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Laboratory efficacy of mycoparasitic fungi (*Aspergillus tubingensis* and *Trichoderma harzianum*) against tropical bed bugs (*Cimex hemipterus*) (Hemiptera: Cimicidae)

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ABSTRACT

Objective: To test the effectiveness of conidial spore formulations [*Aspergillus tubingensis* (*A. tubingensis*) and *Trichoderma harzianum* (*T. harzianum*)] against tropical bed bugs, *Cimex hemipterus*.

Methods: Spore formulations were made from two fungal strains, *T. harzianum* and *A. tubingensis*. The bed bugs were exposed to the conidial spores placed soaked onto a fabric cloth for 1 h and the mortality counts were recorded daily until 14 days.

Results: Mean survival times based on Kaplan–Meier survival analysis showed no significant differences between all the concentrations in both the fungal isolates: *T. harzianum* and *A. tubingensis*. However, the evaluation of both the isolates in terms of virulence resulted in low lethal hours in all the concentrations except for the high concentration of *A. tubingensis* (LT_{50} = 44.629 h) at the conidial exposure of 1×10^6 spores/mL. Rapid mortality of the bed bugs was observed from Day 6 to Day 12, ranging from 13% to 90% in all three concentrations of *A. tubingensis*. With reference to the *T. harzianum* exposure, the concentration of 1×10^4 spores/mL displayed a gradual increase in the percentage mortality of 90 on Day 14.

Conclusions: Approaches to the bed bugs treatment should be explored in-depth using a natural biological agent like fungus especially *A. tubingensis* to reduce this pest population, in order to replace chemical methods.

1. Introduction

The rise of entomopathogenic fungi studies as pest control has implied that recent studies have shown promising improvements in the fungi's effectiveness as biological control agents. The potentials of *Metarhizium anisopliae* (*M. anisopliae*) and *Beauvaria bassiana* (*B. bassiana*) isolates in reducing the survival of major pests and disease vectors have been proven [1–4]. Despite their wide geographical distributions, both of these fungal strains are also capable of infecting a broad range of insect hosts compared to other fungal isolates that are mostly targeted species [5,6]. The combination of the two fungal species also yielded successful treatment in controlling weevils (*Cylas formicarius*) that tend to attack tuber crops in the field, as shown in the study by Reddy *et al.* [4].

Aside from the above major pathogens against insect, other fungal isolates are also currently developed and studied for their uses as promising biological control agents. These include *Trichoderma* sp. which is known for their pathogenic activities against plant diseases but may act on insect vectors like

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mosquito larvae, fly maggots and fruit borer as well [7–9]. The mechanism of these fungi species mainly owes to the broad range of enzyme productions, causing failure in the defensive systems of the insects. Mass production of hydrolytic enzymes, cellulases, hemicellulases, proteases, and β-1,3-glucanase in *Trichoderma* sp. was used in the agricultural industry, plant growth promoters and biofungicides [10,11].

Aspergillus sp. is another potential fungal isolate from the Ascomycota class, which can cause damages to hosts due to their mycotoxins production that usually adheres to the cuticle beforehand [12,13]. Secretion of secondary metabolites, commonly toxins, played a vital role in the interaction between the insect host and pathogens [14]. For example, isolates of *Aspergillus westerdijkiae* infected the ootheca of the American cockroach, *Periplaneta americana* by germinating fungal spores in them [15]. A study by Bawin *et al*. [16] also showed that the virulence of *Aspergillus clavatus* (*A. clavatus*) was similar to the common entomopathogenic fungi when tested against mosquito larvae and produced a high concentration of spores.

Nevertheless, biological control against pests that have medical implications such as bed bugs is still limited to date. Several studies examined the effects of the well-recognized fungi, *M. anisopliae* and *B. bassiana* in terms of their feeding status and the dissemination of conidia in the bed bugs colony which relied on the humidity level [17,18]. Presently, investigations on the bed bugs exposed to other fungal isolates like *Aspergillus* sp. and *Trichoderma* sp. are yet to be reported. Therefore, the objective of this study was to determine the capability and effectiveness of two fungal isolates, *Aspergillus tubingensis* (*A. tubingensis*) and *Trichoderma harzianum* (*T. harzianum*) in controlling tropical bed bugs, *Cimex hemipterus*.

2. Materials and methods

2.1. Insects

The bed bugs were acquired from the sample collection of bed bugs across Peninsular Malaysia. They were reared in the plastic container 8 cm × 8 cm in height and diameter with folded A4 paper as their harborages. A piece of fine net cloth was used to cover the mouth of each container, tied up with rubber bands to prevent the bugs from escaping. The containers were kept in clear plastic tank, covered with black plastic to prevent direct exposure of lights to the bed bugs. The room was maintained at the temperature that ranged within (25 ± 2) °C and humidity level was kept at 50%–60%. The bed bugs were fed every week using expired human blood, obtained from Hospital Pulau Pinang. The artificial feeding setup was modified for easier handling techniques [19].

2.2. Isolation of the fungal strain

The fungi were cultured from the previous isolation of dead bed bugs, but using two fungal strains: *T. harzianum* (isolate TM1) and *A. tubingensis* (isolate AM3) [20]. The isolates were inoculated first by making three points or a single inoculum on malt extract agar plates and incubated in the room conditioned at 25 °C for 7 days. The identification of the fungal species was confirmed by microscopic observations using morphological characteristics outlined by Klich on *Aspergillus* species whereas *Trichoderma* sp. referred to Samuels *et al*. [21,22]. The conidial isolates were then maintained in distilled water with mild agar broth for preservation purposes.

2.3. The preparation of conidia suspension

A suspension was made by mixing the conidia with distilled water and Tween 80 (Sigma–Aldrich) for latter inoculation of the liquid culture medium. Sterilized loose-woven cotton cloth and filter funnel were used in harvesting the conidia, separating excessive mycelia and spores. Spores in the suspension were counted by using a haemocytometer and dried into powder form using a freeze-drying machine. The dried conidia were kept in storage in a zip lock bag, covered with aluminum foil at −10 to −20 °C. Conidial spores were then adjusted into three concentrations of 1×10^6, 1×10^5 and 1×10^4 conidia/mL. They were formulated with a mixture of 60% of mineral oil (Sigma–Aldrich) and 40% mineral spirit prior to the application of conidia to the substrate. Each concentration had three replicates, and blank mineral oil formulation was used for control.

2.4. The application of fungal spores to the exposure surfaces

Spore formulations were applied by using fine mist sprayer at a distance of 15 cm from the fabric cloth substrates, which were made into circles of 9 cm diameter. Spraying activities of conidial spores were conducted in a biosafety cabinet (Class II) at a desirable volume application rate of 10 mL/m^2. Control substrates were sprayed with blank mineral oil formulation only. The treated substrates were placed in 90 mm Petri dishes with the lids covered and were left to dry overnight in a controlled condition in the chamber to prevent contamination of other microorganisms.

2.5. Bed bugs exposure to conidia

Batches of mixed sex adult bed bugs were isolated one day prior to exposure. The bugs were fed 24 h before the testing began. A total of 10 bed bugs were first placed on the sprayed dry substrate in a Petri dish for about 1 h. The experiment was repeated three times, forming triplicates at a time. The bed bugs were transferred onto clean Petri dishes with filter papers inside after the exposure period. Observations on the survived bed bugs were scored daily until Day 14. Survived bugs were maintained in the incubator with a photoperiod of 12:12 h light and dark. Dead bugs were isolated from the survived colonies and incubated in 98% of humidity for further mycosis.

2.6. Data analysis

The number of survived bed bugs was scored daily from the first day of exposure until 14 days. The differences in the bed bugs' survival after being exposed to different concentrations and fungi species were analyzed statistically using the Kaplan–Meier analysis along with a log-rank test (MedCalc Statistical Software version 16.4.3). The mean survival time (MST) was used to compare the three concentrations of the two fungal strains, *Trichoderma* sp. and *Aspergillus* sp. on bed bugs. Lethal time (h) of probit regression was used in determining the speed of killing each fungus against the bed bugs. The effects of

different fungi isolates on the death rate were further evaluated using One-way ANOVA statistical analysis SPSS version 22.0 (IBM Corporation, Armonk, NY, USA).

3. Results

Both fungal isolates, TM1 and AM3 showed no significant differences in the MST of the bed bugs (Table 1). In the fungal strain of TM1, the survival time of the bed bug statistically showed no significant difference between the three concentrations since χ^2 = 1.325 8, 3 degree of freedom, $P > 0.05$. The value of the MST ± SE in the conidial exposure of 1.0×10^6 spores/mL was the lowest [(10.006 ± 0.732) days] compared to the other two concentrations. Bed bugs exposed to a conidial concentration of 1.0×10^5 spores/mL had the highest mean value [(10.511 ± 0.725) days]. Another concentration of 1.0×10^4 spores/mL yielded slightly lower value with (10.296 ± 0.671) days.

Similar outcomes were observed in the fungal isolates of AM3 whereby there were no significant differences in the survival analysis of the three concentrations [χ^2 = 0.882 6, 3 degree of freedom, $P > 0.05$]. Conversely, the highest MST value was observed in the concentration of 1.0×10^6 spores/mL [(10.930 ± 0.765) days] while the concentration of 1.0×10^5 spores/mL exhibited lower survival time than the previous concentration [(10.890 ± 0.664) days]. The lowest survival time of (10.740 ± 0.655) days was showed by 1.0×10^4 spores/mL concentration. Both Figures 1 and 2 show the MST of the bed bugs against the concentrations of each fungus.

The virulence of conidial spores in killing bed bugs was determined by using LT_{50} on each concentration of both the fungal isolates (Table 2). The lowest lethal value was detected in the AM3 fungal strain with a conidial concentration of 1.0×10^6 spores/mL, LT_{50} = 44.629 h (1.9 day). The conidial spores of TM1 took about 182.767 h and 186.382 h to cause mortality in 50% of the population at the concentrations of 1.0×10^4 and 1.0×10^5 spores/mL, respectively. Compared to the remaining concentrations, all of them have high LT_{50} values due to many bed bugs that survived after the exposure period. The following concentrations took more than a week for the fungi to act on half of the population (TM1 isolates, 1.0×10^6 spores/mL, LT_{50} = 218.630 h; AM3 isolates, 1.0×10^4 spores/mL, LT_{50} = 247.002 h and 1.0×10^5 spores/mL, LT_{50} = 275.237 h).

The comparison between the fungal isolates of TM1 and AM3 based on time (h) was statistically performed using One-way ANOVA (Figures 3 and 4). No significant difference was observed between the mortality rates of the bed bugs in hours

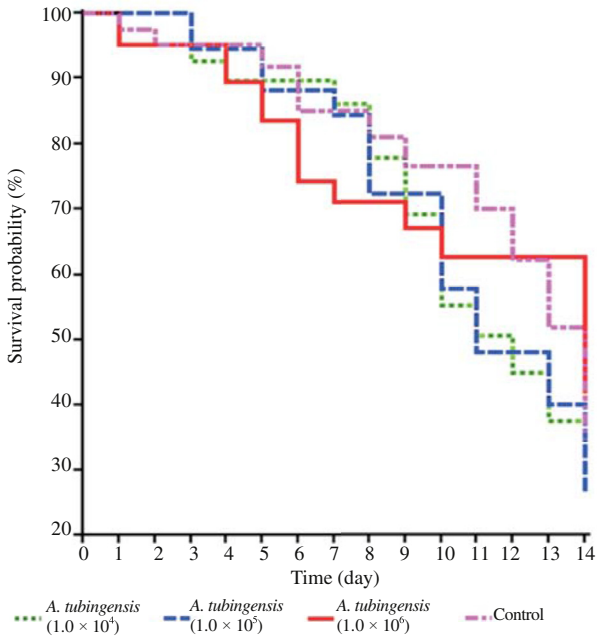

Figure 1. Survival percentage of AM3 isolate on three concentrations (spores/mL).

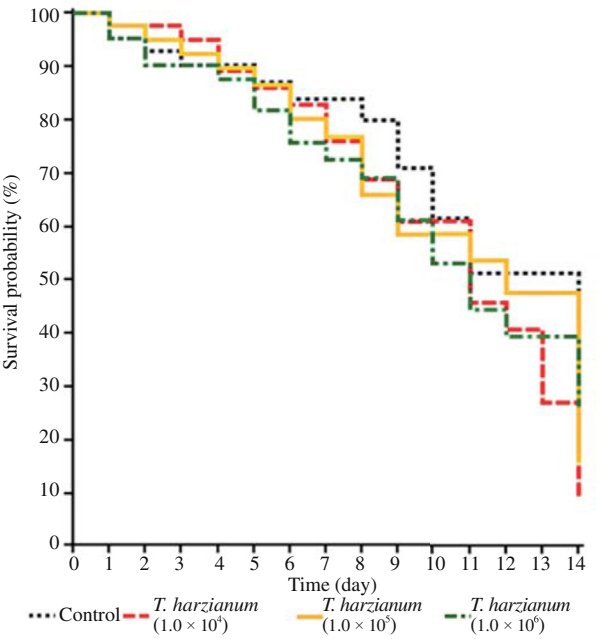

Figure 2. Survival percentage of TM1 isolate on three concentrations (spores/mL).

Table 1
Survival time xddof bed bugs based on concentration of each fungus isolate.

Concentration (spores/mL)		χ^2 value[a]	SE	95% Confidence interval	MST (day)
T. harzianum (TM1)	1.0×10^4	1.325 8	0.671	8.981–11.611	10.296
	1.0×10^5		0.725	9.089–11.933	10.511
	1.0×10^6		0.732	8.572–11.440	10.006
A. tubingensis (AM3)	1.0×10^4	0.882 6	0.655	9.459–12.027	10.743
	1.0×10^5		0.664	9.591–12.193	10.892
	1.0×10^6		0.765	9.433–12.430	10.932

[a]: χ^2 value with associated P value, not significant since $P > 0.05$.

Table 2
LT_{50} of bed bugs after being exposed to fungal spores.

Concentration (spores/mL)		SE	R^2	LT_{50} (h)[*]
T. harzianum (TM1)	1.0×10^4	0.373	0.734	182.767[a] (159.641–206.717)
	1.0×10^5	0.321	0.709	186.382[a] (162.674–213.344)
	1.0×10^6	0.263	0.767	218.630[a] (188.129–262.436)
A. tubingensis (AM3)	1.0×10^4	0.259	0.559	247.002[a] (199.327–333.878)
	1.0×10^5	0.461	0.690	275.237[a] (246.459–320.420)
	1.0×10^6	0.232	0.125	44.629[b] (8.317–76.509)

[*]: Same letters indicate no significant difference between lethal hours. The data in the bracket indicated the time interval.

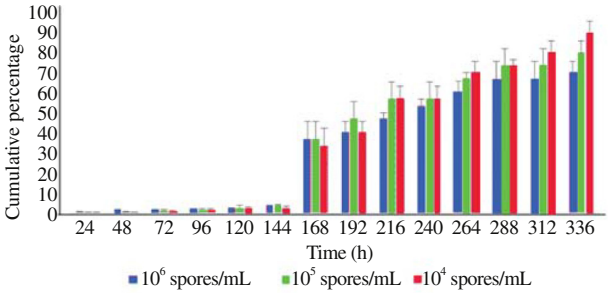

Figure 3. Cumulative percentage of mortality in bed bugs based on time and concentrations of TM1 isolate.

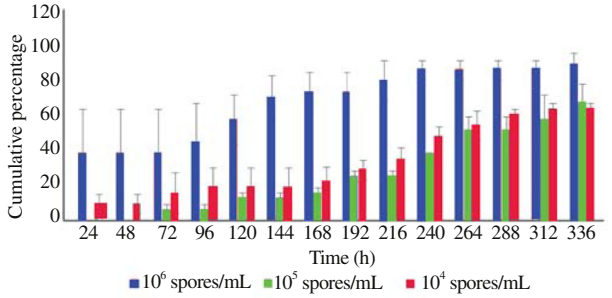

Figure 4. Cumulative percentage of mortality in bed bugs based on time and concentrations of AM3 isolate.

when they were exposed to conidial concentrations of the TM1 isolates since all the *P* values were above 0.05. From Day 1–6, the bed bugs showed a slow mortality rate in all the conidial concentrations that ranged from 0.7% to 3.0%. From Day 7 onwards, there was a gradual increase in the bed bugs' percentage of death and Day 10 marked the time when 50% of the population died. The mortality rate reached between 70% and 90% after the conidial spore's exposure on Day 14 of the treatment (Figure 3).

In contrast, the mortality rate of bed bugs according to hours revealed that there was a significant interaction between the three concentrations of AM3 isolates (Figure 4). The first three days after the exposure of conidial spores of 1.0×10^6 spores/mL resulted in mortality percentage of 40% in bed bugs population. Compared to the other two formulations, their percentage of mortality reached 50% on Day 11. However, rapid mortality of bed bugs was observed from Day 6 to Day 12 in all the concentrations. Significant differences in the three concentrations were observed in the particular hours: 144 h, $F(2,6) = 10.069$; 168 h, $F(2,6) = 13.095$; 192 h, $F(2,6) = 12.412$; 216 h, $F(2,6) = 13.722$; 240 h, $F(2,6) = 31.500$; 264 h, $F(2,6) = 6.529$; 288 h, $F(2,6) = 8.818$ at $P < 0.05$.

Percentage of mortality of bed bugs in the concentration of 1.0×10^6 spores/mL recorded the highest mortality rate of 93% on the 14th day, rather than the other two concentrations, 1.0×10^5 spores/mL and 1.0×10^4 spores/mL, each with the values of 70% and 67% respectively. Cadavers were covered with mycelia, thus showing the presence of mycosis.

4. Discussion

Microbes exist in the airborne spaces, and the capacity of each fungal species is numerous depending on the temperature and humidity level in that particular area [23]. The fungal spores of *Aspergillus* sp. were dominantly found invading the airspace in closed surroundings including food storage area, bakeries, libraries, factories or even in a controlled condition like laboratories [15,23,24]. Thus, initial steps were taken based on previous infection or contamination on the insect to produce control methods using natural biological agents from many fungi species. Therefore, in this study, we obtained fungal isolates from the infected bed bugs culture and identified them as *A. tubingensis* and *T. harzianum* for further investigation.

Our results showed that *A. tubingensis* was able to cause mortality in the bed bugs to half of the population, in short, lethal hours, less than 2 days after the exposure. Although the virulence of this species in killing the bed bugs colony was much slower when compared to the *M. anisopliae* and *B. bassiana* [17], the fungal infection still can be observed gradually between six and seven days from the spores due to mycosis in all the cadavers. Lower concentration experienced even slower mortality rate which was about 7–11 days after the exposure of the fungal strains against the bed bugs. This observation can also be seen in a study by Zhang *et al.* [25] who indicated that the infection of *Aspergillus oryzae* on locusts started after more than 5 days, later than the normal period for fungi to kill its host. Slower mortality in bed bugs was probably affected by nutrient enervation in them but not due to toxin production of the fungus itself [26].

Even though there was no significant interaction between the conidial formulations, a high concentration of AM3 isolates (1.0×10^6 spores/mL) was able to cause a high percentage of mortality in the bed bugs. This is similar to a recent study where a high percentage of mortality in adult mosquitoes was caused by the exposure to high concentrations of *M. anisopliae* and *B. bassiana* [1]. The results obtained contradicted a study which reported that the survival times of the targeted insect can be reduced with the lowest concentration of the fungal species [27]. This, however, was objected by Mnyone *et al.* [1] who argued that different experimental design and procedures involving fungal strains, formulations, targeted insect or arthropods would have affected the end product causing various outcomes between the mycoparasitic fungi studies.

Based on the results obtained, the TM1 isolate was able to infect the bed bugs and caused 70%–90% of mortality rate after a short exposure period regardless of the concentration of conidial spores. Even though the lethal hours were much longer than the AM3 strain, more than half of the treated colonies were killed by using this fungal isolate. Several reports had proven the potential of *Trichoderma* sp. as a natural control agent against some of the targeted insects. For example, it had caused 80% of mortality in cotton leafworm larvae in Egypt. Antibiotics were derived from the extraction of enzymes of *T. harzianum* to prove their pathogenicity against the larvae of mealworm beetle whereas the exposure of the fungal strain had also resulted in the lower production of brood and poor gallery formation of granulate ambrosia beetle, *Xylosandrus crassiusculus* [24,28,29]. Therefore, the studies on mechanisms and enzymes production of *T. harzianum* should be continuously explored to commercialize this fungal species in the outdoor field without causing any harmful effects on human and environment.

Although the percentage of mortality in the AM3 isolates did not achieve 100% mortality rate throughout the treatment period, they are considered as potential mycoparasitic fungi against the common pest. Virulence in the fungal isolates affecting the host might differ depending on the species and targeted insect. For instance, instead of killing the insect host, the fungal strain

might only alter the metabolic activities of the host which resulted in fewer generations in the population. Suliman and Mohammed revealed that the concentrations of *Aspergillus terreus* could reduce the reproductive system in ticks. However, they failed to kill the adult females [30]. The alteration was mainly associated with the secretion of secondary metabolites of *Aspergillus* sp. since it could cause failure in the immune response of the host. However, the possibility of the toxins to become a major causative agent of disease remained unclear [14]. In a recent study, *A. clavatus* was isolated from the cadavers of insect to observe possible damages caused by the toxins involved, via exposure of the spore suspension on the larvae of *Culex* mosquitoes [31]. Within 2 days of exposure time, their results achieved about 70% mortality in a high concentration of *A. clavatus* and the findings revealed that the spores were able to germinate in the digestive tract of the infected larvae.

Barbarin *et al*. demonstrated that the overall mortality in common bed bugs had reached 95% within a shorter period due to the exposure of *B. bassiana* [17]. However, in our findings, both fungal isolates, AM3 and TM1 had slower mortality rate but achieved more than 80% mortality rate during post-treatment. The differences in the concentrations produced a different result of mortality percentages in each fungal isolate. Since the fungi are humidity-dependent microorganisms, the observation of mycosis also showed that high humidity level is required to enhance the germination of the spores while causing the death of the host. Ulrich *et al*. [18] concluded that high humidity of 98% in controlled conditions could cause high mortality rate in bed bugs. Since this is the first study using *A. tubingensis* and *T. harzianum* in bed bugs control, the results obtained can be used for further research to improve the efficacy of fungal isolates as natural agents and can be practically applied in the environment.

In summary, AM3 and TM1 isolates have the potential of becoming biological control agents of the bed bugs. Throughout the post-treatment bioassay, both fungal isolates displayed high mortality rates although they were reduced slowly. The mortality rate was affected by the following factors including fungal strains, concentrations, formulation types, targeted insect and experimental procedures. Nonetheless, further detailed experiments in biological control should be carried out in both laboratory and field environment to control the resistance issue in bed bugs instead of using chemical pesticides.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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