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RESEARCH ARTICLE

Evaluation of entomopathogenic fungi as potential biological control agents of the dengue mosquito, *Aedes aegypti* (Diptera: Culicidae)

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Dengue is a global health concern. Growing insecticide resistance in the primary mosquito vector, *Aedes aegypti*, limits the effectiveness of vector control, so alternative tools are urgently needed. One approach is the use of biopesticides comprising entomopathogenic fungi, e.g., *Beauveria bassiana* and *Metarhizium anisopliae*. These fungi may decrease disease transmission by reducing mosquito vector longevity and also occur worldwide, although many isolates have not been tested for virulence against mosquitoes. Ninety-three isolates of entomopathogenic fungi representing six species (*B. bassiana, M. anisopliae, Isaria fumosorosea, I. farinosa, I. flavovirescens*, and *Lecanicillium* spp.) were screened as potential biological control agents of *Aedes aegypti*. A hierarchical, multi-criteria experimental design was undertaken to find suitable isolates. Initial screening was performed via *in vitro* assays measuring radial growth and spore persistence, eliminating isolates with poor growth or viability on nutrient-rich substrate. Subsequent measurements of spore persistence revealed that only nine of 30 strains tested had half-lives exceeding 3 weeks. Ten isolates were chosen for *in vivo* bioassays against adult *Ae. aegypti*. From these assays, two Australian isolates of *B. bassiana*, FI-277 and FI-278, appeared to be most promising. Both isolates were shown to be virulent against *Ae. aegypti* at 20, 26, and 32°C. Spreading spores manually onto substrate was found to be more efficacious than spraying. *Ae. aegypti* infected by manually-spread spores on cotton substrate were found to have an LT_{50} of 3.7 ± 0.3 days. These characteristics suggest that FI-277 has promise as a dengue mosquito biocontrol agent, either alone or combined with conventional chemical insecticides.

Keywords: *Beauveria bassiana*; *Metarhizium anisopliae*; *Aedes aegypti*; mosquito; dengue; Australia

1. Introduction

Dengue continues to exert a considerable toll on public health, infecting an estimated 50 million people annually in over 100 countries. Australia, for example, has seen its largest epidemic in over 50 years, with over 950 cases in northern Queensland in 2009 (Anonymous 2009). Incidence of dengue hemorrhagic fever is also increasing,

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affecting an estimated 500,000 patients worldwide and causing mortality in approximately 2.5% of cases (WHO 2011). There is currently no available vaccine, so control of mosquito vectors is still the main tool to reduce incidence of this disease. However, insecticide resistance in the primary dengue vector *Aedes aegypti* L. (Hemingway and Ranson 2000; Macoris et al. 2003; Ponlawat, Scott, and Harrington 2005; Cui, Raymond, and Qiao 2006; García et al. 2009) increasingly limits the utility of chemical insecticides, so alternative methods of control are needed.

One potential method is the use of entomopathogenic fungi such as *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metchnikoff) Sorokin. Infective spores of these fungi have been reported to affect larval and adult stages of mosquitoes, but a major limitation had been lack of persistence of the infective spore stage (Alves, Alves, Lopes, Pereira, and Vieira 2002; Scholte, Knols, Samson, and Takken 2004). Regarding the control of terrestrial insects, the use of mineral oil carriers for spores has made the use of entomopathogenic fungi for control of terrestrial insects more feasible (Bateman and Alves 2000). More recently, studies of fungal infection in adult mosquitoes (mostly malaria vectors) have demonstrated potential for fungi to reduce longevity (Scholte, Njiru, Smallegange, Takken, and Knols 2003; Blanford et al. 2005; Scholte et al. 2005; Scholte, Takken, and Knols 2007; de Paula, Brito, Pereira, Carrera, and Samuels 2009), blood-feeding success, fecundity (Scholte, Knols, and Takken 2006) and vector competence (Blanford et al. 2005). Other recent empirical studies demonstrate that fungal pathogens can infect insecticide resistant mosquitoes and reduce expression of insecticide resistance (Farenhorst et al. 2009) and act synergistically with conventional interventions such as bednets (Hancock, Thomas, and Godray 2009; Farenhorst et al. 2010), and chemical insecticides (Farenhorst et al. 2009; Paula, Carolino, Paula, and Samuels 2010), highlighting the potential for using entomopathogenic fungi in novel integrated strategies for adult vector control.

In this study, we present the results of a screening program to identify promising candidate isolates for use in a biopesticide against adult, female *Ae. aegypti*. The initial focus of the project is on developing a product for further testing in Australia, but with potential global application. Accordingly, we screened 93 isolates from six fungus species, including 72 isolates from Australia. We followed a hierarchical multi-criteria screen beginning with *in vitro* assays, such as radial hyphal growth and persistence of spores on a sprayed substrate, under conditions representative of the broad environmental envelope for dengue transmission. Based on these results, we then moved forward with a subset of isolates to investigate virulence against adult female *Ae. aegypti* in simple laboratory bioassays. Virulent strains were then subjected to further assays to measure the impact of temperature, substrate and method of application on mosquito mortality, with the goal of identifying one or more strains with potential for mosquito control.

2. Materials and methods

2.1 Fungal isolates and culture maintenance

Fungus isolates, mostly *M. anisopliae*, *B. bassiana*, were obtained from the USDA Agricultural Research Service Collection of Entomopathogenic Fungi (ARSEF) and the Commonwealth Science and Industry Research Organization (CSIRO) culture

collections (Table 1). Isolates were maintained in culture on potato dextrose agar (PDA) slopes in universal bottles (30 mL) and stored at 4°C. Continuous cultures were maintained on slopes, with sub-cultures grown for 14 days at 25°C, following which the lids were tightly sealed and the cultures stored at 4°C.

2.2 Screen #1: effect of temperature on in vitro *radial colony growth of fungi*

Fungal cultures representing 93 isolates from six fungal species were grown in Petri dishes (90 × 15 mm) containing Sabouraud dextrose agar (SDA) for 2 weeks at 25°C. Following culturing on SDA, conidia were harvested as described previously (Darbro and Thomas 2009). For each isolate, three replicate Petri dishes with SDA were inoculated at the centre with one drop (approximately 10 μL) of a suspension containing 1.0×10^6 spores mL^{-1} of 0.05% Tween 20 in water. Each fungal isolate was incubated at 20, 26, and 32°C (representative temperatures at which dengue transmission can occur) in an incubator with range of ±2°C. The colony radius was measured from the edge of the central inoculation point towards the periphery of each plate every 24 h from day 4 to 15 post inoculation. The hyphal extension of each replicate plate was then plotted against time for each isolate and temperature. General linear models (PROC GLM, SAS 9.1, Cary, NC, USA) were fitted to these data and hyphal extension rates for each isolate at each temperature were obtained.

2.3 Screen #2: effect of temperature on in vitro *conidial persistence on a substrate*

2.3.1 Selection of isolates for screen

Thirty isolates (20 *M. anisopliae*, 10 *B. bassiana*) were selected for spray persistence assays, partly on the basis of screen #1 (i.e., we selected isolates with good growth across a range of temperatures), together with other general criteria such as provenance (particularly isolates from Australia, as native isolates facilitate obtaining regulatory approval for semi-field studies), whether they were originally isolated from dipterans, or whether they were known to show broad biological activity (i.e., infect diverse taxa so likely to infect *Aedes* also).

2.3.2 Biopesticide formulation and application

Fungal cultures were prepared as above, except conidia were suspended in a mineral oil formulation consisting of a mixture of 87.5% ShellSol T and 12.5% Ondina EL (standard proprietary mineral oils) (Darbro and Thomas 2009) instead of Tween 20.

Spray application employed a hand-held artist's air brush (Badger Air-Brush Co., Franklin Park, IL, USA) to produce an aerosol of the spore formulation. The air brush had a nozzle width of 0.5 mm and was held at a distance of 30 cm away from the target surface. Spraying was done with even passes of the airbrush at a hand movement of approximately 12 cm s^{-1}. Eighty-one glass microscope slides (7.62 × 2.54 cm) per fungal isolate were treated with 5 mL of a 1×10^8 spores mL^{-1} oil formulation in a spray hood. Slides were allowed to dry for at 24–26 h at room temperature before being used in germination tests by incubating at either 20, 26, or 32°C. After slides were removed to dry, the hood was disinfected by wiping with 10% bleach solution followed by 95% ethanol solution.

Table 1. Country of origin and host species of entomopathogenic fungus

Species	Isolate code	Geographical origin	Host species
M. anisopliae	ARSEF 5837	Denmark	*Alphitobius diaperinus* (Coleoptera: Tenebrionidae)
M. anisopliae	FI-1265	Australia (QLD)	*Lepidiota frenchi* (Coleoptera: Scarabaeidae)
M. anisopliae	FI-132	Australia (Mackay, QLD)	*Inopus rubriceps* (Diptera: Stratiomyidae)
M. anisopliae	FI-133	" "	" "
M. anisopliae	FI-1386	Australia (Condong, NSW)	*Rhopaea magnicornis* (Coleoptera: Scarabaeidae)
M. anisopliae	FI-1466	Kiribati	Soil sample
M. anisopliae	FI-161	Australia (Bundaberg, QLD)	*Inopus rubriceps* (Diptera: Stratiomyidae)
M. anisopliae	FI-170	" "	Soil sample
M. anisopliae	FI-176	" "	" "
M. anisopliae	FI-179	Australia (Cairns, QLD)	" "
M. anisopliae	FI-190	Australia (Maryborough, QLD)	*Antitrogus parvulus* (Coleoptera: Scarabaeidae)
M. anisopliae	FI-194	Australia (Tirroan, QLD)	*Inopus rubriceps* (Diptera: Stratiomyidae)
M. anisopliae	FI-195	" "	" "
M. anisopliae	FI-209	Australia (Gininnderra, ACT)	*Phaulacridium vittatum* (Orthoptera: Acrididae)
M. anisopliae	FI-224	Australia (Tegege, QLD)	Soil sample
M. anisopliae	FI-24	Australia (SA)	*Aphodius tasmaniae* (Coleoptera: Scarabaeidae)
M. anisopliae	FI-280	Australia (Bussleton, WA)	Soil sample
M. anisopliae	FI-289	" "	" "
M. anisopliae	FI-317	Australia (Sybil Creek, QLD)	" "
M. anisopliae	FI-318	" "	" "
M. anisopliae	FI-319	" "	" "
M. anisopliae	FI-320	Australia (Devereaux Creek, QLD)	" "
M. anisopliae	FI-321	" "	" "
M. anisopliae	FI-322	Australia (Sommerfield, QLD)	*Inopus rubriceps* (Diptera: Stratiomyidae)
M. anisopliae	FI-323	" "	" "
M. anisopliae	FI-324	" "	" "
M. anisopliae	FI-348	Australia	Unidentified Coleoptera
M. anisopliae	FI-349	" "	" "
M. anisopliae	FI-350	" "	" "
M. anisopliae	FI-352	Australia (Walker's Point, QLD)	*Inopus rubriceps* (Diptera: Stratiomyidae)
M. anisopliae	FI-353	" "	" "
M. anisopliae	FI-399	Canada (Quebec)	*Phyllophaga* sp. (Coleoptera: Scarabaeidae)

Table 1 (*Continued*)

Species	Isolate code	Geographical origin	Host species
M. anisopliae	FI-407	Australia (Sommerfield, QLD)	*Inopus rubriceps* (Diptera: Stratiomyidae)
M. anisopliae	FI-409	" "	" "
M. anisopliae	FI-490	" "	" "
M. anisopliae	FI-493	Australia (Tingoora, QLD)	Soil sample
M. anisopliae	FI-494	" "	" "
M. anisopliae	FI-65	Australia (Nambour, QLD)	*Antitrogus mussoni* (Coleoptera: Scarabaeidae)
M. anisopliae	FI-713	Myanmar	Soil sample
M. anisopliae	FI-86	Australia (Cairns, QLD)	Soil sample
M. anisopliae	FI-865	Australia (Central Highlands, QLD)	*Pseudoheteronyx* sp. (Coleoptera: Scarabaiedae)
M. anisopliae	FI-922	Papua New Guinea	Unknown
M. anisopliae	FI-924	" "	" "
M. anisopliae	ARSEF 4620	Australia	*Boreoides tasmaniensis* (Diptera: Stratiomyidae)
M. anisopliae	ICIPE 30	Kenya	*Busseola fusca* (Lepidoptera: Noctuidae)
Beauveria bassiana	FI-528	Australia (Braidwood, NSW)	*Rhopalidia* sp. (Hymenoptera: Vespidae)
B. bassiana	ARSEF 5341	USA	*Musca domestica* (Diptera: Muscidae)
B. bassiana	FI-278	Australia (Pemberton, WA)	Soil sample
B. bassiana	FI-705	New Zealand (Christchurch)	*Aphodius tasmaniae* (Coleoptera: Scarabaeidae)
B. bassiana	ARSEF 5148	Portugal	*Popillia japonica* (Coleoptera: Scarabaeidae)
B. bassiana	FI-696	New Zealand (South Island)	*Costelytra zealandica* (Coleoptera: Scarabaeidae)
B. bassiana	IMI 391510	USA	Coleoptera: Chysomelidae
B. bassiana	FI-277	Australia (Pemberton, WA)	Soil sample
B. bassiana	FI-697	New Zealand (Christchurch)	*Aphodius tasmaniae* (Coleoptera: Scarabaeidae)
B. bassiana	FI-701	New Zealand (Christchurch)	*Aphodius tasmaniae* (Coleoptera: Scarabaeidae)
B. bassiana	ARSEF 2953	USA	*Musca domestica* (Diptera: Muscidae)
B. bassiana	FI-491	Australia	*Inopus rubriceps* (Diptera: Stratiomyidae)
B. bassiana	FI-394	Solomon Islands	*Nilaparvata lugens* (Hemiptera: Delphacidae)
B. bassiana	ARSEF 1151	Canada	*Delia radicum* (Diptera: Anthomyiidae)

Table 1 (*Continued*)

Species	Isolate code	Geographical origin	Host species
B. bassiana	ARSEF 201	USA	*Diabrotica undecimpunctata* (Coleoptera: Chysomelidae)
B. bassiana	ARSEF 1630	France	*Calliphora* sp. (Diptera: Calliphoridae)
B. bassiana	ARSEF 1514	France	*Musca autumnalis* (Diptera: Muscidae)
Paecilomyces farinosus	ARSEF 1349	Poland	Diptera sp.
Paecilomyces flavovirescens	ARSEF 1686	Japan	*Resseliella odai* (Diptera: Cecidomyiidae)
Paecilomyces fumosoroseus	ARSEF 887	France	Diptera sp.
Lecanicillium spp.	ARSEF 5132	United Kingdom	*Delia radicum* (Diptera: Anthomyiidae)

2.3.3 Germination tests

One slide was placed spore-side down onto an SDA agar plate. Each plate was sealed with parafilm and stored in a temperature-controlled incubator. The slide was removed after 21–24 h, and germinated and ungerminated spores were counted along the length of the agar plate. After 20–22 h incubation, conidia were counted to calculate percentage germination under a light microscope (magnification $\times 400$). At least 300 conidia were examined on each plate, and those with a visible germ-tube at least twice the width of the conidium were scored as germinated. Germination tests were performed at 0 days, 1 day, and 1, 3, 6, 10, 14, 18, and 24 weeks with three replicates per strain per time point per temperature.

2.3.4 Data analysis

Germination rates were standardized to control for small variations in initial germination rate by setting all initial (time = 0) germination rates to 100%, then calculating subsequent relative germination rates based on the standardized initial value. Isolates with fewer than 4 non-zero data points (i.e., fell to 0% germination by the third week) were not included for statistical analysis. For longer-lasting isolates, a Gompertz curve, $y = 100 * e^{\left(\left(\frac{\alpha}{\beta}\right)^{*} e^{\beta^{*} t-1}\right)}$, (Clements 1981) was fitted for persistence data using PASW (PASW Statistics 17.0, 2009, Chicago, IL, USA, formerly SPSS). The half-life (in days) of each isolate was solved by setting $y = 0.5$ and calculated as:

$$y = \frac{1}{\beta} * \ln\left(1 + \frac{0.6931 * \beta}{\alpha}\right).$$

2.4 Screen #3: impact of fungal infection on survival of adult Ae. aegypti

2.4.1 Mosquito colony and rearing

Mosquitoes were from a laboratory colony recently colonised from field-collected eggs in Cairns, Australia. All bioassays utilized adults that were no more than three generations removed from the wild. Insecticide resistance has not been reported in the wild population from which our strain was colonised. Larvae were reared at 27°C at a density of 200 larvae per pan in pans containing 2 L of water. Larvae were fed 200 mg of ground fish food per 200 larvae (TetraMin™) daily. Pupae were removed into 14×6 cm containers and left to eclose for 2 days in 60×60 cm cages maintained at 27°C and 70% RH.

2.4.2 Fungus exposure and mortality monitoring

Fungal spores were formulated in mineral oil as described above. All fungus isolates were tested for conidial viability (as above) one day before each bioassay, and only isolates with no less than 80% viability were used in bioassays. Mosquitoes were exposed by spraying 250-mL cardboard drinking cups with formulation. Cups, together with a small piece of cloth mesh (mesh size 0.5 mm) used to cover the cup, were sprayed with the hand-held airbrush with 1.54 mL of spore formulation delivered to each cup, using the same technique as described above. This spray method and volume application rate equated to an application volume of 2×10^{10} spores m^{-2} There were three replicate cups per fungus strain, and three replicate control cups (oil formulation only).

After the cups were allowed to dry for 24 h, 25 female mosquitoes were anaesthetized with ice, placed in each cup using soft forceps, and held at 26°C and 70% RH for an additional 24 h, during which time the mosquitoes had access to cotton soaked with 10% sucrose solution. Only females were assayed in these experiments. The same 25 mosquitoes were then moved into 20×20 cm plastic cages held at the same temperature. Dead mosquitoes were removed at this time and excluded from data analysis. Each cage was given fresh, damp cotton as above. Mortality was measured daily for 21 days. Dead mosquitoes were removed daily at the same time each afternoon, placed in Petri dishes with moist filter paper and sealed with parafilm, stored at 26°C, and observed after 6 days to detect any fungus sporulation.

2.5 Effect of temperature on virulence

Selected isolates that had moderate or strong impact on mosquito survival were assayed again in different environmental chambers at 20, 26, and 32°C at the same dose to assess the impact of fungus at a representative temperatures at which dengue can be transmitted. There were three replicate cups per temperature per strain.

2.6 Effect of concentration on virulence

B. bassiana isolate FI-277 was then assessed, using the same formulation method, over a range of concentrations: 2×10^8, 2×10^9, 2×10^{10}, and 1×10^{11} spores m^{-2} of

substrate (but see later discussion of actual spore concentrations reaching the sprayed substrate), with three replicate cups per dose. To assess the effect of application method, spore formulation was applied in one of two ways: either by spraying with a hand-held artist's air brush (using the same methodology as described above), or by pipetting the formulation around the inside of the cup and then spreading it evenly using a metal spatula. There were three replicate cups per dose for the 'spread' methodology and six replicate cups for the 'spray' methodology. The germination of all formulations was tested, and conidia were confirmed to be $>80\%$ viable. Data were analysed as above.

2.7 Effect of substrate and application methods on virulence

B. bassiana strain FI-277 was used at a high dose (1×10^{11} spores m^{-2} substrate) for this experiment. This experiment was identical to the virulence bioassays described above with the following exceptions.

2.7.1 Treated substrates

Three materials were tested for suitability for fungus application: (a) cardboard drinking cups treated directly (described above), (b) a strip of black cotton drill cloth (17×8 cm) that was treated, then stapled into a cardboard drinking cup, and (c) plywood. Mosquitoes in plywood treatments were exposed in a plastic dish ($16 \times 10 \times 6$ cm) with the walls and floor of the dish lined with cut plywood (total treated surface area per dish was 472 cm^2).

2.7.2 Application methods

Two application methods were tested for paper cup and black cotton cloth substrates: (a) spraying with a 5-L stumpy pneumatic sprayer (Rega™, Brendale, QLD, Australia) at an application rate of 250 mL m^{-2} (spray applications were replicated as Spray 1 and Spray 2), and (b) pouring formulation directly onto substrate and spreading it evenly around the surface by hand while wearing latex gloves (Scholte et al. 2005).

2.7.3 Bioassay

Spores were applied outdoors and substrates were allowed to dry for 24 h in the shade. Substrates were in the shade the entire time. Once dry, substrates were returned to the lab and assembled, after which the standard bioassay protocol was used (see earlier).

2.8 Data analysis

In all mortality bioassays, control mortality was standardized using Abbott's formula (1925) where appropriate. Paper cup assays with excessively high control mortality (defined as 20% by 10 days) were not included in analyses. Median lethal times were estimated using probit regression (PASW 17.0.3, formerly SPSS). Hazard ratios were estimated and evaluated using Cox's proportional hazards

model (PROC TPHREG, SAS 9.1). Multiple pairwise comparisons of hazard ratios were performed using linear contrasts, and *P*-values were adjusted using the Bonferroni method when appropriate. Sporulation rates were arcsine-square root transformed and analysed with ANOVA (PROC GLM, SAS 9.1), and multiple pairwise comparisons of these were controlled in PROC GLM using Tukey's test.

3. Results

3.1 Effect of temperature on in vitro *radial growth of fungi*

Mean growth rates (mm day^{-1}) for all isolates and temperatures measured are presented in Figure 1A, B. *Metarhizium* isolates had significantly faster growth rates than the other species. Both *M. anisopliae* and *B. bassiana* tended to have highest growth rates at 26°C although when least squares means were compared between temperatures for each isolate, mean daily radial growth rates of 50 out of 72 (69.4%) *M. anisopliae* isolates and 5 of 17 (29.4%) of *B. bassiana* isolates did not differ significantly by temperature. Almost half of the *B. bassiana* isolates (8/17) demonstrated significantly slower growth rates at 32°C than at other temperatures measured (Figure 1B).

3.2 Effect of temperature on in vitro *conidial persistence on a substrate*

Of 30 isolates challenged, 21 did not persist for longer than 3 weeks (half life <21 days) at any of the temperatures tested. Table 2 lists non-linear regression parameters, R^2-values and half-lives of the 10 most persistent isolates (one *M. anisopliae*, nine *B. bassiana*). Isolates not listed in Table 2 did not persist long enough for non-linear regression to be possible (i.e., insufficient non-zero data points).

Of isolates analysed, FI-713 (*M. anisopliae)* had the shortest persistence, with a half-life shorter than 3 days at all temperatures assayed. Persistence of *B. bassiana* isolates varied by isolate and temperature. The longest observed half-life for any temperature assayed was 157.3 days at 26°C, although that strain demonstrated considerably shorter spore persistence at 20 and 32°C. Spore persistence tended to be shorter overall at 32°C than for lower temperatures.

3.3 Impact of fungal infection on survival of adult Ae. aegypti

Ten isolates representing two fungal species were assayed (Table 3). All isolates except ICIPE30 were associated with a greater daily mortality rate relative to uninfected controls (i.e., hazard ratio >1). For *B. bassiana* the most virulent isolates were FI-277, FI-278, ARSEF 1151, ARSEF 5341, and FI-491, with median lethal times ranging from 7.7 to 9.4 days and hazard ratios ranging from 4.4 to 8.8, with isolates FI-277 and FI-278 being the most virulent. Most isolates resulted in $>50\%$ sporulation of cadavers with three Australian isolates (FI-277, FI-278, FI-491) resulting in $>80\%$ sporulation, although no significant differences in sporulation between fungal isolates were detectable (Table 3). No mosquitoes in the control group sporulated with *B. bassiana*.

ICIPE30 was the only *M. anisopliae* isolate tested. Spore persistence for this isolate was low (see earlier), but it was included in order to compare with previous

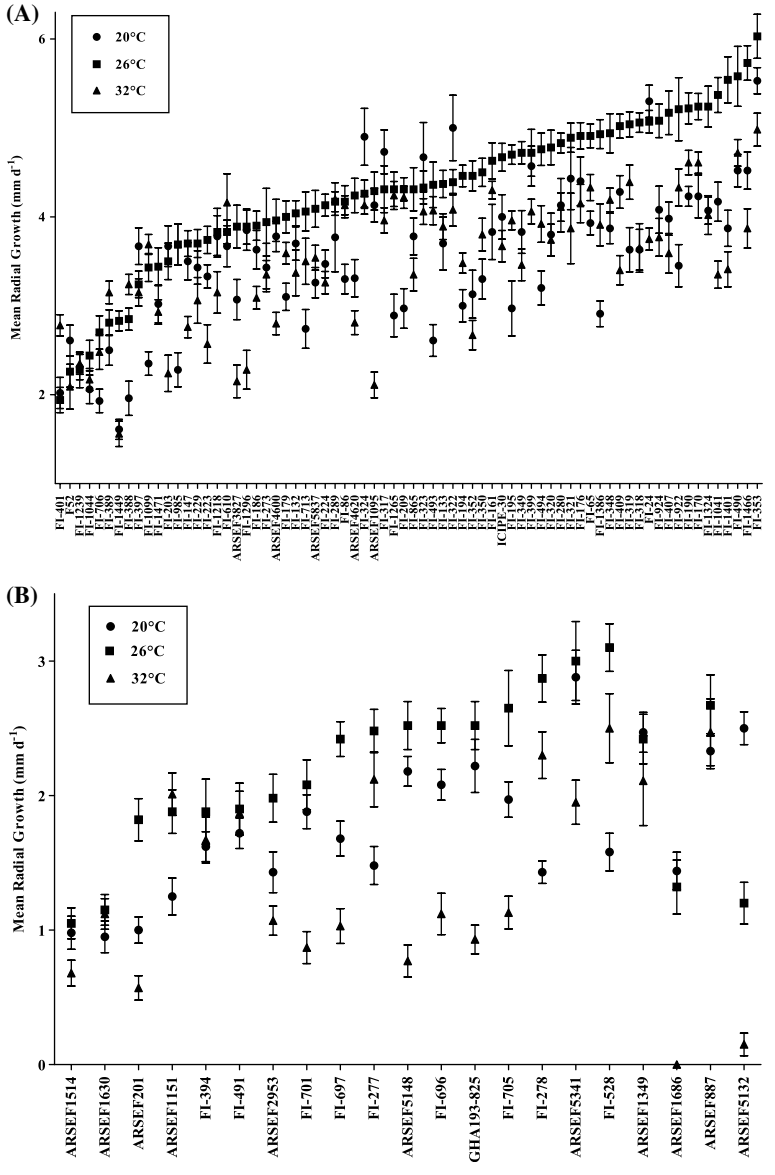

Figure 1. Mean daily radial growth rates (mean ± SE) of entomopathogenic fungal isolates at three temperatures. Bar heights refer to mean radial growth rate (mm/day) on SDA at 20, 26, and 32°C for *Metarhizium anisopliae* (A), *Beauveria bassiana*, *Isaria flavovirescens, I. fumosorosea*, and *Lecanicillium lecanii* (B). For further information on fungus isolates, see Table 1.

studies (Scholte et al. 2005, Farenhorst et al. 2008, 2009). Although sporulation rate was high (73.8%), this isolate had a relatively small impact on mosquito longevity (LT50 = 14.5 days), and the hazard ratio was not significantly different from that of the control.

3.4 Effect of temperature on virulence

The three isolates (two *B. bassiana* and one *M. anisopliae*) reduced survival compared to non-infected controls at all temperatures (Figure 2). Survival curves for unexposed mosquitoes at the three temperatures did not differ significantly (Table 4). Mosquitoes exposed to *B. bassiana* isolate FI-277 at 20°C had a 38% lower risk of death than those at 26°C, and a 64% lower risk of death than those at 32°C. This pattern was similar for mosquitoes exposed to IMI391510: mosquitoes exposed at 20°C had 54 and 32% lower risks of death, respectively, than mosquitoes exposed at 26 and 32°C. In contrast, the risk of death to those infected by ICIPE30 (*M. anisopliae)* increased significantly with each increase in temperature: up by 23% from 20 to 26°C, then by 55% from 26 to 32°C (Table 4).

Sporulation on cadavers occurred in 90–100% of individuals in treatment groups. There was no significant association detected between sporulation frequency and

Table 2. Persistence of a suite of fungal isolates sprayed on glass slides and maintained at 26°C.

Isolate	T (°C)	α^a	β^a	R^2	Half-life (days)[b]
IMI 391510	20	0.002 ± 0.000	0.016 ± 0.007	0.864	117.4
	26	0.002 ± 0.001	0.009 ± 0.002	0.873	157.3
	32	0.002 ± 0.000	0.019 ± 0.002	0.946	106.6
ARSEF1151	20	0.008 ± 0.003	-0.002 ± 0.005	0.642	95.1
	26	0.006 ± 0.001	0.006 ± 0.003	0.910	87.8
	32	0.001 ± 0.001	0.033 ± 0.010	0.812	96.1
ARSEF1514	20	0.001 ± 0.001	0.016 ± 0.005	0.697	155.8
	26	0.003 ± 0.001	0.012 ± 0.005	0.765	110.6
	32	0.003 ± 0.001	0.040 ± 0.007	0.973	58.1
ARSEF5341	20	0.004 ± 0.001	0.009 ± 0.003	0.906	104.4
	26	0.001 ± 0.000	0.018 ± 0.003	0.903	144.5
	32	0.003 ± 0.001	0.044 ± 0.007	0.975	54.8
FI-394	20	0.004 ± 0.002	0.011 ± 0.006	0.668	97.0
	26	0.006 ± 0.002	0.012 ± 0.006	0.871	72.5
	32	0.024 ± 0.004	0.007 ± 0.007	0.960	26.3
FI-491	20	0.035 ± 0.005	-0.008 ± 0.006	0.917	21.6
	26	0.051 ± 0.006	-0.009 ± 0.006	0.974	12.4
	32	0.059 ± 0.021	0.420 ± 0.077	0.968	4.24
FI-277	20	0.007 ± 0.005	0.008 ± 0.008	0.676	72.9
	26	0.003 ± 0.001	0.020 ± 0.005	0.903	86.3
	32	0.005 ± 0.001	0.042 ± 0.007	0.965	45.7
FI-278	20	0.006 ± 0.002	0.013 ± 0.007	0.770	70.4
	26	0.006 ± 0.001	0.015 ± 0.004	0.932	67.0
	32	0.003 ± 0.001	0.054 ± 0.007	0.977	48.2
FI-713	20	4.478 ± 7.031	-2.461 ± 3.997	0.704	0.2
	26	0.184 ± 0.37	-0.057 ± 0.022	0.917	2.1
	32	0.346 ± 0.041	-0.950 ± 0.022	0.976	2.2

Data indicate nonlinear regression (Gompertz) parameters and half-lives for each isolate.
[a]These refer to parameters in the Gompertz equation $y = 100 * e^{((-\alpha/\beta)^* e^{\beta^* t-1})}$, where y = standardized germination rate, t = days post-application and a and are parameters (Clements 1981a).
[b]Half-life was calculated as $t_{1/2} = \frac{1}{\beta} * ln\left(1 + \frac{0.69315 * \beta}{\alpha}\right)$.

Table 3. Median lethal times (LT_{50}), mortality and infection rates of adult *Ae. aegypti* 21 days after exposure to selected isolates of *Beauveria bassiana* and *Metarhizium anisopliae*

Isolate	LT_{50} (days) (95% CI)	Mortality (%±SE)	Hazard Ratio (HR)	Sporulation (%)
None (control)	17.9 (17.0–18.9)	42.0±2.3	1.0a	0a
FI-277	7.8 (7.4–8.1)	100.0±0.0	8.0e	82.4b
FI-278	8.0 (4.4–8.5)	98.8±1.5	8.8e	88.8b
ARSEF1151	8.8 (8.0–9.6)	94.6±1.6	5.3d	73.9b
ARSEF5341	9.4 (8.4–10.3)	88.0±2.8	4.4d	78.9b
FI-491	9.5 (9.0–10.0)	94.0±4.6	5.2d	80.1b
FI-394	12.0 (11.0–12.8)	88.6±5.7	3.5c	69.1b
ARSEF1514	12.6 (11.6–13.6)	85.5±5.3	3.0b	57.7b
IMI 391510	12.6 (11.7–13.5)	81.8±8.7	2.9b	65.4b
ICIPE 30	14.5 (14.1–14.9)	69.3±3.0	1.4a	73.8b

Values followed by identical lowercase letters within a column are not significantly different ($P > 0.05$).

temperature ($F_{2,18} = 2.18$, $P = 0.30$), fungus isolate ($F_{2,18} = 0.20$) or their interaction ($F_{4,18} = 1.96$, $P = 0.15$).

3.5 Effect of concentration on virulence

For *B. bassiana* isolate FI-277, mosquito survival at the lowest sprayed concentration (2×10^7 spores m^{-2}) was not statistically different from the control group (hazard ratio = 1.1, $P = 0.80$), and sporulation rates were lower than 15% (Table 5). The next three higher concentrations were significantly more virulent (2×10^8 spores m^{-2}: HR = 6.8, $P < 0.0003$; 2×10^9 spores m^{-2}: HR = 14.7, $P < 0.0003$; 2×10^{10} spores m^{-2}: HR = 36.2, $P < 0.0003$). Mosquito survival at the two highest concentrations, however, were not statistically different (linear contrast = 0.87, $P > 0.35$). No *B. bassiana* sporulation occurred at the control, whereas detectable sporulation increased with concentration until the last three concentrations, which did not differ significantly from each other (all >90% sporulation, Table 5). The hazard ratios, mortality and sporulation rates between the two sprayed concentration–response experiments were not statistically different.

When spores were directly spread over the cups, hazard ratios were higher for every concentration tested, from HR = 22.0 at 2×10^8 spores m^{-2} to HR = 179.0 at 2×10^{10} spores m^{-2}. Mortality was higher at lower concentrations, but by 2×10^{10} spores m^{-2}, mortality and sporulation was similar for sprayed and manually-spread spores.

FI-277 was more virulent when spread directly onto the substrate. Mortality and sporulation by day 21 were 100% for all concentrations tested. The hazard ratio was 22.0 at the lowest concentration tested (2×10^8 spores m^{-2}) and increased significantly with each concentration, to 179.0 at 1×10^{11} spores m^{-2}.

3.6 Effect of substrate and application method on virulence

All fungus treatments reduced mosquito longevity when compared to untreated controls (Figure 3A–C). Spores applied to cardboard had a greater impact on

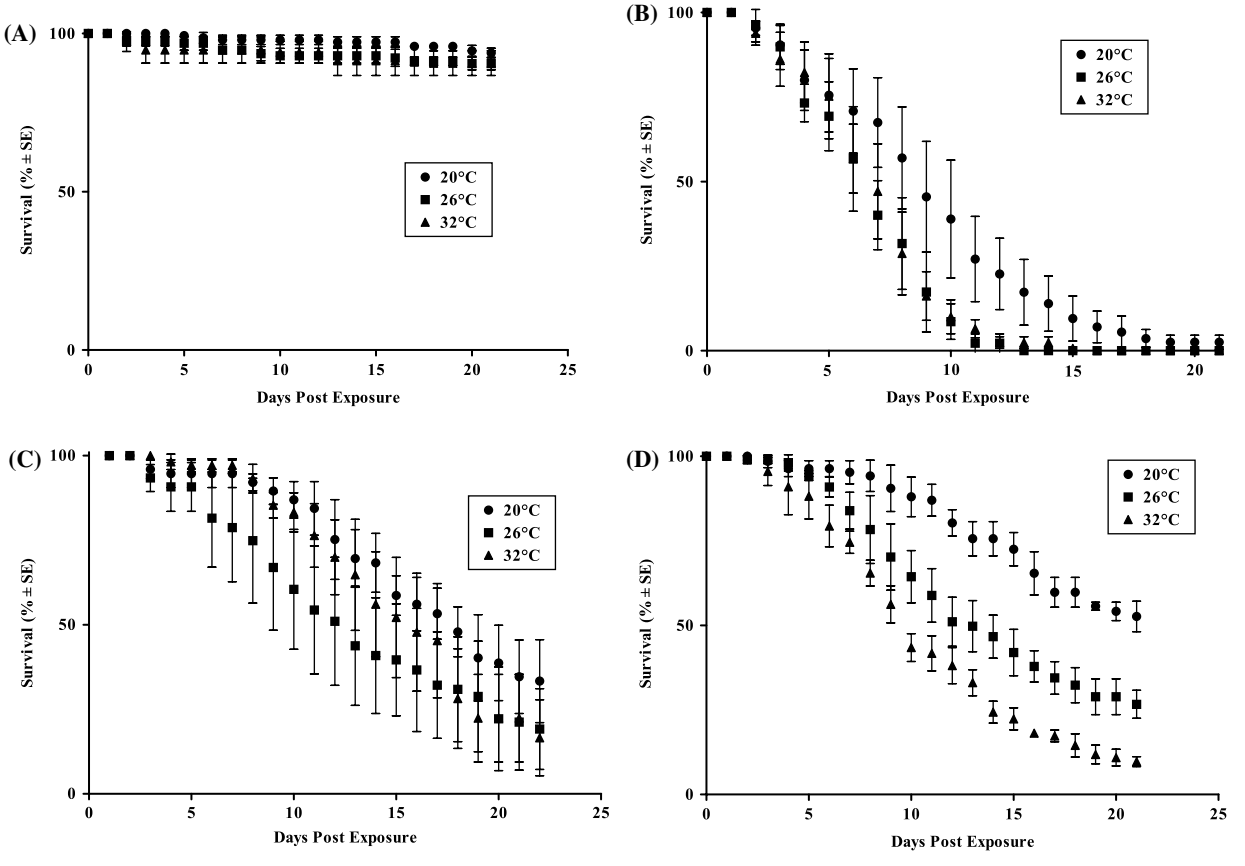

Figure 2. Virulence of selected fungus isolates at three temperatures to *Aedes aegypti*. Survival curves at 20, 26, and 32°C for uninfected controls (A), *Beauveria bassiana* isolates FI-277 (B) and IMI391510 (C), and *Metarhizium anisopliae* isolate ICIPE30 (D). Percent survival (mean ± SE) is shown.

Table 4. Effect of temperature on virulence of a subset of most promising entomopathogenic fungi against adult *Ae. aegypti*.

Isolate	T (°C)	LT_{50} (days) (95% CI)	Mortality (%±SE)	Hazard Ratio[a]	Sporulation %
None	20	>21	6.2±1.5	0.88[a]	0[a]
(Control)	26	>21	7.0±2.0	0.62[a]	0[a]
	32	>21	9.4±4.0	1.00[a]	0[a]
FI-277	20	9.6 (9.1–10.2)	93.2±3.0	0.36[a]	96[b]
	26	6.2 (5.2–7.2)	100±0.0	0.77[b]	98[b]
	32	6.5 (5.9–7.1)	100±0.0	1.00[b]	95[b]
IMI390591	20	16.4 (15.2–17,.6)	65.2±*13.8*	0.68[a]	95[b]
	26	12.0 (11.1–12.9)	80.8±*11.9*	1.22[b]	98[b]
	32	15.0 (14.0–16.0)	82.4±*10.8*	1.00[b]	92[b]
ICIPE30	20	19.0 (18.3–19.8	47.4±*4.5*	0.22[a]	96[b]
	26	14.3 (13.7–14.8)	72.2±*3.0*	0.45[b]	100[b]
	32	9.9 (9.4–10.4)	90.3±*1.5*	1.00[c]	95[b]

Hazard ratios followed by identical lowercase letters are not significantly different ($P>0.05$) from hazard ratios at other temperatures within a given treatment. Sporulation percentages followed by the same lowercase letter are not statistically different ($P>0.05$).
[a]Each Cox regression analysis used 32°C as an arbitrary baseline, so the hazard ratio of each fungus isolate (or uninfected control) was in reference to the hazard ratio for that isolate at 32°C.

mosquito longevity, whether sprayed with a pneumatic sprayer ($LT_{50}=5.3\pm1.3$ days) or spread directly onto the inner surface ($LT_{50}=6.0\pm0.9$ days) (Figure 3A, $\chi^2=0.98$, $P=0.95$). Spores spread directly onto black cotton drill reduced mosquito longevity more than those sprayed onto cloth ($LT_{50}=6.3\pm1.5$ days, Figure 3B) or onto plywood ($LT_{50}=9.6\pm0.7$ days, Figure 3C). Mosquito longevity after being exposed to spores spread directly onto black cotton cloth was reduced in comparison with being exposed to sprayed spores on cloth ($LT_{50}=3.7\pm0.3$ days) (Figure 3B, $\chi^2=5.3$, $P=0.02$). Spores sprayed onto plywood also reduced mosquito longevity ($LT_{50}=9.6\pm0.7$ days).

4. Discussion

From a panel of 93 isolates that were initially screened for a number of traits *in vitro*, we selected a shortlist of 10 isolates for critical assays *in vivo*. Via this hierarchical approach we ultimately identified a virulent, persistent *B. bassiana* isolate FI-277 with potential as a novel biopesticide for use against adult mosquito vectors that transmit dengue. Given there have been no new classes of chemical insecticide registered for control of adult mosquito vectors for more than three decades (Nauen 2007), identification of these candidate fungal isolates represents an exciting development in the progress towards improved integrated vector control strategies.

We began first by assessing isolates using a radial growth assay under different temperatures. Although not necessarily revealing anything about ultimate virulence, this screen was valuable in identifying isolates with capacity to grow across the relevant environmental conditions. Having eliminated certain isolates on the basis of temperature–growth relationships, we then progressed to consider persistence of spores in a spray residue. Persistence is a critical characteristic of any successful

Table 5. Hazard ratios and sporulation rates for *Aedes aegypti* 21 days after exposure to isolate FI-277 (*Beauveria bassiana*) at a range of concentrations and two spore application methods.

	Hazard ratio (95% CI)			Mortality (%±SE) at 21 days			Sporulation rate[b] (%±SE)		
Dose[a]	Spray 1	Spray 2	Spread	Spray 1	Spray 2	Spread	Spray 1	Spray 2	Spread
0	1.0a	1.0a	1.0a	13.2±7.2a	15.9±9.3a	16.6±11.3a	0±0a	0±0a	0±0a
2×10^7	1.1a (0.5–2.3)	N/A	N/A	12.3±6.3ab	N/A	N/A	23.6±14.6b	N/A	N/A
2×10^8	6.8b (3.7–12.3)	4.7b (2.5–8.9)	22.0b (10.5–46.3)	63.8±7.4b	66.7±7.4b	100±0b	65.4±7.6c	79.8±4.6b	100±0b
2×10^9	14.7c (8.2–26.4)	10.5c (5.8–19.1)	47.7c (21.9–103.8)	88.5±9.9bc	89.7±10.3bc	100±0b	81.8±11.7d	100±0c	100±0b
2×10^{10}	36.2d (19.9–66.1)	30.7d (16.4–57.5)	179.0d (78.1–410.0)	100±0	100±0c	100±0b	100±0e	100±0c	100±0b
1×10^{11}	41.7 d (23.0–75.5)	N/A	N/A	100±0	N/A	N/A	100±0e	N/A	N/A

[a]Number of spores mL^{-1} of oil (87.5% ShellSol T, 12.5% Ondina EL).
[b]Percentage of mosquito carcasses that sporulated by 5 days after death.

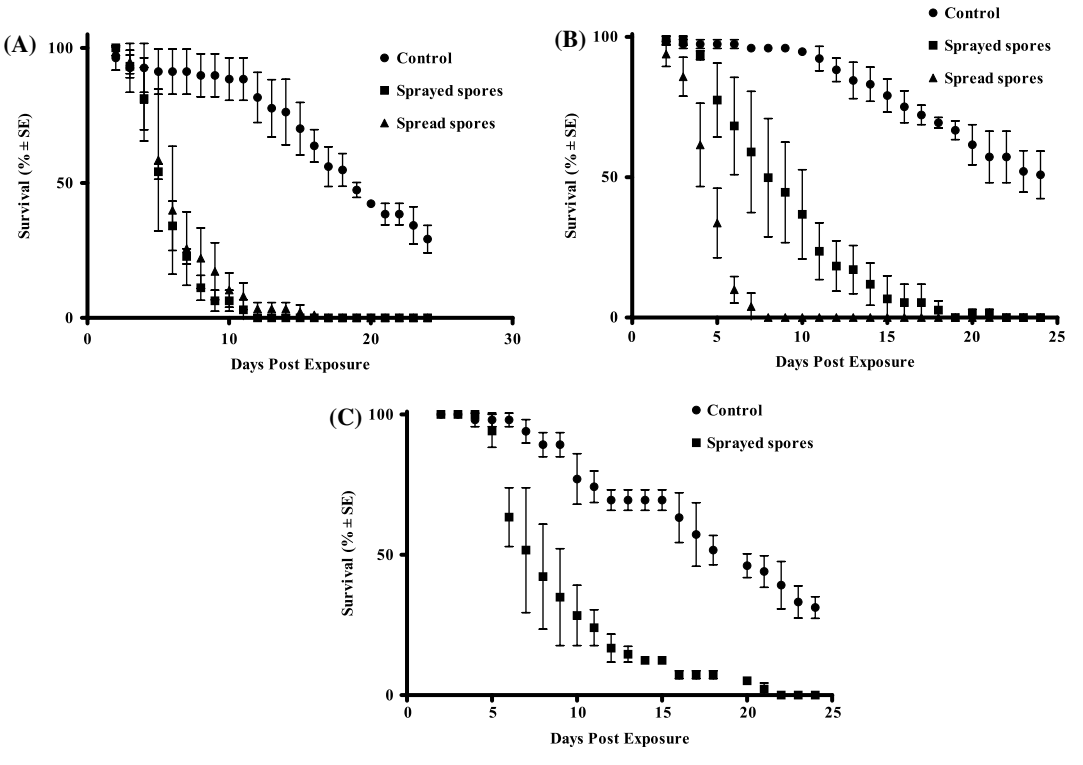

Figure 3. *Beauveria bassiana* infection via different substrates and application methods. Survival of uninfected and infected female *Ae. aegypti* after exposure to different substrates (A, cardboard drinking cup; B, black cotton drill cloth; or C, plywood). Cup and cotton substrates were treated via one of two application methods: pneumatic sprayer ('spray') vs. spreading formulation on substrate by hand ('spread').

residual pesticide; the longer spores persist, the greater the interval between treatments likely required to maintain effective control (Hancock et al. 2009). It is known that for chemical insecticides, the nature of the substrate can affect persistence (e.g., due to interactions with chemical constituents in concrete and also absorption of active ingredient into mud (Baskaran, Kookana, and Naidu 1999; Richman, Tucker, and Koehler 2006) and similar or different interactions may occur with fungal spores. However, as an initial evaluation, we used glass slides as a standard inert substrate to measure intrinsic spore persistence.

Only six isolates tested (all *B. bassiana*) had half-lives of 5 weeks or longer at 20–32°C. Strikingly, none of the *M. anisopliae* isolates persisted beyond 3–5 weeks. Why persistence of this species was so poor is unclear. In other studies, the isolate ICIPE 30 was shown to retain $63 \pm 6.7\%$ spore germination after 3 weeks on pieces of black cloth placed in Tanzanian village huts (Scholte et al. 2005) and lose only a small amount of virulence to *An. gambiae* in bioassays (Mnyone et al. 2009). In the current study, however, *in vitro* viability dropped to zero over the same time frame.

Many factors could contribute to these differences in spore longevity including the different substrates, slightly different culture histories, effects of spore quality from different production media, different conditions of temperature and humidity, different formulations, etc. Such factors make explicit comparison across studies difficult. Nonetheless, even accounting for such variation, there is little evidence to suggest that this, or the other *M. anisopliae* isolates, could exhibit half-lives as long as several of the *B. bassiana* isolates revealed here. Accordingly, we dropped *M. anisopliae* from further screening (with the exception of ICIPE30 which we retained as a standard).

Bioassays with the remaining nine most persistent *B. bassiana* isolates revealed all of them to have some impact on survival of *Ae. aegypti.* While limiting ourselves to persistent isolates admittedly may eliminate some potentially very virulent strains, another goal of our screening strategy would be to develop a candidate that can be mass-produced and applied to the field efficiently, so a persistent, virulent strain would be ideal. Under our test conditions, the most virulent isolates reduced median survival time to about 8 days, compared with about 18 days in the controls.

Further evaluation of five isolates across a range of temperatures revealed that most isolates tended to be more virulent at higher temperatures: *B. bassiana* isolates FI-277 and IMI391510 were least virulent at 20°C, while *M. anisopliae* virulence decreased considerably with each subsequent drop in temperature. Comparing *in vitro* growth with *in vivo* virulence in IMI391510, a temperature increase from 26 to 32°C was associated with both a growth decrease *in vitro* and slight but non-significant loss of virulence *in vivo;* however, a decrease in temperature from 26 to 20°C was also associated with a loss of virulence to mosquitoes, but no change in radial growth *in vitro.* FI-277 grew fastest *in vitro* at 26°C and slowest at 20°C, similar to its relative virulence at these temperatures. Patterns of temperature-dependent virulence in ICIPE30 were even less correlated with temperature-dependent radial growth rates: ICIPE30 grew at a similar *in vitro* rate at all temperatures tested, while its virulence to *Ae. aegypti in vivo* increased with temperature. These results confirm that while *in vitro* growth rate might be informative, it is not necessarily predictive of virulence, as discussed above.

ICIPE30 was not virulent to *Ae. aegypti* when it was tested at 26°C as one of a number of entomopathogenic fungus isolates, but it was virulent when tested

subsequently at 26 and 32°C. The reason for this discrepancy is unclear, given that both formulations were from the same batch of dry fungus spores, and both were tested for conidial viability on agar plates prior to the assay. This may suggest that viability tests, performed on agar as a standard, do not necessarily predict viability on an insect host, although it is noteworthy that replicate trials of *B. bassiana* isolate FI-277 were consistent in all trials.

Sporulation rates for some fungal isolates (e.g., FI-277, IMI390591, ICIPE30) varied between assays, by as much as 65–96% (IMI390591, Tables 3 and 4). Even when allowing carcasses to sporulate in controlled temperatures, factors such as moisture inside the carcass and saprophytic fungi can hinder *Beauveria* or *Metarhizium* sporulation. Recent advances in molecular detection of entomopathogenic fungi (Kouvelis, Ghikas, Edgington, Typas, and Moore 2008; Bell, Blanford, Jenkins, Thomas, and Read 2009) are more sensitive, although more expensive.

Based on these virulence data, we selected isolate FI-277 for further examination of concentration–response relationships. This isolate was selected because it was one of the most virulent at a range of temperatures and, as expected, the impact of infection varied with dose. To test the effect of dose, we first had to address differences in application method. In a recent study, Bell et al. (2009) found that 90–97% of sprayed *Beauveria* and *Metarhizium* spores were lost to the air using a similar application and assay procedure to the one used in the current study. By spraying a haemocytometer with formulation and counting the spores adhering to the surface, we found that as many as 84–99% of spores may be lost to the air in our spray hood (unpublished data).

Perhaps unsurprisingly, mortality and sporulation rates for manually-spread spores were higher than for sprayed spores, particularly at lower doses. At the two highest sprayed doses tested, mortality and sporulation rates were substantial but even doses several orders of magnitude lower still impacted survivorship significantly, relative to controls. Our results for spread of FI-277 spores are similar to those of other studies using different isolates: Scholte et al. (2007) reported an LT_{50} of approximately 4 days for female *Ae. aegypti* and *Ae. albopictus* exposed to 1.6×10^{10} spores m^{-2} of *M. anisopliae* isolate ICIPE30 (2007), and Mnyone et al. (2009) reported an LT_{50} of 4–5 days using 2×10^{10} spores m^{-2} of *B. bassiana* isolate IMI391510). The latter study also found concentrations than 2×10^{10} spores m^{-2} did not noticeably increase virulence to mosquitoes.

To evaluate spore transfer from substrates with potential for field application (e.g., black cotton, plywood), we next measured survival of *Ae. aegypti* infected with a high dose of FI-277 over different application methods. All substrates tested were capable of infecting mosquitoes. Most notably, spores spread manually onto black cotton had the greatest impact on mosquito survival, killing 100% of mosquitoes by day 8. We chose these substrates because they represent potential methods of application of infective spores to *Ae. aegypti* in the field. Black cloth has been used successfully in resting targets for *Ae. aegypti* (Edman, Kittayapong, Linthicum, and Scott 1997; de Paula et al. 2009), so the efficacy of *B. bassiana* on black cloth suggests that the fungus has potential to control mosquitoes in the field using resting targets.

The effect of application on virulence appeared to be substrate-dependent. While spores sprayed onto polyethylene-coated paper cups caused similar virulence as spores spread manually, spores sprayed onto cotton cloth were considerably less

virulent. The results for paper cups seems surprising, given that the previously-discussed dose–response trials demonstrated that spraying loses efficacy compared to applying spores directly. One potential explanation is that there is a dose threshold above which mortality is no longer dose-dependent, and the high dose used in the substrate trial may have exceeded that threshold. The differences between cloth and cardboard could be attributed to a number of factors, including adhesive capability of spores to substrate or the shape of the substrate (flat cloth vs. cylindrical cup) or loss of spores during the spraying process as was noted above.

Another point to consider regarding bioassays is that an exposure time of 24 h, which is adequate for standard screening, may not be achievable in the field. While it may be possible for mosquitoes in resting boxes, such as those designed for use against exophilic *Anopheles arabiensis* in Tanzania (Lwetoijera et al. 2010), to stay exposed to fungus-treated surfaces for several hours or more, mosquitoes landing on a visual target may only rest on spores for as little as a few minutes. Field applications of entomopathogenic fungi should take this into account; one potential solution is to use a higher dose to account for reduced exposure time, although studies by Mnyone et al. (2009) found that increasing concentrations of *B. bassiana* isolate IMI391510 above 2×10^{10} spores m^{-2} and exposure time above 30 min did not appreciably increase mosquito mortality.

Conducting comprehensive screening of the many hundreds (even thousands) of isolates that could potentially prove useful for biocontrol of a particular target pest is extremely challenging. In the current study, we have demonstrated the utility of a hierarchical, multi-criteria screening approach with a series of relatively simple *in vitro* assays used to filter a large number of candidates down to a manageable number for study *in vivo*. The screen, which included evaluation of many Australian fungi, revealed a promising isolate for possible use in novel biopesticides for use against adult mosquito vectors that transmit dengue. Further studies are now underway to fully characterize the overall impacts of this isolate on dengue transmission potential (i.e., using different delivery systems to test high absolute doses and considering not only lethal effects but potentially important pre-lethal effects of infection such as impact on host-seeking behaviour and blood feeding success) and to extend to large field cages to evaluate impact under more realistic semi-field conditions.

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