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Article

Assessing the possibility of using entomopathogenic fungi for mosquito control in Hawaii

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SUMMARY

Currently, the Hawaiian government uses chemical pesticides and Bti, a bacterial agent applied to larval pools, for mosquito control. These methods can pose a threat to the environment and be expensive. Additionally, mosquitoes usually develop a resistance toward these pesticides. An alternative agent for mosquito control is entomopathogenic fungi, which can kill or disable insects while they are non-pathogenic to plants and mammals. The purpose of this research is to investigate the use of entomopathogenic fungi as a more sustainable mosquito-control method. We isolated *Metarhizium anisopliae* from local soil samples in Honolulu, Hawaii. We hypothesized that this entomopathogenic fungus from Hawaii would effectively kill *Culex quinquefasciatus* mosquito larvae. We tested the fungus on the larvae of C. quinquefasciatus at concentrations of 1 x 10^4 and 1 x 10^5 conidia/mL. We found that the more concentrated fungal solution was more virulent to the larvae. Hence, this entomopathogenic fungus may offer a sustainable approach to mosquito control.

INTRODUCTION

Many of the diseases found in tropical areas are mosquito-borne, and studies indicate that global warming will increase disease incidence and mosquito geographic range (1). Mosquito prevalence not only endangers the health of humans, but also poses a threat to the populations of birds, mammals, and even reptiles (1). Examples of major mosquito-borne diseases that affect humans include malaria, yellow fever, and dengue, with malaria alone killing over one million children annually (2).

Hawaii has eight species of mosquitoes, among which six bite humans and two feed only on plants (3). Hawaii's government often uses chemical pesticides and bacterial pesticides to control the mosquitoes (4). However, chemical pesticides pose a threat to the environment, while bacterial pesticides are not as efficient (5).

Biological insecticides, developed from the bacterium *Bacillus thuringiensis*, have been used in watery breeding habitats to suppress mosquito larvae (4). This method is effective to an extent for *Aedes aegypti*, the yellow fever mosquito. However, those methods are not practical for the landscape-level control of mosquito species that breed in rural and forested areas because of the heavy cost and destruction of the environment (6).

Chemical agents reduce the fitness of multiple species aside from the target species, in part due to chemical byproducts and metabolites that accumulate after their application. For example, DDT has been shown to be effective in controlling the genera of numerous species of mosquitoes, but it had a negative effect on numerous non-target species, including humans (7). Chemical agents also contaminate soil and water and lose their effectiveness over time as the mosquitoes develop resistance (7). Chemical agents could also indirectly harm the ecosystem by slowly damaging the food chain. In fact, the use of non-specialized, exotic biocontrol agents to kill Zika- and dengue-carrying mosquitoes has been criticized as harmful to native biodiversity (8).

To overcome the limitations faced by these classes of pesticides in controlling mosquitoes, researchers are exploring another potential source of bio-pesticides: fungi. Entomopathogenic fungi, a type of fungi that infects and kills insects, attack the host more efficiently than bacteria by directly penetrating the cuticle. Other mosquito biocontrol agents like bacteria, microsporangia, and viruses need to be ingested to infect the host (5). Recent studies have investigated entomopathogenic fungi for mosquito control. An entomopathogenic fungus that infected and killed *Anopheles gambiae*, the main vector for malaria, was used in a field study in a rural village in Tanzania. Wild mosquitoes were collected and put in study houses. The researchers observed that the fungus-treated mosquitoes had a shorter lifespan compared to the non-treated mosquitoes (5). Other studies have measured the susceptibility of mosquitoes to entomopathogenic fungi (9, 10). One study showed that there was a 95% mortality rate for malaria mosquitoes treated with *Beauveria* fungus under laboratory conditions (10).

Researchers have shown that certain entomopathogenic fungi, such as *Coelomomyces stegomyiae*, *Metarhizium anisopliae*, *Beauveria bassiana*, and *Entomophthora culicis*, successfully kill the larva, adults, or both of specific mosquito species (11). For example, *Coelomomyces* could infect the larvae of *A. aegypti* and invade the adult of *A. aegypti* by infecting L3 and L4 larvae, which are the third and last stages of larvae development (9). In fact, these fungi could infect the host without being ingested through cuticular penetration (12). The entomopathogenic fungi could also survive in the environment for months as spores and maintain a level of viability, which meant that they had a relatively long persistence in the environment (7).

Additionally, the fungi and their insect hosts often competitively evolve. As a host species develops new barriers for the infection of the fungi, the fungi also evolve new mechanisms for their infection within months. The coevolution of parasitic fungi and their hosts allows the entomopathogenic fungi to adjust themselves to maintain effectiveness (13). Scientists used *M. anisopliae* in a field study in a rural African village to assess whether wild mosquitoes were susceptible

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to the fungi population, and the result was encouraging: the application of fungal spray significantly reduced the lifespan of mosquitoes (10).

However, researchers have not studied this method in the specific landscape of Hawaii. This study focused on *Culex quinquefasciatus* because it spreads avian malaria to bird habitats that were previously mosquito-free, posing a significant threat to the native bird population (4). This research could help suppress the mosquito population in Hawaii and restore a safe habitat for native bird populations.

We hypothesized that entomopathogenic fungi from Hawaii would be effective at killing C. quinequefasciatus larvae. We did not want to introduce any outside species to Hawaii, so it was important to isolate the fungi locally from Hawaii soil. Then, we tested the isolated fungus on *C. quinquefasciatus*, an avian malaria vector to determine whether it killed *C. quinquefasciatus*. Our result shows that the fungus is effective in killing the mosquito larvae.

RESULTS

Entomopathogenic fungus isolation

We isolated *M. anisopliae* from the soil at Ala Wai Dog Park in Honolulu (Lat 21.2838 Long -157.8254), Hawaii using the mealworm insect bait method (**Figure 1**). Mealworms were an ideal bait because they are susceptible to fungal pathogens. After 3 weeks of exposure to the soil, we collected the dead mealworms and found green fungi on their surfaces (**Figure 2**). We scraped the spores from the larvae and put them into malt extract agar (MEA) plates for further isolation. We extracted DNA from the fungi and amplified the internal transcribed spacer (ITS) region using PCR. Sequencing and BLAST analysis of the PCR product confirmed the fungal species as *M. anisopliae*, an entomopathogenic fungus (**Figure 3**).

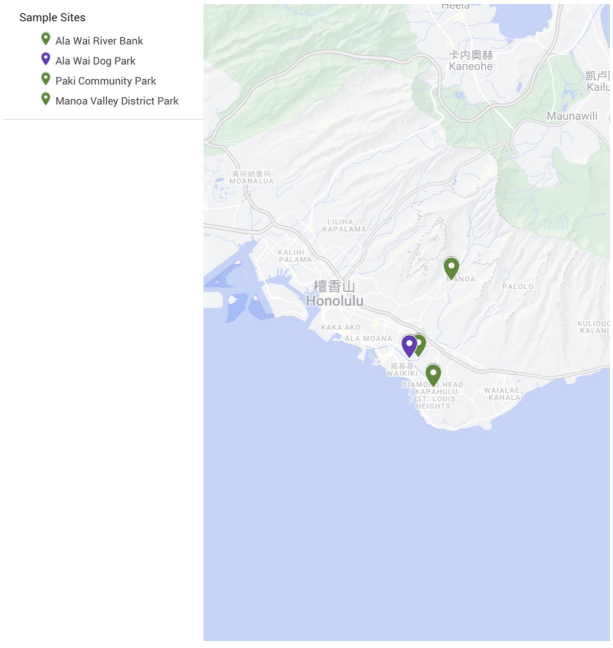

Figure 1. Map of sampling locations in Honolulu, Hawaii for the isolation of entomopathogenic fungi from soil. The pins show the places sampled, while the purple pin shows the sample location with the fungi that was used on the mosquitoes in this study.

Figure 2. Mealworms on malt extract agar. (a) Infected and (b) uninfected by entomopathogenic fungi. The infected mealworm showed fungal growth on its surface. This picture was taken approximately one week after the death of the mealworms.

Larval bioassay

We conducted the bioassay using a protocol proposed by the World Health Organization for testing mosquito larva killing (14). We tested *M. anisopliae* against the 3rd and 4th instar larvae of *C. quinquefasciatus*. These two life stages represent later stages of larval development right before the adult stage, with greater size and robustness compared to earlier instars. We prepared fungal solutions to concentrations of 1 x 10^4 and 1 x 10^5 conidia/mL. Mosquito larvae were exposed to these solutions for six days, with five replicates per concentration. We recorded the mortality daily and compared it to a control group exposed only to water. The fungus showed a dose-dependent mortality of mosquito larvae (**Figure 4**). Survival plots for the fungus-treated groups have a lower median survival (of 4 days and 1 day respectively) than the control group's median survival (>6 days), indicating more rapid mortality.

By the end of six days of treatment, a 90% mortality rate was achieved by the 1 x 10^4 conidia/mL fungal solution, and a 99% mortality rate was achieved by the 1 x 10^5 conidia/mL fungal solution. The survival in the treatment of 1 x 10^4 conidia/mL and 1 x 10^5 conidia/mL was significantly different from the control on day 3 and day 6 ($p<0.05$, one-way ANOVA). The lethal concentration 50 (LC_{50}) is a measure of the concentration of a substance that is required to kill 50% of a test population. In this case, LC_{50} was the concentration at which half of the mosquitoes died after three days of exposure. The LC_{50} for *M. anisopliae* against *C. quinquefasciatus* larvae was 3.16 x 10^4 conidia/mL (**Figure 5**).

DISCUSSION

In this study, we isolated the fungal pathogen *M. anisopliae* from soil in Honolulu, Hawaii, and tested for the killing of *C. quinquefasciatus* larvae. We found that the fungus considerably increased mosquito mortality in their 3rd and 4th larval stages. Although potato dextrose agar is a more commonly used and more efficient way to grow fungal cultures (15), malt extract agar (MEA) also managed to sustain the

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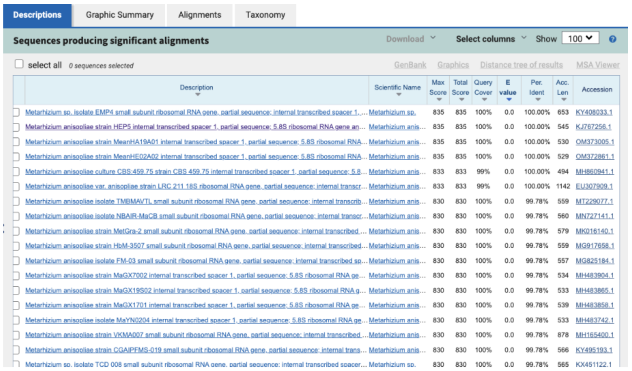

Figure 3. BLAST results for fungi samples. Sequencing of the PCR product from the fungal DNA sample showed that it belonged to Metarhizium anisopliae with 100% query cover and 100% identical nucleotides.

fungal culture. We used MEA since it was more accessible in the school lab.

In this study, the daily survival rates of *M. anisopliae*-infected *C. quinquefasciatus* larvae throughout the experiment were lower than that of the control group. This shows that the dose of fungal conidia was effective in reducing the mosquito life span. In all experimental groups, the larvae did not turn into adults, which meant the fungus also arrested the development of mosquitoes (data not shown). In comparison, mosquitoes in the control group typically took three to five days to turn into adults. By controlling the number of adults developed from mosquito larvae, this fungus can limit any vector potential for spreading diseases in the Hawaiian environment and community.

The correlation between the increasing concentration of conidia and the mortality rate suggests that they are directly proportional. This was reasonable because a higher concentration of conidia likely results in a higher frequency of infection, which could lead to a more rapid and severe disease progression in the mosquito host. In the present study, a higher dose of conidia resulted in a greater mortality rate. The lower concentration took around three days to reach a 50% mortality rate, while the higher concentration reached a

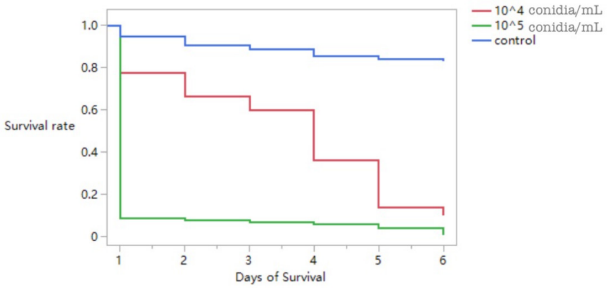

Figure 4. *M. anisopliae* is lethal to *C. quinquefasciatus* larvae. Kaplan-Meier Curve showing the cumulative proportional survival of 3rd and 4th instar *C. quinquefasciatus* larvae after exposure to two concentrations (in conidia/mL) of fungal solution containing the entomopathogenic fungi *M. anisopliae*. The lines indicate the cumulative proportional survival of 3rd and 4th instar *C. quinquefasciatus* larvae. The data was pooled from 100 larvae in each experimental group. The control group received pure water.

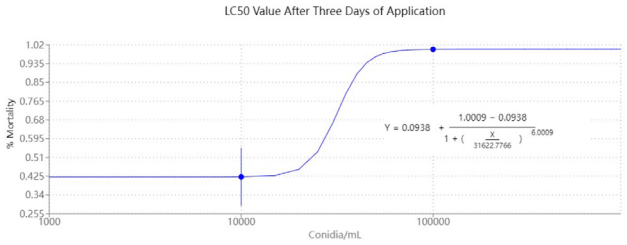

Figure 5. LC_{50} graph for fungal bioassay. LC_{50} graph and function showing the results after three days of application with 1 x 10^4 and 1 x 10^5 conidia/mL concentrations. LC_{50} for M. anisopliae against C. quinquefasciatus larvae is between 10^4 and 10^5 after three days of exposure.

90% mortality rate after the first day of application. Compared to the virulence of *M. anisopliae* against other mosquito larvae such as *Aedes albopictus* (16), this test against *C. quinquefasciatus* shows a higher virulence. Although this experiment only used *M. anisopliae* as the mosquito-control agent, it was shown to be the more effective fungi species against *C. quinquefasciatus* compared to *Verticillium lecanii* and *B. bassiana* (17).

A limitation of this study is that only two concentrations of *M. anisopliae* conidia (1 x 10^4 and 1 x 10^5 conidia/mL) were tested against the mosquito larvae. Testing additional intermediate concentrations spanning across the lethal range would allow a more accurate determination of LC_{50} based on the full dose-response curve rather than two points.

We can apply this fungus to waters around Hawaii; however, this application poses challenges in terms of cost and manpower. This is offset by the fungus, as a living organism, can reproduce and spread following its natural lifecycle. This mechanism can reduce the frequency of repeated applications, which are necessary for the "non-living" chemical pesticides.

In this study, we investigated the effectiveness of *M. anisopliae* as a biocontrol agent against *C. quinquefasciatus* mosquito larvae. However, it is important to acknowledge that the efficacy of this fungus may vary depending on the mosquito species being targeted. Previous studies have shown that different mosquito species can have varying susceptibility to fungal infections(11, 16). Therefore, testing *M. anisopliae* on other mosquito species will likely produce varying results.

Furthermore, the effectiveness of *M. anisopliae* may also depend on the specific strain of the fungus being used. Different strains of the fungus may have varying levels of virulence or pathogenicity against different mosquito species (11). We isolated the strain used in this experiment from Honolulu, Hawaii, but strains from other regions of the world may show different virulence for *C. quinquefasciatus*.

On the other hand, if this experiment is adapted to a field study, then results may differ from what was obtained in the laboratory for several reasons. This is a direction for future research. In a field study, it may be difficult for the fungal conidia to be applied to adult mosquitos. In addition, keeping track of the viability of such a large amount of fungi would be challenging. Environmental conditions such as temperature and humidity can also influence both the efficacy of fungal

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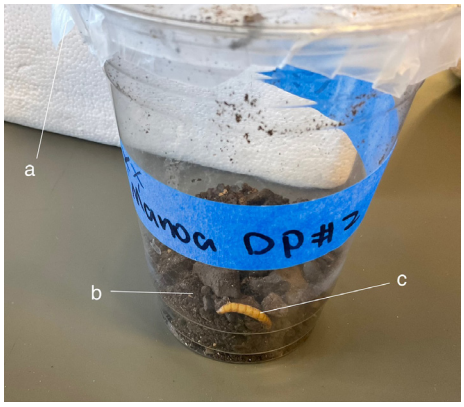

Figure 6. Insect bait method setup. Plastic cup containing 100 g of soil with five mealworms placed within the soil. The cup is covered with a parafilm, creating a sealed environment, with holes added for air exchange. This setup was used for the Insect Bait Method, a technique employed for the isolation of entomopathogenic fungi: A plastic cup was covered with parafilm (a), loaded with 100g of soil (b) and 5 mealworms (c).

biocontrol agents and the mortality of mosquitoes. The mosquitoes may also be exposed to other sorts of diseases that might influence the experimental results. It is also more difficult to collect data for dead mosquitoes in the environment without introducing bias.

In this experiment, we did not compare the effect of the application of synthetic chemical insecticides and *M. anisopliae*. However, previous research shows that *M. anisopliae* is more environmentally friendly and easy to produce, and the fungus is nonpathogenic to birds, fish, and humans (18). Additionally, the coevolution of the fungus with its host, in this case, *C. quinquefasciatus*, can prevent the mosquitoes from developing a resistance against *M. anisopliae* (13).

In conclusion, we successfully isolated the entomopathogenic fungus *M. anisopliae* from Hawaiian soil samples. The study showed that *C. quinquefasciatus* larvae are susceptible to *M. anisopliae*. The larval stage of mosquitoes is one of the most crucial and effective stages for biocontrol agents, and we showed that the life span of *C. quinquefasciatus* was greatly reduced if contacted with the conidia. By killing the mosquitoes before they enter their adult stage, this method can lower the risk of disease transmission. As a result, this mosquito control method may help mitigate the threat to Hawaii's bird population. Although the efficiency of chemical pesticides still outweighs that of the fungal biocontrol method, the fungi provide more benefits in the long run, making the mosquito-control process more sustainable. Therefore, this approach is promising for development into a more environmentally friendly, target-specific, and cheaper mosquito control tool.

Figure 7. The pure cultures of *Metarhizium anisopliae* on malt extract agar. Fungi isolated from Honolulu, Hawaii (Ala Wai Dog Park).

MATERIALS AND METHODS

Isolating entomopathogenic fungi in Hawaii

The species of entomopathogenic fungi differ from region to region. Some areas in Oahu, Hawaii possess a greater number of fungi species because of local biodiversity (19). The entomopathogenic fungi are commonly found in the soil in natural environments. Since mosquito-pathogenic fungi in Hawaii have not been the focus of previous literature studies, the first step of this research was to isolate entomopathogenic fungi from the soil samples collected in different sites in Honolulu.

To isolate entomopathogenic fungi of unknown species straight from soil samples, the insect bait method was used. Soil samples were collected from four locations in Honolulu (**Figure 1**). After the samples were collected, samples were dried and holes were punched in the lids of the containers to allow air exchange, and 10 mealworms (*Tenebrio molitor* larvae) were added to each sample (**Figure 6**). Containers were incubated at 20-22 Celsius in the dark for three weeks (20). To allow the larvae to contact every surface of the soil, the containers were inverted every day during the first week. The condition of the container and larvae was inspected and recorded seven days from the start of incubation. This process was repeated every three to four days for three weeks after the initial baiting.

Fungal cultures

After a larva was discovered to have died due to an entomopathogenic fungus, the dead larvae were rinsed with clean water and placed into a petri dish containing MEA (Criterion, Cat. C6201) medium to promote the growth of entomopathogenic fungi. The petri dishes were incubated at room temperature to allow the fungi to grow (17). Upon the emergence of fungal colonies, the fungi of different colors were isolated on new MEA plates. This process was repeated every 2-3 weeks to obtain a pure culture (**Figure 7**).

DNA extraction, PCR amplification, and sequencing

The fungal DNA was extracted from spores scraped from each culture using a DNA extraction kit (SYNERGY 2.0 Plant DNA Extraction Kit), following the protocol of the manufacturer. The DNA was stored at 4 C.

PCR was performed in 25 µL volumes consisting of 12.5 µL PCR mixture 2 × Taq PCR Master Mix with red dye (BioMix, Cat# BIO-25006), 10.5 µL of water, 0.5 µL of ITS-1, the forward primer, and ITS-4, the reverse primer, and 1 µL of DNA template (21). Thermocycling conditions consisted of an initial denaturation temperature of 95 C for 10 minutes,

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followed by 95 C for 15 seconds, 52 C for 30 seconds, 72 C for 15 seconds for 35 cycles, and a final extension of 72 C for 7 minutes (Bio-Rad, Cat# 1861096). Verification of PCR products was carried out on a Bio-Rad electrophoresis system (Bio-Rad, Cat# 1704422), and amplicons were verified using a 100 bp DNA ladder (TriDye, Cat# N3271).

The PCR products were sent for Sanger DNA sequencing. The generated sequences were then compared on GenBank using the BLAST algorithm to establish that the PCR products belonged to the fungal species *M. anisopliae* (**Figure 3**). After the PCR, only the fungus from Ala Wai Dog Park was confirmed to be *M. anisopliae* as shown by the purple pin on the map (**Figure 1**).

Larvicidal bioassay

Conidia from *M. anisopliae* were harvested by scraping the surfaces of a two-week-old culture with a plastic spatula and were suspended in ultra-pure water containing 0.1% Triton X-100 (Calbiochem, Cat# 648462). The mixture was put on a vortex machine (VWR, Cat# 10153-842) to suspend all the spores. The conidial concentration of the suspension was determined using a hemocytometer, and the fungal solutions were preserved at 4 C until the bioassay.

The fungi were tested on *C. quinquefasciatus* larvae. Dilutions were made of the conidial concentrate, and an appropriate amount of ultra-pure water was added to attain the desired concentrations (1×10^4 and 1×10^5 conidia/mL). In each bioassay, 20 larvae of the 3rd and 4th instar, which were identified by comparing to standard reference images, were added to a 50 mL centrifuge tube (LabServ, Cat# 14955161) containing 20 mL of the test concentration of fungi. Each bioassay was conducted five times at two different concentrations (1×10^4 and 1×10^5 conidia/mL). Data was obtained over the course of six days. Control groups consisted of 20 larvae in 20 mL of ultra-pure water. After the first application of the fungi on the experimental group, the conditions in water were observed daily. Larvae were considered dead if there was no observed movement when the centrifuge tubes were gently agitated to disturb the larvae. Lack of response to this stimulus, such as floating to the surface or exhibiting swimming motions, indicated termination of feeding behavior and locomotor reflexes characteristic of live larvae.

Statistical analysis

Treatment results after three days and six days of application were compared by one-way ANOVA. A threshold of $p<0.05$ was considered to meet statistical significance in all the cases.

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