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OCCURRENCE OF ENTOMOPATHOGENIC FUNGUS, *Metarhizium anisopliae* ISOLATED FROM ISLAND, BRIS AND COASTAL SOILS OF TERENGGANU, MALAYSIA

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Abstract: Entomopathogenic Fungi (EPF) are environmental friendly biological control agent that can be used to control insect pests. These fungi can be found naturally from the soils of natural forest and agricultural areas. The main aims of this study were to isolate and determine the occurrence of EPF from soils of island, BRIS (Beach Ridges Interspersed with Swales) and coastal areas in Terengganu, Malaysia. The EPF were isolated from the soil samples by using insect baiting method with the larvae of mealworm, *Tenebrio molitor*. Physico-chemical analyses of soil such as soil texture, pH, water content and nitrogen analysis were also carried out in this study. *Metarhizium anisopliae* was the only EPF species found from the soil samples of the island of Taman Tropika Kenyir (three isolates) and BRIS soil of Marang (four isolates). No EPF was found in the coastal soil samples of Tok Jembal Beach. Significantly, larger spore size of *M. anisopliae* from island soil was recorded compared to BRIS soil ($F=5.816$, $p<0.05$). On Potato Dextrose Agar, the colony of *M. anisopliae* was whitish yellow and turned to dark green when matured; slow growing with floccose mycelium. Lower water content and pH value were significantly associated with the lower occurrence of EPF in the island and BRIS soils. Further research can be carried out on the pathogenicity of *M. anisopliae* against insect pests.

KEYWORDS: Entomopathogenic fungi, *Metarhizium anisopliae,* soils, isolation, occurrence.

Introduction

Entomopathogenic Fungi (EPF) are fungi that specifically infect insects and kill them. They were among the first organisms to be used as a biological control agent against pests. More than 700 species of fungi from around 90 genera are pathogenic to insects such as *Metarhizium anisopliae, Beauveria bassiana and Paecilomyces* sp. (Gindin *et al.,* 2006; Khan *et al.,* 2012). EPF are being evaluated against many pests belonging to Coleoptera, Isoptera, Lepidoptera and Hemiptera (Altre & Vanderberg, 2001; Hashim & Ibrahim, 2003; Van *et al.,* 2007; Orduño-Cruz *et al.,* 2011; Cheong *et al.,* 2013).

The use of EPF as biocontrol agents against insect pests have been well studied and established in some countries. These insect-pathogenic fungi play important roles in regulating the population of insect. EPF have potential to control the insects because they can directly infect the insect by penetrating the spore into the cuticle (Gindin *et al.,* 2006). EPF contain dextruxin which paralyze the insect and subsequently death after three to fourteen days depending on size and type of insects (Vänninen, 1996).

EPF can be found naturally from the soils of natural forest and agricultural areas which are free of fertilizers and pesticides. Soil is considered an important natural habitat where EPF can survive longer in it for their several life cycle stages (Medo & Cagáň, 2011). Soil is also believed to be a good habitat as soil protects the fungi from UV radiation and other biotic and abiotic influences (Rocha *et al.,* 2013). Therefore, soil is the most suitable environment to obtain and isolate EPF.

Much effort has been put in research on the occurrence and characterization of EPF especially in agricultural and inland soils. Climate, temperature, soil properties and cropping systems are the several factors that have been studied by scientists which affect the occurrence of EPF (Ignoffo, 1992; Rath *et al.,* 1992; Sun & Liu, 2008). It has been suggested that the geographical factor becomes a major factor affecting the occurrence of EPF (Vänninen, 1996). Rath *et al.* (1992) reported that certain abiotic factors such as soil types and rainfall affect the distribution of EPF, while soil pH, altitude, temperature and conductivity showed minor or no effects. However, Hussain *et al.* (2009) found that temperature and relative humidity also affect the ability of fungi to survive. EPF require high humidity for germination. The temperature for EPF to survive is between 10 - 30˚C. The temperature above 30˚C could inhibit growth and development of EPF (Ignoffo et al., 1977). Therefore, it is important to investigate the relationship of physico-chemical properties of soil with the occurrence of EPF.

In Malaysia, the use of EPF as a means of biological control for insect pests is still in the early stages of evaluation, but it could be useful in an integrated pest management and ultimately benefit our agriculture industry. The bio-insecticide *M. anisopliae* based product was researched by Malaysian Palm Oil Board (MPOB) namely ORY-X. This product can be applied by spraying the solution on the breeding sites and eventually helps to greatly reduce the *Oryctes rhinoceros* beetle. The other product of *M. anisopliae* is the granule formulation to combat the rhinoceros beetle too (Ramle *et al.,* 2009). Another product of *M. anisopliae* is Vigor-MA commercialized by Star Ag Corporation. Vigor-MA used to control insects from various order such as Coleoptera, Orthoptera and Lepidoptera. Currently, all these bio-insecticides products were in powder form which needs to be diluted with surfactant and water before being sprayed at the infestation areas.

In order to develop a control strategy using fungi as a biological agent, it is necessary to investigate the natural occurrence of the indigenous EPF from the soils taken from various locations in Malaysia, particularly in the east coast of Peninsular Malaysia. The sampling sites in this study were mainly in Terengganu due to the scarcity of information available on the occurrence of EPF in soils of Terengganu. Understanding of the EPF occurrence would help to identify the species or strains that are best suited to a particular environment which would improve bio-control efficiency. Thus, the objectives of this study are to isolate and determine the occurrence of EPF from the island soil of Kenyir Tropical Garden, BRIS soil of Jambu Bongkok Reserve Forest and the coastal soil of Tok Jembal Beach, as well as to evaluate the occurrence of EPF in relation to the soil physico-chemical parameters.

Materials and Methods

Collection of Soil Samples

The soil samples were collected from three different regions in Terengganu to represent different types of soils based on locality (Figure 1). Each region is represented by one type of soil: island soil from Taman Tropika Kenyir (TTK) (5˚02´ N, 102˚44´E), BRIS or 'Beach Ridges Interspersed with Swales' soil from Jambu Bongkok Reserve Forest, Marang (JBFR) (4˚55´ N, 103˚21´E), and coastal soil from Tok Jembal Beach, Kuala Terengganu (TJB) (5˚24´ N, 103˚05´E). Each site had four plots of soil sampling areas. The soil samples were collected to a depth of 15 cm using a cylindrical Polyvinyl chloride (PVC) core tube (15 cm height, 12 mm diameter). Ten soil cores were collected in a zigzag pattern within the area in each plot. The distances between sampling points varied ranged from 26 m to 755 m, depending on the locality of the plots. The samples then were mixed and homogenized to get representative composite samples. All the soil samples were stored in polypropylene zip bag and stored in a refrigerated room at 5-10˚C for further processing.

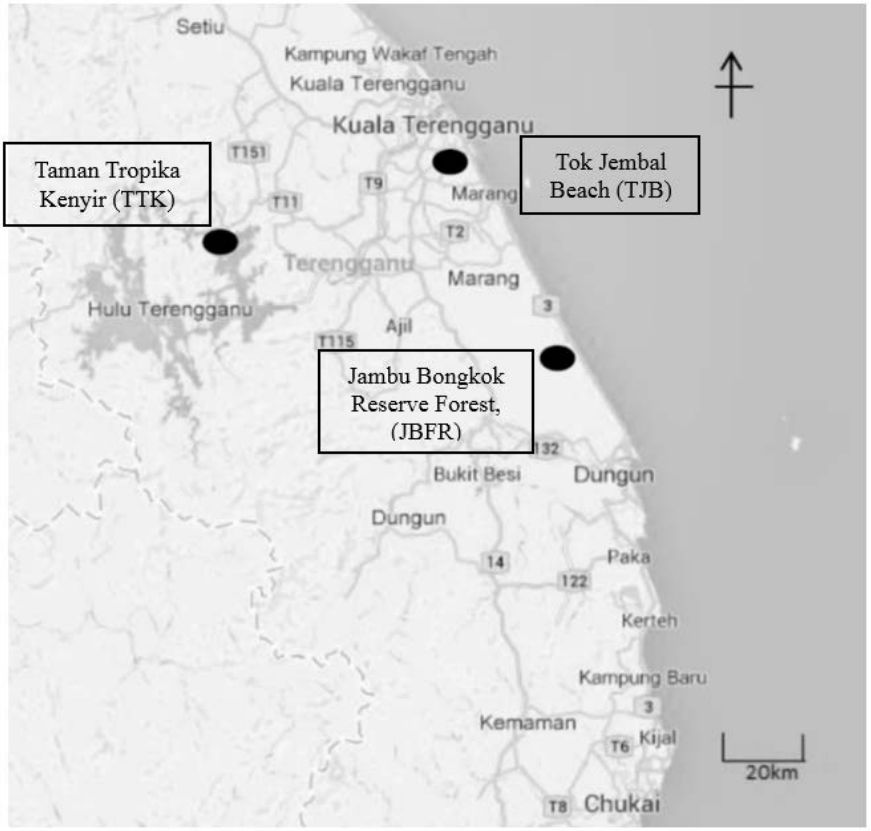


Figure 1: The location of soil sampling sites in Terengganu (black dots).

Physico-Chemical Analysis of Soil

The extraneous materials like leaves, debris and twigs were removed from soil samples and mixed on a clean tray and air-dried in laminar flow hood at room temperature. After that, the soils were ground using mortar and pestle and then sieved using 2.0 mm sieve. The soil samples were stored for further analysis. For the soil pH, 20 g of soil samples were weighed and placed in a 100ml beaker. Then 40 ml of water was poured into the beaker. The solution was shaken for 5 minutes using reciprocating mechanical shaker. The electrode of pH meter was immersed in soil water suspension and the reading was recorded (Patiram *et al.,* 2007). The nitrogen content (%) of each soil sample was determined by combustion method using an elemental analyzer (Elementar Analysen systeme GmbH). The soil textures were classified based on USDA soil texture triangle (Staff, 1996). Water content of each soil sample was determined by the moisture in the soil after it is dried in an oven at 125°C for 24 hours. The formula was as follows (Singh & Ratnasingam, 1971):

[Mass of Dry Sample / Mass of Wet Sample] x 100

Isolation of Entomopathogenic Fungi

The soil samples were filled up until three-quarters of the plastic containers (6.5 cm x 9 cm). Five mealworm larvae, *Tenebrio molitor* were placed on the soil surface and these mealworm larvae used were obtained from pet stores in Kuala Terengganu. The soil containers were incubated at room temperature for one to two weeks. After one week, the dead larvae were taken out and placed in another small container containing moist filter paper and incubated for a few days until EPF had fully grown on the body

of larvae. The larvae infected by EPF had to undergo disinfection with 2 % bleach, followed by rinsing three times with distilled water. After that, the larvae were placed onto Potato Dextrose Agar (PDA) plates and incubated at 28 °C. After five days of incubation, the EPF growing on the PDA plates were transferred into another PDA plate to get pure culture plates.

Morphological Identification of Entomopathogenic Fungi

The fungi grown on the PDA plates were observed for colony colour and texture margin. The microscopic images of the fungi were also observed under light microscope (Nikon 80i) and captured using microscope camera. The size of the conidia for each fungus was also measured. The fungi were identified based on Bischoff *et al.* (2009) and Lacey (1997).

Data Analysis

T-test was used to determine the differences of spore size between soil samples of island and BRIS since no EPF was found in coastal soil. Chi-square ($\chi 2$) test was used to test the effect of soil properties on the occurrence of EPF. The analyses were conducted using SPSS 21.0 statistical software.

Results and Discussion

Physico-Chemical Analysis of Soil

Physico-chemical properties of each soil sample from different locations were analyzed in terms of their texture, water content (%), pH and nitrogen content (%). The soil texture of island soil from TTK was clay and loam, BRIS soil from JBFR ranged from sandy and loamy sandy, whereas the coastal soil samples from PTB were all categorized as sandy (Staff, 1996). Island and BRIS soils showed lower pH values (< 7) which were acidic. In TTK (island soil), the pH ranged from 4.98 to 4.71, while in JBFR (BRIS soil) the pH range was from 3.82 to 4.18. The highest values of pH were recorded at TJB (beach soil), ranging from 7.94 to 8.25, which were alkaline. The water content of all soil samples ranged from 26.74 % to 70.06 %. The highest nitrogen content recorded was 0.101 % (beach soil), while the lowest nitrogen content was 0.051 % (island soil) (Table 1). However, there were no significant differences of physico-chemical parameters between moisture, pH and nitrogen content among all soil type ($p > 0.05$).

Table 1: Results of physico-chemical analyses of soils for each study site in Terengganu

Location / Type of Soil	Plot	Soil Texture	Moisture (%)	pH	Nitrogen (%)
Taman Tropika Kenyir (TTK) / Island soil	1	Clay loam	41.41±1.51	4.98±0.03	0.06±0.01
	2	Medium loam	46.91±1.67	4.84±0.06	0.07±0.01
	3	Medium loam	42.16±3.25	4.71±0.02	0.07±0.09
	4	Clay loam	46.74±3.21	4.93±0.09	0.05±0.08
Jambu Bongkok Forest Researve (JBFR) / BRIS soil	1	Sandy loam	53.30±0.34	3.82±0.02	0.01±0.01
	2	Sandy loam	44.44±2.05	4.18±0.03	0.09±0.02
	3	Sandy	46.17±1.31	4.07±0.04	0.09±0.02
	4	Sandy	56.20±0.87	3.95±0.02	0.07±0.01
Tok Jembal Beach (TJB) / Beach soil	1	Sandy	68.70±1.21	7.94±0.06	0.10±0.02
	2	Sandy	63.36±1.55	7.98±0.01	0.07±0.02
	3	Sandy	55.70±2.52	8.09±0.01	0.09±0.01
	4	Sandy	70.06±0.91	8.25±0.05	0.09±0.02

Morphological Identification of Entomopathogenic Fungi

After 2 weeks of soil baiting, only 14 out of 120 larvae (11.66 %) were infected by EPF. For island soil of TTK, only 6 larvae from a total of 40 larvae (15 %) were infected, whereas BRIS soil of JBFR, 8 larvae (20 %) were infected. No infected larvae were detected from the coastal soil of TJB. Figure 2 shows the infected larvae after four days of soil baiting. During the first day of infection, white hyphae could be observed forming around the integuments of the larvae (Figure 2A). After the fourth day, the green spores were found forming on the whole body of the larvae (Figure 2B). Infected larvae from all soil samples were transferred to PDA and identified as *M. anisopliae* based on the morphological characteristics of the fungi.

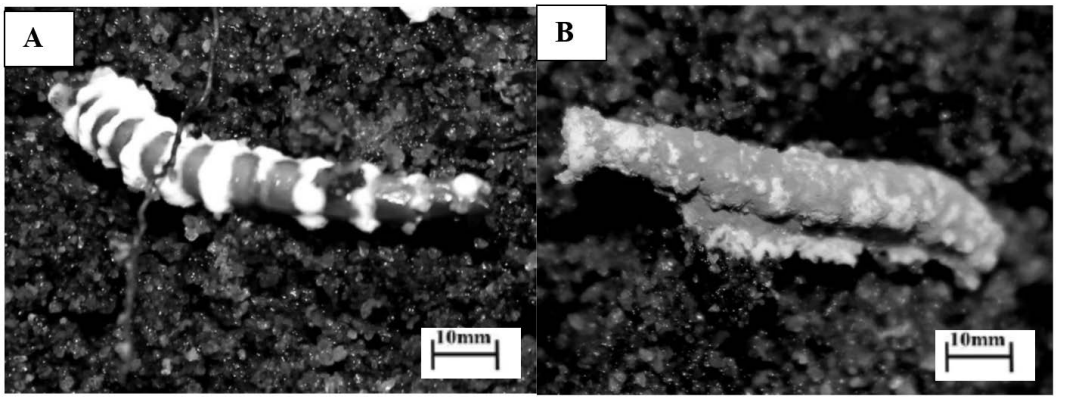


Figure 2: The infected larvae of first day of infection (A) and the infected larvae after 4 days of infection (B)

Figure 3 shows the plates of EPF isolates from infected larvae of island and BRIS soils. *Metarhizium* colonies included in this study were mostly dark green and light green. From the plates, most of the isolates sporulated in the centre and formed green spores with irregular forms at the edge of the plates. Most isolates presented a white edge of variable thickness, whereas at the rear end of the plate they were brownish or yellow. Green spores were forming at the edge of the colony with irregular forms, white to yellow colour in the centre on PDA plate. The most significant morphological characters of this species are based on the conidia size and shape (Figure 4). The conidia of *M. anisopliae* are cylindrical in shape and the sizes were in the range of 5-8 µm long. The colony of *M. anisopliae* was relatively slow growing, which took 7 – 14 days to form the conidia.

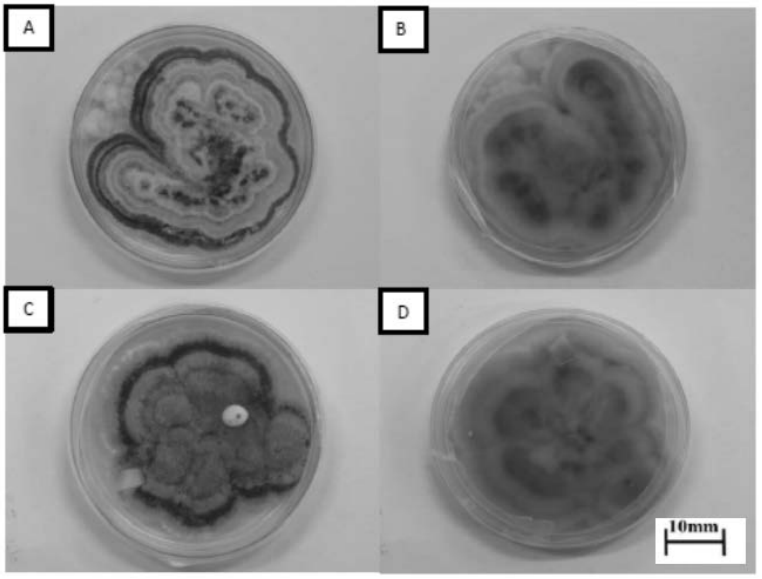
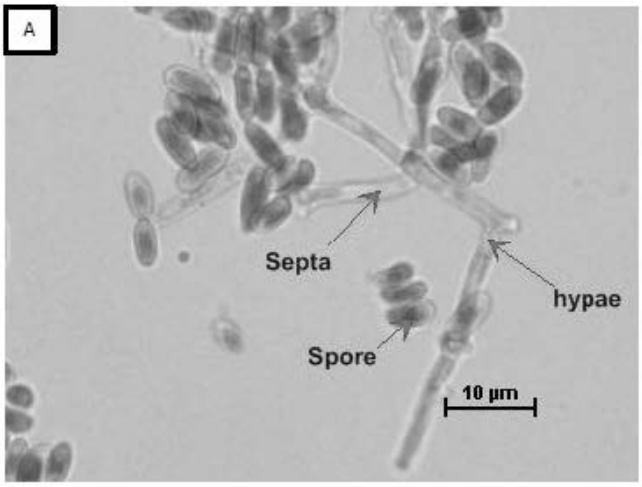


Figure 3: The front view (A) and back view (B) of isolated EPF from island soil of Taman Tropika Kenyir (TTK). The front view (C) and back view (D) of isolated EPF from BRIS soil of Jambu Bongkok Forest Reserve (JBFR).

Occurrence and Infectivity of Metarhizium anisopliae

A total of seven isolates of *M. anisopliae* were successfully isolated: four isolates were from inland soil and three isolates were from BRIS soil. However, EPF isolate was not found from the coastal soil of PTJ. In addition, other fungi such as *Penicillium* sp., *Aspergillus* sp. and *Tricoderma* sp. were also isolated from all types of soils. Table 2 shows the total number of infected larvae, total number of isolates and spore size for each isolate. The spore size was significantly different with locations ($F= 5.816$, $df = 6$, $p \leq 0.05$). Isolates from the inland soils of TTK had the longest spore size ranging from 6.00 µm to 7.54 µm, whereas the spore size for BRIS soil of JBFR ranged from 5.80 µm to 6.20 µm. There was a significant difference of spore size with a number of isolates ($F = 21.427$, $df = 6$, $p \leq 0.05$).



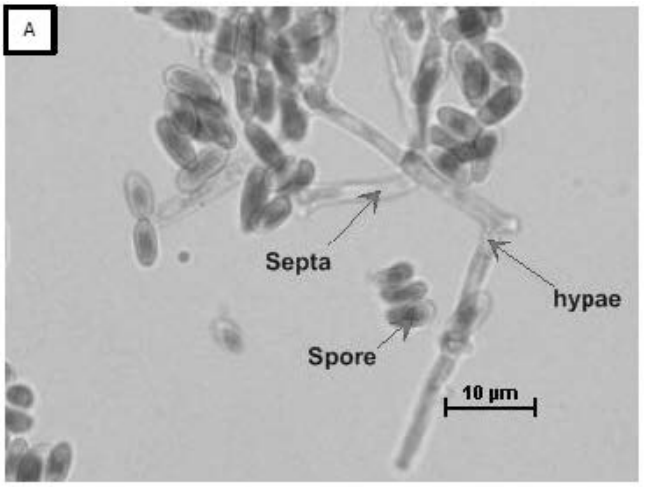


Figure 4: Microscopic view of cylindrical spore of *Metarhizium anisopliae* under advance light microscope at 1000x magnification and the spore size were ranging between 5µm to 8µm.

Table 2: Total number of infected larvae of mealworm *Tenebrio molitor,* total number of isolates and spore size of each isolate. Note that similar alphabet showed no significant difference between the isolates ($p > 0.05$).

Location / Type of Soil	Plot	No. Of infected larvae	Percentage of infection (%)	Days of Infected	Isolates	Spore size (µm)
Taman Tropika Kenyir (TTK) / Island soil	1	0	0	0	Nil	Nil
	2	3	7.5	14	Isolate A	6.83 ± 1.13^{a}
					Isolate B	6.61 ± 0.62^{a}
	3	3	7.5	16	Isolate C	7.54 ± 0.83^{b}
					Isolate D	$6.00\pm0.88^{a,c}$
	4	0	0	0	Nil	Nil
Jambu Bongkok Forest Researve (JBFR) / BRIS soil	1	0	0	0	Nil	Nil
	2	4	10	9	Isolate E	5.80 ± 0.77^{c}
	3	4	10	9	Isolate F	6.42 ± 0.94^{b}
					Isolate G	$6.20\pm0.17^{b,c}$
	4	0	0	0	Nil	Nil
Tok Jembal Beach (TJB) / Beach soil	1	0	0	0	Nil	Nil
	2	0	0	0	Nil	Nil
	3	0	0	0	Nil	Nil
	4	0	0	0	Nil	Nil

In this study, no EPF isolate was found in alkaline and high water content soils (beach soil). The EPF isolates could be found in acidic condition which ranged from 4.07 to 4.84 and in soil moisture ranging from 44.46 % to 49.91% (island & BRIS soils). However, chi-square test revealed that the occurrence of EPF had no significant association ($p> 0.05$) with all the soil properties. The finding of this study was similar with several previous studies, which reported that there was no effect between soil properties and the occurrence of EPF in soil (Ali-Shtayeh *et al.,* 2003; Asensio *et al.,* 2003). According to Vega *et al.* (2012), *Metarhizium* sp. were

easily isolated from soil in tropical countries as compared to *Beauveria* sp. that was more commonly found in temperate countries. Thus, this indicates that the *Metarhizium* sp. is more tolerant to warmer environment as claimed by various workers (Meyling & Eilenberg, 2006; Medo & Cagáň, 2011)

Soil of TTK is classified as clay-loam soil and the soil type is "Renggam" series (Alia *et al.,* 2013). The soil was acidic with pH ranging from 4.71- 4.98. The soil moisture ranged from 26.74% to 41.41% and the nitrogen content ranged from 0.05% to 0.07%. A study was conducted by Alia *et al.* (2013) to evaluate the effect of land clearing on soil microbial functional diversity of TTK. Based on their study, the highest soil moisture recorded in TTK was 44.88% and pH ranging from 4.3-4.6, whereas the nitrogen contents ranged from 0.24-0.31%. Results from this study and the one conducted by Alia *et al.* (2013) were mostly similar except for the nitrogen values which were slightly lower in this study.

The EPF isolates from island and BRIS soils had higher mortality of infected larvae which were relatively high in nitrogen content. It has been reported that conidiogenesis on insect surface needs high soil moisture. The lower soil moisture could lead to slow conidia production and horizontal transmission of spores is reduced which can slow down the transfer of infection (Chee, 2008). This finding was similar to that of Oddsdottir *et al.* (2010) as high diversity of soil arthropod or microorganisms were found in soils with high nitrogen content.

In this study, the isolates from acidic soil had higher mortality of infected larvae than alkaline soil. This result was in line with the study of Asensio *et al.* (2003). According to Padmavathi et al. (2003), *Metarhizum* sp. has a better adaptation to grow in acidic soils. Baath and Anderson (2003) stated that soil pH may control biomass composition of fungi and bacteria by providing optimum condition to these microbials. However, the effects of soil pH were minimal as many fungi can survive in a wide range of pH (Inglis *et al.,* 2001).

Findings in this study demonstrate that the high occurrence of EPF in BRIS and island soil suggest that the sufficient availability of nutrients and organic matters for the fungi to grow (Ingham, 2000). There are many factors affecting the occurrence of EPF which include vegetation, geographical condition, temperature and others (Vänninen, 1996; Sun & Liu, 2008). The study did not examine the possible factors as it requires further investigation. Thus, a high number of soil sampling sites are also required in order to investigate the influence of soil properties on the occurrence of EPF, particularly the *M. anisopliae.*

A study by Grace *et al.* (2017) on EPF isolated from the soils of Terengganu as potential bio-pesticides against the Red Palm Weevil, *Rhynchophorus ferrugineus* revealed that two isolates of indigenous *M. anisopliae* showed promising results as both strains achieved 100% cumulative by 20th day of inoculation. Thus, it is clearly show that indigenous EPF has acclimatized to harsh environment and therefore has the potential of bio-pesticide against the insect pests specifically in the tropical climate of Malaysia that are susceptible to sunlight and rainfall.

Conclusion

In this study, only one type of Entomopathogenic Fungus (EPF) was successfully isolated and identified as *M. anisopliae* from the island, BRIS and beach soils of Terengganu, Malaysia. The occurrence of EPF was slightly higher in BRIS soil as compared to island soil, but no EPF was recorded from beach soil. This was probably due to low water content, high soil temperature, high pH value and low in nitrogen content. This study acts as a baseline for a better understanding of the soil parameters on the occurrence of EPF in soils of the East Coast of Peninsular Malaysia. This study will be further extended on the pathogenicity of *M. anisopliae* against different insect pests. The outcome will be used to develop a potential indigenous biological pest

control agent which will ultimately contribute to a sustainable agriculture system in Malaysia.

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