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New insights into the *in vitro* development and virulence of *Culicinomyces* spp. as fungal pathogens of *Aedes aegypti*

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ABSTRACT

Culicinomyces spp. (Hypocreales: Cordycipitaceae) are facultative fungal pathogens affecting the larval stages from a range of mosquito species and are especially notable in their ability to infect hosts through the digestive tract after conidial ingestion. While *Culicinomyces* spp. were studied mainly in the 1980s, little is yet known about inter- and intraspecific variability of the *in vitro* development of these fungi at different temperatures, and nothing is known about the impact of serial host-passage on the development or virulence against *Aedes aegypti* larvae. The development of ten isolates of *C. clavisporus* (ARSEF 372, 582, 644, 706, 964, 1260, 2471, 2478, 2479 and 2480) and one of *C. bisporalis* (ARSEF 1948) was assessed on solid SDAY/4 and liquid SDY/4 at 15, 20, 25, 30 and 35 °C. Based on the results of these assays, three isolates were selected (ARSEF 644, 964 and 2479) for three serial host-passage/reisolation cycles, and comparison of the reisolates with the original stock isolates for their virulence, vegetative growth and conidiogenesis. The highest germination rates (≥95%) after 48 h incubation were obtained at 25 and 20 °C, and the lowest germination (≤12%) at 35 °C after the same time. The optimal temperature for radial growth was 25 °C (≥11.8 mm), followed by 20 °C for all isolates. ARSEF 706, 582 and 372 showed the greatest vegetative growth (≥20 mm). In general, there was little radial growth of colonies at 30 °C (≤2.5 mm), and none at 35 °C. Isolates, especially ARSEF 964, 2479, and 644, generally produced the highest numbers of conidia at 25 °C (≥1.42×10^5 conidia/plate) after 15 days. After two host-passages, conidiogenesis increased significantly on SDAY/4 for ARSEF 2479 but not for ARSEF 644 or 964. All larvae exposed to these three isolates of *C. clavisporus* died within 7 days regardless of the concentration or host-passage; *C. bisporalis* was not tested in these experiments. The virulence of ARSEF 964 increased at lower concentrations (10^6–3×10^5 conidia/ml) after the first host-passage.

1. Introduction

The genus *Culicinomyces* (Hypocreales: Cordycipitaceae) facultatively infects aquatic larval stages of a range of culicid dipterans, including such important vectors of human diseases as *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles* spp. (Knight, 1980). Unlike other entomopathogenic fungi, however *Culicinomyces* species are known to invade their hosts through the digestive tract after conidial ingestion (Sweeney, 1975). *Culicinomyces clavisporus*, the most widely distributed and most studied species, was first reported in 1972 from Sydney, Australia (Sweeney et al., 1973), and from Chapel Hill, North Carolina, United States of America (Couch et al., 1974). In both cases, *C. clavisporus* was isolated from laboratory-reared anopheline larvae exposed to field-collected water samples (Sweeney et al., 1973; Couch et al., 1974). *C. clavisporus* was described as a new monotypic genus and species by Couch et al. (1974), and since that time only one additional species, *C. bisporalis*, has been described (Sigler et al., 1987). Some isolates of *C. clavisporus* were studied in the 1970s and 1980s, but after initially encouraging findings, the interest in this pathogen decreased due to issues related to the high conidial inoculum necessary for reasonable infection rates, the uncertain tolerance of the fungus to higher temperatures (Sweeney, 1975), and its low persistence in the field (Knight, 1980); such complications tended to discourage further studies of the potential of these entomopathogens for biological control of mosquitoes. However, recent severe outbreaks of dengue, chikungunya and Zika fevers in tropical and subtropical Latin America and Asia have renewed the search for additional non-chemical methods to control *A. aegypti*, and have increased the general interest in entomopathogenic fungi for biological control. To date, approximately 20 isolates of *C. clavisporus* and one of *C. bisporalis* have been available for study. These isolates originated from regions with subtropical, tropical and temperate climates in North America, and Australia, and all isolates

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were obtained from culicid or ceratopogonid larvae. Little is yet know about either the inter- or intraspecific variability of isolates within this genus.

For the development of mycoinsecticides based on *Culicinomyces* spp. against *A. aegypti*, it is crucial to select highly virulent isolates that are adapted to elevated temperature and to test cultural conditions that allow the highest conidial production. It is well known that subculturing entomopathogenic fungi on artificial media may induce phenotypic degeneration and cause attenuation of virulence in susceptible host insects (Ibrahim et al., 2002; Ryan et al., 2002; Butt et al., 2006). Even so, the virulence of these fungi was eventually restored or even increased after one or more (serial) passages through a suitable host (Adames et al., 2011; Butt et al., 2006). While the phenotypic properties and pathogenicity of *C. clavisporus* were maintained through years of continuous subculturing on solid medium (Sweeney, 1981); nothing is yet known about the effects of host-repassage in this genus or as a result of prolonged periods of cryopreservation, (as was reported by Hajek et al., 1990, for an entomophthoralean fungal pathogen of gypsy moth larvae).

The present study reports the impact of temperature on *in vitro* germination, growth and conidial production of *C. clavisporus* and *C. bisporalis* isolates as well as the effect of serial passages of selected isolates through *A. aegypti* on virulence against larvae or their overall capacity to produce conidia.

2. Material and methods

2.1. Origin and preparation of the fungi

A total of ten *C. clavisporus* and one *C. bisporalis* isolates was tested. All fungi were obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, NY, USA), and information about their origins appears in Table 1. The isolates were routinely grown for 15 days 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 and 75 ± 5% relative humidity (RH). For the assays, 2 ml of sterile distilled water were added onto the culture, and aerial conidia were harvested by scraping with a spatula. The conidial suspensions were filtered through hydrophilic cotton and then transferred into microcentrifuge tubes (2 ml). The number of conidia was calculated with a hemocytometer, and the suspensions were adjusted to required concentrations.

2.2. Origin, rearing and preparation of Aedes aegypti

Adults of *A. aegypti*, Rockefeller strain (originally from Cuba), were reared in the laboratory at 27 ± 1 °C and 75 ± 10% RH in cages (40 × 40 × 30 cm) and fed *ad libitum* with cloth pads saturated with 10% saccharose (sucrose) solution. Females were artificially fed once per week on membranes placed over fresh, defibrinated cow's blood. For oviposition, filter papers (10 × 15 cm) were set on amber-colored glass flasks containing 100 ml of tap water and placed in the mentioned cages. Each 5 days the filter papers were retrieved from the cages and dried for 24 h. Eggs on filter papers, which at this time bore fully developed larvae, were stored in plastic bags under the same conditions as the adults (see above) until use. For the assays, filter papers with eggs were transferred into a plastic bowl with 500 ml of tap water, and larvae were fed daily with small quantities of powdered dry cat food (Whiskas, Mars, Incorporated, McLean, USA) until reaching the needed larval stage.

2.3. Effects of temperature on conidial germination

Twenty µl of a conidial suspension of 10^6 conidia/ml (a total of 2×10^4 conidia) of the isolates were dropped on the center of SDAY/4 medium in two Petri dishes for each isolate, and the dishes subsequently incubated at 15, 20, 25, 30 or 35 ± 1 °C, 75 ± 5% RH in scotophase. At 24 or 48 h of incubation, the germination was stopped with a drop of 50% acetic acid, examined with a light microscope (Olympus BX 51, Olympus USA), and photographed digitally (CFscan camera, JenOptik) at 400× magnification for subsequent assessment. At least 300 conidia per dish were evaluated and scored as non-germinated or germinated if the germ tube length was greater than the conidial diameter.

2.4. Effects of temperature on vegetative growth and conidial production on solid medium

Twenty µl of a conidial suspension of 10^7 conidia/ml (total of 2×10^5 conidia) of each isolate were dropped on SDAY/4 medium in the center of Petri dishes, and these incubated at 15, 20, 25, 30 or 35 ± 1 °C in scotophase at 75 ± 5% RH. The colonial diameter of each isolate was measured daily with a graduated ruler for 15 days. Subsequently, aerial conidia were harvested and quantified as mentioned. The viability of new conidia was checked by inoculating 20 µl of each suspension onto SDAY/4 medium, and incubating at 25 °C and 75 ± 5% RH for 48 h. The germination was then quantified microscopically as noted in Section 2.1.

2.5. Vegetative growth and conidial production in liquid medium

One small piece (ca. 1 mm^3) of the SDAY/4 medium with a 15-day fungal culture of each isolate was cut with a scalpel and transferred into

Table 1
Culicinomyces isolates and their respective collection information.

Species	ARSEF[a]	Geographical origin	Climate[b]	Year	Host
Culicinomyces bisporalis	1948	Milla Milla, Australia	Cfa	1984	*Aedes kochi*[c]
C. clavisporus	372	Chapel Hill, USA	Cfa	1979	*Anopheles quadrimaculatus*[c]
	582	Chapel Hill, USA	Cfa	1981	*An. quadrimaculatus*[c]
	644	Sydney, Australia	Cfa	1981	*An. amictus hilli*[c]
	706	North Carolina, USA	Cfa	1982	Culicíde larva[c]
	964	Devon, Canada	Dfb	1982	*Culiseta inornata*[c]
	1260	Devon, Canada	Dfb	1984	*Cu. inornata*
	2471	Mittagong, Australia	Cfb	1981	*Ae. rupestris*[c]
	2478	Mittagong, Australia	Cfb	1983	*Dasyhelea* sp.[d]
	2479	Mallacoota, Australia	Cfb	1984	*Cu. inconspicua*[c]
	2480	Milla Milla, Australia	Cfa	1985	*Forcipomyia marksae*[d]

[a] Obtained from USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, New York).
[b] Köppen-Geiger climatic classifications (Geiger, 1961): Cfa = temperate/tropical hot summer; Cfb = maritime/oceanic temperate; Dfb = warm summer continental.
[c] Diptera: Culicidae.
[d] Diptera: Ceratopogonidae.

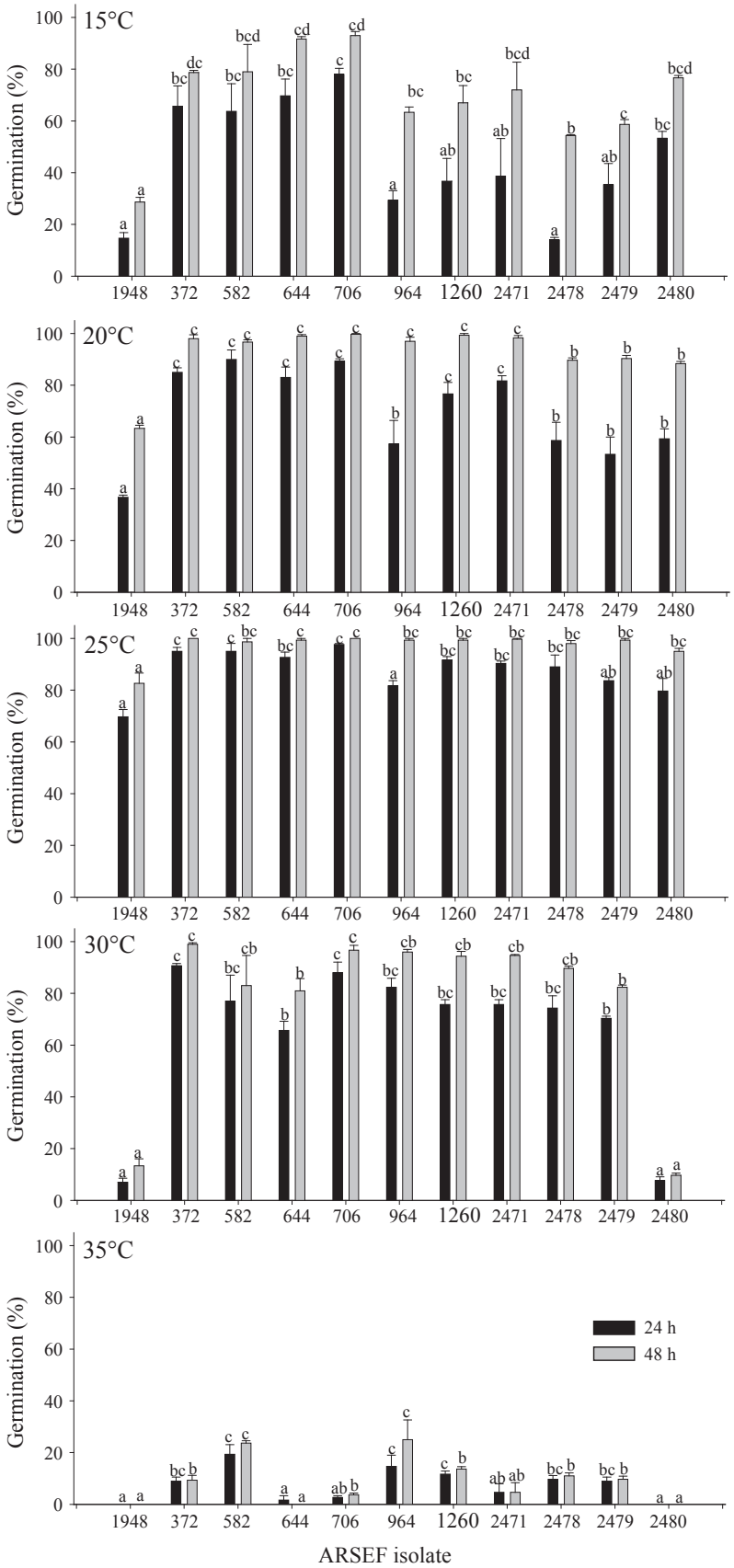

Fig. 1. Relative cumulative germination of *Culicinomyces clavisporus* (ARSEF 372, 582, 644, 706, 964, 1260, 2471, 2478, 2479 and 2480) and *C. bisporalis* (ARSEF 1948) conidia inoculated on SDAY/4 and incubated at 15–35 °C up to 48 h.

tissue culture flasks (Corning® 25 cm^2 flask, Corning Costar Corporation, Cambridge, USA) containing 10 ml of SDY/4 broth, and flasks were incubated at 15, 20, 25, 30 or 35 ± 1 °C for 15 days in scotophase. Each flask was shaken daily by hand for 10 s to submerge the developing mycelium, and the mycelial growth was scored visually as either none, low or abundant. Every 5 days, each culture was shaken by hand to assume a homogeneous distribution of conidia before removing a 250-µl aliquot with a pipette for conidial quantification; conidia were quantified as mentioned.

2.6. Passage through host and reisolation of C. clavisporus

The three isolates–ARSEF 644, 964 and 2479–with the highest conidial production on solid medium in the previous assays were selected for further studies. They were grown on SDAY/4, and aerial conidia harvested with 5 ml of distilled sterile water as noted above. A final 20 ml of suspension (10^7 conidia/ml) was prepared for each isolate. Ten third-instar larvae (L3) were exposed to this suspension, and incubated at 25 ± 1 °C and 75 ± 5% RH. After 24 h, newly deceased larvae were transferred into 20 ml of distilled sterile water and incubated in the same temperature and humidity. After 48 h, the larvae were transferred onto SDAY/4 plus chloramphenicol (0.05% by volume; Sigma Chemical Company, St Louis, USA), and incubated as noted in Section 2.1. The fungal growth on larval bodies was checked daily, and after 5 days, the reisolated fungus was transferred to SDAY/4, grown for 15 days, and then stored at 4 °C. *A. aegypti* larvae were exposed to conidia from a newly reisolated strain, and this procedure was repeated serially up to a total three reisolations for each strain.

2.7. Virulence and conidial production of reisolated C. clavisporus

Aerial conidia were harvested from 15-day cultures of the original (non-repassaged) isolates and also from cultures originating from the first, second, and third reisolations of ARSEF 644, 964 and 2479. Suspensions of 10^5, 3.3×10^5, 10^6, 3.3×10^6 and 10^7 conidia/ml were prepared for each isolate. Ten L3 were exposed to 20 ml of each suspension and incubated at 25 ± 1 °C and 75 ± 5% RH. Control larvae were inoculated with 20 ml of sterile distilled water without conidia. Larvae were fed each 2 days with a small amount of cat food (as noted above), and mortality was monitored daily for up to 10 days. Dead larvae were transferred into 20 ml of sterile distilled water, and the fungal development on the larvae was monitored for 5 days.

Additionally, the vegetative growth and conidial production of each isolate in liquid (SDY/4) or solid (SDAY/4) media were evaluated after 15 days at 25 ± 1 °C as mentioned above.

2.8. Development of C. clavisporus in low-oxygen environment

Nitrogen gas was added to flasks aseptically by removing the cap of each tissue culture flask containing 10 ml of SDY/4 broth, and immediately lowering each flask individually into nitrogen vapor in a cryogenic dewar to displace the air in the flasks; these nitrogen-filled flasks were stored at 25 ± 1 °C for 24 h to allow gas equilibration. Then 100 µl of conidial suspension (10^7 conidia/ml) of ARSEF 644, 964 or 2479 were added, and incubated at the same conditions mentioned above for 15 days. Each 5 days the conidial production was quantified by a hemocytometer as noted above, and the flasks were re-charged with nitrogen gas.

2.9. Analysis of data

All tests were carried out with three independent repetitions. Values of germination, colony diameter, conidial production 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 to be 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% C.I.) were calculated by probit analysis (Mathematica 7.0, Wolfram, Champaign, USA) for dependent and independent data, respectively (Preisler and Robertson, 1989; Throne et al., 1995).

3. Results

3.1. Effect of temperature on in vitro germination

In general, *Culicinomyces* spp. were slow to germinate, regardless of the isolate or temperature tested. Germination after 24 h of incubation ranged between 68% (ARSEF 1948) and 95% (ARSEF 706); only ARSEF 372 and 706 reached a 100% germination after 48 h (Fig. 1). There was a significant effect of the incubation time on conidial germination at different temperatures tested ($F_{1,4} \geq 3.4$; $P \leq 0.045$: 48 h > 24 h), and a highly significant effect of the temperature (ranked from highest to lowest rates: 25 °C = 20 °C > 30 °C > 15 °C > 35 °C) on germination for all 11 tested *Culicinomyces* isolates at 24 h ($F_{4,10} \geq 12.6$; $P \leq 0.002$) and 48 h ($F_{4,10} \geq 42.5$; $P < 0.001$). Germination rates, regardless of the temperature, varied significantly among the isolates tested ($F_{10,22} \geq 8.3$; $P < 0.001$). *C. clavisporus* isolates germinated at the highest rates at 25 °C (≥79.7% after 24 h and ≥95% after 48 h), followed by 20 °C (≥53.3% after 24 h and ≥88.3% after 48 h; Fig. 1). All conidia of ARSEF 372 and 706 germinated after 48 h at 25 °C. There was no significant effect of the climatic classification defined by Geiger (1961) where fungi originally had been isolated (Table 1) on germination at these temperatures ($F_{2,8} \leq 2.2$; $P \geq 0.2$). *C. bisporalis* ARSEF 1948 germination was 63 ± 1.2% and 82 ± 4% at 20 °C and 25 °C, respectively, after 48 h (Fig. 1). At 35 °C, some conidia (< 20%), regardless of the isolate and species tested, showed some swelling (an initial stage of germination) and began to form a germ tube that never became long enough to score as germinated. No conidia of ARSEF 2480 (*C. clavisporus*) or 1948 (*C. bisporalis*) germinated at 35 °C, and the germination of all the other isolates at this temperature was ≤23.6% (Fig. 1).

3.2. Vegetative growth and conidial production in liquid or solid media

In liquid medium (SDY/4), the vegetative growth of *Culicinomyces* spp. was first observed after 3 days of incubation at 20–25 °C. At these temperatures, mycelial growth was abundant regardless of the isolate tested; maximal biomass was found after 8–10 days of incubation. At 15 °C, first growth was only visible after 4–5 days and abundant after 12 days. At 30 °C, mycelium became visible for the same isolates after 3 or 4 days; however, growth was not abundant in any of the isolate; the isolates ARSEF 1948, 644 and 2480 showed only little visible growth at 30 °C. No vegetative growth was observed at 35 °C for any isolates. For isolates tested in low oxygen environment at 25 °C (ARSEF 644, 964 or 2479), the rate and quality of vegetative development was similar to those tested at ambient level of oxygen.

There were highly significant effects of temperature ($F_{4,10} \geq 90.2$; $P \leq 0.001$) and isolate ($F_{10,22} \geq 15.3$; $P \leq 0.01$) tested on the vegetative growth rates on the solid medium (mm/day) of *Culicinomyces* spp. The highest growth rates occurred at 25 °C for all isolates tested, and ranged from 1.6 mm/day (ARSEF 706) to 0.9 mm/day (ARSEF 644 and 2471; Fig. 2). At this temperature and after 15 days of incubation, ARSEF 706, 582 and 372 presented the largest colonies (≥20 mm) whose sizes differed statistically from all other isolates ($F_{10,22} \geq 15.4$; $P < 0.001$; Fig. 3a). At 20 °C, the growth rates ranged from 1.4 mm/day (ARSEF 706) to 0.6 mm/day (ARSEF 644), with a final colony size ≥17.7 mm (Figs. 2 and 3a). For isolates incubated at 15 °C, the growth rate was between 0.72 to 0.27 mm/day. At 30 °C there was little radial extension (≤2.5 mm), and no growth by any tested isolates at 35 °C after 15 days (Fig. 3a). There was no significant effect of the climatic classification of the fungal origin (Table 1) on radial growth regardless

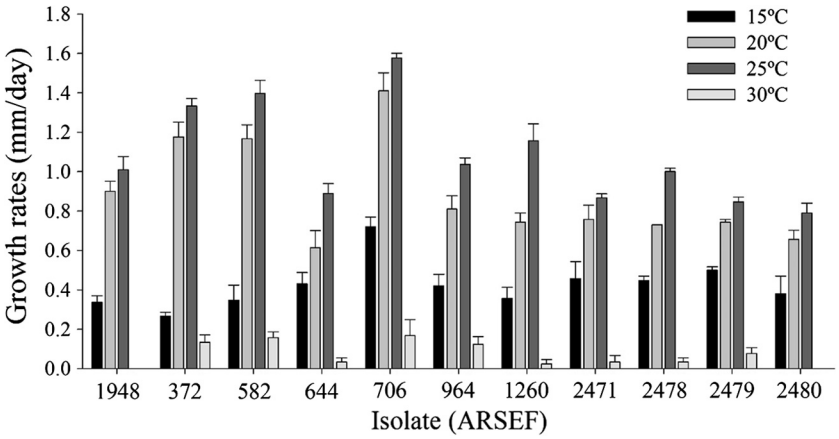

Fig. 2. Daily growth rate of *Culicinomyces clavisporus* (ARSEF 372, 582, 644, 706, 964, 1260, 2471, 2478, 2479 and 2480) and *C. bisporalis* (ARSEF 1948) conidia inoculated on SDAY/4 and incubated at 15–35 °C for 15 days.

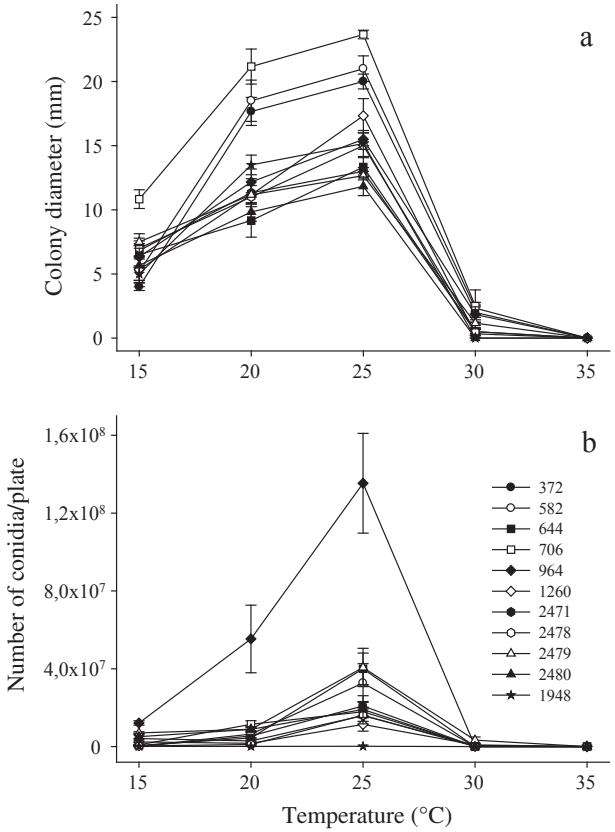

Fig. 3. Colony diameter (a) and conidial production (b) of *Culicinomyces clavisporus* (ARSEF 372, 582, 644, 706, 964, 1260, 2471, 2478, 2479, 2480) and *C. bisporalis* (ARSEF 1948) on SDAY/4 incubated for 15 days at 15–35 °C. See Supplementary Tables 1 and 2 for the data presented in these graphs.

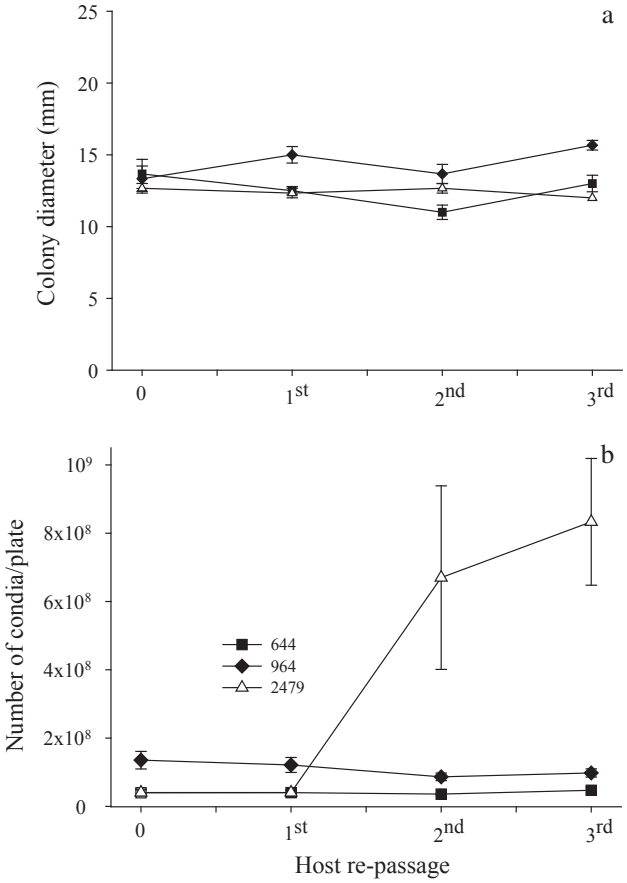

Fig. 4. Colony diameter (a) and conidial production (b) of *Culicinomyces clavisporus* (ARSEF 644, 964, 2479) no or up to three host-passage, on SDAY/4 incubated for 15 days at 15–35 °C.

of the temperature ($F_{2.8} \leq 2.5$; $P \geq 0.78$) Fig. 4.

All isolates produced aerial conidia on SDAY/4 and on the surface of liquid SDY/4 when incubated without agitation. In flasks that were shaken daily, no aerial or submerged conidial production was observed, regardless of the isolate, temperature or oxygen availability. The best conidial production on solid medium, regardless of the isolate, was found at 25 °C ($\geq 1.42 \times 10^5$ conidia/plate). At this temperature, ARSEF 964 produced the most number of conidia (10^8 conidia/plate; Fig. 3b), and the number of conidia of this isolate was significantly higher than those of all other isolates of *C. clavisporus* or *C. bisporalis* ($F_{10,22} = 14.5$; $P < 0.001$). At 15 °C and 20 °C, the general conidial production was $\geq 6 \times 10^4$ and $\geq 9.7 \times 10^4$ conidia/plate, respectively; few conidia ($\leq 10^5$ conidia/plate) were produced at 30 °C; no conidia at all were formed at 35 °C. There was no significant effect of the climatic classification for the sites of origin of these isolates on the number of conidia ($F_{2.8} \leq 2.6$; $P \geq 0.13$).

3.3. Vegetative growth, conidial production and virulence of *C. clavisporus* reisolates

No changes of color or colony size of any isolate after host repassage were observed, even after three serial repassages through a host. The vegetative growth rates of ARSEF 644, 964 or 2479 passed through *A.*

aegypti larvae (1–3 passages) were similar to the rates of the wild types, and there was no significant effect of the host-passage on vegetative growth on SDAY/4 ($F_{2,7} \leq 2.5$; $P \geq 0.09$) (Fig. 4a). However, conidial production by ARSEF 2479 was 4.1×10^7 conidia/plate for the wild type and first repassage was noted to increase significantly after the second passage in *A. aegypti* larvae (6×10^8 conidia/plate), but remained without further significant increase after the third passage (8.3×10^8 conidia/plate; $F_{3,8} = 6.5$; $P = 0.02$; Fig. 4b). There was no significant effect of the host-passage on conidial production by ARSEF 644 and 964 ($F_{3,8} \leq 1.9$; $P \geq 0.24$). In the liquid medium assays, the vegetative growth was similar for all passages, and there was no conidial production by *C. clavisporus*, even after three host-passages.

The mortality of L3 started at the first day after exposure to the highest concentration of conidia (10^7 conidia/ml) of *C. clavisporus*, regardless of the isolate (ARSEF 644, 964 or 2479) or host-passage (0–3 passages), but all larvae died within the first 48 h. For all other concentrations (10^5–3.3×10^6 conidia/ml), there was 100% mortality within 7 days. In the control, the mortality was always ≤10% at the same moment. The mortality of L3 exposed to ARSEF 964 increased at lower concentrations (3.3×10^5–10^6conidia/ml) after the first host-passage ($F_{3,8} \geq 7$; $P < 0.001$), and showed significant reductions of lethal concentrations (conidia/ml; Table. 2) and lethal times (days; Table 3). For other isolates (ARSEF 644 and 2479), there was no significant effect of host-passage on larval mortality ($F_{3,8} \leq 2.1$; $P \geq 0.1$), and did not affect lethal time (LT_{50}/LT_{90}) or lethal concentration (LC_{50}/LC_{90}), even after the third passage through *A. aegypti*.

Table 3
Lethal time (days) to kill 50% (LT_{50}) or 90% (LT_{90}) with their respective confidence interval (C.I.) and slope ± standard error of the mean (S.E.) for *Aedes aegypti* third instar larvae after treatment with *Culicinomyces clavisporus* conidia (original isolate and up to three host-passages) and incubation at 25 ± 1 °C.

ARSEF	Host-passage	Lethal time (C.I. 95%)		Slope ± S.E.
Isolate		50%	90%	
644	0	1 1.3 (1–2.7)a	4.7 (2.2–5.6)a	0.01 ± 0.02
	1	0.8 (0.7–2.1)a	1.6 (1.1–2.9)a	0.06 ± 0.02
	2	0.9 (0.7–2.3)a	1.8 (1.2–3)a	0.06 ± 0.02
	3	0.8 (0.7–1.9)a	1.6 (1.2–2.8)a	0.06 ± 0.02
964	0	1.1 (1–1.5)a	2.7 (2.1–4.1)a	0.05 ± 0.01
	1	1.1 (0.9–1.3)a	2.4 (2–3.2)a	0.04 ± 0.02
	2	0.8 (0.7–1.1)ab	1.3 (1.1–2.3)ab	0.12 ± 0.04
	3	0.7 (0.5–0.9)b	1.2 (1–1.5)b	0.06 ± 0.02
2479	0	1 (0.6–1.3)a	2 (1.6–2.6)a	0.05 ± 0.02
	1	0.8 (0.7–2)a	1.6 (1.4–2.5)a	0.06 ± 0.02
	2	0.9 (0.7–1.8)a	1.8 (1.3–2.8)a	0.05 ± 0.02
	3	0.8 (0.7–2)a	1.7 (1.3–2.4)a	0.1 ± 0.02

Values based on four repetitions 1.0×10^6 conidia/ml.

4. Discussion

For the application of a fungus as a biocontrol agent, it is important to choose an isolate with high virulence against a target pest. However, this fungus should produce large quantities of conidia to incorporate specific formulation. Temperature clearly influenced *in vitro* germination, growth and subsequent conidial production of both fungal species tested here. At test conditions, 25 °C was the optimal temperature for supporting the most rapid and highest levels of germination, the maximal vegetative growth, and the highest values of conidial production on solid medium for all isolates of both *C. clavisporus* and *C. bisporalis*. This temperature also permitted the maximal vegetative growth in liquid medium. This optimal temperature was similar to what has been reported for other hypocrealean fungi such as *Beauveria bassiana* (Uma Devi et al., 2005) and *Metarhizium anisopliae* (Ouedraogo et al., 1997). There was no correlation between climatic conditions in the original isolation sites for these fungi and their *in vitro* development and larvicidal patterns found at different temperatures. The colony diameter and the number of conidia produced by different fungi depended on temperature, but diameter values and number of conidia were not correlated; the isolates with the largest colonies (ARSEF 372, 582 and 706) were not the best for overall conidial production; ARSEF 644, 964 and 2479 produced smaller colonies but were the best producers of conidia.

Phenotypic degeneration–e.g., changes in color, growth rate, or reduced sporulation–has been suggested to occur in a wide range of fungi, including entomopathogens, after continuous subculturing (Butt et al., 2006). For example, after several years of successively subculturing on artificial media (Hajek et al., 1990) and cryopreservation (Hajek et al., 1995), isolates of the entomophthoralean pathogen *Entomophaga maimaiga* lost some important phenotypic properties (most notably, an apparent loss of virulence in direct proportion to time spent in cryogenic storage at −196 °C). Submerged conidiogenesis in subatmospheric oxygen concentrations has been observed to be common for recently isolated *Culicinomyces* spp., for which there seems to be a preference for conidia to be produced in agar or liquid culture media rather than on aerial hyphae (R. Humber, personal information). Some degree of submerged conidial production by *Culicinomyces* isolates tested was, therefore, expected in liquid or submerged in solid medium but was not observed. After long periods of cryogenic storage all *Culicinomyces* isolates appear to have lost their previous ability to

Table 2
Lethal concentration (conidia/ml)[a] to kill 50% (LC_{50}) or 90% (LC_{90}) with their respective confidence interval (C.I.) of *Aedes aegypti* third instar larvae after treatment with five concentrations[b] of *Culicinomyces clavisporus* conidia (no or up to three host-passages) and incubation at 25 ± 1 °C.

ARSEF	Host-passage	Lethal concentration (C.I.)	
Isolate		50%	90%
644	0	3.3×10^5 (2.3×10^5–4.6×10^5)a	1.5×10^6 (1.1×10^6–2.2×10^6)a
	1	4.5×10^5 (3.6×10^5–5.6×10^5)a	9.1×10^5 (7×10^5–1.5×10^6)a
	2	2.5×10^5 (1.8×10^5–3.3×10^5)a	7.5×10^5 (5.2×10^5–1.4×10^6)a
	3	2.3×10^5 (1.5×10^5–3.1×10^5)a	9.1×10^5 (6.2×10^5–1.8×10^6)a
964	0	3.7×10^5 (2.5×10^5–5.1×10^5)a	1.6×10^6 (1.1×10^6–2.3×10^6)a
	1	1.6×10^5 (2.5×10^4–4.1×10^5)ab	1×10^6 (4×10^5–3.7×10^6)a
	2	1.5×10^5 (2×10^4–3.8×10^5)ab	9.6×10^5 (3.6×10^5–3.6×10^6)a
	3	1.3×10^5 (7.5×10^4–1.9×10^5)b	5.7×10^5 (3.6×10^5–1.5×10^6)a
2479	0	2.5×10^5 (1.7×10^5–3.5×10^5)a	1×10^6 (7×10^5–2×10^6)a
	1	2.2×10^5 (1.5×10^5–3×10^5)a	7.3×10^5 (5.2×10^5–1.3×10^6)a
	2	2.8×10^5 (1.9×10^5–3.7×10^5)a	1.1×10^6 (7.4×10^5–1.9×10^6)a
	3	2.5×10^5 (1.7×10^5–3.6×10^5)a	1.1×10^6 (8×10^5–1.9×10^6)a

[a] Values based on cumulative mortality scored after 2 d incubation in conidial suspensions.
[b] 10^5, 3.3×10^5, 10^6, 3.3×10^6 and 10^7 conidia/ml.

produce submerged conidia. The degree of degeneration for any given fungus probably varies among different isolates, and repassage of a pathogen through a susceptible host might reverse some of the phenotypical changes, and might also improve some other characteristics (Butt et al., 2006). Unfortunately, none of the cryopreserved isolates of *Culicinomyces* used in this study passaged was able to produce submerged conidia after even all three repassages through *A. aegypti* larvae.

This is the first report on the effects of repeated short-term repassage and subsequent reisolation of *Culicinomyces* spp. from a susceptible host. Although *Culicinomyces* has never been isolated from field-collected specimens of *A. aegypti*, the susceptibility of this important mosquito species to this genus is well-known based on laboratory tests (Cooper and Sweeney, 1982).

After repeated serial repassages through *A. aegypti* larvae, the lethal times (LT_{50} and LT_{90}) and lethal concentrations (LC_{50} and LC_{90}) of ARSEF 964 decreased, but it was not clear whether the repassages improved the virulence or might have restored some of the original insecticidal activity. We cannot feel certain from these experiments whether host-repassage has affected the observed virulence, whether the strains might have retained their full pathogenic potential but cannot be induced to express this, or whether these isolates had undergone irreversible changes as has been suggested by Butt et al. (2006).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2017.03.012.

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