Journal of Asia-Pacific Entomology 24 (2021) 24–29

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Full length article

Pathogenicity of entomopathogenic fungus, *Metarhizium anisopliae* MET-GRA4 isolate on dengue vectors, *Aedes albopictus* and *Aedes aegypti* mosquito larvae (Diptera: Culicidae)

Wan Fatma Zuharah [a,b,*], Md Rodzay Rohaiyu [a], Wahizatul Afzan Azmi [c], Hideyuki Nagao [b]

[a] Medical Entomology Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[b] Tropical Mycology Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[c] School of Marine and Environmental Sciences, Universiti Malaysia Terengganu, 21030 Kuala, Terengganu, Malaysia

ARTICLE INFO

Keywords:
Aedes albopictus
Aedes aegypti
Biological control
Entomopathogenic fungus
Metarhizium

ABSTRACT

Considering the rapid transmission of the dengue virus, substantial efforts need to be conducted to ward-off the epidemics of dengue viruses. The control effort is depending on chemical insecticides and had aroused undesirable conflicts of insecticide resistance. Here, we study the entomopathogenic fungus, *Metarhizium anisopliae* as a promising new biological control agent for vector control. The pathogenicity effects of *Metarhizium anisopliae* against field and laboratory strains of *Aedes albopictus* and *Aedes aegypti* larvae were tested using the larvicidal bioassay technique. The results demonstrate that the treatments using *M. anisopliae* isolate MET-GRA4 were highly effective and able to kill 100% of both *Ae. albopictus* and *Ae. aegypti* mosquito larvae at a conidia concentration of 1×10^6/ml within 7 days of the treatment period. The fungus displayed high larvicidal activity against laboratory and field strain of *Ae. aegypti* larvae with LC_{50} values (9.6×10^3/ml, 1.3×10^3/ml) and LC_{95} values (1.2×10^6/ml, 5.5×10^5/ml) respectively. For *Ae. albopictus*, LC_{50} values for laboratory and field strains were (1.7×10^4/ml, 2.7×10^4/ml) and the LC_{95} values were (2.1×10^6/ml, 7.0×10^5/ml) respectively. Interestingly, the susceptibility of field strain towards *M. anisopliae* was higher as compared to the laboratory strain *Aedes* larvae. In which, the causative agents of all the dead larvae were verified by the virulence of *M. anisopliae* and caused morphological deformities on larval body. The findings from this study identify this isolate could be an effective potential biocontrol agent for vector mosquitoes in Malaysia.

Introduction

The upsurge of dengue infections in Malaysia continued to worsen with an increment of 100, 028 dengue cases and 231 deaths recorded within the year 2016 (World Pacific Region Organization, 2017). It is predicted that the magnitude of dengue infections will continue to increase, creating a more intense scenario, if no effective control strategies are enforced (Pang and Loh, 2016). Basically, the mode of vector control strategies is to disrupt the spreading of dengue infections by making it more efficient, cost-effective, ecologically sound and sustainable (Hii et al., 2016, World Health Organization, 2012).

The primary vector responsible for the spreading of the dengue virus in Malaysia is *Aedes aegypti*. This mosquito larvae is a peri-domestic vector found mostly in artificial containers associated with human dwellings and adult mosquito feeds preferentially on human blood (Hopp and Foley, 2001). Due to their intensive biting activity on man, this collectively contributes to the capacity of *Ae. aegypti* to cause spreading of dengue virus in urban areas (Ritchie, 2014). Aided by an advance international trade, slowly and irrevocable *Aedes albopictus* rises as the second primary vectors of dengue, spreading its distribution from Asia to Africa, Americas and Europe (Knudsen, 1995, World Health Organization, 2009). Even though *Ae. albopictus* plays a minor role in dengue virus transmission compared to *Ae. aegypti*, however, the possibility of *Ae. albopictus* to change the transmission dynamics has called for considerable concern (Lambrechts et al., 2010).

The important elements to curb the outbreaks of dengue infections are by employing better vector control strategies. The National Dengue Strategic Plan (NDSP) introduced by the Ministry of Health in 2011, stressed the demand of finding promising control tools and strategies to achieve sustained reduction of dengue incidences (Mudin, 2015). The

* Corresponding author at: Medical Entomology Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.
E-mail address: wfatma@usm.my (W.F. Zuharah).

https://doi.org/10.1016/j.aspen.2021.04.008
Received 16 December 2020; Received in revised form 16 March 2021; Accepted 2 April 2021
Available online 8 April 2021

virulence of *Metarhizium anisopliae* against many insect pests had drawn research attention to studying on their development as biological control for global medically important vectors (Zimmermann, 2007). Since 1980s, the usage of entomopathogenic fungi in the microbial control of mosquito larvae had been considered in a range of studies (Riba et al., 1986). Significant larvae mortality had been observed by these fungi when conducting larvicidal assays against *Culex pipiens*, *Ae. aegypti* and *Ae. albopictus* with the percentage of mortality reaching up to more than 90% (Benserradj and Mihoubi, 2014, Pereira et al., 2009, Bilal et al., 2012). However, the virulence of these fungi depends on the isolate used, concentration and exposure time (Scholte et al., 2003, Blanford et al., 2005, Benserradj and Mihoubi, 2014).

In an attempt to evaluate the action of *M. anisopliae*, isolated from the native environment in Malaysia, as an approach to control *Aedes* populations, we explored the bio-potential of this fungus towards the larvae stage of *Ae. aegypti and Ae. albopictus* mosquitoes. In order to study the dynamic impacts and effectiveness of this biological control against the field strain, we performed a comparative study against laboratory strain *Aedes* mosquito with field strains from two areas; urban and sub-urban regions of Penang Island, Malaysia. In Malaysia, the usage of *M. anisopliae* is still among the pioneer and it is only adapted for the controlling of agricultural pests. Besides, there it is still lack of study conducted to try the possibility of this fungus as one of mosquito control effort in Malaysia.

Materials and methods

Collection and rearing of Aedes aegypti and Aedes albopictus field strains

Aedes aegypti field strain was collected from Sungai Dua area (N5°.348508, E100°.300104), which is a small urban residential area located on the east part of Penang Island, Malaysia. Due to the dominance of *Ae. aegypti* as a principle dengue vector in this urban residential area, we chose Sungai Dua as our study sites to collect our *Ae. aegypti* field strains. The residential neighborhood area is packed with apartments and flats to accommodate high human populations within this area. In this area, the sanitation efforts of communities are lacking creating numerous artificial containers influencing the proliferation of potential breeding sites for *Ae. aegypti* mosquitoes.

Whereas, *Ae. albopictus* field strain was collected from in the Batu Maung area (N5°.283881, E100°.279320) found to be the dominant *Aedes* species in this area. Batu Maung is a small sub-urban area located in the south-eastern part of Penang Island, Malaysia consists of few organized residential areas and several small villages. The housing distributions are not crowded, garbage collection is regular and the sanitation efforts among the communities are good. Although the availability of breeding containers is low, *Ae. albopictus* can be found abundantly in this area.

Eggs of both field strains, *Ae. aegypti* and *Ae. albopictus* were obtained by setting up ovitraps in the selected sites two weeks prior to the experiment. Ovitraps is made up of 250 ml black tin containers filled with de-chlorinated tap water together with paddles made up of wood soaked inside as an oviposition substrate. After four days, ovitraps container together with paddles were collected and brought into the laboratory for rearing purposes. The larvae obtained within ovitraps were transferred into enamel trays. As *Aedes* eggs are capable to thrive in dry conditions by having reasonable desiccation resistance (Faull and Williams, 2015), we dried the collected paddles for 24 h to kill any other insect eggs might inhabit on top of the paddles. Then, the paddles with *Aedes* eggs were further soaked in an enamel tray filled with dechlorinated water to let them hatched. After the occurrence of hatching, the newly hatched larvae were pipetted into new enamel trays. In order to avoid competition and stressful conditions for larval development, each new tray was restricted to 100 larvae. Then, the larval trays were covered with white netting to avoid cross-contamination of other mosquito species being reared. The hatching larvae were fed daily with 0.5 gm of food larvae made of dog biscuits, beef liver, yeast and milk powder at the ratio of 2:1:1:1 volume. The larval culture maintained at temperature of 28 ± 2 °C and 70–85% humidity. Larvae were identified by using key from Becker et al. (2003).

Rearing of laboratory strains Aedes aegypti and Aedes albopictus

The laboratory strains of *Ae. aegypti* and *Ae. albopictus* used in this study had been established and maintained continuously at the Vector Control Research Unit (VCRU), School of Biological Sciences, Universiti Sains Malaysia since the 1970s. The rearing procedures were same as field strains of both *Aedes* species mentioned above. As our study focuses on larvicidal assays, larvae for all strains were reared until L3 and early L4 stages before being subjected to bioassays test.

Isolation and identification of Metarhizium anisopliae

Metarhizium anisopliae var. *anisopliae*, isolate MET-GRA4 was isolated from agricultural soils in Felda Tenang, Setiu, Terengganu (N05°32.079′, E102°31.626). The *M. anisopliae* colonies of the given batch were cultured on Potato Dextrose Agar (PDA) plates and maintained at 28 °C. After 5 days, the fungi colonies of new isolated batches were identified and authenticated by fungi taxonomist from the Department of Agrobiology, Universiti Sains Malaysia.

Metarhizium anisopliae culture

Prior to the bioassay procedure, stock samples of agar-cultured conidia were cultured on new PDA plates and incubated at 28 °C. The isolate of *M. anisopliae* used in this study is from MET-GRA4 strain as in Ishak et al. (2020). This strain has conidiophores in compact patches and broadly branches as in individual conidiophores under 100x10 compound microscope. Molecular assay showed at 600 bp labelled by 100 bp marker with specific primers (Ishak et al. 2020). The 14 days old fungus culture of MET-GRA4 strains was harvested by scraping the upper layers of conidia spores with an inoculation loop. Next, the inoculations were suspended in a solution of 0.01% Tween-20 in sterilized distilled water. The conidial suspension was vortexed for 3 min and filtered passing through double sheet cotton gauze to remove the supernatant. A serial dilutions of conidial concentrations were prepared at six different concentrations which is 10, $10^{\wedge 2}$, $10^{\wedge 3}$, $10^{\wedge 4}$, $10^{\wedge 5}$ and $10^{\wedge 6}$ conidia/mL. The conidial concentrations of the final suspension were determined and adjusted by direct counts using a haemacytometer. The total conidial spores counted were then multiplied by 10,000 (104) indicated the current conidial suspension concentrations. While the other suspension concentrations were multiplied subjected to the dilution ratio prepared.

Larvicidal assays

The bioassays were designed to test the bio-activity of *M. anisopliae* against mosquito larvae. As mentioned in the previous section, the desired concentrations were prepared and adjusted by using a haemacytometer prior to bioassay. Twenty mosquito larvae of the 3rd and early 4th instar were introduced into testing cups (6 cm × 10.5 cm × 8.5 cm) containing a mixture of 200 ml deionized water and fungal suspensions. Prior to the introduction of fungal suspension in deionized water, the final concentration used in the bioassay was premeasured to confirm the desired concentrations of 10, $10^{\wedge 2}$, $10^{\wedge 3}$, $10^{\wedge 4}$, $10^{\wedge 5}$ and $10^{\wedge 6}$ conidia/ml. Each assay was replicated three times together with control. Experiments were done separately for each species (*Ae. albopictus* and *Ae. aegypti*) with both field and laboratory strains. The experiments were continued until all larvae in the control treatment emerged as adults (World Health Organization, 2005). To avoid cannibalism and scavenging behavior of larvae due to long exposure treatment, food powder was provided at two day intervals at 1.0 mg/ml until the end of the experiments. The larval mortality was recorded every day until the

complete emergence of adults in the control cup. The experiment was run in laboratory conditions at 28 °C, 75% RH humidity and 12L:12D.

Verification of causative agents

Verification of causative agents were identified using two techniques; (i) morphological deformities under compound microscope and (ii) plating technique using PDA. In order to verify the cause of the death of larvae infected with *M. anisopliae*, all dead larvae were removed from the treated container and placed on new PDA plates. The external body of dead mosquito was first swap on the PDA agar and wash with sterilized saline before the same dead mosquito crushed on PDA agar to confirm the internal infection. These plates were sealed and incubated at 28 °C. After 3 days the fungal growth was checked and identified under a compound microscope to ensure the presence of *M. anisopliae*. The plates will be left for another 7 days and recheck for further confirmation.

Statistical analysis

To determine the effective lethal dose of *M. ansiopliae*, the lethal concentrations (LC_{50}) and (LC_{95}) for the treatments were analyzed using probit analysis in SPSS program version 20.0. Whereas, the significant differences between the variation in fungal concentrations, different strains, and *Aedes* mosquito species, were analyzed using multivariate analysis of variance (MANOVA). The larval mortality rates were subjected as dependent variables, whereas fungal concentration, types of *Aedes* species and strains were subjected as independent variables. Prior to statistical analysis, Shapiro-Wilk test was carried out to verify the normality of the data. The data were log-transformed to fulfill the assumption of MANOVA. The statistically significant difference was set at $P < 0.05$.

Results

Pathogenicity effect of Metarhizium anisopliae towards Aedes larvae

The results from the bioassay test showed the pathogenicity of entomopathogenic fungi, *M. anisopliae* were effective against *Aedes* larvae. It was observed the percentage mean mortality exhibited by *Aedes* larvae was up to 100% mortality within the treatment period. Interestingly, the virulence of *M. anisopliae* towards *Ae. aegypti* was higher and significantly different than *Ae. albopictus* ($F = 68.549$, $df = 1$, $P < 0.05$). For *Ae. aegypti* laboratory strain, more than 50% mortality rate was recorded at 4 days post-inoculation (pi) (Fig. 1C) and 5 days pi for *Ae. albopictus* (Fig. 1A). Whereas at 3 days pi, >60% mortality rate was recorded for a field strain of *Ae. aegypti* (Fig. 1D) and greater than 50% mortality rate for and *Ae. albopictus* (Fig. 1B). Among these treatments, *M. anisopliae* showed the fastest virulence at 6 days pi causing 100% mortality on the *Ae. aegypti* field strain (Fig. 1D), followed by 7 days pi for the other treatments (Fig. 1A, B, C). These observations showed that the virulence of *M. anisopliae* was higher for the field strain of *Ae. aegypti* larvae. As mentioned, the field strains also showed statistically faster larvicidal response than the laboratory strains ($F = 4.154$, $df = 1$, $P = 0.042$).

In response to significant differences of conidial concentration exposure during the bioassay, the maximum percentage of larval mortality was at a significantly highest conidial concentrations of 10^6 ($F = 321.533$, $df = 1$, $P < 0.05$). As shown in Fig. 1, *Aedes* larval mortality gradually increased in relation to the treated period. The maximum 100% larval mortality recorded was at the end of the treated period of day 7 and it was significantly increased from day 1 with no mortality recorded ($F = 193.210$, $df = 6$, $P < 0.05$). The results indicated that *M. anisopliae* MET-GRA4 was highly effective in killing 100% larvae with increasing treatment period. No mortality was recorded in the control treatment.

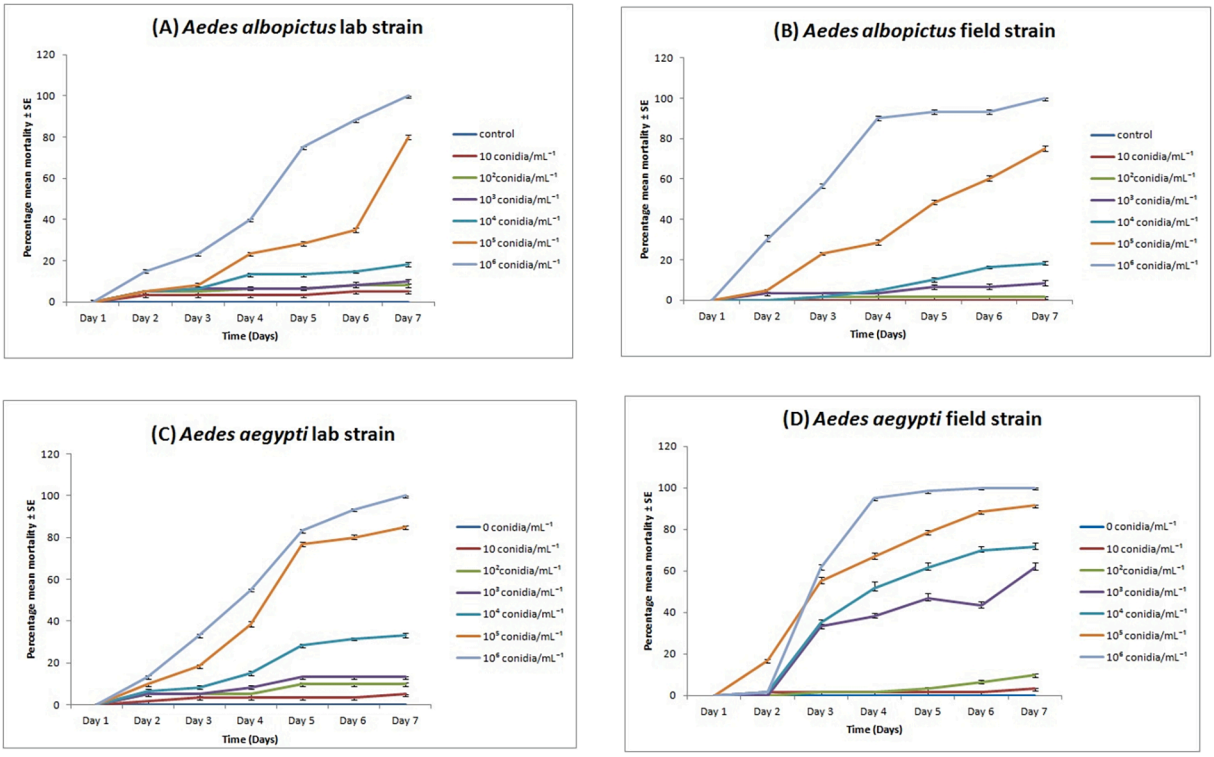

Fig. 1. Mean percentage of *Aedes* larval mortality of; (A) *Aedes albopictus* laboratory strain, (B) *Aedes albopictus* field strain, (C) *Aedes aegypti* laboratory strain, and (D) *Aedes aegypti* field strain at different concentrations of *Metarhizium anisopliae* from day 1 until day 7.

The effective lethal dose of M. anisopliae to exterminate Aedes larvae

Based on the statistical probit analysis, the LC_{50} and LC_{95} values decreased corresponding with the increasing times of treated periods (Tables 1 and 2). No mortality was recorded on the first day of bioassay. Both *Ae. albopictus* and *Ae. aeegypti* strains showed the highest LC_{50} and LC_{95} values on second day of treatment ranging from a 1.2×10^7 conidia/ml to 3.9×104 conidia/ml and 5.4×10^9 conidia/ml to 2.2×104 conidia/ml, respectively. In contrast, at the end of treated period the LC_{50} and LC_{95} values recorded relatively lower than the second day with probit values ranged from 1.3×10^3 conidia/ml to 2.7×104 conidia/ml and 1.2×105 conidia/ml to 2.1×10^6 conidia/ml, respectively.

On the last day of the treated period, our results revealed that *Ae. aegypti* of both laboratory and field strains showed strong susceptibility to the fungal infections with LC_{50} values of 9.6×10^3 and 1.3×10^3, while LC_{95} values were 1.2×10^6 and 5.5×105 respectively (Table 2). Meanwhile, the LC_{50} values for laboratory and field strains of *Ae. albopictus* for the last treated periods were 1.7×104 and 2.7×104, whereas the LC_{95} values were 2.1×10^6 and 7.0×105 respectively (Table 1). Based on the probit analysis, it can be observed that the field strains have relatively lower LC_{50} and LC_{95} values compared to laboratory strains for both *Aedes* species.

Confirmation of causative agent and effects on larvae

The results from morphological deformities that occur in treating larvae indicated the virulence of *M. anisopliae* on both *Aedes* larvae (Fig. 2B & 2C) in comparison to normal healthy larva (Fig. 2A). The infected larvae were completely morphologically disrupted with swollen body (Fig. 2B) and high conidia concentrated in the larva's body (Fig. 2C) after the treatment. All the dead larvae also shown the deformities as in Fig. 2B and 2C after treating with *M. anisopliae* fungus. All these dead larvae were then surface sterilized for internal checked of fungus and kept on the new PDA plate, the fungal germinations were observed after 3 and 7 days incubation period. Based on colony observations, all the fungal species isolated from the treated larvae grow into white color at the initial development (3–4 days incubation) and becomes olive greenish as the conidia mature (7–10 days incubation). Then, the precise identity of these fungal isolates was identified under microscope. We found that both of external and internal bodies of all dead mosquito larvae consisted of MET-GRA4 fungus on PDA agar. The characteristics appeared to be *M. anisopliae* fungus based on the arrangement of the phialides bearing chains and slightly ovoid in shape of the conidia. This identification also was further verified by fungi taxonomists.

Table 1
The effective lethal dose of *Metarhizium anisopliae* against laboratory and field strains of *Aedes albopictus* from Day 1 until Day 7.

Strain	Time (Days)	LC_{50} conidia/ml	LC_{95} conidia/ml	Probit equation
Ae. albopictus Laboratory	Day 1	–	–	–
	Day 2	1.2×10^{12}	2.2×10^{21}	0.178x – 2.147
	Day 3	2.8×10^{10}	2.8×10^{18}	0.206x – 2.149
	Day 4	3.0×10^7	1.36×10^{12}	0.326x – 2.305
	Day 5	3.1×105	4.4×10^8	0.523x – 2.880
	Day 6	1.1×105	1.1×10^8	0.557x – 2.825
	Day 7	1.7×104	2.1×10^6	0.784x – 3.318
Ae. albopictus Field	Day 1	–	–	–
	Day 2	1.2×10^7	5.4×10^9	0.62x – 4.395
	Day 3	8.7×105	1.7×10^8	0.717x – 4.257
	Day 4	1.7×105	6.4×10^6	1.034x – 5.398
	Day 5	8.0×104	3.3×10^6	1.020x – 5.001
	Day 6	5.1×104	2.4×10^6	0.982x – 4.624
	Day 7	2.7×104	7.0×105	1.16x – 5.141

Table 2
The effective lethal dose of *Metarhizium anisopliae* against laboratory and field strains *Aedes aegypti* treated from Day 1 until Day 7.

Strain	Time (Days)	LC_{50} conidia/ml	LC_{95} conidia/ml	Probit equation
Ae. aegypti Laboratory	Day 1	–	–	–
	Day 2	3.2×10^{12}	1.3×10^{22}	0.171x – 2142
	Day 3	6.4×10^7	1.52×10^{13}	0.306x – 2.389
	Day 4	3.1×105	8.5×10^8	0.479x – 2.632
	Day 5	2.5×104	1.1×10^7	0.626x – 2.753
	Day 6	1.5×104	2.9×10^6	0.720x – 3.005
	Day 7	9.6×10^3	1.2×10^6	0.785x – 3.129
Ae. aegypti Field	Day 1	–	–	–
	Day 2	3.9×10^14	2.2×10^24	0.169x – 2.461
	Day 3	8.1×104	3.3×10^8	0.456x – 2.238
	Day 4	9.5×10^3	2.1×10^6	0.700x – 2.784
	Day 5	4.2×10^3	5.5×105	0.774x – 2.802
	Day 6	2.6×10^3	1.8×105	0.899x – 3.033
	Day 7	1.3×10^3	1.2×105	0.845x – 2.644

Discussion

Since the facts of high-level resistance notably by field strain mosquitoes due to the continuous exposure towards chemical insecticides, we have predicted that the pathogenicity of *M. anisopliae* is effective in controlling the laboratory strains of *Ae. albopictus* and *Ae. aegypti* rather than field strain. However, based on our results, the study showed the pathogenicity of this fungus can cause faster 100% larval mortality on field strain as compared to the laboratory strains of *Aedes* larvae, especially on *Ae. aegypti,* which suggested high susceptibility of field strains towards this fungus. Similar to reported by the previous studies, the biology of these dengue vectors of *Ae. albopictus* and *Ae. aegypti* appeared to be susceptible to fungus treatment (Lee et al., 2015, Pereira et al., 2009, Bilal et al., 2012, Vyas et al., 2015, Ravindran et al., 2015). The virulent of fungi are favorable depend on fungal isolates, susceptibility of mosquito species, time and dosage applied (Pereira et al., 2009, Silva et al., 2004, Benserradj and Mihoubi, 2014).

In this assay, the fungal isolates showed different levels of larvicidal activity ranging from 0% mortality on the first day of the treated period to 100% at 6 days post-inoculation for *Ae. aegypti* field strain and 7 days post-inoculation for others depending on the dosage applied. As reported in the present studies, the highest percentages of larval mortality were at the highest conidial concentration of 1×10^6 of *M. anisopliae* MET-GRA4 isolation which obviously lower as compared to Lee et al. (2015) study with the highest virulence concentration of 1×10^7 (conidia/ml) against *Ae. albopictus* larvae. Meanwhile for *Ae. aegypti*, the highest percentages of larval mortality also at conidial concentration of 1×10^6 (ml) which relatively lower than Pereira et al. (2009) which was at 1×10^8 (conidia/ml). As the fungal isolates take time to kill the

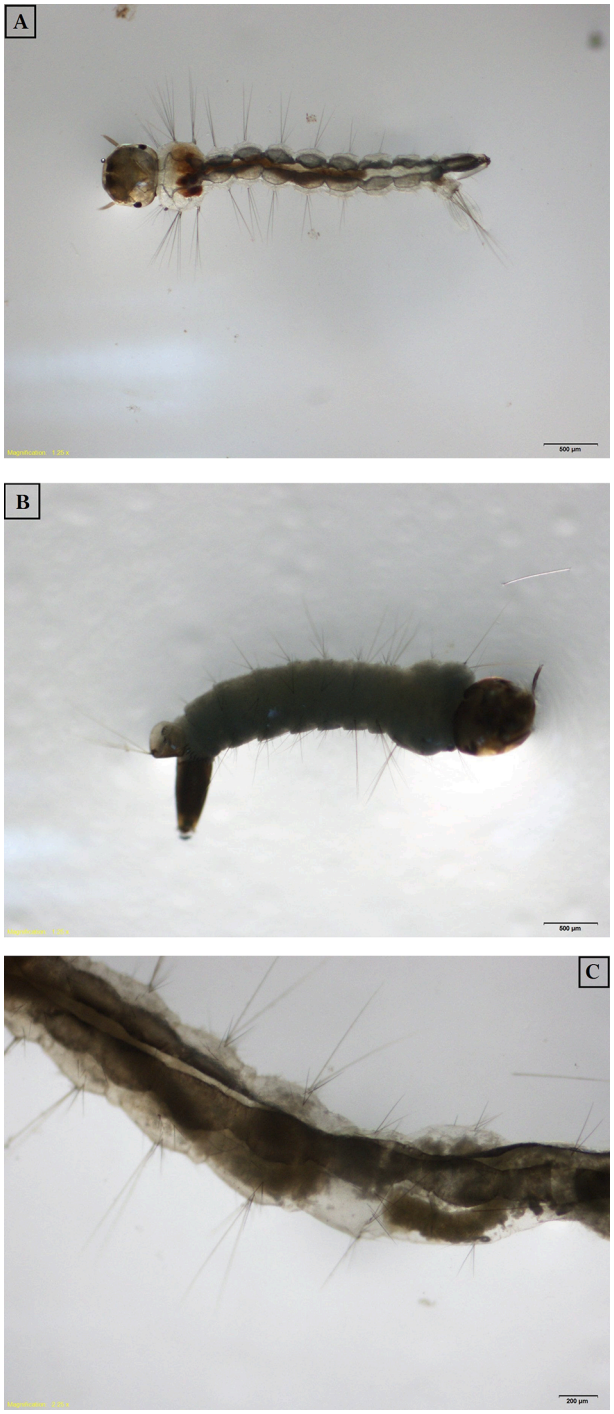

Fig. 2. Morphological deformities induced by *Metarhizium anisopliae* on treating larvae; (A) normal larvae, (B) larvae *Aedes aegypti* with a swollen body after the treatment, (C) conidia concentrated in epithelial gut of larvae *Aedes albopictus* after treatment.

larvae, the number of LC_{50} and LC_{95} values will be decreased from time to time (Bilal et al., 2012) which is similar to present findings. The LC_{50} value for laboratory strain *Ae. albopictus* and *Ae. aegypti* observed in this study are lower than those cited in Bilal et al. (2012), (1.09 × 105 conidia/ml), for *Ae. albopictus* and Maldonado-Blanco et al. (2014), (8.28 × 105 conidia/ml), for *Ae. aegypti*. As the treatment period increases, the virulence of this fungal isolate was elevated. This clarified the maximum larvae mortality recorded at the end of the treatment period in this study.

Up to our knowledge, there are no available references distinguished from the study on the comparative efficacy of *M. anisopliae* towards field strain larvae of both *Ae. albopictus* and *Ae. aegypti*. Impressively our findings showed the LC_{50} and LC_{95} values for field strain were significantly lower than laboratory strains indicating the higher susceptibility of field strains to *M. anisopliae*. As susceptibility might vary according to geographic variation, evaluation of the efficacy of *M. anisopliae* verified the pathogenicity of this fungi might be effective against resistant mosquito populations in our sampling locations. In fact, the different strains of same species mosquito might possess different susceptibility towards control agent.

The expressions of pathogenicity by *M. anisopliae* might vary according to larvae species. In this study, it was observed that the susceptibility *Ae. aegypti* towards these fungal isolates is higher than *Ae. albopictus*. The variation of pathogenicity of fungal isolates towards different larvae species possibly due to the immunity defenses mechanism and genetic variations adapted to the local conditions (Dubovskiy et al., 2013). Besides, the distinct physical characteristics hold by these two *Aedes* species (Ahbirami et al., 2014) reflected the different susceptibility levels of these species towards the fungal isolate in the study. In contrast to Bukhari et al. (2011), the treatment *M. anisopliae* to different species of Anopheline larvae, *Anopheles gambiae* and *An. stephensi* was found to be equally effective. Although the action on *M. anisopliae* against *Ae. albopictus* is slower compared to *Ae. aegypti* in this study, the proportion of larvae mortality is at the highest concentration of 10^6conidia/ml was equal. Thus indicated that both of these dengue vectors are still highly susceptible to virulent of this *M. anisopliae* fungal MET-GRA4 isolate.

Microscopy study represented by Butt et al. (2013) described the presence of conidia being concentrated inside the gut lumen of the larvae and some of them hydrated and swollen. Corroborates the findings of Butt et al. (2013), microscopy observation of our treated larvae also showed higher conidia concentration in epithelial gut of the larvae (Fig. 2C) and some of them have swollen body (Fig. 2B). Besides, all the dead cadaver verified the existence of causative agents of *M. anisopliae* that caused the larvae mortality in all samples. The filter feeders of both *Ae. aegypti* and *Ae. albopictus* typically generates water current by rotating their mouthpart and browsing by scraping to filter out the food suspension towards the mouth (Becker et al., 2003). Other than that, the scrapping behavior of these *Aedes* species on the substrate increases the chances of taking in the *M. anisopliae* conidia through ingestion. On the basis of this feeding behaviour, possibly it has increased the chances of taking in the *M. anisopliae* conidia through ingestion. As colonization and germination of fungus increased with time, it explained the time lag occurs between initial exposure to the treatment to earlier mortality can be notified after 24 h. Thus, the colonization and germination of this fungus in the body of both *Aedes* larvae increased with time either via mouth intake or attachment on the cuticle.

Despite being entered the larva's body through the mouth or siphon, the conidia might attach to the cuticle parts and the interior parts of the larvae (Bukhari et al., 2011). Generally, the route of entomopathogenic fungi invasion triggered when the conidia attached to a susceptible host, germinating and producing serial enzyme activity reaction between both of the host and fungal parasites (Shah and Pell, 2003). However, the stages of the invasion process might be different depends on the cuticle composition that will initiate the production of the enzymes to degrade it (Schrank and Vainstein, 2010). Hydrophobic tension created between conidia and the cuticles of mosquito larvae constantly changes the events of colonization of conidia on mosquito larvae cuticle. Besides the occluding of conidia inside the larval body, the pathogenicity determinants of *M. anisopliae* correlated with the enzyme activities expressed in the treated larvae which are protease subtilisin (*Pr1*), trypsin (*Pr2*) and adhesin (*Mad1*), adhesin (*Mad2*). Enzymology assays done by Butt et al. (2013) and Greenfield et al. (2014) analyzed the expressions of protease and adhesins shown to be higher in the infected larvae suggested these genes involves in initial binding conidia to the

cuticle providing further developing and fungal pathogenesis.

Conclusions

To our knowledge, this is the first evaluation of the comparative effects of *M. anisopliae* on both laboratory strains and field strains *Ae. albopictus* and *Ae. aegypti* mosquito larvae under the laboratory conditions. In fact, the effectiveness of these fungi towards field strains is stronger than in laboratory strain. Even though the laboratory strain was known as insecticide-free strains and have low genetic diversity due to their inbreeding in the laboratory, conversely the present studies showed the higher susceptibility of the field strain towards this *M. anisopliae* fungi. It is worthy to note that Malaysian strain, *M. anisopliae* MET-GRA4 isolate have high potential to inhibit the development of *Aedes* larvae and the usage of this fungus to combat dengue vector in Malaysia can be considered.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to the Vector Control Unit and School of Biological Sciences, Universiti Sains Malaysia for generous supports during the study. This project was funded by Research University Grant USM (1001/PBIOLOGI/8011066), Fundamental Research Grant Scheme, Ministry of Higher Education Malaysia (203/PBIOLOGI/6711629), and myBrain15 Ministry of Higher Education for post-graduate study of Rohaiyu Rodzay.

References

AhbiRami, R., Zuharah, W.F., Thiagaletchumi, M., Subramaniam, S., Sundarasekar, J., 2014. Larvicidal efficacy of different plant parts of railway creeper, *Ipomoea cairica* extract against dengue vector mosquitoes, *Aedes albopictus* (Diptera: Culicidae) and *Aedes aegypti* (Diptera: Culicidae). J. Insect Sci. 14 (1), 180.

Becker, N., Zgomba, M., Petric, D., Dahl, C., Boase, C., Lane, J., Kaiser, A., 2003. Mosquitoes and Their Control. Kluwer Academic/Plenum Publisher, New York, pp. 133–152.

Benserradj, O., Mihoubi, I., 2014. Larvicidal activity of entomopathogenic fungi *Metarhizium anisopliae* against mosquito larvae in Algeria. Int. J. CurrMicrobiol. App. Sci. 3 (1), 54–62.

Bilal, H., Hassan, S.A., Khan, I.A., 2012. Isolation and efficacy of entomopathogenic fungus (*Metarhizium anisopliae*) for the control of *Aedes albopictus* Skuse larvae: suspected dengue vector in Pakistan. Asian Pac. J. Trop. Biomed. 2 (4), 298–330.

Blanford, S., Chan, B.H., Jenkins, N., Sim, D., Turner, R.J., Read, A.F., Thomas, M.B., 2005. Fungal pathogen reduces potential for malaria transmission. Science 308 (5728), 1638–1641.

Bukhari, T., Takken, W., Koenraadt, C.J., 2011. Development of *Metarhizium anisopliae* and *Beauveria bassiana* formulations for control of malaria mosquito larvae. Parasites Vectors 4 (1), 23.

Butt, T.M., Greenfield, B.P., Greig, C., Maffeis, T.G., Taylor, J.W., Piasecka, J., Dudley, E., Abdulla, A., Dubovskiy, I.M., Garrido-Jurado, I., Quesada-Moraga, E., 2013. *Metarhizium anisopliae* pathogenesis of mosquito larvae: a verdict of accidental death. PLoS ONE 8 (12), e81686.

Dubovskiy, I.M., Whitten, M.M., Yaroslavtseva, O.N., Greig, C., Kryukov, V.Y., Grizanova, E.V., Mukherjee, K., Vilcinskas, A., Glupov, V.V., Butt, T.M., 2013. Can insects develop resistance to insect pathogenic fungi? PLoS ONE 8 (4), e60248.

Faull, K.J., Williams, C.R., 2015. Intraspecific variation in desiccation survival time of *Aedes aegypti* (L.) mosquito eggs of Australian origin. J. Vector Ecol. 40 (2), 292–300.

Greenfield, B.P., Lord, A.M., Dudley, E., Butt, T.M., 2014. Conidia of the insect pathogenic fungus, *Metarhizium anisopliae*, fail to adhere to mosquito larval cuticle. R. Soc. Open Sci. 1 (2), 140193.

Hii, Y.L., Zaki, R.A., Aghamohammadi, N., Rocklöv, J., 2016. Research on climate and dengue in malaysia: a systematic review. Curr. Environ. Health Rep. 3 (1), 81–90.

Hopp, M.J., Foley, J.A., 2001. Global-scale relationships between climate and the dengue fever vector, *Aedes aegypti*. Clim. Change 48 (2–3), 441–463.

Ishak, I., Ng, L.C., Haris-Hussain, M., Jalinas, J., Idris, A.B., Azlina, Z., Wahizatul, A.A., 2020. Pathogenicity of an indigenous strain of the entomopathogenic fungus *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) (MET-GRA4 Strain) as a potential biological control agent against the Red Palm Weevil (Coleoptera: Dryophthoridae). J. Econ. Entomol. 113 (1), 43–49.

Knudsen, A.B., 1995. Global distribution and continuing spread of *Aedes albopictus*. Parasitologia 37, 91–97.

Lambrechts, L., Scott, T.W., Gubler, D.J., 2010. Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. PLoS Negl. Trop. Dis. 4 (5), e646.

Lee, S.J., Kim, S., Yu, J.S., Kim, J.C., Nai, Y.S., Kim, J.S., 2015. Biological control of Asian tiger mosquito, *Aedes albopictus* (Diptera: Culicidae) using *Metarhizium anisopliae* JEF-003 millet grain. J. Asia-Pac Entomol. 18 (2), 217–221.

Maldonado-Blanco, M.G., Gallegos-Sandoval, J.L., Fernández-Peña, G., Sandoval-Coronado, C.F., Elías-Santos, M., 2014. Effect of culture medium on the production and virulence of submerged spores of *Metarhizium anisopliae* and *Beauveria bassiana* against larvae and adults of *Aedes aegypti* (Diptera: Culicidae). Biocontr. Sci. Technol. 24 (2), 180–189.

Mudin, R.N., 2015. Dengue incidence and the prevention and control program in Malaysia. Int. Med. J. Malaysia 14 (1), 5–9.

Pang, E.L., Loh, H.S., 2016. Current perspectives on dengue episode in Malaysia. Asian Pac. J. Trop. Biomed. 9 (4), 395–401.

Pereira, C.R., de Paula, A.R., Gomes, S.A., Pedra Jr., P.C.O., Samuels, R.I., 2009. The potential of *Metarhizium anisopliae* and *Beauveria bassiana* isolates for the control of *Aedes aegypti* (Diptera: Culicidae) larvae. Biocontr. Sci. Technol. 19 (8), 881–886.

Ravindran, K., Rajkuberan, C., Prabukumar, S., Sivaramakrishnan, S., 2015. Evaluation of Pathogenicity of *Metarhizium anisopliae* TK-6 against developmental stages of *Aedes aegypti* and *Culex quinquefasciatus*. Pharm. Biol. Eval. 2 (5), 188–196.

Riba, G., Keita, A., Soares Jr., G.G., Ferron, P., 1986. Comparative studies of *Metarhizium anisopliae* and *Tolypocladium cylindrosporum* as pathogens of mosquito larvae. J. Am. Mosq. Control Assoc. 2 (4), 469–473.

Ritchie, S.A., 2014. 24 Dengue Vector Bionomics: Why *Aedes aegypti* is Such a Good Vector. Dengue Hemorrh. Fever. CABI Publishing, EU, pp. 455–480.

Scholte, E.J., Takken, W., Knols, B.G., 2003. Pathogenicity of five east African entomopathogenic fungi against adult *Anopheles gambiae* ss mosquitoes (Diptera, Culicidae). Proc. Neth. Entomol. Soc. Meet. 14, 25–29.

Schrank, A., Vainstein, M.H., 2010. *Metarhizium anisopliae* enzymes and toxins. Toxicon 56 (7), 1267–1274.

Shah, P.A., Pell, J.K., 2003. Entomopathogenic fungi as biological control agents. Appl. Microbiol. Biotechnol. 61 (5), 413–423.

Silva, R.O., Silva, H.H., Luz, C., 2004. Effect of *Metarhizium anisopliae* isolated from soil samples of the central Brazilian cerrado against *Aedes aegypti* larvae under laboratory conditions. Rev. Pat. Trop. 33, 207–216.

Vyas, N., Dua, K.K., Prakash, S., 2015. Larvicidal Activity of Metabolites of *Metarhizium anisopliae* against *Aedes* and *Culex* Mosquitoes. Entomol. Ornithol. Herpetol. 4 (4), 1.

World Health Organization. 2005. Guidelines for laboratory and field testing of mosquito larvicides WHO/CDS/WHOPES/GCDPP/2005.13. World Health Organization, Geneva, Switzerland, pp, 10–11.

World Health Organization. 2009. Dengue: guidelines for diagnosis, treatment, prevention and control WHO/HTM/NTD/DEN/2009.1. World Health Organization, Geneva, Switzerland, pp, 3–10.

World Health Organization. 2012. Handbook for Integrated Vector Management WHO/HTM/NTD/VEM/2012.3. World Health Organization, Geneva, Switzerland, pp, 1–4.

World Pacific Region Organization. 2017. Dengue Situation Update Number 507. Manila, Philippines, pp, 1–5.

Zimmermann, G., 2007. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontr. Sci. Technol. 17 (9), 879–920.