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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)

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Abstract

Metarhizium anisopliae Metchnikoff (Hypocreales: Clavicipitaceae) is a fungal pathogen that causes disease in various insect pests, and it can be exploited and developed as a biological control agent to combat the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Dryophthoridae). The study on indigenous isolates is crucial especially for development of bioinsecticides in the future. The *M. anisopliae* strain called MET-GRA4 was tested for pathogenicity against adult red palm weevil and treated in vitro with different spore viabilities. The isolates exhibited pathogenicity with 100% mortality 21 d postinfection. The median lethal time (LT_{50}) for 85% viable spores was 8.6 d,

biological methods to combat red palm weevil (Abbas 2019). Current research has turned to entomopathogenic fungi (EPF) as a means of biological control which will be able to target specific species of insects which prove detrimental (Sapna-Bai et al. 2013). Hussain et al. reported that 700 fungi species are pathogenic to insects. EPF have an intriguing way of attacking, as their spores can reach a host's hemocoel without being ingested by the host through the cuticle or mouth. Moreover, the ingested fungal spores can maintain their dormant stage inside a host's gut until it is expelled with the insect's feces (Charnley and Collins 2007). Interestingly, there are some fungi that have specific host ranges. Two EPF, *Metarhizium anisopliae* and *Beauveria bassiana*, are well characterized as pathogens for several insects and have been used as biological control agents to control pest infestation worldwide (van Lenteren et al. 2017). *Beauveria bassiana* is an EPF species that has already been implemented in the IPM program for controlling red palm weevil in Mediterranean and other palm-growing regions (Güerri-agulló et al. 2011). There are fewer reports on *M. anisopliae* as a pathogen of red palm weevil, with recent studies conducted outside Malaysia (Cito et al. 2014, Yasin et al. 2017). However, Kachatourians and Qazi (2008) reported that the EPF isolates used in one particular region may not be suitable in all localities.

Some commercialized products have used *M. anisopliae* as an active ingredient; such

The MET-GRA4 strain was isolated from clay loam soil in Felda Tenang (05°20′N, 102°57′E), Kuala Terengganu. The pure culture of *M. anisopliae*, strain MET-GRA4, was used in this study (Grace et al. 2017). A loopful of germinated spores was transferred and streaked on a potato dextrose agar (PDA) plate and incubated at 28°C for 14 d. In order to maintain the viability and virulence of *M. anisopliae*, the red palm weevil was infected after every 6 mo to ensure the spores' robustness. In addition, adult red palm weevils were chosen as the candidates to be infected since they are mobile, cryptic, and can be lured into traps. With those criteria, the infected adult red palm weevils were expected to infect other individuals and populations, especially during mating, as the infected red palm weevils take a few days to die. This can eventually lead to population reduction.

Preparation of Solid Substrate and Inoculation of Rice

Rice (45 g) was washed thoroughly under running tap water and dried under room temperature for about 12 h. Rice (15 g) was soaked in a 250-ml conical flask containing 15 ml distilled water, 3% yeast, and 0.02% chloramphenicol, for 18 h. The rice was then cooked in a microwave for 4 min per flask, in casserole rice auto mode. The cooked-rice flasks were autoclaved under 15 psi at 121°C for 20 min. The rice substrates, covered with aluminum foil, were allowed to cool at room temperature

Adult red palm weevils were collected from the Department of Agriculture (DOA) institutions in several districts of Terengganu, Malaysia (i.e., Dungun, Kuala Berang, and Rantau Abang) using the pheromone mass trapping technique (Wahizatul et al. 2014). In total, 75 adult red palm weevils were selected. Adults were evaluated prior to use to ensure they had complete body parts, were of the same size, and were able to mate. Adult red palm weevils were sterilized by dipping in 70% ethanol solution, 0.5% bleach solution and they were rinsed with sterilized distilled water. Then, they were acclimatized in a plastic container with a perforated lid and fed sugarcane before undergoing bioassay. The experiment was conducted with five red palm weevil adults per replicate and three replications per treatment (15 red palm weevil individuals as control, 60 as red palm weevil individuals being treated). The in vitro assay took place in a controlled laboratory condition at 25 ± 1°C and 75 ± 5% RH. Adult red palm weevils were dipped into the prepared conidial suspension for about 60 s, with 108 conidia per ml (with different spore viability, 85, 68, 56, and 39%), and then placed into the container individually (Dembilio et al. 2010). Meanwhile, the red palm weevils for the control were dipped in sterilized distilled water containing 0.02% of Tween 80. Both the infected and the control red palm weevils were incubated at room temperature, and the mortality of the red palm weevils was observed on a daily basis for 21 d. Red palm weevil cadavers were placed in a petri dish containing moistened filter paper to confirm mycosis

were extracted through a Buchner funnel powered by a vacuum filtration machine from a 3-d-old culture and crushed into fine powder with a mortar and pestle under liquid nitrogen. Digested genomic DNA (25 mg) from *M. anisopliae* was subjected to polymerase chain reaction (PCR) in order to amplify the internal transcribed spacer (ITS) region of nuclear ribosomal DNA using the following ITS primers: ITS 1-F (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS 4-B (5′ TCC TCC GCT TAT TGA TAT GC 3′).

PCRs were performed in a 25-µl reaction with 2.5 µl of Buffer Taq + KCl (10×), 0.5 µl of dNTP, 1.5 µl of $MgCl_2$, 1.0 µl of forward primer and reverse primer, 0.5 µl of DNA template having a concentration of 50 ng/µl, and 0.5 µl of Taq Polymerase. The reaction mixtures were then transferred into a 0.2-ml PCR tube and amplified in a thermo-cycler machine (T100 Thermal Cycler, Bio-Rad, Hercules, CA). Both PCR and gel electrophoresis were carried out based on Wahidah (2017), with some modifications. The reaction was completed at 72°C with a 10-min extension. Nine microliters of PCR product was analyzed in 1.2% agarose gel prepared in a 1× Tris-Acetate EDTA (TAE) buffer containing 2.0 µl ethidium bromide, in order to visualize the purity and length of the amplified DNA. The molecular weights of the fragments were determined by comparison with 1 µl of gene ruler 100-bp DNA ladder (Promega, Madison, WI). The gel was run for 100 min at 80 V. The band pattern was pictured under an ultraviolet transilluminator and a Gel Doc XR Gel Documentation

Growth and Viability of *M. anisopliae*

The growth of *M. anisopliae* spores on PDA plates was recorded at 6-h intervals for 48 h. The spore size ranged from 6 to 8 µm. The gradual extension of germ tube length started after 6 h of incubation and lasted for 36 h. The growth started to slow down between 42 and 48 h of incubation. After the initial 6 h of incubation, most of the spores still maintained their original shape. As the time increased to 12 h, the spores started to produce a germ tube. The spores grew longer in subsequent hours, with a few budding germ tubes produced in each spore. Spore germination increased with a time of incubation and this is supported by the Pearson correlation (R = 0.77), which supported a strong positive correlation of incubation time with the development of germ tubes. As time increased from 6 to 18 h, the average germ tube length increased 11 µm, followed by 15 µm at 24 h, 28 µm at 30 h, and 64 µm at 36 h. When the incubation time reached 42 and 48 h, germ tube growth slowed to 21 and 9 µm, respectively.

Pathogenicity Against Adult Red Palm Weevil

The pathogenicity test on adult red palm weevils was conducted using different spore viability: 85, 68, 56, and 39%. ANOVA showed that each treatment had

treatments ($P < 0.05$, Tukey's HSD test).

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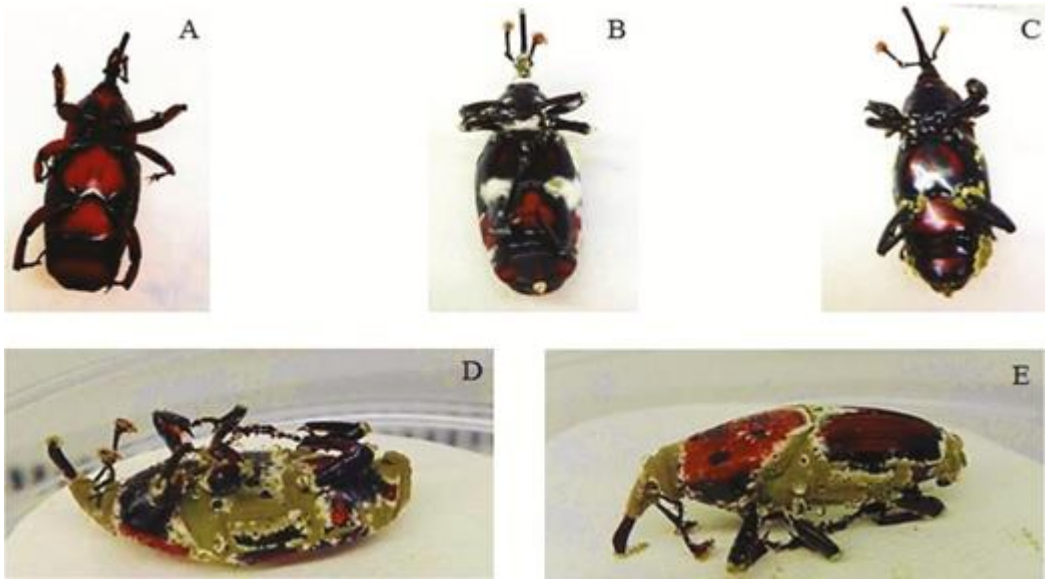
The native *M. anisopliae* strain was found to be infective and virulent on adult red palm weevils within 21 days after treatment (DAT) with 92% mycoses in adult red palm weevil. The highest spore viability (85%) infected 92% of the population, with two red palm weevil cadavers being found dead and infected at 3 d of postinoculation. The least viable spore treatment only infected 16.6% of adult red palm weevils.

Figure 1 shows that the infected red palm weevil cadavers at 14 d after death were almost fully covered with olive-green conidia and the whitish mycelia of *M. anisopliae*. On the first day after death, the red palm weevil cadaver retained its color and condition. Three to four days after death, white hyphae emerged from the leg (tarsus), antenna, and integument between the head and the thorax of the infected cadavers. From day 5 onwards, olive-green spores covered the red palm weevils' whole body. The emergent spores were observed under a microscope to determine the spore morphology, and they were inoculated on new PDA plates with the same results as the *M. anisopliae* MET-GRA4 strain. The color of the infected cadaver's



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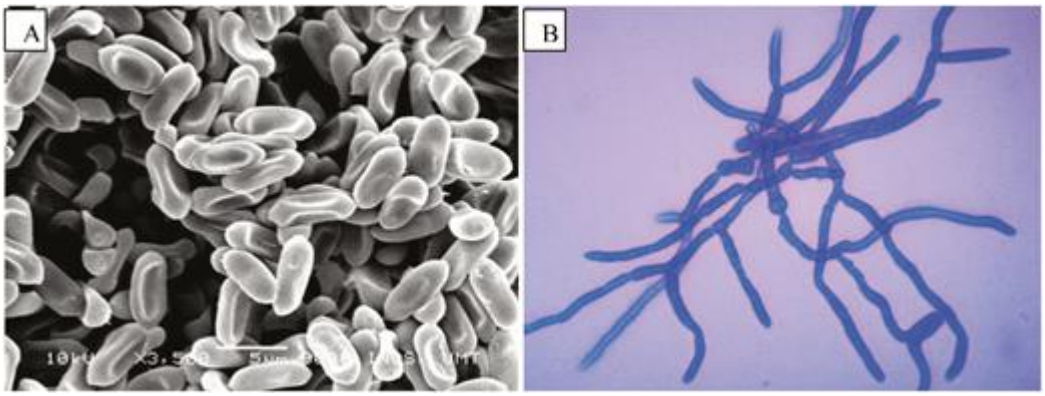
Mycoses of red palm weevil cadavers, on day 1 (A); day 4—the hyphae start growing out from the cadaver (B); day 7—conidia emerged from the cadaver (C); day 14—the spores grow over the cadaver body (D); lateral view and dorsal view of infected red palm weevil (E).

Morphological and Molecular Identification of *M. anisopliae*



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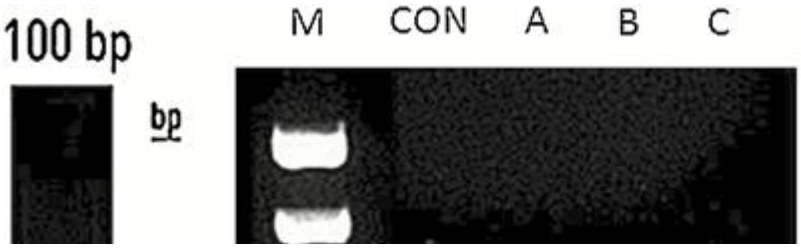
Conidia cylindrical-laterally adherent conidial chains (A) and conidiogenous cells (phialides) forming a dense layer (hymenium) of *M. anisopliae* (B). Note that magnification for (B) is 100 × 10 under compound microscope.

Fig. 3.



Discussion

Growth and Viability of *M. anisopliae*

In this study, the conidial germination rate of *M. anisopliae* was clearly influenced by the incubation period of the substrates. A study by Samuels et al. (1989) demonstrated that the conidia germination rate and the virulence of EPF strain against the target pest were highly correlated. The germination rate of virulent strains is significantly faster than less virulent strains, suggesting that the conidial germination rate is a criterion to screen an efficient strain for field application (Ummidi et al. 2013). Besides, the favorable conditions such as suitable temperature (28 ± 1°C) and food source (the PDA medium) enhance the growth of *M. anisopliae*. Maniania et al. (2008) reported that the temperature range 25–30°C is optimum for EPF radial growth. A study conducted by Faria et al. (2015) claimed that the *B. bassiana* conidia germination rate significantly leads to shorter survival times of the host.

It has been reported that insect-specific resistances continuously to evolve, and important variations in host infectivity have been recorded between different strains (Kouassi et al. 2016). Different EPF strains allow selecting the most

current study showed that with 68% of spore viability, 50% of adult red palm weevils at 11.2 DAT were killed. In order to develop marketable bioinsecticide, it would be necessary to select strains that remain highly infectious toward pest.

Ramle et al. (2006) observed that *M. anisopliae* killed 100% of the *O. rhinoceros* larvae as early as 12 DAT. A study conducted by Lestari and Rao (2016) reported that *Tenebrio molitor* larvae treated with *M. anisopliae* and *B. bassiana* took 2 wk after inoculation to kill 100 and 80% of larvae, respectively. In comparison with the aforementioned previous study, the *M. anisopliae* MET-GRA4 strain killed 100% of the treated population of adult red palm weevil with hardened exoskeleton and elytra within 21 d. In addition, it was found that the robustness and susceptibility of entomopathogens depends on the type of insect species and developmental stage (Goettel et al. 2005). We chose to test this strain on adults because their cuticle is more difficult for EPF to penetrate. We predict that this strain will be even more virulent against the larval stage of red palm weevil.

In this study, the spores of *M. anisopliae* took longer to germinate on the weevils than in PDA culture. This may be due to the fact that the spores took a longer time to adhere and produce hydrolytic (chitinase and protease) enzymes for penetration through the insect cuticle. As the time posttreatment increased from 36 to 48 h, the

et al. 2016).

Morphological and Molecular Identification of *M. anisopliae*

The conidia morphological characteristics of MET-GRA4 strain correspond with the *M. anisopliae* previously described (Humber 1994). In terms of molecular analysis, the DNA sequences for all three samples (pure isolate, mass-produced spores, and spores from the infected red palm weevil cadaver) were confirmed as *M. anisopliae*. Previous studies have proved that the range of *Metarhizium* sp. is between 500 and 650 bp (Henri et al. 2004), where PCR amplification yielded 540 bp for *M. anisopliae*, 600 bp for *M. anisopliae* var. *anisopliae*, 650 bp for *M. album*, and 600 bp for *M. flavoviride*. In order to commercialize insecticides, a molecular method is needed to identify, ensure the stability, protect the strain patent as well as provide a foundation for environmental impact assessments in field application (Henri et al. 2004). Henri et al. (2004) investigated *Metarhizium* isolates and discovered that the ITS-5.8S rDNA regions of *M. anisopliae* var. *anisopliae*, *M. album*, and *M. flavoviride* strains produced different PCR products. The DNA-based technology systems can be ideal for strain identification studies. The extracted fungal DNA from infected insects can be evaluated in laboratory and field studies by using primer sets rapidly, swiftly, and precisely (Sepulveda et al. 2016). The genetic data for promising

Ern Lin for the pure culture of *M. anisopliae*, META-GRA4 strain, and Mr. Zazali Chik and his supportive team from the Department of Agriculture, Terengganu, for supplying the red palm weevils. Also, the authors are indebted to the Universiti Malaysia Terengganu for providing the laboratory facilities.

References Cited

Abbas, T. S. M. 2010. IPM of the red palm weevil, *Rhynchophorus ferrugineus*. *Integr. Manag. Plant Pest Dis* . 5: 209–233.

Charnley, K. A., and S. A. Collins. 2007. Entomopathogenic fungi and their role in pest control, environmental and microbial relationship. *The Mycota IV* . 2: 159–187.

Cito, A., G. Mazza, A. Strangi, C. Benvenuti, G. P. Barzanti, E. Dreassi, T. Turchetti, V. Francardi, and P. F. Roversi. 2014. Characterization and comparison of *Metarhizium* strains isolated from *Rhynchophorus ferrugineus*. *FEMS Microbiol. Lett* . 355: 108–115.

Beauveria Bassiana conidia on their viability and pathogenicity to the onion thrips, *Thrips Tabaci* Lind. (Thysanoptera: Thripidae). *J. Plant Protec* . 49: 97–104.

Faleiro, J. R. 2006. A review of the issues and management of the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Rhynchophoridae) in coconut and date palm during the last one hundred years. *Int. J. Trop. Insect Sci* . 26: 135–154.

Faria, M., R. B. Lopes, D. A. Souza, and S. P. Wraight. 2015. Conidial vigor vs. viability as predictors of virulence of entomopathogenic fungi. *J. Invertebr. Pathol* . 125: 68–72.
Google Scholar Crossref PubMed

Fong, J. H., N. K. A. Siti, and A. A. Wahizatul. 2018. Virulence evaluation of entomopathogenic fungi against the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Dryopthoridae). *Malays. Appl. Biol. J* . 47: 25–30.

Goettel, S., J. Eilenberg, and T. Glare. 2005. Entomopathogenic fungi and their role in

Henri, R., R. Destéfano, S. A. L. Destéfano, C. L. Messias, and S. Paulo. 2004. Detection of *Metarhizium anisopliae* var. *anisopliae* within infected sugarcane borer *Diatraea saccharalis* (Lepidoptera, Pyralidae) using specific primers. *Genet. Mol. Biol* . 27: 245–252.

Google Scholar Crossref

Humber, R. A. 1994. Special considerations for operating a culture collection of fastidious fungal pathogens. *J. Ind. Microbiol* . 13: 195–196.

Google Scholar Crossref

Kachatourians, G. G., and S. S. Qazi. 2008. Entomopathogeneic fungi: biochemistry and molecular biology, pp. 33–61. In *A. A. Brakhage and P. F. Zipfei (eds.), Human and animal relationships, 2nd ed., the mycota VI. Springer-Verlag, Berlin.*

Kergunteuil, A., M. Bakhtiari, L. Formenti, Z. Xiao, E. Defossez, and S. Rasmann. 2016. Biological control beneath the feet: a review of crop protection against insect root herbivores. *Insects* . 70: 1–22.

Google Scholar Crossref PubMed

Ramle, M., W. Mohd Basri, K. Norman, A. A. Siti Ramlah, and H. Noor Hisham. 2006. Research into the commercialization of *Metarhizium anisopliae* (Hyphomycetes) for biocontrol of the rhinoceros beetle, *Oryctes rhinoceros* (Scarabaeidae) in oil palm. *J. Oil Palm Res* . 18: 37–49.

Samuels, K. D. Z., D. E. Pinnock, and P. G. Allsopp. 1989. The potential of *Metarhizium anisopliae* (Metschikoff) Sorokin (Deuteromycotina: Hyphomycetes) as a biological control agent of *Iinopus rubriceps* (Macquart) (Diptera: Stratiomyidae). *Austral. J. Entomol* . 28: 69–74.

Google Scholar Crossref

Sandhu, S. S., A. K. Sharma, V. Beniwal, G. Goe, P. Batra, A. Kumar, and S. Malhotra. 2012. Myco-biocontrol of insect pests: factors involved, mechanism, and regulation. *J. Pathog* . 2012: Article ID 126819. doi: https://doi.org/10.1155/2012/126819

anisopliae. *Springerplus* . 5: 1748.
Google Scholar Crossref PubMed

Ummidi, V. R. S., U. Josyula, and P. Vadlamani. 2013. Germination rates of *Beauveria bassiana* and *Metarhizium anisopliae* its possible correlation with virulence against *Spodoptera litura* larvae. *Int. J. Adv. Res* . 2: 625–630.

Wahidah, N. M. R. 2017. *Identification, bioactive compound profiling and potential uses of saprophytic macrofungi collected from Kapas Island, Terengganu* . *Dissertation Master's Degree of Science* , School of Food Science and Technology, Universiti Malaysia Terengganu, Malaysia, p. 102.

Wahizatul, A. A., N. D. Shahrol, M. H. Haris, K. W. Yong, C. Zazali, and S. S. Ahmad. 2014. Field trapping of adult red palm weevil *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) with food baits and synthetic pheromone lure in a coconut plantation. *Philipp. Agric. Sci* . 97: 409–415.

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