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Virulence evaluation of entomopathogenic fungi to subterranean termites, *Globitermes sulphureus* (INSECTA: ISOPTERA)

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ABSTRACT

Aims: Subterranean termites, *Globitermes sulphureus* is one of the significant pests for agricultural crops such as coconut and oil palm, and occasionally attacks building structure in Malaysia. Efforts to control subterranean termite infestations depended heavily on liquid termiticide applications. Natural pathogen of termites such as entomopathogenic fungi (EPF) is a promising alternative to chemical control. The objective of this study was to determine the most virulent EPF such as *Metarhizium anisopliae*, *Beauveria bassiana* and *Paecilomyces* sp. against the subterranean termites, *G. sulphureus* for the development of bio-insecticide for future use of termite control.
Methodology and results: Subterranean termites were collected from mound-building termites found in Universiti Malaysia Terengganu (UMT). Cultivating, harvesting and counting of conidia of EPF were carried out to prepare the desired concentrations for screening test and bioassays, which were 1×10^3, $1x10^5$, 1×10^7 and control. The pathogenicity testing was observed daily within 1 week under laboratory conditions. Screening showed that *M. anisopliae* was found to be the most virulent compared to *B. bassiana* and *Paecilomyces* sp., achieving 100% mortality within 3-4 days. *M. anisopliae* was then further tested on termites and it was found that concentration of 1×10^7 showed the lowest LT_{50} value, while LC_{50} of *M. anisopliae* in 1 day was 2.0151×10^6.
Conclusion, significance and impact of study: It could be concluded that *M. anisopliae* is the most virulent EPF against termites and the most effective concentration was $\pm10^7$ followed by strains of *B. bassiana* and *Paecilomyces* sp. However, there is limited field evaluation of EPF against termites in Malaysia. Evaluation on the efficiency of *M. anisopliae* in the field should be conducted so that its efficacy could be proven and marketed.

Keywords: entomopathogenic fungi, *Metarhizium anisopliae*, *Beauveria bassiana, Paecilomyces* sp., subterranean termites

INTRODUCTION

Termites belong to the insect order Isoptera. They are medium-sized, soft-bodied, light-coloured, polymorphic, cellulose-eating social insects living in large communities of several hundred to several million individuals (Collins, 1988; Thorne and Carpenter, 1992). Termites are widely dispersed throughout the tropics as well as some temperate regions and achieve their highest diversities and abundance in the rain forests of Africa, South America and South-east Asia (Collins, 1988; Bignell and Eggleton, 1998).

Termites play an important role in economic by two different ways, that are beneficial and yet extremely damaging to man's item. They are horrible destroyer since the principle food of their caste is cellulose (Krutmuang and Mekchay, 2005). Termites digest cellulose, the hard structural component of wood and other plant tissues. They also will feed on nearly any sources of cellulose including wood, roots, twigs, mulch, paper, cardboard and fabrics made of cotton and other plant-based materials (Ogg *et al.,* 2010).

At present, the use of chemical to control soil insects causes problems such as having ground water contamination (Krutmuang and Mekchay, 2005). In last decade, many entomopathogens for instance virus, bacteria, protozoa and fungi have been tested against termites with each procedure having a different success rate (Shahid *et al.,* 2012; Sajap *et al.,* 2014). All the entomopathogens listed show the potential for application in biological control of termites (Yu *et al.,* 2006) and serve as an alternative to broad spectrum chemical insecticides (Sindhu *et al.*, 2011) and more environmentally acceptable (Culliney and Grace, 2000). Fungi are important natural biological control agents of many insects and other arthropods and frequently cause

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epizootics that significantly reduce host populations (Carruthers and Soper, 1987). Fungal biocontrol agents are promising as they act by contact and do not require ingestation, they can be mass-produced very easily and are host specific (Shahid *et al.*, 2012).

The chemical methods are the sole strategy currently applied to control the termites in Malaysia. However, there are many deleterious effects of chemical pesticides in the control of crop production. These chemical pesticides cause many health hazards, contamination of food and water, resurgence of primary pest, replacement of primary pest with secondary one, destruction of natural enemies and changing pest behaviour, dispersal, development and fecundity (Jayaraj, 1986). In order to reduce the usage of chemical pesticides, biological control agent can be used as an alternative. The aim of this study was to test the susceptibility of the subterranean termites, *G. sulphureus* to selected entomopathogenic fungi (EPF), *M*. *anisopliae*, *B*. *bassiana* and *Paecilomyces* sp. in order to develop formulations and application strategies suitable for future use in biological control.

MATERIALS AND METHODS

Collection of samples

Subterranean termites, *G*. *sulphureus* were collected from mound-building termites found in Universiti Malaysia Terengganu (UMT), Kuala Terengganu, Terengganu. Termites with their substrates were placed in Petri dishes lined with moistened filter paper and reared in the laboratory condition at the temperature of 20 ± 5 °C; humidity of 70-75% and 24 h in the dark.

Preparation of entomopathogenic fungi (EPF) culture

Stock of *B*. *bassiana* was provided by Mohd. Farid, A. from Forest Research Institute Malaysia (FRIM). *M. anisopliae* isolated from coastal soil of Rantau Abang was provided by Grace, L.E.L. from UMT, while *Paecilomyces* sp. strains isolated from the soil in Malaysian Palm Oil Board (MPOB) of Hulu Paka was provided by Pong, K. K. From UMT. Potato Dextrose Agar (PDA) was prepared in the laboratory by using 3.9% (m/v) of PDA powder with 0.3% (m/v) of chloramphenicol (to prevent bacteria contamination). Total plates prepared were around 40 to 50 plates. Three strains of EPF (*M. anisopliae*, *B. bassiana* and *Paecilomyces* sp.) were used in this study. Conidia of EPF were cultivated using a modified method described by Singha *et al.,* (2010). All inoculated plates were incubated for 2 weeks in an incubator at 28 °C.

Preparation of conidia suspension

Matured EPF were harvested by using 30 mL sterile distilled water containing 0.02% Tween 80 (Singha *et al.,* 2011). The conidia suspension was centrifuged and the pellet was resuspended with 30 mL of sterile distilled water containing 0.02% Tween 80.

Serial dilution and spore counting

Serial dilution was carried out as described by Sajap *et al.* (2014) with some modifications. The conidia suspension (stock) were vortexed for one min. After that, 3 mL of stock solution was transferred to centrifuge bottle containing 27 mL of sterile distilled water and 0.02% Tween 80 and vortexed for another one min. For spore counting, 10 µL from EPF spore solution was placed onto the chamber in an improved Neubauer haemocytometer. The conidia spores were counted under Leica compound microscope. After the number of conidia spores in the stock was determined, the desired number of spores was prepared from each EPF strains tested.

Virulence screening of *M. anisopliae*, *B. bassiana* and *Paecilomyces* sp.

The desired concentration of 1×10^7 for each strain of *M. anisopliae, B. bassiana* and *Paecilomyces* sp. were prepared and applied on 10 termites in each plate. The termites were surface sterilised with 1% $NaClO_4$ and were washed with sterile distilled water for 2 to 3 before EPF strains were applied. Screenings were conducted in the laboratory conditions at temperature 25 °C, humidity of 70 – 75% and 24 h in the dark and were observed daily for one week. The most virulent EPF strain that caused highest mortality of termites in a short time (by day) was chosen for bioassay. During observation, the external parts (fungal sporulation on surface; changes in colour) of termites were observed daily for one week using Olympus Zoom Stereo Microscope with Dino Eye-Piece Camera (Model AM-4023X ANMO).

Effect of conidia numbers of *M. anisopliae* on virulence to termites

The method of bioassay was obtained from Hussain *et al.* (2011) with some modifications. There were three different concentration of spore solution for the most virulent EPF, *M. anisopliae* (1×10^5, 1×10^7 and 1×10^9) and distilled water with 0.02% Tween 80 was used as control. Ten worker termites were used for each replicate with the total of five replicates for each concentration. Hence, 200 individuals of worker termites were used in this experiment. As described above, ten worker termites were first surface sterilized with 1% $NaClO_4$ and washed with distilled water for two to three times on a sterile filter paper in a sterile petri dish. Spore solution was sprayed on termites and they were dried for 2 min. Treated termites were transferred to wet filter paper and sealed with parafilm. Laboratory bioassays were conducted in the laboratory conditions. Signs and symptoms of infection and the mortality rate were monitored and recorded daily within 7 days. Once the termintes died, they were removed to another petri dish with PDA. The petri dishes were maintained at 27±2 °C to observe the fungal growth.

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Statistical analysis

Graph of cumulative percentage mortality against days was plotted using Microsoft Excel 2010 to determine the Lethal Time (LT_{50}) for each EPF strains and the Lethal Concentration (LC_{50}) value for *M. anisopliae*. Mortality data recorded were analysed using analysis of variance (ANOVA) with $P \leq 0.05$. Tukey Post-hoc test was carried out for multiple comparisons between different concentrations of *M. anisopliae* at $P \leq 0.05$. All analyses were performed with the Statistical Package for the Social Sciences (SPSS) software version 20.0.

RESULTS AND DISCUSSION

Selection of the most virulent pathogenic entomopathogenic fungi (EPF)

Three isolates of potential EPF, *M. anisopliae, B. bassiana* and *Paecilomyces* sp. were subjected to initial assay in order to select EPF with high virulence against the termites. The tested EPF differed in their virulence to the termites. At 5 days of post-inoculation, mortality of treated termites with *M. anisopliae* was 100%, and 70-100% for *B. bassiana* and *Paecilomyces* sp. (Figure 1). Thus, it shows that *M. anisopliae* was more virulent than *B. bassiana* and *Paecilomyces* sp. *Metarhizium anisopliae* killed the termintes relatively fast, with LT_{50} at 2.4 days, in comparison to *B. bassiana* and *Paecilomyces* sp. with LT_{50} at 4.1 days.

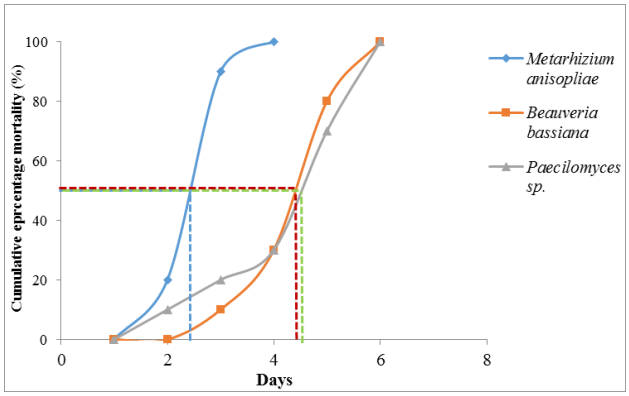

Figure 1: Comparison of cumulative percentage mortality of termites between *M. anisopliae, B. bassiana* and *Paecilomyces* sp.

A study by Singha *et al.* (2011) revealed that the dosage mortality test illustrated stronger correlation between dose and time. Thus, the same 1×10^7 concentration for each of the EPF was used for this screening to allow unbiased comparison. This study indicates that *M. anisopliae* strains caused earlier mortality (±3 days) compared to *B. bassiana* and *Paecilomyces* (±5 days). This observation is in accordance to earlier reports (Yanagawa *et al.*, 2008; Singha *et al.*, 2011) on similar patterns of pathogenicity by *M. anisopliae*, *B. bassiana* and *Paecilomyces* sp. isolates. In this study, *M. anisopliae* achieved 50% mortality in lesser time compared to the *B. bassiana* and *Paecilomyces* sp.

Morphological observation of entomopathogenic fungi (EPF)

Dead termites were removed daily and placed in a new Petri dish lined with moistened filter paper and incubated at 25 ± 2 °C in the dark. The termites were observed daily to provide evidence that the cadaver of termites were killed by the targeted EPF. Generally, the sign of penetration of the termites cuticle by *M. anisopliae* appeared faster than *B. bassiana* and *Paecilomyces* sp. After the penetration, the EPF appeared to break through the cuticle and conidia emerged on the surfaces of the cadavers (Figures 2, 3 and 4).

Figure 2: Dead termites infected by *M. anisopliae* at 2nd day (A), 3rd day (B), 5th day (C) and 7th day (D).

Based on the observation of the cuticle of dead termites infected by *M. anisopliae,* white hyphae started to grow on the 3rd day on the thorax, legs and antennae of the termites (Figure 2). On the 4th day, greenish mycelia started to appear. Green conidia could be seen clearly on the 5th day and became matured on the 7th day and covered the whole body of dead termites. For termites infected by *B. bassiana*, white hyphae could be seen at the 4th until 6th day mostly at the anterior part of termites (Figure 3). Mycelia growth could be seen on the 6th day and became more abundant on the 7th day. Conidia appeared on the 7th day and turned from white to yellow in colour and covered the anterior part of the termites. Similar observation was recorded on the dead termites infected by *Paecilomyces* sp. White hyphae could be seen on the 4th day and covered most of the anterior part of termites (Figure 4). During the 5th to 6th day, pink mycelia could be seen and covered all of the termite's body. Mycelia became more abundant on the 7th day and the conidia started to appear.

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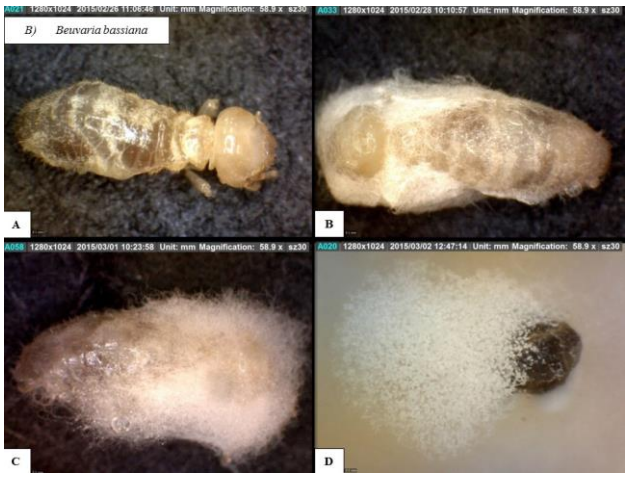

Figure 3: Dead termites infected by *B. bassiana* at 3rd day (A), 5th day (B), 6th day (C) and 7th day (D).

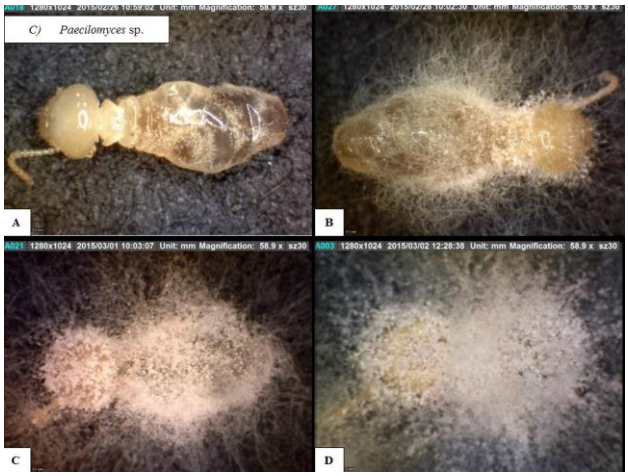

Figure 4: Dead termites infected by *Paecilomyces* sp. at 3rd day (A), 5th day (B), 6th day (C) and 7th day (D).

Initially, the infected termites showed slow locomotory response. Finally, termites died and those termites that were alived showed a lot of grooming activities on dead termites. Yamamoto *et al.* (2012) reported that subterranean termites which live in soil usually were infected by various microorganisms including entomopathogens like fungi. Thus, to maintain the stability of termites' colony, termites had social behaviors called allogrooming by mouth to clean the attached entomopathogens from the surface of their nestmates (Yanagawa and Shimizu, 2005). Termites started to change in colour after few days of infection (darkened), had softened bodies and became more fragile when touched. Hyphae only could be seen after ±one day after *M. anisopliae* treatment and after ±3 days after treatment for *B. bassiana* and *Paecilomyces* sp. This finding was supported by Sajap and Kaur (1990), who found that mycelia can be seen from the cuticle until approximately ±4 days post inoculation, even though termites were dead for ±2 days after inoculation. Fungal hyphae mostly started to grow at the germ tube (Sajap and Kaur, 1990), legs and at the tips of antenna closer to head of the termites.

For *M. anisopliae*, the mycelia initially appeared pale yellowish white and finally turned into olive-green in colour. Olive-green conidia could be seen as early on the 5th day and becoming more matured on the 7th day. On the 7th day, powdery conidia of *M. anisopliae* could be seen covering almost all of the termites' body. For *B. bassiana*, the mycelia initially appeared as pale yellowish white on the 6th day and more abundantly found on the 7th day. Similar to *Paecilomyces* sp., the appearance of mycelia could be observed on the 6th day and more abundantly on the 7th day. However, the *Paecilomyces* sp. conidia initially appeared as pale purplish white and finally turned into purple in colour.

Effect of conidia numbers of *M. anisopliae* on virulence to termites

The bioassay on termites were conducted with *M. anisopliae,* which was shown to be as the most virulent to the termites than *B. bassiana* and *Paecilomyces* sp. Bioassay was carried out to determine the most effective concentration of conidia of *M. anisopliae* against termites. Hundred percent of mortality was recorded at the concentration of 1×10^5 conidia/mL and 1×10^7 conidia/mL (Figure 5). However, the difference was not statistically differed. Significantly ($P < 0.05$) lower percentage of mortality (50%) was recorded at the concentration of 1×10^3 conidia/mL and control compared to 1×10^5 conidia/mL and 1×10^7 conidia/mL (Figure 5). This result showed that the lowest and higher concentrations used were significantly ($P < 0.05$) influenced the mortality rate of termites where higher concentration killed more termites in a short time and vice-versa.

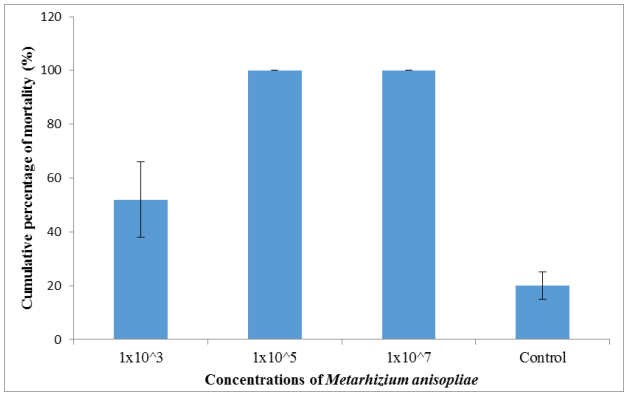

Figure 5: Virulence of different concentrations of conidia of *M. anisopliae* towards subterranean termites. Bars indicate standard deviation of the mean. Different letter indicates significant differences between groups (Tukey Post-Hoc Test, $P \leq 0.05$).

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Based on the observation, some termites were stressed (allogrooming activity) and eventually affected the rate mortality of the termites. After inoculation, allogrooming activities could be observed. Some termites had been bitten by other termites and eventually caused death. After a few days, fungal hyphae could be seen on those bitten termites. Most of the previous studies showed that grooming among nest mates for avoidance and sealing-off the diseased termites were the main reason for the failure of pathogens in field conditions (Rath, 2000). However, in this study, plenty of grooming activity could be seen which caused the termites being cannibalised and followed by death. Chouvenc and Su (2012) reported that the exposed termites died from the infection, but no secondary infection was observed in the rest of the group after 90 days because all of the cadavers were quickly cannibalised. This report showed that the cannibalisation occured during this study was not due to the lack of food supply, but it was the behaviour of the social insects. However, the cannibalisation in this study does not refer to the dead termites. It could be seen that the bitten termites were still and had movements. Thus, it can be suggested that grooming activities could cause death in a termite colony too.

LT_{50} analysis showed that concentration 1×10^3 conidia/mL of *M. anisopliae* took a longer time ($\pm$ 9.38 days) to kill the termites compared to concentrations 1×10^5 conidia/mL ($\pm$ 2.20 days) and 1×10^7 conidia/mL ($\pm$ 0.32 days). Concentration 1×10^7 conidia/mL showed the lowest in LT_{50} value which then can be suggested that 1×10^7 conidia/mL is the most effective concentration compared to 1×10^3 conidia/mL and 1×10^5 conidia/mL. The results show that the higher mortality depends on the conidia concentration. The higher the concentration, the higher the mortality rate of termites. This was supported by previous study, Ahmed *et al*. (2008) who reported that when subjected to lower concentration, longer durations was observed for a significant mortality.

There was a strong negative correlation between LT_{50} value and concentrations of *M. anisopliae*. Thus, it could be concluded that the lower concentration is needed for the higher LT_{50} value to achieve 50% mortality of termites. The LC_{50} of *M. anisopliae* in this current study against termites for one day was 2.0151×10^6. Several previous studies stated that as termites live in conducive environment for the pathogen, which is a humid warm with minimal diural temperature fluctuations and crowded environment with considerable social interactions, it caused them to be a good candidate for biocontrol (Kramm *et al.,* 1982; Ignoffo, 1992; Rath, 2000). Milner and Staples (1996) reported that *M. anisopliae* was recommended for practical control of termites as bioinsecticide because (i) it has robust conidia that are easy to formulate and store; and (ii) has conidia that can survive >18 months in termite nests.

Furthermore, it was shown by other researchers that rapid killing by *M. anisopliae* of its host could caused not only through direct physical invasion of the hyphae, but also possibly due to some enzymatic mechanisms or toxic metabolites produced by the fungus (Jiang *et al.,* 2002, 2003). Besides, these fungi are relatively inexpensive to grow in the quantity sufficient to be used in the field (Grace, 1997; Jackson *et al.,* 2000). However, Chouvenc and Su (2012) reported that even though *M. anisopliae* may be a virulent pathogenic fungus to termites at the individual level, it does not have the capability to trigger an epizootic at the colony level.

CONCLUSION

In this study, *Metarhizium anisopliae* was the most virulent entomopathogenic fungi (EPF) against subterranean termites, *G. sulphureus* compared to *B. bassiana* and *Paecilomyces* sp. As the result in this study are preliminary, field trial should be conducted as the efficacy between laboratory and field condition could differ. Thus, integrated pest management (IPM) of termites should focused on this EPF and further verified in the field. Hence, the effectiveness of *M. anisopliae* could be proved and marketed for the development of eco-friendly bio-insecticide for future use of termites control.

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