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Efficacy of *Culicinomyces* spp. against *Aedes aegypti* eggs, larvae and adults

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ABSTRACT

The aquatic fungal genus *Culicinomyces* attacks dipteran larvae but little is known about its efficacy against *Aedes aegypti*. Here we report on the activity of both described species–*Culicinomyces clavisporus* and *Culicinomyces bisporalis*–on larvae, eggs and adults, and on trans-stadial transmission. Ten *C. clavisporus* isolates (ARSEF 372, 582, 644, 706, 964, 1260, 2471, 2478, 2479 and 2480) and *C. bisporalis* ARSEF 1948 were screened against larvae of this important vector of viral diseases. ARSEF 644, 964 and 2479 had the lowest LC_{50} ($\leq 3.6 \times 10^5$ conidia/ml) after a 3-day exposure and shortest LT_{50} (≤ 1.3 days) at 10^6 conidia/ml against larvae; none of these isolates affected either eggs or adults treated topically with conidia. However, adults fed on a conidial (10^6 conidia/ml) suspension in 10% sucrose died ($\leq 26.6 \pm 3.3\%$ mortality, 5 days after feeding) but no fungal development was detected on dead adults. No pupae or adults obtained following treatment of fourth instar larvae with 10^5 or 10^6 conidia/ml showed any indication of fungal presence. *C. clavisporus*–especially ARSEF 644, 964 and 2479–is the first choice for control of *A. aegypti* and has high potential in control strategies targeting aquatic larvae.

1. Introduction

Aedes aegypti is the primary vector of the important arboviruses causing dengue, Zika and chikungunya fever in the tropics (Weaver et al., 2018). This highly urbanized mosquito species is frequently found in domestic and peri-domestic areas and breeds in small or medium-sized water collections (Araújo et al., 2015).

Sanitation measures that reduce the number of mosquito breeding sites and that involve applications of chemical insecticides–usually insect growth regulators against larvae and pyrethroids against adults (Baldacchino et al., 2015)–aim to reduce the vector populations and are the principal tools of integrated control to prevent the diseases they spread (Araújo et al., 2015). Entomopathogenic fungi presenting low risks to human health and environment have gained considerable interest as biological control agents against *A. aegypti,* but more efforts are necessary to explore their real potential and to develop specific mycoinsecticides that will serve as sustainable tools against mosquito larvae (Scholte et al., 2004; Baldacchino et al., 2015; Lacey, 2017; Deshayes et al., 2017).

The conidial fungal genus *Culicinomyces* consists of two known species, *Culicinomyces clavisporus* and *C. bisporalis*. Both are known only as pathogens of dipterans. Only few isolates of *C. clavisporus* from Australia and Northern America and a single *C. bisporalis* isolate from Australia are available, all of which were obtained from infected culicid or ceratopogonid larvae. In contrast to other entomopathogenic fungi, *Culicinomyces* spp. infect their hosts mainly through the gut after ingestion of conidia rather than by direct penetration through the cuticle (Scholte et al., 2004). Although these fungi have been recognized for decades as mosquito pathogens there is still very little information on key questions about them such as inter- and intraspecific variability of virulence, activity against eggs and adults and trans-stadial transmission, topics that were investigated here.

2. Materials and methods

2.1. Origin, rearing and preparation of Aedes aegypti

The *A. aegypti* colony originated from larvae collected in Goiânia, Brazil, in 2012, and was maintained in the laboratory at 27 ± 5 °C, 75 ± 10% relative humidity (RH) and natural photophase. Adults were fed *ad libitum* on cloth pads saturated with 10% sucrose solution. Females were fed twice per week following the method described by Lima et al. (2009), a technique approved by the Committee of Ethics for the Use of Animals, UFG, CEUA 40399. For oviposition, an amber-

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Table 1
Culicinomyces spp. isolates used in this study.

Species	ARSEF	Country	Climate[a]	Year	Dipteran host larva
Culicinomyces bisporalis	1948	Milla Milla, Australia	Cfa	1984	*Aedes kochi*[b]
C. clavisporus	372	Chapel Hill, USA	Cfa	1979	*Anopheles quadrimaculatus*[b]
	582	Chapel Hill, USA	Cfa	1981	*A. quadrimaculatus*[b]
	644	Sydney, Australia	Cfa	1981	*Anopheles amictus hilli*[b]
	706	North Carolina, USA	Cfa	1982	unspecified culicid
	964	Devon, Canada	Dfb	1982	*Culiseta inornata*[b]
	1260	Devon, Canada	Dfb	1984	*C. inornata*[b]
	2471	Mittagong, Australia	Cfb	1981	*Aedes rupestris*[b]
	2478	Mittagong, Australia	Cfb	1983	*Dasyhelea* sp.[c]
	2479	Mallacota, Australia	Cfb	1984	*C. inconspicua*[b]
	2480	Milla Milla, Australia	Cfa	1985	*Forcipomyia marksae*[c]

[a] Köppen-Geiger climatic classifications (Kottek et al., 2006): Cfa = temperate/tropical hot summer; Cfb = maritime/oceanic temperate; Dfb = warm summer continental.
[b] Culicidae.
[c] Ceratopogonidae.

colored glass flask (100 ml) containing 80 ml of distilled water and filter papers (90 mm diameter) was set in the cage (Luz et al., 2007). Twice per week the filter papers with eggs were retrieved from the cage and transferred to a humid chamber (RH > 98%). After 48 h, the filter papers and eggs were dried for 12 h, and then stored at 25 ± 5 °C and RH 75 ± 10% until use. For assays with larvae, filter papers with eggs were transferred to a plastic bowl (29 cm width, 15 cm height) with 1000 ml of tap water, and larvae were fed with triturated cat food (Bom Preço®, Salto de Pirapora, Brazil) until reaching the needed larval stage. For the tests with adults, larvae were fed until pupation, and pupae then were transferred to a plastic cup (50 ml) containing 30 ml of tap water and placed in a plastic container (500 ml) covered with a mesh to collect the emerging adults.

2.2. Origin and preparation of *Culicinomyces spp.*

One isolate of *C. bisporalis* and ten *C. clavisporus* isolates (Table 1) were obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, NY, USA). The isolates were routinely grown on quarter-strength Sabouraud dextrose agar plus yeast extract (SDAY/4; 0.25% peptone, 1% dextrose, 0.25% yeast extract, 1.5% agar) in small Petri dishes (60 mm diameter) at 25 ± 1 °C, RH 75 ± 5% and 12 h photophase for 15 days. For the assays, 5 ml of sterile distilled water were added to the surface of the culture, and aerial conidia harvested by scraping with a spatula. Conidial suspensions were filtered through hydrophilic cotton and then transferred to glass tubes (10 ml). The number of conidia per ml was calculated with a hemocytometer, and the suspensions adjusted with sterile distilled water to the chosen conidial concentrations. At the start of each assay, the viability of the conidia (> 95% of germination) was checked by inoculation of 20 µl of a suspension (10^6 conidia/ml) of each isolate onto SDAY/4, and incubated at 25 ± 1 °C, RH 75 ± 5% and 12 h photophase. After 24–36 h of incubation, germination was stopped by adding lactophenol, and the number of germinated conidia scored with a light microscope. At least 300 conidia per dish were evaluated and scored as non-germinated or as germinated if the germ tube length was greater than the conidial diameter.

2.3. Evaluation of larvicidal activity

Five conidial suspensions of each of the 11 isolates were prepared, as described above, and adjusted to 10^5, 3.3×10^5, 10^6, 3.3×10^6, and 10^7 conidia/ml. Ten third-instar *A. aegypti* larvae (L3) were exposed to 25 ml of each suspension and incubated at 25 ± 1 °C, RH 75 ± 5% and 12 h photophase. For the controls, L3 were maintained in 25 ml of water without conidia under the same conditions. Larvae were fed each 2 days with a small amount of cat food (as noted above). Mortality was monitored daily for up to 10 days. Dead larvae were transferred to another container with 20 ml of sterile distilled water and incubated at the same conditions. Fungal development on larvae was monitored daily for 5 days, and at the end of the test, about 30% of the dead larvae were mounted in drops of Amman's solution on slides. The development of hyphae and conidiogenesis were checked with a light microscope (400× magnification).

2.4. Evaluation of ovicidal activity

For this assay, filter papers were cut into squares with a 1 cm^2 surface area and then autoclaved for 20 min at 121 °C (Luz et al., 2007). Thirty *A. aegypti* eggs aged to 3–5 days after oviposition were carefully placed with a brush on each filter paper (two filter papers per isolate). The filter papers containing the eggs were exposed for 15 min to ultraviolet light (UV-C Lamp Germicidal Ultraviolet G30T8, Royal Philips Electronics, Amsterdam, Netherlands) in order to reduce the number of microorganisms on the surface of the eggs. Suspensions of 10^8 conidia/ml were prepared for the isolates ARSEF 644, 964 and 2479 that were selected as the most favorable for further studies. For each isolate 50 µl of suspended conidia at a final concentration of 5×10^6 conidia/cm^2 were applied topically with a semi-automatic pipette onto eggs. For the controls, 50 µl of distilled sterile water were applied to eggs. The filter papers with treated eggs were then moved to Petri dishes and dried at room temperature for 1 h. Afterwards, the Petri dishes with the eggs were placed in a moist chamber (RH > 98%) and incubated at 25 ± 1 °C and 12 h photophase for either 10 or 15 days. Eggs were examined microscopically after 18–24 h of incubation (25× magnification), and only 20 eggs showing no visible damage of the chorion were left on any filter paper. Subsequently eggs were checked daily for signs of desiccation or deformation, premature eclosion of larvae on the filter paper, and for fungal growth on the chorion. After a 10-day and 15-day incubation, eggs were submerged in 20 ml of sterile water adjusted at 35 ± 1 °C to stimulate eclosion and then incubated at 25 ± 1 °C, RH 75 ± 5% and a 12 h photophase. Eclosion was checked daily for up to 10 days, and newly eclosed larvae were transferred to another container with 20 ml of sterile distilled water, and held as above. The survival and development of these larvae were followed for up to 20 days. Fungal development on dead larvae was assessed as described.

2.5. Evaluation of adulticidal activity

Activity of *C. clavisporus* in adults was tested using two methods. Adults were either treated by exposure to a substrate pre-treated with conidia or fed on a sucrose solution containing conidia.

For the first test, a conidial suspension of ARSEF 644, 964 and 2479

Table 2
Cumulative relative mortality ± standard error (SE) and lethal time (days), with respective confidence interval (CI) to kill 50 or 90% (LT_{50} e LT_{90}) *Aedes aegypti* third-instar larvae exposed to *Culicinomyces* spp. conidia (10^6 conidia/ml) and incubated at 25 ± 1 °C, 75 ± 5% relative humidity and 12 h photophase for up to 10 days.

Isolates (ARSEF)	Mortality ± SE			Lethal time (CI)		Slope ± SE
	3rd day	5th day	10th day	LT_{50}	LT_{90}	
1948	0a	17 ± 3.3a	97 ± 3.3ab	6.9 (6–7.9)c	9.9 (8.9–11.6)c	0.43 ± 0.07
372	83 ± 3.3d	100e	100b	1.1 (0.9–1.5)a	2.6 (1.9–4.3)ab	0.49 ± 0.1
582	90d	100e	100b	1.6 (1.3–3.6)b	4.1 (2.9–6.5)ab	0.36 ± 0.01
644	97 ± 3.3de	100e	100b	1.3 (1–1.5)ab	2.6 (2.1–3.8)a	0.75 ± 0.1
706	60 ± 2.9c	73 ± 2.9c	100b	2.6 (2–12.2)bc	6.6 (4.4–98.2)bc	0.21 ± 0.06
964	100e	100e	100b	1.2 (0.8–1.5)a	2.8 (2.2–3.9)a	0.1 ± 0.01
1260	57 ± 2.9c	73 ± 2.9c	100b	2.2 (1.9–3.8)b	7.2 (5.4–11.2)bc	0.25 ± 0.06
2471	57 ± 2.9c	80 cd	100b	2.3 (1.7–3.6)b	6.8 (5.1–10.3)bc	0.27 ± 0.06
2478	53 ± 2.9c	83 ± 3.3d	100b	1.9 (1.6–3.4)b	7 (5.1–11.2)b	0.24 ± 0.06
2479	93 ± 3.3de	100e	100b	1 (0.5–1.5)a	2.6 (2.1–3.8)a	0.76 ± 0.1
2480	20b	53 ± 6.6b	93 ± 3.3a	5.4 (4.1–6.8)c	9.8 (8.2–12.4)c	0.29 ± 0.05
$F_{10,22}$	173	135	2.3	–	–	–
P	< 0.001	< 0.001	0.05	–	–	–

Cumulative control mortality was < 10% at a 10-day incubation.
Values in the same column followed by different letters (a–e) were significantly different based on ANOVA and SNK test (cumulated mortalities) or their CI (LT).

was prepared, and the concentration adjusted to 7.2×10^8 conidia/ml. Then, 50 µl of each suspension (a total 3.6×10^7 conidia) were mixed with 200 mg of sterile magnesium silicate as a carrier, homogenized with a spatula, and dried in a desiccator with silica gel for 24 h at 4 ± 1 °C. The total inner surface of polypropylene cups (360 cm^2, PPT505, Copobras, São Ludgero, Brazil) was roughened either vertically and horizontally with a sandpaper (A-257 Gr180, Norton Saint-Gobain Abrasivos, Guarulhos, Brazil). The cups were exposed for 15 min to ultraviolet light, and then the conidial magnesium silicate mixture applied with a hair brush at a final concentration of 10^5 conidia/cm^2. Then five female and five male adult *A. aegypti*, aged to 24–72 h after emergence, were transferred into each covered treated cup. For the control, adults were exposed to cups treated with magnesium silicate without conidia or maintained inside cups without any added ingredients. The cups with adults were then incubated in a moist chamber (RH > 98%) at 25 ± 1 °C and 12 h photophase for up to 15 days. Mortality was assessed daily, and dead adults were transferred onto water-agar (WA) medium (thiabendazole 4 mg, chloramphenicol 1 g, agar 10 g, pH 5.5; Leles et al. 2013); the growth of mycelium and new production of conidia was evaluated for 10 days.

For the second test, conidial suspensions of ARSEF 644, 964 and 2479 at 2×10^6 conidia/ml were prepared. A 20% sterile sucrose solution (5 ml) was mixed with 5 ml of each conidial suspension (at final 10^6 conidia/ml), vortexed and then pipetted on cloth pads and offered to adults arranged in polypropylene cups as in the first test. For the control, sucrose solution without conidia was offered. The adults were then incubated at 25 ± 1 °C, RH > 98% and 12 h photophase for up to 15 days. Mortality was checked daily, and dead adults were processed as mentioned previously.

2.6. Evaluation of trans-stadial infection

Ten fourth-instar larvae (L4), unfed during the previous 24 h, were exposed to 25 ml of a conidial suspension (10^5 or 10^6 conidia/ml) of ARSEF 964 and then incubated at 25 ± 1 °C, RH 75 ± 5% and 12 h photophase. After 24 h the L4 were transferred to another cup containing 25 ml of sterile water and fed until pupation with ground food as mentioned. For the controls, larvae were kept permanently in the conidial suspension (at the same concentration) or in sterile water without conidia for the positive and negative control, respectively; they were also fed as noted above until reaching the pupal stage. Pupae were transferred to a new cup with 20 ml distilled water, which was placed in a polypropylene container (500 ml) covered with a screen. Emerging adults were fed with 10% sucrose solution. Mortality of larvae, pupae and adults was checked daily for up to 15 days. Dead larvae and pupae were kept in sterile water. Dead adults were set on WA medium. Growth of mycelium and development of conidia on cadavers were checked daily up to 15 days, and fungal structures examined with a microscope.

2.7. Analysis of data

All tests were carried out with three independent repetitions. Values of eclosion and mortality were analyzed with ANOVA and the Student-Newman-Keuls multiple range test for comparison of means (Statistica 7.1; StatSoft, Tulsa, USA). Means were considered significantly different at $P < 0.05$. Lethal times (LT_{50} and LT_{90}), lethal concentrations (LC_{50} and LC_{90}) and their respective confidence intervals (95% CI) were calculated by probit analysis (Mathematica 7.0; Wolfram, Champaign, USA) for dependent and independent data, respectively (Throne et al., 1995; Pacheco and Rabelo, 2013; Savi et al. 2017).

3. Results

3.1. Activity of Culicinomyces spp. in larvae

Larvae treated with conidia as well as control larvae did not change their movements in water in the first 4 h regardless of the fungal species, isolate or conidial concentration tested. In the following 20 h, some larvae treated with *C. clavisporus* isolates, but not those exposed to *C. bisporalis* ARSEF 1948, showed signs of paralysis at higher conidial concentrations (≥ 3.3×10^6 conidia/ml), and the first larvae died at this time. Paralysis was observed in fungus-treated larvae but never in the control larvae. Cumulative mortality 3 days after treatment with 10^6 conidia/ml ranged from 0% (ARSEF 1948) to 100% (ARSEF 964) and generally increased in the next days with a high significant effect of the isolate on mortality ($F_{10,22} \geq 135$; $P < 0.001$) at day 3 and day 5. Most larvae (≥93 ± 3.3%) had died by 10 days after treatment (Table 2). Increases in cumulative mortality depended on the fungal species ($F_{1,163} = 66.7$; $P < 0.001$), isolate (Table 2), conidial concentration ($F_{4,160} = 16.3$; $P < 0.001$, 3 days after treatment) and incubation time ($F_{9,1640} = 154.5$; $P < 0.001$), and are presented in Fig. 1 for selected isolates that induced distinctly lower (ARSEF 1948, 2480) or higher mortalities (ARSEF 372, 644, 964, 2479). The LT_{50} at the same conidial concentration (10^6 conidia/ml) varied between 1 (ARSEF 2479) and 6.9 (ARSEF 1948) days, and values of LT_{90} varied between 2.6 (ARSEF 372, 644 and 2479) and 9.9 days (ARSEF 1948; Table 2). Lethal conidial concentrations to kill 50% and 90% of the larvae, 3 days

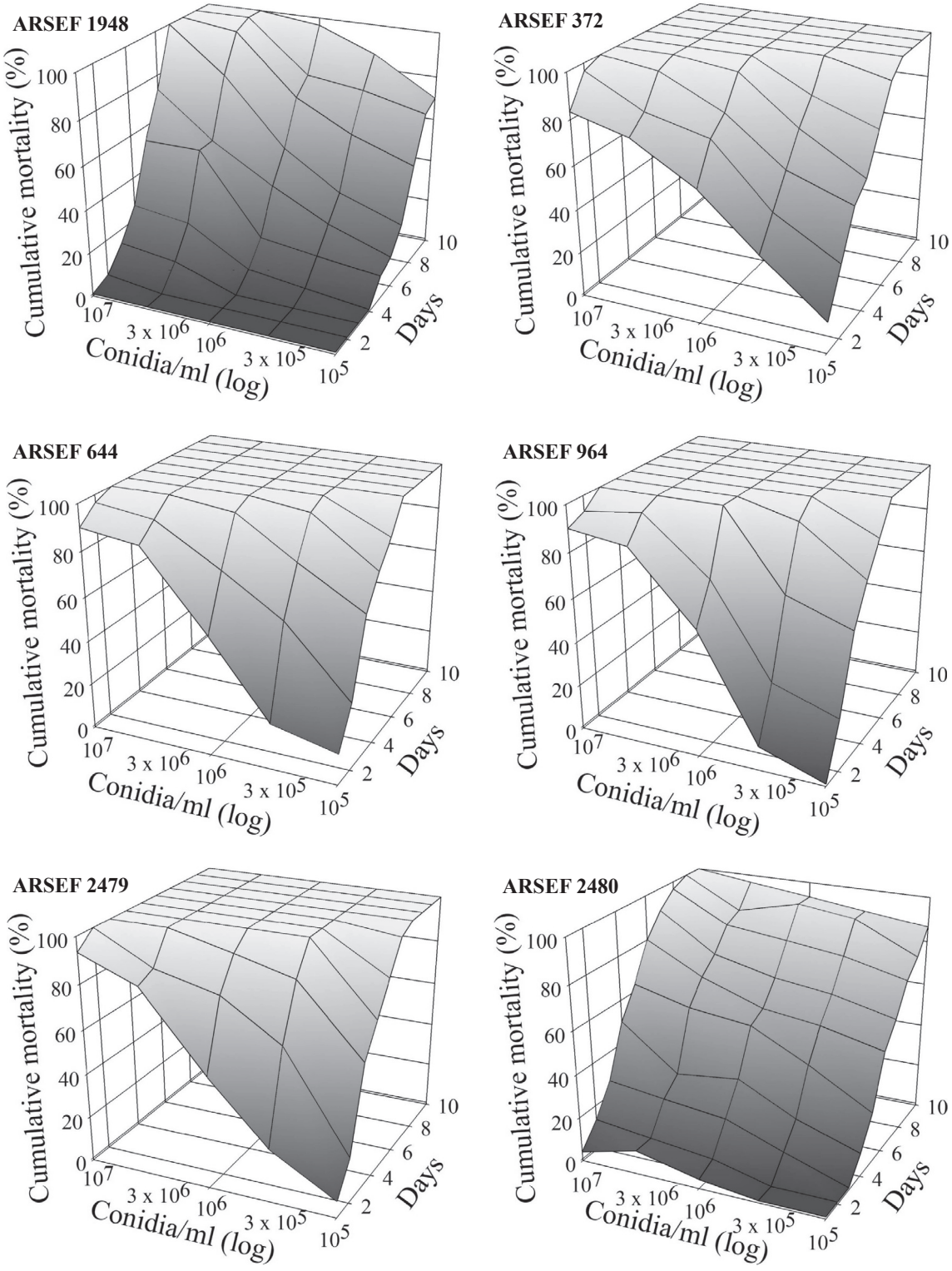

Fig. 1. Relative cumulative mortality of *Aedes aegypti* third-instar larvae exposed to five conidial suspensions (10^5–10^7 conidia/ml) of *Culicinomyces bisporalis* (ARSEF 1948) or *Culicinomyces clavisporus* (ARSEF 372, 644, 964, 2479 or 2480) and incubated at 25 ± 1 °C and 12 h photophase for up to 10 days.

after treatment, reached lowest LC_{50} 2.5×10^5 and LC_{90} 1.9×10^6 conidia/ml with ARSEF 2479 and highest LC_{50} 8.2×10^6 (ARSEF 1948) and LC_{90} 4.4×10^8 conidia/ml (ARSEF 2480; Table 3). Control mortality was ≤10% at the end of the experiments.

The exoskeleton of about 50% of the dead test larvae, treated previously with fungus and kept in distilled water, darkened after staining with Amman's solution, and hyphae and conidiogenous cells with characteristic conidia of *C. bisporalis* or *C. clavisporus* were observed.

3.2. Activity of Culicinomyces clavisporus in eggs and further developing stages

Most eggs (≥92.5 ± 2.5%) treated with *C. clavisporus* ARSEF 644, 964 or 2479 or with water (control) and held at high moisture

Table 3
Lethal concentration (conidia/ml), with respective confidence interval (CI) to kill 50 or 90% (LC_{50} and LC_{90}) *Aedes aegypti* third-instar larvae three days after initial exposure to *Culicinomyces* spp. conidia at five concentrations (10^5–10^7 conidia/ml) and incubated at 25 ± 1 °C, 75 ± 5% relative humidity and 12 h photophase.

ARSEF	LC_{50}	LC_{90}	Slope ± SE
1948	8.2×10^6 (4.6×10^6–3.9×10^7)b	1.9×10^8 (5×10^7–2×10^{10})b	13.9 ± 0.08
372	4.8×10^5 (2.4×10^5–7×10^5)a	3.6×10^6 (8×10^5–7.5×10^6)a	9.6 ± 0.12
582	5.1×10^5 (3×10^5–10^6)a	6.8×10^6 (9×10^5–1.5×10^7)a	8.3 ± 0.07
644	3.6×10^5 (1.9×10^5–6.4×10^5)a	2.5×10^6 (9.8×10^5–10^7)a	9 ± 0.81
706	2.5×10^6 (5×10^5–2.8×10^7)ab	9.3×10^7 (6.4×10^7–2×10^9)b	11.5 ± 0.03
964	3×10^5 (1.5×10^5–5.4×10^5)a	2.2×10^6 (1.3×10^6–8.9×10^6)a	9.6 ± 0.09
1260	1.4×10^6 (8×10^5–5×10^6)ab	6.1×10^7 (1.4×10^7–6×10^8)ab	13.5 ± 0.15
2471	2×10^6 (9.5×10^5–5×10^6)ab	10^8 (8×10^7–6.3×10^8)ab	10.2 ± 0.81
2478	2.4×10^6 (8.8×10^5–5×10^6)ab	9×10^7 (4.8×10^7–2.8×10^8)ab	14.5 ± 0.5
2479	2.5×10^5 (1.3×10^5–6.2×10^5)a	1.9×10^6 (8×10^5–4×10^7)a	12.4 ± 0.12
2480	1.4×10^6 (9×10^5–5.4×10^6)b	4.4×10^8 (4.3×10^7–6×10^{10})b	10.5 ± 0.07

Values in the same column followed by different letters (a–b) were significantly different based on their CI.

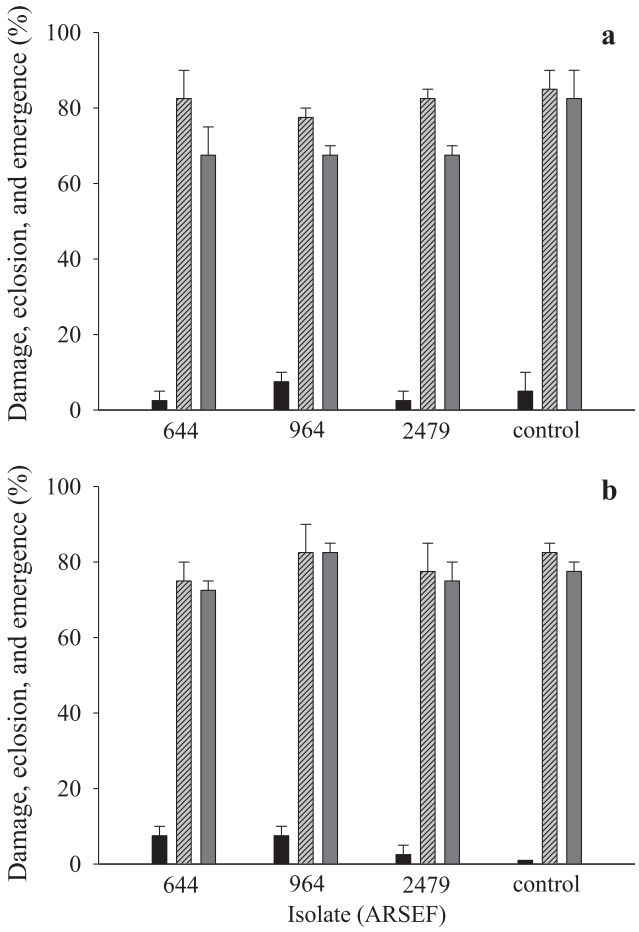

Fig. 2. Relative cumulative numbers (± standard error) of damaged *Aedes aegypti* eggs ( ), eclosed larvae ( ) and posterior emergence of adults ( ) (percentage related to the initial number of eggs) after topical application of *Culicinomyces clavisporus* conidia (5×10^6 conidia/cm^2) or water (control) on eggs and incubation at 25 ± 1 °C and RH > 98% for 10 (a) or 15 (b) days and subsequent submersion of eggs in water for 20 days.

(RH > 98%) generally showed no signs of damage such as shrinkage after desiccation ($F_{3,8} \leq 1$; $P \geq 0.44$; Fig. 2) or growth of mycelium and conidia of the inoculated fungus on the chorionic surface at up to 15 days of exposure. A saprobic *Penicillium* sp. did develop on between 10% and 15% of the *C. clavisporus*-treated and untreated (control) eggs. No larvae eclosed prematurely from fungus- or water-treated eggs held on the filter paper at RH > 98%, 25 ± 1 °C and 12 h photophase up to 15 days.

After submerging eggs in water, the first larvae eclosed immediately, and eclosion continued over the next days with highest rates, ≥30%, in the first 2 days. For eggs treated with conidia or water only and held for 10 days at RH > 98% prior to submersion in water, the cumulative eclosion ranged from 77.5 ± 2.5% (ARSEF 964) to 85 ± 5% (control). At a 15-day exposure, eclosion varied from 75 ± 5% (ARSEF 644) to 82.5 ± 7.5% (ARSEF 964 and control; Fig. 2). There was no significant effect of the isolate on cumulative eclosion of larvae ($F_{3,8} \leq 1.7$; $P \geq 0.31$), regardless of the incubation periods tested.

The cumulative mortality of larvae, related to the number of eclosed larvae, ranged from 3.1 ± 3.1% (control) to 19.2 ± 5.8% (ARSEF 964) after a previous 10-day incubation of eggs and, after a 15-day incubation, from 3 ± 3% (ARSEF 2479) to 6.5 ± 6.5% (ARSEF 644; Fig. 3). Larval mortality was significantly different between the isolates and the control ($F_{3,8} = 4.3$; $P = 0.04$) at a 10-day incubation, but not after a 15-day incubation ($F_{3,8} = 0.2$; $P = 0.94$). No development of *C. clavisporus* mycelium or conidia on dead larvae was observed.

The cumulative emergence of adults, based on the initial number of eggs, 20 days after submersion of eggs in water, ranged from 67.5 ± 7.5% (ARSEF 644) to 82.5 ± 7.5% (control) for eggs with a previous 10-day incubation at RH > 98% and, with 15 days of incubation, from 72.5 ± 2.5% (ARSEF 644) to 82.5 ± 2.5% (ARSEF 964; Fig. 2). There was no significant effect of the isolate on the cumulative emergence of adults ($F_{3,8} \leq 1.8$; $P \geq 0.1$), regardless of the previous incubation period.

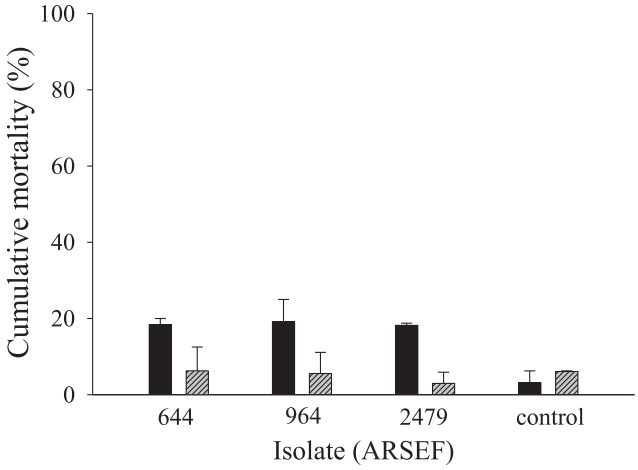

Fig. 3. Relative cumulative mortality (± standard error) of *Aedes aegypti* larvae (calculated on the total number of eclosed larvae) 20 days after submersion of eggs in water; before submersion, eggs were treated with *Culicinomyces clavisporus* conidia (5×10^6 conidia/cm^2) or water (control) and incubated for either 10 ( ) or 15 days ( ) at 25 ± 1 °C and RH > 98%.

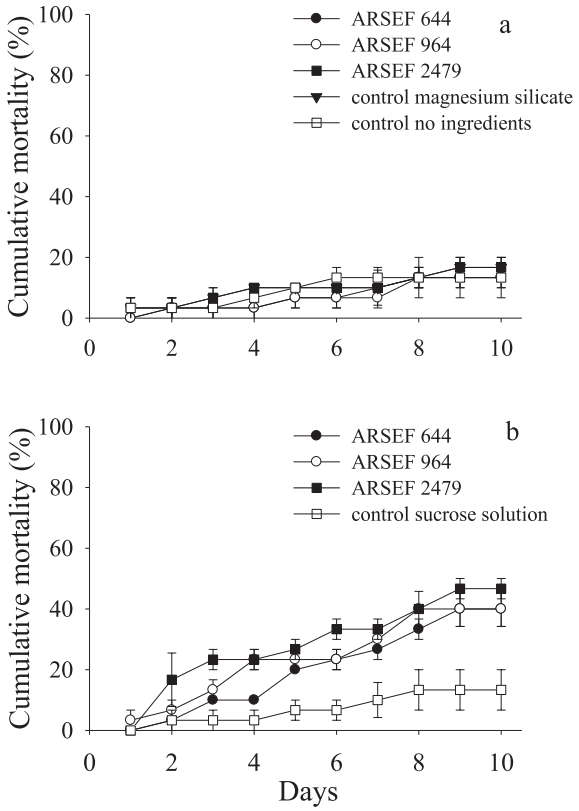

Fig. 4. Relative cumulative mortality (± standard error) of *Aedes aegypti* adults exposed to *Culicinomyces clavisporus* ARSEF 644, 964 or 2749 at 10^5 conidia/cm^2 prepared in magnesium silicate (MS), MS only or no ingredient (controls) (a), or fed a 10% sucrose solution with 10^6 conidia/ml of the same isolate, 10% sucrose solution only (control) (b). Adults were incubated at 25 ± 1 °C, RH > 98% and 12 h photophase for up to 10 days.

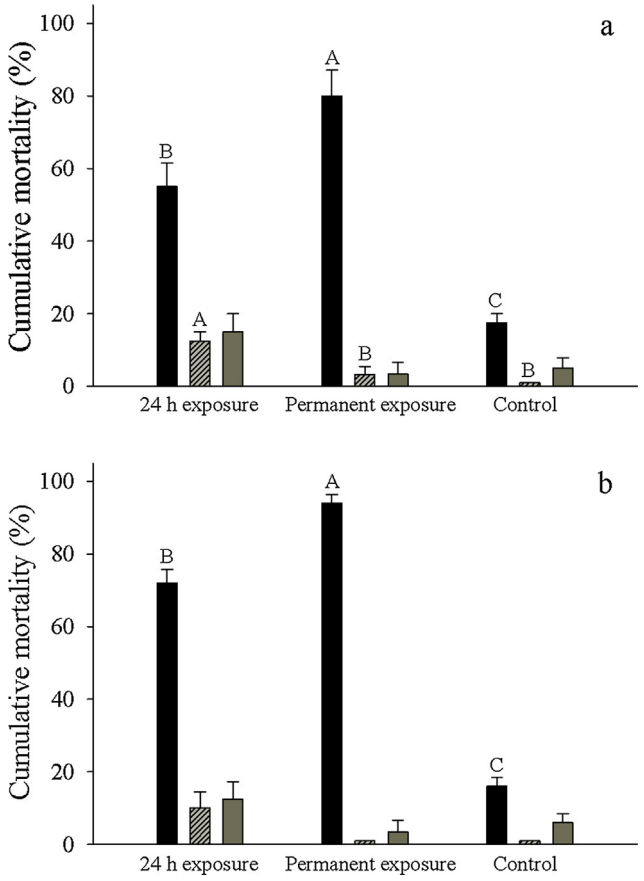

Fig. 5. Relative cumulative mortality (± standard error) of *Aedes aegypti* fourth-instar larvae (L4) ( ), pupae ( ) and adults ( ) 15 days after initial treatment of L4 with *Culicinomyces clavisporus* ARSEF 964 and exposure at 25 ± 1 °C, RH > 98% and 12 h photophase. Larvae were exposed either for 24 h or permanently to 10^5 conidia/ml (a) or 10^6 conidia/ml (b) or permanently to distilled water (control). Relative mortality of larvae, pupae and adults was calculated based on the initial number of L4. Values of mortality in columns within the same stage (L4, pupae or adults) and part of the figure (a–b) with different letters (A–C) were significantly different (ANOVA, SNK, $P < 0.05$).

3.3. Activity of Culicinomyces clavisporus in adults

The mortality of adults treated indirectly with *C. clavisporus* conidia (at 10^5 conidia/cm^2) ranged from 13.3 ± 6.7% (ARSEF 644 and 964) to 16.7 ± 3.3% (ARSEF 2479) at a 10-day exposure to conidia mixed with magnesium silicate (MS) on the cup's inner surface. A similar mortality (≤13.3 ± 3.3%) was obtained with the controls exposed in cups treated with MS only or in untreated cups (Fig. 4a). There was no effect of the treatment on adult mortality ($F_{4,10} = 0.3$; $P = 0.87$).

Adults fed on 10% sucrose solution containing conidia (10^6 conidia/ml) began to die within 2 days and, after 8 additional days, cumulative mortality ranged between 40 ± 5.7% (ARSEF 644 and 964) and 46.7 ± 3.3% (ARSEF 2479; Fig. 4b). Control mortality after this period was 13.3 ± 3.3% and was significantly lower than groups treated with conidia ($F_{3,8} = 11$; $P = 0.003$), but there was no effect of the isolate on mortality ($F_{2,6} = 0.6$; $P = 0.6$). Saprobic fungi were observed growing on a few individuals, but no growth of *C. clavisporus*, regardless of the inoculation technique or isolate, was observed on dead adults set on WA medium and held at 25 ± 1 °C for up to 10 days.

3.4. Trans-stadial infection with Culicinomyces clavisporus

The first fourth-instar larvae began to die within the first 24 h, regardless of exposure time (24 h or permanently in the positive control) to ARSEF 964 conidia or concentration tested (10^5 or 10^6 conidia/ml). Pupation also began in the first 24 h, and the first dead pupae were found on the second day. The first adults emerged by day 3 after treatment.

At the low conidial concentration (10^5 conidia/ml), the exposure time of larvae to conidia (0, 24 h or permanent) had a significant effect on mortality of larvae and pupae ($F_{2,12} \geq 14.9$; $P \leq 0.002$) but not of newly emerged adults ($F_{2,12} = 2.9$; $P = 0.13$; Fig. 5a).

At a 24-h initial exposure of L4 to conidia at 10^5 conidia/ml, the larval mortality reached 55 ± 6.5% after 20 days of incubation. Based on the initial number of L4 exposed, pupal mortality was 12.5 ± 2.5%, and 15 ± 5% of the adults died after emerging (Fig. 5a). When larvae were continuously exposed to the conidial suspension (positive control), larval mortality was 80 ± 6.7%, and 2.5 ± 2.5% at pupal stage and 3.3 ± 3.3% as adults (Fig. 5a). Mortality of larvae, pupae and adults in the negative control without fungal treatment was 17.5 ± 2.5%, 0%, and 5 ± 2.8%, respectively.

In L4 exposed for 24 h or continuously to higher conidial concentration (10^6 conidia/ml) pupal mortality after 20 days was 10 ± 4.5% and 0%, respectively, and adult mortality was 12.5 ± 4.8% and 3.3 ± 3.3% at the same time without significant difference from the control (6 ± 2.4%; $F_{2,12} \leq 2.9$; $P \geq 0.13$; Fig. 5b). *C. clavisporus* developed on dead larvae held in water but not on larvae placed on WA medium. No development was found on or inside dead pupae or adults set on WA medium regardless of the exposure time.

4. Discussion

Both known *Culicinomyces* species, *C. clavisporus* and *C. bisporalis*,

after initial intense exploration in the 1980s, subsequently received very little attention as pathogens of mosquito and biting midge vectors (Panter and Frances, 2003; Scholte et al., 2004; Unkles et al., 2004; Singh and Prakash, 2012; Rodrigues et al., 2017). The distinct specificity of *Culicinomyces* spp. as entomopathogens known to attack aquatic larval stages of culicids and ceratopogonids is a further benefit as a candidate for a mycoinsecticide to control mosquitoes. In the last decade, research on entomopathogenic fungi for the biological control of anopheline and aedine vectors increased distinctly (Lacey, 2017).

Culicinomyces clavisporus is comparatively well studied from 27 known isolates from northern America and Australia and, with a demonstrably remarkable virulence for some isolates in *A. aegypti* larvae, has substantial potential for the control of mosquito larvae. The only two *C. bisporalis* isolates are known from Australia; all isolates of these two species are deposited in the USDA-ARS Collection of Entomopathogenic Fungi (ARSEF) in Ithaca, NY, USA. The small number of *Culicinomyces* isolates is probably related to their cryptic development on dead larvae (C. Luz, C. Bergamini, C. Montalva, J. Rodrigues and R. A. Humber, unpublished data) and the difficulty of detecting *Culicinomyces* spp. infecting mosquito larvae in the field. The few records, all from North America and Australia (Humber et al., 2014) and from diverse temperate and tropical climates, did not show any correlation between the isolatés virulence in *A. aegypti* larvae and the original climate or host. On culture medium *Culicinomyces* spp., like most other entomopathogenic fungi, develop best in a 20–25 °C temperature range (Ferron et al., 1991; Rodrigues et al., 2017).

Both species present a singular means to infect their hosts through the gut after ingestion of conidia. The distinct virulence, especially at higher conidial concentrations detected for some of the tested *C. clavisporus* isolates but not of the *C. bisporalis* isolate, is obviously related to toxic metabolites released by the germinating conidia in the gut that kill larvae quickly before any hyphae reach the haemocoel (Sweeney, 1981; Panter and Russell, 1984). There are no studies on metabolites of the genus *Culicinomyces* and their toxic activity in mosquitoes. Recently, Singh and Prakash (2012) reported on the activity of *C. clavisporus* culture filtrates in *A. aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi* adults. Based on the results of our extensive screening on virulence, ARSEF 964, 644, 2479 are the most promising candidates to consider for further development of control of *A. aegypti* larvae. Furthermore, these isolates produced the highest numbers of conidia on culture medium in a previous comparative study (Rodrigues et al., 2017).

Tests with selected isolates made clear that *C. clavisporus* conidia affect solely larvae and have no ovicidal or adulticidal activity for *A. aegypti*. Apparently, conidia must be ingested in order to be able to develop, and only the larval stage of the mosquito is able to ingest solid food filtered from the water. It is not clearly understood how germination of conidia is triggered in the larval gut but conidia applied onto the cuticle of the adults or the chorion of eggs were not stimulated to germinate under the test conditions. *Culicinomyces* caused no deformation or obvious damage of the chorion, the embryo or any developing larva inside the egg. Damage to the chorion and a premature eclosion of larvae from fungus-treated *A. aegypti* eggs without any previous contact with water was reported for other entomopathogenic fungi such as *Metarhizium anisopliae* and *Tolypocladium cylindrosporum* (Luz et al., 2007; Sousa et al., 2013; Flor-Weiler et al., 2017). However, intact larvae that eclosed from fungus-treated eggs and ingested their conidium-contaminated chorion or conidia suspended in the surrounding water were susceptible to infection with the fungus in this study.

The isolates of *C. clavisporus* did not affect adults when conidia were applied onto the cuticle, probably due to the inability of the fungus to develop on the cuticle and reach to the inner haemocoel. Adults however, died after ingesting conidia, but without any later visible growth of *C. clavisporus* on the cadavers placed on WA, so no infection of adults was confirmed. Currently, a control of this vector by oral application of conidia in adults does not appear to be feasible.

Infections by *C. clavisporus* in some late-surviving larvae eventually persist to the following developmental stages, so that the fungus may injure and kill pupae and adults that developed from infected larvae. The development of mycelia and subsequent sporulation of the fungus on dead pupae and adults on WA was not especially strong under our test conditions, and development of the pathogen was restricted to the interior of dead individuals on WA. The fungus is probably disseminated by contaminated or infected adult females dispersing by flying to new breeding sites and succumbing there to fungal infection exposed to water. Fungal infection in a larval population in a breeding site is transmitted to healthy larvae by conidia developing on larvae killed by the fungus. The rapid death of larvae after ingestion of the conidia of highly virulent *C. clavisporus* isolates, probably caused by toxic metabolites, could affect any further development of the fungus and production of new conidia in the dead larvae, and might subsequently hamper the transmission of the infection. The lack of sporulation on these dead larvae could increase the safety–and, potentially, increase the profitability–of a mycoinsecticide based on a highly virulent isolate. The dynamics of the dispersal and persistence of this fungus in larval populations have not yet been studied.

Up to now, and based on the results on larvicidal activity of the present study and previous findings on virulence and *in vitro* development (Rodrigues et al. 2017), *C. clavisporus* ARSEF 964, and also ARSEF 644 and 2479, have high potential for biological control of *A. aegypti* larvae.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jip.2018.08.010.

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