

ENTOMOPATHOGENIC FUNGI ISOLATED FROM THE SOIL OF TERENGGANU, MALAYSIA AS POTENTIAL BIO-PESTICIDES AGAINST THE RED PALM WEEVIL, *Rhynchophorus ferrugineus*

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Abstract: Red Palm Weevil, *Rhynchophorus ferrugineus* (RPW) is a new invasive species that has infested coconut trees along the coastline of Terengganu. It has also shown signs of plague in the inland of Peninsular Malaysia. Entomopathogenic fungi (EPF) is a natural antagonist of insects that has huge potential to be developed as bio-insecticides. Indigenous EPF has acclimatized to harsh environment and therefore would be effective to combat RPW. In this study, seven isolates of EPF (MetGra-1 – MetGra-7) were isolated from the soil samples and bioassay was performed against the adult RPWs by evaluating the mortality and hyphae growth on RPW cadavers. MetGra-4 and MetGra-7 showed promising results as first mortalities were observed on the 4th and 5th day respectively. Both strains achieved 100% cumulative mortality by 20th day of inoculation. The ET₅₀ was achieved on eighth day for MetGra-7 and tenth day for MetGra-4. This study on indigenous EPF served as a platform to search for potential bio-pesticide against the RPW threat that could affect the economics of coconut and palm oil industries.

Keywords: Entomopathogenic fungi, red palm weevil, *Rhynchophorus ferrugineus*, pathogenicity, bio-pesticide.

Introduction

Agriculture is an important industry which provides 11.1% of employment in Malaysia (World Fact Book, 2014). The top ten crop production in the year 2012 includes oil palm (117,560,180 tonnes), paddy (2,750,404 tonnes), rubber (970,000 tonnes), sugarcane (820,000 tonnes) and coconut (606,530 tonnes) (Agriculture and Agri-Food Canada, 2014). Among these, oil palm and coconut palm classified under Arecaceae family are commonly affected by the pest infestation. Rhinoceros beetle (*Oryctes rhinoceros*), Red Palm Weevil (*Rhynchophorus ferrugineus*), Coconut Leaf Beetle (*Brontispa longissima*) and Coconut Leaf Moth (*Artona catoxantha*) are some of the severe palm pests in Malaysia (Masilamany *et al.*, 2012).

The infestation of the invasive pest, the Red Palm Weevil, *R. ferrugineus* Olivier (Coleoptera: Dryophthoridae) has for the first time been recorded on coconut palm in the East

Coast of Peninsular Malaysia. A total of 465 ha areas planted with coconut palms have been destroyed by *R. ferrugineus* in just over the span of nine years, since the first detection in 2007, by the Department of Agriculture (DOA) in all seven Terengganu districts (DOA, 2016). The Malayan Tall, MAWA, MATAG and aromatic dwarf or 'kelapa pandan' are among the cultivars that have been under severe attack (Wahizatul *et al.*, 2013). The RPW is also being detected in pheromone traps in oil palm plantations, though the infestation is yet to be confirmed as plant deaths caused by *R. ferrugineus* may take up to two years (DOA, 2016).

In Malaysia, pest management and control has widely utilized insecticides such as Cypermethrin which is being sprayed every two weeks (DOA, 2016). Naphthalene is administered to dwarf coconut palms as a preventive and for defensive measures, while Monocrotophos or Dichlorovos are applied through trunk injection or root feeding

(Masilamany & Tang, 2013). The pheromone traps with food baits are applied to reduce the population of adult RPW in the vicinity of the plantation and the traps are commonly allured with chemicals such as Ferrolure™ (Costa Rica) or Sime RB™ (India) (Masilamany *et al.*, 2012). These however are unfeasible as a long term solution due to the unwanted environmental consequences.

Green biocontrol is a feasible way to manage the infestation of *R. ferrugineus* due to its effectiveness on the target pest, rapid decomposition and the ability to maintain high and safe crop yield without any toxic effects to the ecosystem. Many natural antagonist agents of the RPW can play significant role as pest biocontrol such as fungi (*Metarhizium anisopliae*, *Beauveria bassiana*), virus (*Baculovirus oryctes*, Cytoplasmic Polyhedrosis Virus), bacteria (*Bacillus* sp., *Pseudomonas aeruginosa*) and nematodes (*Heterorhabditis indica*, *Steinernema abbasi*) (Wraight *et al.*, 2001; Masilamany & Tang, 2013; Mazza *et al.*, 2014). The EPF is especially ideal for the reason that infection can occur by direct contact with the insect cuticle through the combination of mechanical pressure and enzymatic degradation without ingestion (Reithinger *et al.*, 1997; Sanheedep *et al.*, 2011). Upon infection, EPF such as the *Metarhizium* sp., could transmit horizontally (from infected pest or cadavers to the untreated pest) and vertically (from infected pest to succeeding developmental stages via the new generation of spores) (Lacey *et al.*, 1999; Quesada-Moraga *et al.*, 2004).

The EPF can be found naturally from the soils of natural forest and agriculture areas. Having the ability to acclimatise easily, *Metarhizium anisopliae* are ubiquitous and has been commercialised as branded products such as GreenMuscle (LUBILOSA, South Africa) to exterminate locust and grasshopper, ORY-X (MPOB, Malaysia) to kill rhinoceros beetle (*Oryctes rhinoceros*) and Met52 (Novozyme, Denmark) to eradicate ticks and white flies. Native natural enemy should be considered when invasive alien species are detected in any particular country before any biocontrol measures are to be introduced and applied in the field. Most countries impose stringent law and regulation when it comes to importing and introducing any alien natural enemies for pest control (Mazza *et al.*, 2014).

The objectives of this study were to isolate and characterize the indigenous *fungus isolates* from selected areas in Terengganu and to evaluate the pathogenicity of the isolated fungus against the adult *R. ferrugineus*.

Materials and Methods

Soil Sampling

Soil samples were collected at the end of January 2012 from six sites in Terengganu, Malaysia with high infestation - three from inland agricultural soil of FELDA oil palm plantations (Belara, Chalok Barat and Tenang) and three from sandy soil of coastal areas (Batu Rakit, Merchang and Rantau Abang). GPS coordinate reading was taken at each location (Table 1). Soil was sampled at about 10-15 cm soil depth and the

Table 1: Soil sampling sites in Terengganu, Malaysia

Sampling Site	GPS Coordinate	Soil Texture
FELDA Belara	05°20' N, 102°57' E	Clay Loam
FELDA Chalok Barat	05°25'N, 102°48'E	Clay Loam
FELDA Tenang	05°31'2"N, 102°32'E	Clay Loam
Pantai Batu Rakit	03°28'N, 103°02'E	Sandy
Pantai Merchang	04°58'N, 103°20'E	Sandy
Pantai Rantau Abang	04°52'N, 103°23E	Sandy

leaf litter layers were removed. The soils were then kept in zip-lock plastic bags and brought to the Mycology and Pathology Laboratory, Forest Research Institute Malaysia (FRIM) and stored in the dark at room temperature (± 25 °C).

EPF Soil Baiting

Soil samples from each site were transferred into four 250 ml plastic containers with punched lids for air ventilation. Then, each mealworm, *Tenebrio molitor* larvae was surface sterilized with 2% sodium hypochlorite (NaOCl), subsequently rinsed with sterile distilled water for three times and finally air dried on sterile filter paper. Five larvae of mealworm were introduced into each container and incubated in the dark at room temperature (± 25 °C) for 21 days. These containers were observed daily and dead larvae were collected. Harvested cadavers were surface sterilized by dipping consecutively in 70% ethyl alcohol, 1% NaOCl and lastly sterile distilled water for 3 times. The infected mealworms were then transferred into a petri dish containing Potato Dextrose Agar (PDA) and incubated in the dark room at ± 25 °C. Fungal growth from the cadavers were isolated, identified, subcultured and kept in PDA slant for further studies.

Morphological Characterization of Isolates

The growth rate of fungal isolates from the infected cadaver of *T. molitor* larvae was measured. A disc of fungal mycelium (1 cm x 1 cm) was transferred to the center of the PDA plate and incubated at 25 °C for 21 days. Five replicates were prepared for each fungal isolate. Fungal growth rate was measured every 3 days. Culture characteristic and morphology and the sporulation rate was observed daily. In addition, conidia suspensions were prepared when each isolate reached its maturity, ranging from 7 to 21 days. The length of 50 conidia was measured for each fungal isolate.

Pathogenicity Against Adult RPW

Adult RPWs were trapped using pheromone trapping method at the sites of infestation in Terengganu (Haris *et al.*, 2014) and were later

transported to the Mycology and Pathology Laboratory, FRIM. Each adult was placed into 250 mL plastic containers with fresh sugarcane pieces as food source. Prior to the pathogenicity test, each isolated fungal strain was sub-cultured in PDA and left for three weeks to grow. The three week old pure culture was then used as a 'transient' inoculation source. The adult RPWs were introduced to the petri dish for 5 mins and then transferred back to the container. *Metharizium anisopliae* from ORY-X and *Beauveria bassiana* provided by FRIM were also used to investigate their virulence against the adult RPWs. Adult RPWs were introduced to a fresh PDA dish for 5 mins as control. A total of 100 RPWs were used in this experiment and food baits were replaced every five days. Observation was made daily and mortality was recorded when there was no sign of movement of the RPWs even when disturbed.

Data Analysis

The median effective time (ET_{50}) of the fungal isolate was determined by using the probit analysis method. One way ANOVA was carried out to determine whether there were significant differences in the total mortalities of the RPW between the different treatments used. This was carried out by using a statistical software SPSS 21.0.0.

Results and Discussion

EPF Soil Baiting

The mortality of mealworm associated with EPF from FELDA Tenang soil occurred as early as three days after incubation. For FELDA Belara and FELDA Chalok Barat soils, the larvae mortality was observed on seventh day after incubation. In general, the infected larvae were less active on the third day after incubation as compared to the healthy larvae. The highest larvae mortality was from FELDA Belara soil samples with 80% infection, out of 20 mealworm baits. However, only 25% and 20% of the dead larvae were associated with EFP after incubation in the soils obtained from FELDA Tenang and FELDA Chalok Barat respectively (Table 2).

For coastal soils, only 5% mortality were observed on soils sampled from Pantai Rantau Abang. The mealworm exhibited weak symptoms associated with EPF infection five days after incubation. For Pantai Batu Rakit soil, only one mealworm cadaver associated with EPF was found and the mortality was recorded on the twelfth day of incubation (Table 2). Some larvae were found killed due to infection by bacteria and nematodes. The symptoms of larvae infected by the micro-organisms include the moist and the darkened and softened morphology. In comparison, symptomatic *T. molitor* larvae associated with EPF infection had its original colour maintained with drier and stiffer morphology than the surviving larvae. In more advanced stages, the dead larvae may often be mummified by white hyphae and later with an olive green velvet-like crust seen on the white hyphae.

Physical Morphology of Isolated EPF

Growth performance of *EPF* isolated on PDA did not show significant difference between each isolate within 7 days of incubation. MetGra-3, MetGra-4 and MetGra-6 had an average growth rate of 1.71 mm/day, while MetGra-1, MetGra-2, MetGra-5 and MetGra-7 had an average growth rate of 1.69 mm/day.

The culture characteristics and sporulation rate however differed between each isolate (Figure 1). For MetGra-1, MetGra-2 and MetGra-3, the mycelia mat observed was white and smooth. After the fifth day of incubation, dark green conidia mass with zonation was often observed. MetGra-4 and MetGra-5 exhibited similar morphological characteristics as described above, but the sporulation rate was slower and observed only after seventh day. The culture of MetGra-6 and MetGra-7 were clearly distinct as compared to the rest where the mycelia mat was plumose at the center, yellowish white and woolly with zonation. The conidia mass produced was also dark green and, only slightly noticeable after 3 weeks of incubation. The conidia formed was according to the zonation, by growing from the center and spreading towards the outer margin (Figure 1).

The conidia of all isolates appeared to be similar but the conidia length within isolates were significantly different (Table 2). Conidia are single cell, cylindrical in shape with rounded ends and colourless. The longest conidia at 7.24 μm were observed on MetGra-1 and the smallest was MetGra-6 at 5.08 μm length. For MetGra-2, MetGra-3, MetGra-4 and MetGra-5, the length were 6.48 μm - 6.62 μm , with no significant difference among the four isolates. All isolates therefore had equal growth rate

Table 2: Sampling sites and morphological data of Entomopathogenic fungi (EPF) isolated from the soils in Terengganu

Sampling Site	No. of Infected Mealworm	No. of EPF Isolated	Reference Code	Conidium Length (μm)	Growth Rate (mm day^{-1})
FELDA Belara	16	2	MetGra-1	7.24 \pm 0.11 ^a	1.69
			MetGra-2	6.49 \pm 0.12 ^b	1.69
FELDA Chalok Barat	4	1	MetGra-3	6.54 \pm 0.11 ^b	1.71
			MetGra-4	6.62 \pm 0.11 ^b	1.71
FELDA Tenang	5	2	MetGra-5	6.48 \pm 0.09 ^b	1.67
			MetGra-6	5.08 \pm 0.09 ^c	1.71
Pantai Batu Rakit	1	1	NIL	-	-
Pantai Merchang	0	0			
Pantai Rantau Abang	1	1	MetGra-7	5.65 \pm 0.11 ^d	1.69

*Values followed by the same lower case alphabets in the same column are statistically equivalent ($p < 0.05$) according to the Tukey test.

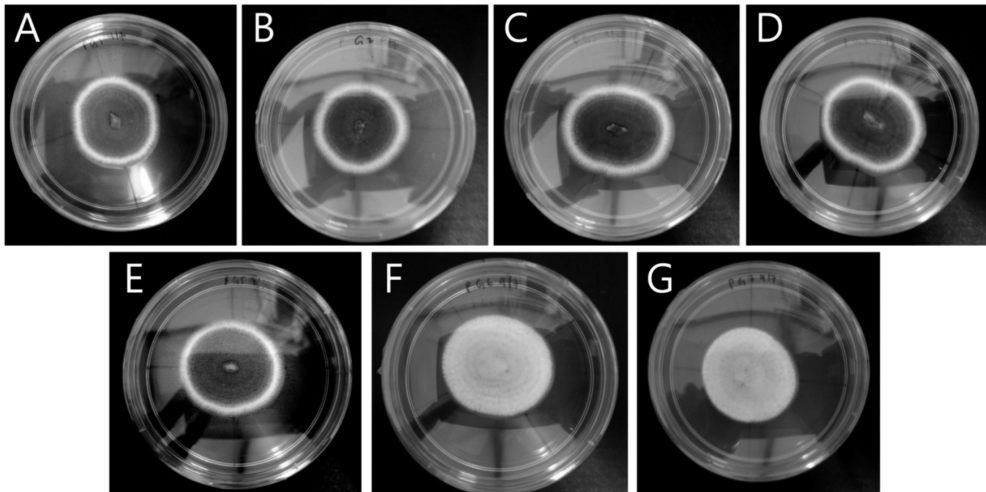


Figure 1: Ten day old cultures of EPF isolated from soils sampled in Terengganu on PDA: A) MetGra-1; B) MetGra-2; C) MetGra-3; D) MetGra-4; E) MetGra-5; F) MetGra-6; and G) MetGra-7

on the same media but different sporulation rate. As MetGra-6 and MetGra-7 were isolated from coastal sandy soil, the optimum condition for maturation and the C:N ratio provided in PDA were insufficient for optimum growth contributing towards their poor sporulation potential. This is in agreement with the study which evaluates the effect of Carbon/Nitrogen ratio (C:N ratio) on the growth, sporulation and germination of *M. anisopliae* and *B. bassiana* isolated from different insect cadaver (Mustafa *et al.*, 2009). The radial growth may not have positive correlation with the yield of conidia (Ekesi *et al.*, 2005).

At this stage, the isolates can be tentatively described as that of *M. anisopliae* and agreeable to the description by Tulloch (1976). The *M. anisopliae* has been subdivided into two varieties (*M. anisopliae* var. *anisopliae* and var. *major*) based on the length of conidia. The *M. anisopliae* var. *anisopliae* has smaller conidia size (5-8 μm in length) than var. *major* (10-14 μm) (Tulloch, 1976). Different culture media may also produce a slight variation in conidia size. However, the physical morphology such as the length of conidia, the colour of mycelia and sporulation rate are unreliable to determine the species (Bridge *et al.*, 1993). Hence, DNA profiling and molecular identification hold the key for a more conclusive identification of *M.*

anisopliae isolated from the soil. This will be the subject of future studies.

Pathogenicity Against Adult RPW

Signs of infection exhibited by adult RPW were often recognised when they were less attracted to the fresh supply of food baits or when their food uptake were observed much reduced. The healthy and uninoculated RPW were often active and responded very quickly to the fresh food bait. Two to five days following the symptoms, the RPW activities were greatly reduced and they became less aggressive and exhibiting the symptoms of shivering or moving backwards with hind legs twisted, which was followed by death. Three days after death, white mycelia could be detected around the joints of the RPW cadaver, especially between the head and the thorax, the joint legs, antennae and mouthpiece. In more advanced stages, dark green conidia were often observed on the cadavers. The dead RPW also showed unique characteristic in not having the distinct rusty red colour on its body not disappearing, as compared to when meeting its natural death (Figure 2).

The screening test revealed that MetGra-7 and MetGra-4 were the most pathogenic. Mortality of RPW first occurred on day four after treatment with MetGra-4. After 5 days,

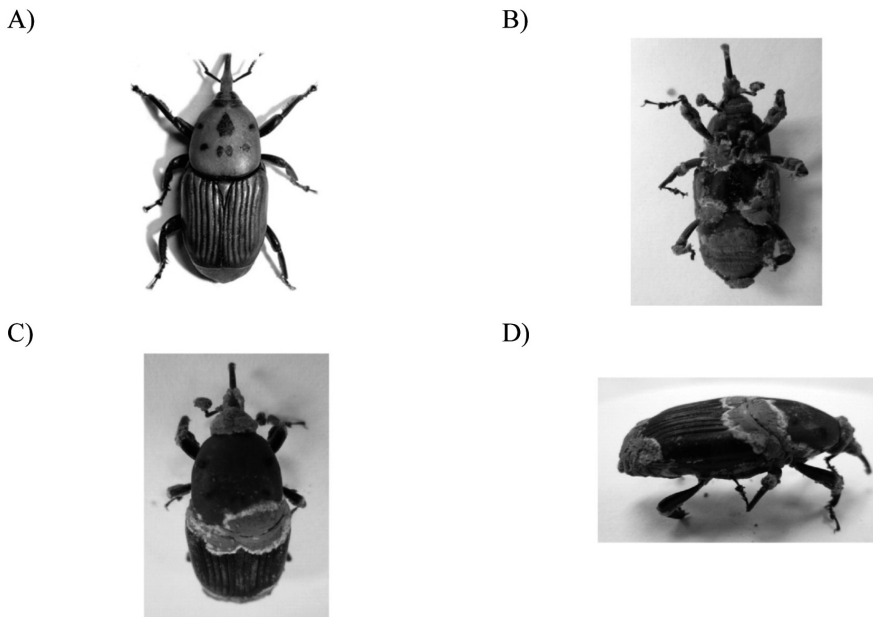


Figure 2: Adult RPW infected with MetGra-4 after 21 days treatment. White hyphae and sporulation can be observed at most of the joints of RPW. A) Healthy RPW; B) Posterior view; C) Anterior view; and D) Side view

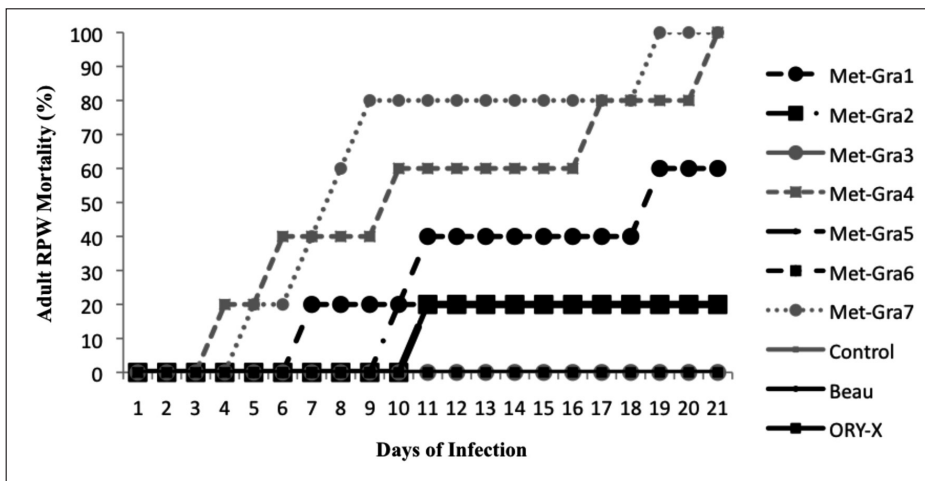


Figure 3: Cumulative mortality of adult RPW inoculated with EPF obtained from Terengganu soils

RPW treated with MetGra-7 showed some incidence of mortality. The RPW inoculated with MetGra-4 and MetGra-7 was 100% killed within 19 and 20 days after inoculation respectively. For MetGra-1 treatment, 60% mortality was recorded, for MetGra-2, MetGra-5 and MetGra-6, only 20%, while for MetGra-3, no death was recorded (Figure 3). The median

effective time (ET_{50}) for MetGra-7 was 8 days, which was the fastest and most effective. The ET_{50} for MetGra-4 and MetGra-1 was 10 and 19 days respectively. Interestingly, no infection was observed on RPW in uninoculated control treatment or when treated with ORY-X and *B. bassiana*.

Insect baiting method should be paired with soil suspension on selective media in order to isolate a wider range of indigenous EPF from the soil. However, *T. molitor* has been proven to be effective in EPF detection as well as to facilitate isolation of EPF selectively. The most frequent fungus isolated from dead larvae of *T. molitor* was tentatively identified as *M. anisopliae* while others such as *Beuveria* spp., *Peacilomyces* spp. etc. were rarely identified. The failure could be due to the rapid growth of saprophytic fungi that may dominate over the growth of other EPF. This finding is in agreement with Nicolai (2007) where EPF often takes longer time to adapt and develop as compared to saprophytic fungi. Both *M. anisopliae* and *B. bassiana* are weak competitors for organic sources as compared to the opportunistic saprophytic fungi in the soil (Keller & Zimmerman, 1989).

Cito *et al.* (2014) have shown that the *M. pingshaense* isolated from Vietnam at an infested RPW area could cause high mortality rate in adult RPW attributable to its unique protease activity as well as toxin production. The pathogenicity of locally isolated *M. anisopliae* against adult RPW in Italy has reportedly achieves LT_{50} in 13 days with 90% of cumulative mortality RPW (Francardi *et al.*, 2012). In India, for the spore suspension treatment attains 100% cumulative adult RPW mortality in 28-35 days and 12-21 days for the dry rice based formulation. This may suggest that locally isolated, indigenous EPF strains such as that from Peninsular Malaysia, are promising to be applied locally against RPW infestation. In our study, both MetGra-4 and MetGra-7 exhibited the ability to achieve 100% mortality in less than 21 days, whilst achieving the ET_{50} on eighth day for MetGra-7 and tenth day for MetGra-4, earlier than that reported in previous studies.

Chemical pesticides are much more effective in terms of the level of efficiency and economically. They are simple for application, fast action, easily available and have long shelf life (Faria & Wraight, 2001). Nevertheless chemical pesticides may come with severe environmental hazards and risks. EPF as bio-

pesticides have many advantages such as high specificity, contact transmission, natural dispersion, safety for non-target organisms and the ability to maintain lasting control once established in the environment (Van Driesche & Hoddle, 2007). A well implemented IPM strategy will boost the effectiveness of bio-pesticides as it could diminish the risk of pest resistance to the chemical molecules. One of the challenges for field application is the survivability of EPF under unpredictable environment such as temperature, humidity and UV exposure. *M. anisopliae* has a superior survivability in soil than *B. bassiana* as the latter is more sensitive to soil microbiota (Bidochka *et al.*, 1998). Under wide range of soil temperature and moisture, the conidia of *M. anisopliae* has exhibited its pathogenicity against the sugar cane pest (Raid *et al.*, 1992). The virulence variability, even within a single fungal species is natural and may be caused by genomic variability (Bidochka *et al.*, 1994) and physiological factors within the host (Miranpuri & Khachatourians, 1995). Adhesion of fungal conidia to the cuticle of insect pest is crucial for infection. Hence, the hydrophobicity of fungal spores have to be taken into consideration when formulating the suspension. In monitoring the application of aquatic spore suspension, the dry spores of *M. anisopliae* have shown higher efficiency in both larvae and adult RPW life stages (Gindin *et al.*, 2006). More studies are needed to understand the virulence and physiological factors and the general physical environment in agriculture ecology especially with regards to the coconut palm and oil palm plantation.

Conclusion

In this study, MetGra-4 was exceptionally effective and can be considered as the best EPF isolate for further investigation to curb the infestation of RPW on coconut palm as well as oil palm plantation in Malaysia. The development of possible new bio-control agent of the invasive coconut pest is now possible with a better understanding on the potential use of indigenous entomopathogenic fungi.

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