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Research Article

Evaluation of biocontrol potential of *Metarhizium anisopliae* strains against larvae and adults of *Aedes aegypti* (L.)

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ABSTRACT: In the present study, we exposed larvae and adult females of *Aedes aegypti* (L.) to fungal suspensions of three virulent strains of *Metarhizium anisopliae* (Metsch.), *viz.*, M34412, M34311 and M81123. The strains resulted in 7 to 37% survival as a result of fungal infection over the 8 d test period in case of adults and 2 to 90% survival for larvae. Mean survival period varied between 3 and 4 d for treated adults, whilst control survival exceeded 28 d. Values of LC_{50} varied from 5.92×10^3 conidia/ml for M34412, 3.49×10^4 conidia/ml for M34311 and 5.12×10^5 conidia/ml for M81123. Based on virulence and stability, the most promising strain, *M. anisopliae* M34412 was used for lethal exposure time determination. An exposure time of only 4 h was necessary to cause 50% mortality. Supplementation with *M. verrucaria* chitinase enzyme increased the larval mortality at lower conidial concentrations.

KEY WORDS: Entomopathogen, *Metarhizium anisopliae*, *Aedes aegypti*, biocontrol, Diptera, Culicidae

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INTRODUCTION

Vector borne diseases like malaria, filariasis, dengue and chikungunya caused by mosquito genera such as *Anopheles stephensi* Liston, *Culex quinquifasciatus* Say and *Aedes aegypti* (Linn.) have a significant impact on human health. An important criterion for successful transmission of disease is the longevity of insect vectors and thus control efforts are focused on methods that cause a rapid reduction in adult survival (Smith and McKenzie, 2004). Efforts to control such insect vectors largely depend on the use of chemical pesticides like organochlorines, organophosphates and pyrethroids with a quick knock-down effect (Das and Amalraj, 1997; Farenhorst *et al.*, 2010). Among the bacterial species evaluated for vector control, *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* are commercially used as larvicides exerting control by ingestion (Fillinger *et al.*, 2008; Geissbuhler *et al.*, 2009). The use of entomopathogenic fungi, such as *Metarhizium anisopliae* which are effective by contact of conidia have been demonstrated too. Furthermore, *Beauveria bassiana* and *M. anisopliae* are reported to be highly effective against insecticide resistant mosquitoes such as *A. gambiae* (Kikankie *et al.*, 2010; Farenhorst *et al.*, 2009; Howard *et al.*, 2010).

The conidia of entomopathogenic fungi germinate and penetrate through the cuticle. Subsequent development of the fungi within the hemocoel causes internal damage, depletion of nutrients and production of toxic metabolites by the fungus resulting in eventual death (Gillespie and Clayton, 1989). In case of mosquito larvae that proliferate in aqueous milieu, conidial adherence to the cuticle as well as ingestion followed by germination and subsequent disruption of the gut chitin with the aid of chitinolytic enzymes have been reported (Lacey *et al.*, 1988; Silva *et al.*, 2004; Seye *et al.*, 2009). In addition to the lethal effect of entomopathogenic fungi on mosquitoes, they were also reported to cause reduction of vectorial capacity, feeding, fecundity, flight or dispersal capacity as well as predator escape responses (Scholte *et al.*, 2006; Thomas and Read, 2007; Blandford *et al.*, 2009; Read *et al.*, 2009). Studies on entomopathogenic fungi for control of adult *A. aegypti* included *M. anisopliae* (Scholte *et al.*, 2007; Paula *et al.*, 2008) while for larvae, *Tolypocladium cylidrisporum* (Goettel, 1987; 1988) and *B. bassiana* were reported (Clark *et al.*, 1968; Miranpuri and

Kachatourians, 1991). Furthermore, Paula *et al*. (2011) reported that sub-lethal concentrations (0.1ppm) of imidacloprid in combination with the *M. anisopliae* conidia significantly reduced survival rates in adult *Ae. aegypti.*

Other fungal products such as extracellular chitinases from deuteromycetous fungus, *Myrothecium verrucaria* and keratinase, an extracellular fungal metabolite from *Trichophyton entagrophytes* were reported to have larvicidal potential against mosquito larvae (Mendosa *et al.,* 1996; Murugesan *et al.,* 2009). In a recent study, Halder *et al.* (2012) reported the effect of bacterial chitinolytic enzymes of *Aeromonas hydrophila* on *Cx. quinquifasciatus*, a vector of filariasis.

The present studies were carried out to evaluate the effect of 3 most effective strains of *M. anisopliae*, *viz.*, M34412, M34311 and M81123 against *Ae. aegypti* along with and without supplementation of CDE complex of *M. verrucaria*. The effect of repeated subculturing of *M. anisopliae* (M34412) conidia was also studied on the survival of *Ae. aegypti* larvae.

MATERIALS AND METHODS

Organisms

M. anisopliae strains M34412, M34311 and M81123 were maintained on potato dextrose agar (PDA) as described earlier (Kulkarni *et al.,* 2008). After repeated subculturing, the 40th sub-culture of *M. anisopliae* M34412 was grown on PDA at 28°C for 7 days. The conidia were produced in solid state fermentation on rice for 14 d at 28°C and 75-80% RH, harvested and kept in –80°C until use (Kulkarni *et al.,* 2008). Fungal suspensions were initially prepared in Tween 80 (0.1% w/v) and conidial concentrations were determined using a Neubauer hemocytometer. The conidial suspensions were vortexed vigorously before use.

For the production of cuticle degrading enzymes, *M. verrucaria* was grown for 7 days in a medium which contained (g/l): KH_2PO_4, 3.0; $KHPO_4$, 1.0; $MgSO_4$, 0.7; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5; $CaCl_2$, 0.5; bacto-peptone, 0.5 and chitin, 0.5; trace metal solution, 1ml of (mg/ml): $FeSO_4$, 5.0; MnSO4, 1.56; $ZnSO_4$, 3.34; $CoCl_2.2H_2O$, 2.0 and pH, 6.0 under shaking conditions as described earlier (Vyas and Deshpande, 1989). The culture supernatant was collected by centrifugation at 5000×g for 10 min and was lyophilized to dryness and stored at –20°C until use.

Enzyme activities

Total chitinase activity in the culture supernatant was estimated colorimetrically using acid-swollen chitin as a substrate prepared from crabshell chitin, as described by Vyas and Deshpande (1989). The *N*-acetylglucosamine (GlcNAc) residues produced were estimated colorimetrically at 585 nm with p-dimethylamino benzaldehyde (DMAB) as described by Reissig *et al.* (1955). One international unit was defined as the activity which produced 1 µmol of GlcNAc per min. Protease activity was measured using Hammerstein casein as a substrate (Vyas and Deshpande, 1989). One unit of enzyme liberated 1 µmol of tyrosine per min. Lipase activity was determined as described by Pignede *et al.* (2000). The substrate emulsion was prepared with olive oil (50 ml) and gum arabic (50 ml, 10%, w/v, Sigma). Enzyme activity was determined by titration of the fatty acids released with 50 mM NaOH. One unit of lipase is the amount of enzyme that released 1 µmol of fatty acids per min.

Protein was estimated according to Lowry *et al.* (1951) using bovine serum albumin as the standard.

Insect culture

Virus free adults of *A. aegypti* were obtained from Vector Control Research Centre (VCRC), Pondicherry, India and reared in cages under laboratory conditions at 28°C, 75 ± 5% RH and a photoperiod of 16:8 (L:D). Larvae were maintained in trays filled with water and fed with pet food and yeast powder (3:1).

Bioassays

To evaluate fungal efficacy, *i.e*., the dose of conidia required, as well as the lethal exposure time and stability of the conidia, different bioassays were carried out against the larvae and adults of *A. aegypti*. Bioassays were also carried out to evaluate the efficacy of *M. anisopliae* (M34412) 1st and 40th subculture conidia and *M. anisopliae* (M34412, 1st subculture) conidia in combination with *M. verrucaria* enzyme against larvae of *A. aegypti.*

With larvae

Twenty 3rd instar larvae of *A. aegypti* were transferred to 50 ml glass beaker containing 20 ml of *M. anisopliae* conidial suspensions (1×10^3 to 1×10^7 conidia/ml) of 3 strains, *viz.*, M34412, M34311 and M81123 and 40th subculture of M34412 in tap water. This was replicated 3 times to ensure reproducibility. The larvae in water without conidia served as control. Total survival was recorded for all the replicates of each treatment at 24 h intervals up to 7 d.

The varying concentration of *M. verrucaria* enzyme complex measured as (0.5-1.5 U/ml total chitinase activity)

singly and in combination with *M. anisopliae* (M34412) conidia at a concentration of 1×10^3/ml were used for bioassay. Twenty 3rd instar larvae of *Ae. aegypti* were transferred to 50 ml glass beaker containing 20 ml of *M. anisopliae* (M34412) total conidial count 2×10^4 and *M. verrucaria* enzyme complex (10- 30 units) in tap water. The observations were recorded for 7d at intervals of 24h.

With adults

The 3 strains of *M. anisopliae* were evaluated against adult females of *Ae. aegypti* by indirect contact method using filter paper impregnated with conidial suspensions. Sterile filter paper (Whatman No. 1, 8×6 cm) was immersed in suspensions of 1×10^7 to 1×10^{10} conidia/ml which were equivalent to 1.39×10^6 to 5.9×10^8 conidia/cm^2 (estimated by determining conidial concentrations following re-suspension of conidia from randomly sampled 1 cm^2 of filter paper). The filter papers impregnated with conidia (conidial cards) were subsequently allowed to dry at 28°C, 70% RH for 16-18 h, before being placed in 500 ml glass beakers. The size of the conidial card was such that it rested almost vertically within the beaker and allowed free movement of the adult mosquitoes in the beaker. Ten adults (2-3 d old) were released in each beaker and for each strain, 30 adults were tested. Control treatments were carried out by treating the filter papers without conidia. All the experiments were conducted three times at separate time intervals and appropriate controls (filter papers treated with 0.1% (w/v) Tween 80) were used for all experiments. Insects were fed with 10% sucrose in cotton swabs and placed inside the beaker. The observations were taken for 8 days.

For determining the mean lethal exposure time, adult mosquitoes were exposed to filter papers impregnated with *M. anisopliae* M34412 (1×10^{10} conidia/ml) formulated in 0.1% (w/v) Tween-80 for different time periods *viz*., 1 h, 4 h, 12 h, 24 h and 48 h and survival was recorded every 24 h for a period of 8 d.

The stability of conidia of *M. anisopliae* M34412 on impregnated filter paper was studied. The stability of conidia was evaluated by exposing adult females of *Ae. aegypti* to the same treated filter papers after every 1 week upto 4 weeks. Sterile filter paper (Whatman No. 1, 8×6 cm) was immersed in suspensions of 1×10^{10} conidia/ml which were equivalent to 5.9×10^8 conidia/cm^2 and kept at 28°C until use.

The dead larvae and adults of *Ae. aegypti* treated with conidia of *M. anisopliae* were transferred to wet filter paper and maintained at 70-80% RH and 28°C for fungal growth. Conidial germination on cuticle was observed under an inverted light microscope. Hyphal growth, indicative of fungal infection was observed after 3-4 d.

Statistical analysis

Differences in survival between larval and adult mosquito of fungus-infected and control groups were analyzed using the Kaplan-Meier method to plot cumulative survival factors by treatment with pair-wise comparison conducted using the log-rank test (SPSS 13) (Pignede *et al.*, 2000). Mean lethal concentration (LC_{50}) was calculated using non-linear regression function in the SPSS software 13 and compared using one-way ANOVA with Tukeys Multiple comparison plot test.

RESULTS AND DISCUSSION

The present study demonstrated the ability of *M. anisopliae* strains M34412, M34311 and M 81123 to infect both the larvae and adults of *A. aegypti*.

Effect of *M. anisopliae* conidia on *Ae. aegypti* larvae

Among the 3 *M. anisopliae* strains, M34412 was most effective and virulent particularly to the larvae of *A. aegypti*. In the presence of 1×10^7 conidia/ml, 3rd instar larvae of *A. aegypti* were susceptible to all the 3 strains of *M. anisopliae* with survival <5% at the end of 7 d of exposure (Table 1). At the lowest concentration of 1×10^3 conidia/ml, percent survival ranged between 68% and 90% for all the 3 strains (Table 1). Percent survival was significantly lower as compared to control (M34412 – $F_{5, 36}$=6.75, p<0.0001; M34311– $F_{5, 36}$=7.22, p<0.0001 and M81123 - $F_{5, 36}$=5.16, p<0.001). Values of LC_{50} varied from 5.92×10^3 conidia/ml for M34412, 3.49×10^4 conidia/ml for M34311 and 5.12×10^5 conidia/ml for M81123 (Table 1).

Fig. 1a depicts the day-wise survival of *A. aegypti* larvae with *M. anisopliae* strains. The median survival time of larvae (MST) in the control group was more than the experimental duration of 8 d and was significantly higher as compared to any of the treatments (Table 2). The MST varied between 2.18 d to 2.6 d for M34412, 2.50 d to 2.94 d for M34311 and 2.97 d to 4.51 d for M81123 at the concentrations evaluated, i.e., 1×10^7 to 1×10^4 conidia/ml. Exposure of larvae of *A. aegypti* to concentrations of 1×10^6 and 1×10^7 conidia/ml resulted in significantly lower MST's in the presence of strains M34412 and M81123. At the lowest concentration of 1×10^4 conidia/ml, the MST was significantly higher for all the 3 strains. Silva *et al.* (2004) reported 15 out of 80 *M. anisopliae* soil isolates to be highly virulent (>90 % mortality) against 2nd instar larvae of *A. aegypti* (Silva *et al.*, 2004), while Seye *et al.*

Table 1: **Cumulative percent survival and LC_{50} of 3rd instar larvae of *Aedes aegypti* after treatment with different concentrations of conidia of *Metarhigium anisopliae* strains M34412, M34311 and M81123 over a 7 d period**

M. anisopliae strains	Concentration (conidia/mL)	Cumulative per cent survival (±SE)	LC_{50}
M34412	1×10^7	1.66±1.49	5.92×10^3
	1×10^6	3.33±0.56	
	1×10^5	6.66±1.13	
	1×10^4	21.67±0.56	
	1×10^3	68.31±2.26	
M34311	1×10^7	3.33±1.49	3.49×10^4
	1×10^6	5.00±0.00	
	1×10^5	6.66±2.04	
	1×10^4	31.66±1.13	
	1×10^3	81.65±3.15	
M81123	1×10^7	5.00±1.69	5.25×10^5
	1×10^6	6.66±1.49	
	1×10^5	18.33±4.42	
	1×10^4	25.00±4.49	
	1×10^3	90±1.69	

Table 2: **Kaplan-Meier pair wise comparisons of the median survival times (MST) of larvae of *Aedes aegypti* exposed to different concentrations (1×10^7, 1×10^6, $1\times10^5$and 1×10^4 conidia/mL) of *Metarhizium anisopliae* strains M34412, M34311 and M81123**

Concentration	MST (±SE)	1×10^7 conidia/mL	1×10^6conidia/mL	1×10^5conidia/mL	1×10^4conidia/mL
M34412					
Control	–	÷2=87.47*p<0.001	÷2=64.96*p<0.001	÷2=65.90*p<0.001	÷2=30.40*p<0.001
1×10^7	2.18±0.10		÷2=7.76*p<0.01	÷2=18.19*p<0.001	÷2=36.40* p<0.001
1×10^6	2.40±0.90			÷2=1.56	÷2=11.70*p<0.001
1×10^5	2.60±0.30				÷2=5.94*p<0.01
M34311					
Control	–	÷2=54.79*p<0.001	÷2=52.20*p<0.001	÷2=49.49*p<0.001	÷2=20.78*p<0.001
1×10^7	2.50±0.12		÷2=0.67	÷2=2.29	÷2=17.91*p<0.001
1×10^6	2.81±0.04			÷2=0.5	÷2=13.10*p<0.001
1×10^5	2.94±0.05				÷2=9.03*p<0.01
M81123					
Control	–	÷2=63.47*p<0.001	÷2=41.86*p<0.001	÷2=28.37*p<0.001	÷2=23.74*p<0.001
1×10^7	2.97±0.10		÷2=10.35*p<0.01	÷2=26.51*p<0.001	÷2=32.04*p<0.001
1×10^6	3.68±1.12			÷2=4.33*p<0.05	÷2=6.97*p<0.01
1×10^5	4.51±1.17				÷2=0.41

(2009) reported *Aspergillus clavatus* to be the most virulent among the 4 species isolated from the locust, *Oedaleus senegalensis* against larvae of different mosquito species with > 95% mortality against both *A. aegypti* and *Cx. quinquefasciatus* and 95% against *An. gambiae* (Seye *et al.,* 2009).

In the present study, the concentration of conidia was positively correlated with mortality and maximum reduction in the survival of larvae was achieved at a concentration of 1×10^7 conidia/ml. At these concentrations, the MST was short indicative of rapid infection and consequent mortality. For *A. aegypti* larvae, LC_{50} of 5.92×10^3 conidia/ml of *M. anisopliae* M34412 after 7 d was observed. Of the several *M. anisopliae* isolates evaluated, Alves *et al.* (2002) reported a LC_{50} of 1.97×10^4 conidia/ml for isolate 1037 while Pereira *et al.* (2009) reported a LC_{50} of 3.16×10^5 conidia/ml and a MST of 5 d for the most virulent strain CG144.

Attenuation of virulence of conidia has been observed in nearly all the taxa of entomopathogenic fungi. Attenuated conidia may germinate and infect their hosts marginally slower than non-attenuated conidia, which may partly be due to the lack of the right set of enzymes to facilitate host penetration. The data for percent survival of *Ae. aegypti* at concentrations 1×10^3, 1×10^5, and 1×10^7 conidia/ml for 1st and 40th subculture conidia are presented in Fig. 2. It is evident that though there is <10% survival at 1×10^5, and 1×10^7 conidia/ml concentrations for both the subcultures, a significant increase in survival (~20%) was observed for 40th subculture as compared to 1st subculture at 1×10^3 conidia/ml concentration. Decrease in mortality for 40th subculture may be attributed to decrease in the levels of enzyme activities and concomitant decrease in virulence during *in vitro* repeated transfers from the 1st to 40th sub-culture as demonstrated previously for *Helicoverpa amigera* (Hubner) (Nahar *et al.,* 2008).

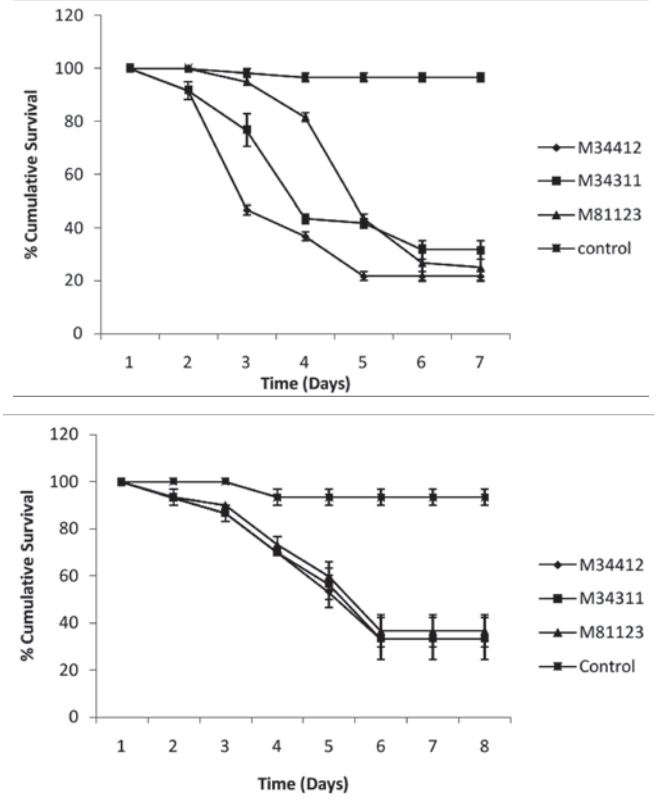

Fig. 1 (a and b): Mean daily survival rate of a) larvae and b) adult of *Aedes aegypti* inoculated with three strains of *Metahizium anisopliae* at concentration ($1x10^4$ conidia/ml) and ($1x10^7$ conidia/ml) respectively (±SE).

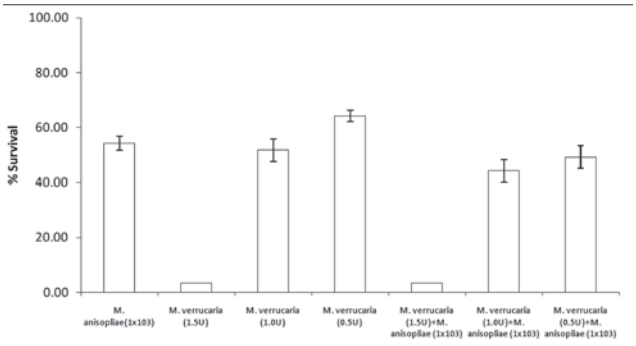

Fig. 2: Percent survival of *A. aegypti* 3rd instar larvae with *M. anisopliae* conidia (1×10^3conidia/ml) and *M. verrucaria* enzyme at different combination determined upto 4 days (±SE)

Myrothecium verrucaria has been shown to produce a cuticle degrading enzyme (CDE) complex containing chitinase, protease and lipase enzymes with potential application in the control of *A. aegypti* (Mendonsa *et al.,* 1996). In the present study, the *M. verrucaria* CDE complex was used in different combinations with *M. anisopliae* conidia for bioassay with larvae. The enzyme activities in the CDE complex were – chitinase, 1.5 U/ml; protease, 0.07 U/ml and lipase, 0.415 U/ml. In our bioassay with *M. verrucaria* chitinase 0.5, 1.0 and 1.5 U/ml enzyme complexes, the percent survival were 64.17%, 51.16% and less than 5%, respectively. For treatment with *M. anisopliae* (1×10^3 conidia/ml) singly, the percent survival was 54.17%. For *M. verrucaria* CDE complex, the survival was less than 5% at 1.5U/ml chitinase concentration. Combination of *M. anisopliae* conidia (1×10^3/ml) with *M. verrucaria* chitinase activity at 0.5 and 1 U/ml showed significant reduction in percent survival (Fig. 3).

Effect of *M. anisopliae* conidia on *A. aegypti* adults

All fungal strains significantly reduced adult *A. aegypti* survival compared to untreated control after 8 d

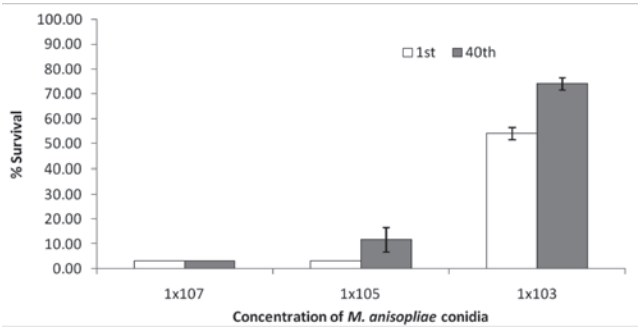

Fig. 3: Percent survival of *A. aegypti* 3rd instar larvae with *M. anisopliae* (M34412) 1st subculture and 40th subculture conidia at different concentration determined upto 4 days (±SE)

exposure. At higher concentrations of $>1\times10^9$ conidia/ml, survival were 6.6%, 13.3% and 16.6% in adults exposed to M34412, M34311and M81123 strains respectively (Table 3). At lower concentrations of 1×10^8 and 1×10^7conidia/ml, it varied from 23.3-36.6% among the 3 strains. At these concentrations, per cent survival was significantly lower than control (M34412 – $F_{4, 35}$=3.87, p<0.01; M34311 – $F_{4, 35}$=3.90, p<0.01; M81123 – $F_{4, 35}$=3.90, p<0.01). The concentration of conidia that resulted in 50% mortality (LC_{50}) was 6.92×10^8 conidia/ml for M34412, 5.03×10^9 conidia/ml for 34311 and 8.22×10^9 conidia/ml for M81123 (Table 3). Fig. 1b depicts the day-wise mortality of *Ae. aegypti* adults with *M. anisopliae* strains.

The median survival time (MST) of adult mosquitoes in the control group extended beyond the experimental duration and was significantly higher compared to the different concentrations of the 3 strains (Table 4). The MST varied between 3.55 d to 4.38 d for M34412, 4.05 d to 4.30 d for M34311 and 4.15 d to 4.42 d for M81123 at the concentrations evaluated, i.e., 1×10^{10} to 1×10^8 conidia/ml. However, no significant difference was observed in the MST values between the different concentrations in all the 3 strains except for comparisons between concentrations 1×10^{10} and 1×10^7 conidia/ml in strain M34311. There were no differences between concentrations of 1×10^8 and 1×10^7 conidia/ml for M34412 (3±0.8, $\div^2$ = 0.13, p = 0.71), M34311 (3.3±0.5, $\div^2$ = 0.29, p = 0.59) and M81123 (3.7±0.6, $\div^2$ = 0.49, p = 0.48).

In the case of adults, we observed survival of 10% with MST of 4.25 d when exposed to M34412 while, it was 16% s when exposed to M34311 and M81123 at a concentration of 1×10^9 conidia/ml (1.2×10^7 conidia/cm^2 on Whatman no. 1 filter paper) with median survival time (MST) 4.25 d and 4.17 d respectively were observed. Mnyone *et al.* (2009) reported the median survival time (MST) of *An. gambiae* infected with *M. anisopliae* ICIPE-30 varied between 4 d at 2×10^{10} conidia/m^2 conidia and 10 days at 1×10^7conidia/m^2. Similarly, Lawetojera *et al.* (2010) reported a MST of 2 d when *An. gambiae* adults were exposed to 3.9×10^8 conidia/cm^2 of *M. anisopliae*, while in our study a MST of 3.55 d was observed when *Ae. aegypti* were exposed to M34412 at a concentration of 1×10^{10} conidia/ml (5.9×10^8 conidia/cm^2 on Whatman no. 1 filter paper).

Table 3: Cumulative percent survival and LC_{50} of adult females of *Aedes aegypti* after treatment with different concentrations of conidia of *Metarhizium anisopliae* strains M34412, M34311 and M81123 over a 8 d period

M. anisopliae strains	Concentration (conidia/mL)	Cumulative percent survival (±SE)	LC_{50}
M34412	1×10^{10}	6.66±6.79	6.92×10^8
	1×10^9	10.00±5.88	
	1×10^8	23.33±3.40	
	1×10^7	33.33±8.99	
M34311	1×10^{10}	13.33±3.40	5.03×10^9
	1×10^9	16.66±3.40	
	1×10^8	26.66±6.79	
	1×10^7	33.33±8.99	
M81123	1×10^{10}	16.66±3.40	8.22×10^9
	1×10^9	16.66±3.40	
	1×10^8	30.00±5.88	
	1×10^7	36.66±6.79	

Table 4: Kaplan-Meier pair wise comparisons of the median survival times (MST) of adult females of *Aedes aegypti* exposed to different concentrations (1×1010, 1×109, 1×108 and 1×107 conidia/mL) of *Metarhizium anisopliae* strains M34412, M34311 and M81123

Concentration	MST (±SE)	1×10^7 conidia/mL	1×10^6conidia/mL	1×10^5conidia/mL	1×10^4conidia/mL
M34412					
Control	–	÷2=21.62*p<0.001	÷2=20.47*p<0.001	÷2=12.49*p<0.001	÷2=8.73*p<0.01
1×10^{10}	3.55±0.06		÷2=0.90	÷2=2.96	÷2=6.05* p<0.05
1×10^9	4.25±0.17			÷2=0.82	÷2=3.38
1×10^8	4.38±0.11				÷2=3.38
M34311					
Control	–	÷2=16.80*p<0.001	÷2=14.86* p<0.001	÷2=11.15* p<0.001	÷2=8.84* p<0.01
1×10^{10}	4.05±0.09		÷2=0.17	÷2=0.66	÷2=2.62
1×10^9	4.25±0.13			÷2=0.17	÷2=1.53
1×10^8	4.30±0.11				÷2=0.47
M81123					
Control	–	÷2=16.00*p<0.001	÷2=15.09* p<0.001	÷2=10.07* p<0.01	÷2=7.95* p<0.01
1×10^{10}	4.15±0.27		÷2=0.0002	÷2=0.47	÷2=2.33
1×10^9	4.17±0.13			÷2=0.44	÷2=2.28
1×10^8	4.42±0.19				÷2=0. 97

Selection of *M. anisopliae* M34412 and the concentration (1×10^{10} conidia/ml) were based on results obtained with bioassays on fungal efficacy. Survival of mosquitoes exposed to M34412 was significantly reduced as compared to control (p<0.0001, Table 5). The MST of mosquitoes after 12 h and above was significantly lower than mosquitoes exposed for 1 h and 4 h. In adults, an exposure time of 4 h resulted in 50% survival and increasing the exposure time to 48 h resulted in 7% survival. Similarly, Paula *et al.* (2008) demonstrated that a 3.5 h exposure of *A. aegypti* adults to *M. anisopliae* isolate at 1×10^9 conidia/ml resulted in 50% survival and increasing the exposure time to 48 h resulted in 10.7% survival with MST of 3 d. The increased exposure time possibly allow the mosquitoes to settle on the conidial card sufficiently thereby increasing the mortality. While Stevenson (2008)

Table 5: Kaplan-Meier pair wise comparisons of the median survival times (MST) of adult *Aedes aegypti* females exposed to *Metarhizium anisopliae* strain M34412 at 1×10^{10} conidia/mL for different time exposures (1h, 4h, 12h, 24h and 48h)

Exposure time	Cumulative per cent survival (±SE)	MST (±SE)	1h	4h	12h	24h	48h
Control	93.33±3.39	–	÷2=1.13	÷2=3.89* p<0.05	÷2=6.42* p<0.05	÷2=10.47* p<0.001	÷2=18.68* p<0.01
1h	76.67±3.39	12.50±0.86		÷2=0.002	÷2=3.84* p<0.05	÷2=9.22* p<0.01	÷2=10.42* p<0.01
4h	50±5.88	7.82±0.38			÷2=4.07* p<0.05	÷2=6.08* p<0.05	÷2=7.01* p<0.01
12h	30±3.39	4.38±0.20				÷2=0.001	÷2=0.30
24h	16.67±3.39	4.50±0.19					÷2=0.25

observed an increase in mortality of *An. stephensi* exposed to *M. anisopliae* beyond 6 h, Scholte *et al.* (2003) reported no significant difference in *An. gambiae* exposed to *M. anisopliae* for 24 h, 48 h or continuous exposure. Mnyone *et al.* (2009) observed exposure times as short as 15 and 30 min to be sufficient to reduce the survival of *An. gambiae* mosquitoes.

The MST's of adult *A. aegypti* exposed to conidial card treated with 1×10^{10} conidia/ml of *M. anisopliae* M34412 at 1, 2, 3 and 4 weeks post inoculation were significantly lower than control (Table 6) and survival of mosquitoes was lower for 1 week post application compared to those exposed 3 and 4 weeks later. However, Scholte *et al.* (2005) observed a decline in infectivity of *M. anisopliae* conidia impregnated on black sheets with a reduction in germination from 95% after day 1 to 63% after 3 weeks (Scholte *et al.*, 2006). Based on the data collected, they developed a model which suggested that fungus impregnated sheets would have a significant impact on parasite transmission.

The present study documents the ability of *M. anisopliae* strains M34412, M34311 and M81123 in reducing the survival rate of *A. aegypti*. It has several characteristics that are important for achieving successful control of *A. aegypti* – a) it kills both the larvae and adults of *A. aegypti* b) shows stability and c) increased mortality in combination with the *M. verrucaria* enzyme. In our view, either of the *M. anisopliae* strains M34412, M34311 and M81123 should preferentially be applied in integrated control programs in order to gain maximum impact both on larval and adult mosquito populations.

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REFERENCES

Alves SB, Alves LFA, Lopes RB, Pereira RM, Vieira SA. 2002. Potential of some *Metarhizium anisopliae* isolates for control of *Culex quinquefasciatus* (Diptera: Culicidae). *J Appl Entomol.* **126**: 504–509.

Blanford S, Read AF, Thomas MB. 2009. Thermal behaviour of *Anopheles stephensi* in response to infection with malaria and fungal entomopathogens. *Malaria J.* **8**: 72.

Clark TB, Keller WR, Fukuda T, Lindgren JE. 1968. Field and laboratory studies on the pathogenicity of the fungus *Beauveria bassiana* to three genera of mosquitoes. *J Inv Pathol.* **11**: 1–7.

Das PK, Amalraj D. 1997. Biological control of malaria vectors. *Indian J Med Res.* **106**: 174–197.

Farenhorst M, Knols BGJ, Thomas MB, Howard AFV, Takken W, Rowland M, N'Guessan R. 2010. Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes. *PLoS One*. **5**: 8.

Farenhorst M, Mouatcho JC, Kikankie CK, Brooke BD, Hunt RH, Thomas MB, Koekemoer LL, Knols BGJ, Coetzee M. 2009. Fungal infection counters insecticide resistance in african malaria mosquitoes. *Proc Nat Acad Sci.* **106**: 17443–17447.

Fillinger U, Kannady K,William G, Dongus S, Nyika D, Geissbuhler Y, Chaki P, Gorella N, Mathenge E, Singer B, Mshinda H, Lindsay S, Tanner M. 2008. A tool box for operational mosquito larval control: preliminary results and early lessons from the Urban

Table 6: Stability of conidia in suspension and impregnated on filter paper for 1-4 weeks of *Metarhizium anisopliae* strain M34412 (1×10^{10}conidia/mL) measure as MST of *Aedes aegypti*

Exposure time	Cumulative per cent survival (±SE)	MST (±SE)	Week 1	Week 2	Week 3	Week 4
Control	93.33±3.39	-	÷2 = 23.74*	÷2=12.67*	÷2 = 8.08*	÷2=6.49*
			p<0.001	p<0.001	p<0.01	p<0.01
Week 1	6.66±6.79	3.50±0.27		÷2=2.88	÷2=5.94*	÷2=7.30*
					p<0.05	p<0.01
Week 2	28.58±3.39	4.16±0.39			÷2= 0.72	÷2=1.23
Week 3	42.87±3.39	5.08±0.83				÷2=0.08

Malaria Control Programme in Dar es Salaam, Tanzania. *Malaria J.* **7**: 20.

GeissbuhlerY, Kannady K, Chaki P, Enidi B, Gorella NJ, Mayagaya U, Kiama M, Mtasiwa D, Mshinda H, Lindsay SW, Tanner M, Fillinger U, MC, Killeen GF. 2009. Microbial larvicide application by a large scale, community based program reduces malaria infection prevalence in urban Dar es Salam, Tanzania. *PLoS One* **4**: e5107.

Gillespie AT, Clayton N. 1989. The use of entomopathogenic fungi for pest control and the role of toxins in pathogenesis. *Pestic Sci.* **27**, 203–215.

Goettel MS. 1987. Studies on bioassay of the entomopathogenic hypomycete fungus, *Tolypocladium cylindrosporum* in mosquitoes. *J Amer Mos Cont Assoc.* **3**: 239–245.

Goettel MS. 1988. Pathogenicity of the hypomycete *Tolypocladium cylindrosporum* in the mosquito *Aedes aegypti*. *J Inv Pathol.* **51**: 259–274.

Halder SK, Maity C, Jana A, Pati BR, Mandal KC. 2012. Chitinolytic enzymes from the newly isolated *Aeromonas hydrophila* SBK1: study of the mosquitocidal activity. *Biocontrol* **57**: 441–449.

Howard AFV, Koenraadt CJM, Farenhorst M, Knols BGJ, Takken W. 2010. Pyrethroid resistance in *Anopheles gambiae* leads to increased susceptibility to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *Malaria J.* **9**: 168.

Kikankie C, Brooke B, Knols B, Koekemoer L, Farenhorst M, Hunt R, Thomas M, Coetzee M. 2010. The infectivity of the entomopathogenic fungus *Beauveria bassiana* to insecticide-resistant and susceptible *Anopheles arabiensis* mosquitoes at two different temperatures. *Malaria J.* **9**: 71.

Kulkarni SA, Ghormade V, Kulkarni G, Kapoor M, Chavan SB, Rajendran A, Patil SK, Shouche Y, Deshpande MV. 2008. Comparison of *Metarhizium isolates* for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. *Biocontrol Sci Technol.* **18**: 809–828.

Lacey CM, Lacey LA, Roberts DR. 1988. Route of invasion and histopathology of *Metarhizium anisopliae* in *Culex quinquefasciatus*. *J Inv Pathol.* **52**: 108–118.

Lwetoijera D, Sumaye R, Madumla E, Kavishe D, Mnyone L, Russell T, Okumu F. 2010. An extra-domiciliary method of delivering entomopathogenic fungus, *Metharizium anisopliae* IP 46 for controlling adult populations of the malaria vector, *Anopheles arabiensis*. *Parasite Vectors* **3**: 18.

Mendonsa ES, Vartak PH, Rao JU, Deshpande MV. 1996. An enzyme from *Myrothecium verrucaria* that degrades insect cuticles for biocontrol of *Aedes aegypti* mosquito. *Biotechnol Lett.***18**: 373–376.

Miranpuri GS, Khachatourians GG. 1991. Infection sites of the entomopathogenic fungus *Beauveria bassiana* in the larvae of the mosquito *Aedes aegypti*. *Ent Exp Applic.* **59**: 19–27.

Mnyone L, Lyimo I, Lwetoijera D, Mpingwa M, Nchimbi N, Hancock P, Russell T, Kirby M, Takken W, Koenraadt C. 2009. Exploiting the behaviour of wild malaria vectors to achieve high infection with entomopathogenic fungus. *Malaria J.* **9**: 23.

Murugesan A, Sathesh Prabu C, Selvakumar C. 2009. Biolarvicidal activity of extracellular metabolites of the keratinophilic fungus *Trichophyton mentagrophytes* against larvae of *Aedes aegypti* - a major vector for Chikungunya and dengue. *Folia Microbiol.* **54**: 213–216.

Nahar PB, Kulkarni SA, Kulye MS, Chavan SB, Kulkarni G, Rajendran A, Yadav PD, Shouche Y, Deshpande MV. 2008. Effect of repeated *in vitro* sub-culturing on the virulence of *Metarhizium anisopliae* against *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Biocontrol Sci Technol.* **18**: 337–355.

Paula A, Carolino A, Paula C, Samuels R. 2011. The combination of the entomopathogenic fungus *Metarhizium anisopliae* with the insecticide imidacloprid increases virulence against the dengue vector *Aedes aegypti* (Diptera: Culicidae). *Parasite Vectors* **4**: 8.

Paula A, Carolino A, Silva C, Samuels R. 2008. Susceptibility of adult female *Aedes aegypti* (Diptera: Culicidae) to the entomopathogenic fungus *Metarhizium anisopliae* is modified following blood feeding. *Parasite Vectors* **4**: 91.

Pereira CR, De Paula AR, Gomes SA, Pedra PCO, Samuels RI. 2009. The potential of *Metarhizium anisopliae* and *Beauveria bassiana* isolates for the control of *Aedes aegypti* (Diptera: Culicidae) larvae. *Biocont Sci Technol.* **19**: 881–886.

Pignede G, Wang HJ, Fudalej F, Gaillardin C, Seman M, Nicaud JM. 2000. Characterization of an extracellular

lipase encoded by LIP2 in *Yarrowia lipolytica*. *J Bacteriol.* **182**: 2802–2810.

Read AF, Lynch PA, Thomas MB. 2009. How to make evolution-proof insecticides for malaria control. *PLoS Biology* **7**: e1000058.

Reissig JL, Strominger JL, Leloir LFA. 1955. A modified colorimetric method for the estimation of *N*-acetylamino sugars *J Biol Chem.* **217**: 959–966.

Scholte EJ, Ng'habi K, Kihonda J, Takken W, Paaijmans K, Abdulla S, Killeen GF, Knols BGJ. 2005. An entomopathogenic fungus for control of adult African malaria mosquitoes. *Science* **308**: 1641–1642.

Scholte E-J, Njiru B, Smallegange R, Takken W, Knols B. 2003. Infection of malaria (*Anopheles gambiae* s.s.) and filariasis (*Culex quinquefasciatus)* vectors with the entomopathogenic fungus *Metarhizium anisopliae*. *Malaria J.* **2**: 29.

Scholte EJ, Knols BGJ, Takken W. 2006. Infection of the malaria mosquito *Anopheles gambiae* with the entomopathogenic fungus *Metarhizium anisopliae* reduces blood feeding and fecundity. *J Inv Pathol.* **91:** 43–49.

Scholte E-J, Takken W, Knols BGJ. 2007. Infection of adult *Aedes aegypti* and *Ae. albopictus* mosquitoes with the entomopathogenic fungus *Metarhizium anisopliae*. *Acta Tropica.* **102**: 151–158.

Seye F, Faye O, Ndiayel M, Njie E, Afoutou JM. 2009. Pathogenicity of the fungus, *Aspergillus clavatus*, isolated from the locust, *Oedaleus senegalensis*, against larvae of the mosquitoes *Aedes aegypti*, *Anopheles gambiae* and *Culex uinquefasciatus*. *J Insect Sci.* **9**: 53.

Silva RO, Silva Heloisa HG, Luz C. 2004. Effect of *Metarhizium anisopliae* isolated from soil samples of the central Brazilian cerrado against *Aedes aegypti* larvae under laboratory conditions. *Rev Pathol Trop.* **33**: 207–216.

Smith DL, McKenzie FE. 2004. Statics and dynamics of malaria infection in *Anopheles* mosquitoes. *Malaria J.* **3**: 13.

Stevenson JC. 2008. The use of fungi against adult malaria mosquitoes (PhD Thesis). London, School of Hygiene and Tropical Medicine.

Thomas MB, Read AF. 2007. Fungal bioinsecticide with a sting. *Nature Biotechnol.* **25**: 1367–1368.

Vyas P, Deshpande MV. 1989. Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation. *J Biol Chem.* **35**: 343–350.