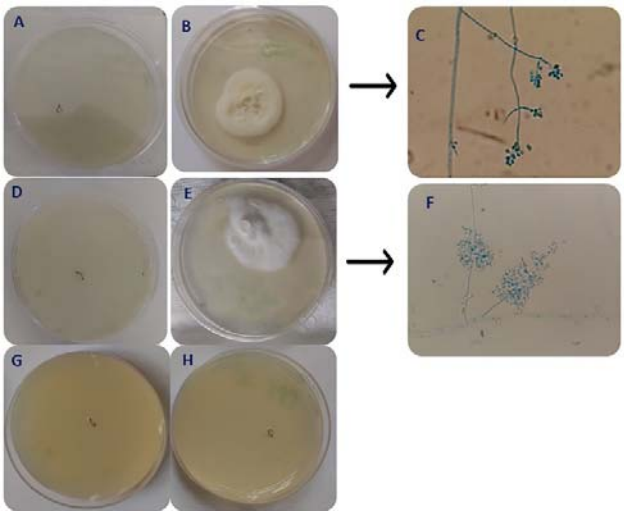

Fig. 3 Death *Aedes aegypti* larvae after exposure to blastopores (A) or conidia (D) *B. bassiana* CG 479 isolate. (B; E) Fungal growth from death larvae after 7 days. (C) Micro cultivation of *Beauveria* spp. conidia after re-isolation from *Aedes aegypti* larvae (400×). *Aedes aegypti* larvae from aqueous control (G) and oily control (H)

D. Re-Isolation of Entomopathogenic Fungi from Dead Aedes aegypti Larvae

The isolated fungal colonies universally presented the key morphological features consistent with *B. bassiana*; *A. aegypti* larvae from the aqueous control and oily control groups did not exhibit fungal growth. The fungal re-isolation was observed in Fig. 3.

IV. Discussion

B. bassiana is a filamentous fungus widely studied for mosquito biological control [9], [30]-[34]. Here, fungus aqueous suspensions or oil-in-water formulations were effective to control *A. aegypti* larvae.

Both fungal oil-in-water propagules formulations yielded better results than blastospores aqueous suspensions, in agreement with other studies [25], [35]-[37] suggesting that even this very low mineral oil concentration (i.e., 0.1%) is a promising adjuvant alternative [38].

It is interesting to note that conidia aqueous suspension did not differ in comparison to both propagules' fungal formulations, showing high fungal performance; nevertheless, both fungal oil-in-water propagules' formulations reduced the survival rate of the larvae by 90% in 3 days (Fig. 2) suggesting, fungal formulations based on mineral oil are promising for the reduction of the action time necessary to kill the larval population.

Despite the selection of virulent isolates is fundamental in the process of a bioproduct development, the screening different formulation types, is also necessary to optimize the fungus performance and minimize the negative effects caused by abiotic factors [22]. Accordingly, vegetable or mineral oils involve conidia or blastospores in micelles, facilitating fungal adhesion in the host's lipophilic tegument [25], [26], [37], [38].

Mineral oil is a highly refined petroleum derivate [39] used as a humectant in fungi-based formulations that dissolve the host's cuticle and facilitate fungal infection [38]. Even the results of conidia aqueous suspension did not differ statistically in comparison to formulated propagules. This study, showing that the use of mineral oil as adjuvant, is an advantage due to the short time to action of the fungus formulated against mosquito larvae.

B. bassiana fungi produces aerial conidia (terrestrial lipophilic and hydrophobic propagules) and after penetration into host's hemocoel, these fungi start producing hyphal bodies (=blastospores) that are characterized as hydrophilic propagules [13]. These hydrophobic aerial conidia cannot adhere firmly on the mosquito surface in the aquatic environment [40], neither they germinate into the mosquito's digestive tract; consequently, fungi are eliminated by fecal pellets [41]. However, blastospores have hydrophilic characteristics that allow these propagules to adhere on the host surface even in the aquatic environment [11], [20].

Several papers reported higher efficacy of blastopores in comparison to aerial conidia against *A. aegypti* larvae [11], [19], [20]. Despite the high efficacy of blastospores has been reported by other studies, here, larvae survival rate yielded by blastospores was the same observed for aerial conidia suggesting that not every fungal isolate will produce blastospores more effectively than aerial conidia. In the present study, both CG 479 propagules were equally effective, accordingly, it is necessary to consider that fungal virulence differs among the isolates and also regarding the host's population.

The death of mosquito larvae, after fungal exposure, was reported by multiple factors, such as protection mechanisms activated by the conidium in an inhospitable environment and/ or asphyxiation caused by the obstruction of the respiratory siphon [41]. The same authors reported that, the fungus would not be adapted to the aquatic environment, despite other studies showed that blastospores would be able to act in an aquatic environment [19], [20].

It is suggested that here mineral oil allowed the fungal propagules to adhere in the surface of the aquatic host (i.e., mosquito larvae) [42]; also favoring the fungal propagules adhesion in the respiratory siphon. Furthermore, the mineral oil could protect the propagules from the extreme high humidity, allowing fungal viability during the time to contact with the mosquito larvae [29], [38].

It is important to emphasize that, despite our results are consistent with the results obtained by other authors [25], [35]-[37], in oil-in-water formulations and aqueous suspensions that were tested here, using both B. *bassiana* CG 479 propagules (i.e., conidia or blastospores) showed potential to control of *A. aegypti* larvae.

V. Conclusion

B. bassiana propagules of isolate CG 479 has potential to control of *A. aegypti* larvae. Nevertheless, here, the effectiveness of blastospores suspension does not differ in comparison to conidia suspension.

Interestingly, this study showed that fungi oil-based formulations at mineral oil are a promising form of emulsification that can be used in controlling the *A. aegypti* mosquito. Nonetheless, we need deeper researches to understand all the mechanisms involved and to develop effective formulations to mosquito control.

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