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Effectiveness of the Pathogenic Fungus (*Penicillium* sp.) Against Mortality of the Termite (*Coptotermes curvignathus*)

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ABSTRACT

This research presents a novel and innovative approach to termite control using the fungus *Penicillium* sp. as an environmentally friendly alternative to chemical compounds. The study aims to evaluate the effectiveness of this method and the symptoms exhibited by *C. curvignathus* termites infected with the fungus. The experimental design utilised a Completely Randomised Design (CRD) to assess the impact of the fungus on the termites. Probit analysis determined the Lethal Concentration (LC50) and Lethal Time (LT50) values. The findings indicated that the LC50 value of the fungus *Penicillium* sp. is 1×10^7 conidia/mL, with an LT50 of 5.4 days. The highest mortality rate observed in *C. curvignathus* due to the fungus was 77.71% at the highest conidia density of 1×10^9 conidia/mL. Symptomatology observed in the termites included changes in body colour and the growth of fungal mycelium covering their bodies.

1. INTRODUCTION

Termites are insects that significantly threaten wood and wood-related buildings in Indonesia, leading to substantial economic losses [1, 2]. This attack occurs because wood contains lignocellulosic material and serves as food for termites [1, 3]. Furthermore, the losses caused by termites reached IDR 3.73 trillion in 2000, increasing to more than IDR 18.68 trillion in 2015. The urgency of finding effective termite control methods is evident from these staggering figures [4, 5].

The species of termites recorded as destroying wood in Indonesia are *Coptotermes curvignathus* Holmgren, *Schedorhinotermes javanicus* Kemner, *Macrotermes gilvus* Hagen, *Microtermes inspiratus* Kemner, and *Cryptotermes cynocephalus* Light [6]. Genus Coptotermes is classified as the most vicious in attacking wood and buildings in Indonesia and several other countries [7]. The losses caused by termites are certainly alarming for humans, while their control has been carried out using chemical pesticides, such as organochlorine insecticides and methyl bromide. Using this material has the potential to endanger human health, pollute the environment, and impact non-target organisms [8]. Another alternative currently being developed to control termites is to use biological control agents as termite entomopathogens.

Entomopathogens play a crucial role as natural regulators in controlling insect populations. In addition to nematodes and bacteria, fungi exhibit significant potential as biological control agents. Among the approximately 700 known species of entomopathogenic fungi, only 10% have been or are currently being developed for insect control [8, 9]. Fungi are particularly promising as biological control agents due to their rapid contact action and ability to be mass-produced quickly and with specificity [10]. Entomopathogenic fungi that are currently widely cultivated are *Bauveria bassiana* and *Metarhizium anisopliae*. These two fungi have been widely used to control insects, including termites. In addition to these two fungi, several are considered capable of being used as entomopathogens, including *Penicillium* sp. [11]. Several studies show that Penicillium can kill beetles, mosquitoes and aphids (aphids) [12-14]. Penicillium species can produce chitinase enzymes, which degrade the exoskeletons of insects, leading to their colonisation and eventual death. Examples of Penicillium strains known for chitinase production include *Penicillium ochrochloron* [15], *Penicillium chrysogenum* [16], and *Penicillium oxalicum* [17]. *Penicillium* species can also produce various mycotoxins that are toxic to insects [18]. This research is prompted by the need for more research on Penicillium's ability as a termite pathogen. Therefore, it is hoped that this research will obtain data on the effectiveness of *Penicillium* sp. fungus as an entomopathogen in controlling the termite *Coptotermes curvignathus.*

As a wood-eating social insect, the *C. curvignathus* poses a severe threat to buildings in Indonesia. This species also attacks the plantation industry and causes losses that continue to increase yearly. Pesticides have caused severe problems for humans and the environment, which pose risks to children and pregnant women and can also harm non-target organisms [19], so an alternative, environmentally friendly, biological control

technology is needed. Therefore, it is necessary to develop environmentally friendly biological control agents, including the fungus *Penicillium* sp., which is an entomopathogenic agent of termites.

This study aims to determine the effectiveness of the pathogenic fungus *Penicillium* sp. as a biological control agent for *C. curvignathus* by determining the Lethal Concentration (LC50) and Lethal Time (LT50) values for termite mortality and by observing the symptomatology of *C. curvignathus* infected with the fungus. Besides that, we will provide initial information on developing the *Penicillium* sp. fungus as an environmentally friendly biological control agent for *C. curvignathus*. The study results are anticipated to provide insights into the potential advantages of using Penicillium sp. as a biological control agent, which could help decrease pesticide use and enhance environmental quality.

2. MATERIALS AND METHODS

This research employed a Completely Randomised Design (CRD) as its experimental approach. Probit analysis was conducted to determine the LC50 (lethal concentration) and LT50 (lethal time) using five treatment levels and three replications, resulting in 18 experimental configurations. Additionally, supplementary probit analysis was performed with the same five treatment levels and three replications, leading to 15 experiments. The primary goal of both studies was to assess the impact of fungal treatment on the mortality of *C. curvignathus*.

The CRD approach was chosen for its flexibility in handling various treatment and replication quantities. In this investigation, the termites used were relatively uniform in age and size, and environmental conditions were consistently maintained [20]. The only variable was the treatment, specifically the conidial density per mL, allowing any observed responses to be directly attributed to the treatment's effects.

2.1 Media and propagating fungi

This study used Potato Dextrose Agar (PDA) as the growth medium, selected for its well-balanced nutritional composition, which promotes optimal fungal growth. Moreover, PDA's widespread availability enhances its accessibility for a broader range of users [21]. While the growth rate of Penicillium on PDA may vary compared to that of sabouraud dextrose agar (SDA) and malt extract agar (MEA), PDA continues to be a suitable option [22].

To prepare the PDA medium, 39 grams of PDA powder were measured and transferred to an Erlenmeyer flask. Gradually, one litre of distilled water was added, and the mixture was stirred until uniform. The flask was then covered with aluminium foil and wrapped in plastic film before being sterilised in an autoclave at 121℃ and 15 psi (approximately 101 kPa) for 20 minutes [23].

Penicillium sp. was isolated from termite nests collected at the Suaq Balimbing Research Station in Gunung Leuser National Park, Sumatra. The fungal isolation followed the method described by Lisa et al. [24], which involved cutting a 1×1 cm² section of the termite nest and inoculating it onto a PDA medium. These isolates were then incubated in a Memmert incubator at 37℃ for 24 to 72 hours. The fungi were re-inoculated onto a fresh PDA medium to obtain a pure culture. The fungi were subsequently propagated by inoculating them onto PDA and incubating them at room temperature for 14 days. Microscopic observations were performed using the slide culture technique [25].

2.2 Propagation of fungi on rice media

Making rice media was carried out using a method modified from the study [26] by washing the rice until clean, then steaming it for 15 minutes and cooling it. Next, 50 grams of rice media were weighed and placed in heat-resistant plastic, then sterilised using an autoclave. Inoculation was carried out by transferring pieces of fungus from PDA media into rice media. Next, the fungus was incubated for 21 days at room temperature.

2.3 Taking test termites

The termites used in this study were *Coptotermes curvignathus* (worker caste), collected from termite nests in Banda Aceh, Sumatra. To maintain a controlled and stable environment, the termites were acclimatised for one week at room temperature in a storage box at the Biosystematics Laboratory, Universitas Syiah Kuala, Indonesia. During this acclimatisation period, the termites were fed paper bait.

2.4 Preparation of inoculum suspension

The inoculum suspension is prepared by taking 100 grams of rice media with fungus growing on it and placing it in a mortar. Then 100 mL of distilled water and 1 mL of Tween 80 solution were added. The mixture of fungi, destilled water, and Tween 80 solution was crushed until soft and homogeneous. It was then filtered using filter paper and collected into an Erlenmeyer flask. After obtaining the inoculum suspension, multilevel dilution was carried out. Dilution was done by taking 1000 µL of the inoculum suspension using a micropipette and placing it in a test tube containing 9 mL of distilled water. Dilution was performed to obtain 10^8, 10^7, 10^6, and 10^5 conidia counts for the LC50 and LT50 tests.

2.5 Calculation of conidia density

The calculation of conidia density follows the method described by Sudarjat et al. [27], which involves taking 1 mL of the treatment suspension and dropping it into a Neubauer chamber hemocytometer. Conidia density was calculated under a microscope with 400x magnification using Eq. (1) [27, 28].

$$C = \frac{t}{n \times 0.25} \times 10^6 \quad (1)$$

where,

C = Conidia density per mL

T = The total number of conidia in the sample box is observed

n = Number of sample boxes (5 large boxes × 16 small boxes)

0.25 = The correction factor uses a small-scale sample box on the haemocytometer

2.6 Testing the virulence

Before being applied to termites, the diluted fungus

suspension is put into a spray bottle at a rate of 2 mL per bottle [29]. The dilutions tested were those with a conidia density of 10^8-10^5 for testing the LC50 and LT50 values. A total of 20 individual worker caste termites were placed in Petri dishes with a diameter of 18.5 cm lined with filter paper, which had been dripped with sterile distilled water to keep them moist. Virulence testing is carried out by spraying the suspension into a petri dish. The suspension was sprayed 20 times at 5 cm from the top of the cup until all test termites were wet with the fungal conidia suspension. Observations were carried out every 24 hours for seven days. Dead termites were observed using a Dyno lite AM211 connected to a computer. Termite mortality is calculated using Eq. (2) [30].

$$Mortality = \frac{\text{Number of termite deaths}}{\text{Number of termite tests}} \times 100\% \quad (2)$$

2.7 Parameters

This research aims to determine the Median Lethal Concentration (LC50) value and Median Lethal Time (LT50) value of the *Penicillium* sp. fungus, most effectively killing 50% of *C. curvignathus*. It also aims to know the average mortality value of *C. curvignathus* at each *Penicillium sp.* fungus conidia density.

2.8 Data analysis

The determination of Lethal Time (LT50) and Lethal Concentration (LC50) values from fungal conidia treatment was analysed using probit analysis. Data from observations of the mortality of the *C. curvignathus* caused by the fungus *Penicillium* sp. were analysed by analysis of variance (ANOVA) and F test at the 5% level using the SPSS program. If the results show a real difference, further analysis is conducted using the Duncan test at the 5% level [31].

3. RESULTS AND DISCUSSIONS

3.1 Lethal concentration (LC50) pathogenicity

We found that the LC50 value obtained for the fungus isolate *Penicillium* sp. is 1×10^7 conidia/mL (Table 1), higher than that conducted by Maketon et al. [32], who received the LC50 value of the fungus *Penicillium citrinum* to kill *Culex quinquefasciatus* was 3×10^5 conidia/mL. Isolate of the fungus *Penicillium* sp. shows that the higher the number of conidia, the higher the deaths of termites. This follows research by Maketon et al. [32], which stated that the higher the conidia density of *Aspergillus* spp., then the higher the mortality of termites.

Table 1. The average number of termite deaths and the LC50 value of the *Penicillium* sp.

Fungal Isolate	Conidia Density (conidia/mL)				LC50 Value (conidia/mL)
	10^5	10^6	10^7	10^8	
Penicillium sp.	8	9	15	18	1×10^7

Differences in LC50 values could be due to differences in the fungus isolate's virulence and the host type [33]. Furthermore, fungus genera can influence termite mortality caused by conidia's attachment to a host's cuticle [34, 35]. Fungal spores typically invade the host's body within 24 hours of contact with the insect cuticle, remaining susceptible to environmental factors such as temperature, humidity, and insect health [36]. They also encounter new challenges within the host, including humoral factors influenced by factors like food plant suitability and resource allocation. The sex and developmental stage of the insects also influence the death of test animals caused by entomopathogenic fungi.

In terms of gender differences, female mosquitoes experience higher mortality compared to male mosquitoes when exposed to the *Penicillium marneffei*. The fungus's action mechanism on adult male and female mosquitoes is through penetration at the thin areas of the cuticle after the adult mosquitoes have been sprayed with the fungus. In addition, the smaller the growth phase, the more deaths occur. This is because the immune system of insects is not yet fully developed, and their body proportions are smaller and thinner [37].

3.2 Lethal time (LT50) pathogenicity

The research results on the average number of termite deaths and LT50 values at various conidia densities (Table 2) show that in isolates of the fungus *Penicillium* sp., the higher the number of conidia, the faster it takes to kill termites. During seven days of observation, the time required to kill 50% of termites with a conidia density of 10^7 conidia/mL was 5.4 days.

Table 2. The average number of termite deaths and LT50 values at various conidia densities

Fungal Isolate	Conidia Density (conidia/mL)	(Days to)							LT50 Value (days)
		1	2	3	4	5	6	7	
Penicillium sp.	10^5	1.3	2.67	3.67	4.00	6.00	7.33	9.00	9.08
	10^6	2.3	3.00	5.00	6.00	8.33	8.67	9.00	8.4
	10^7	2.0	4.00	5.00	6.33	9.33	14.00	15.30	5.4
	10^8	7.00	9.33	11.30	14.70	16.30	17.30	17.30	3.5

The highest conidia density, 10^8 conidia/mL, killed 50% of termites in 3.5 days, while the lowest conidia density, 10^5 conidia/mL, took longer, 9.08 days. This aligns with research conducted by Kang et al. [38], which found that the higher the number of *Isaria javanica* conidia, the less time it takes to kill the *Myzus persicae* insect. The difference in termite mortality over time can be influenced by chemical compounds produced by fungi, such as secondary metabolites [11]. In addition, the secretion of cuticle-degrading enzymes such as chitinase, lipase, N-acetylglucosaminidase, and esterase is how entomopathogenic fungi infect insects [39].

The ability of entomopathogenic fungi to infect different hosts varies. This can be based on their ability to penetrate, release enzymes, and form spore propagules quickly. The fungus of *M. anisopliae* requires a shorter time to kill *A. glycines* compared to other test fungi, such as *B. bassiana* and

L. lecanii. In addition, the emulsion used to apply the mushrooms also affects the time of death [40].

3.3 Termite mortality

Based on the Lethal Concentration (LC50) value obtained, it is known that the conidia density used to test the pathogenicity of each isolate is different. The analysis of variance (ANOVA) test results in Table 3 shows that all treatments of conidia density of *Penicillium* sp. isolates significantly affect the mortality value of termites. Based on Table 3, the control treatment, 10^5 conidia/mL, and 10^6 conidia/mL were not significantly different. Treatments with conidia densities of 10^7/mL, 10^8/mL, and 10^9/mL were not significantly different, but treatments 10^8/mL and 10^9/mL were significantly different from control treatments, 10^5/mL and 10^6/mL.

Table 3. The average percentage of mortality of *C. curvignathus* after applied by *Penicillium* sp.

Conidia Density (conidia/mL)	Average Termite Mortality (%)
Control	$29.92^{a}\pm3.32$
10^5	$42.00^{ab}\pm13.60$
10^6	$41.14^{ab}\pm21.04$
10^7	$66.93^{bc}\pm25.69$
10^8	$76.92^{c}\pm22.65$
10^9	$77.71^{c}\pm10.64$

The highest mortality percentage obtained was 77.71%, with a conidia density of 10^9/mL. The lowest mortality value was obtained at a conidia density of 10^6/mL with a mortality percentage of 41.14%. This value is slightly lower than the conidia density of 10^5/mL with a percentage value of 42.00%, but these two conidia densities are not significantly different. The higher the conidia density of the *Penicillium* sp., the higher the mortality percentage of termites. This aligns with research by Guswenrivo et al. [11], which stated that the higher the conidia density of the *Penicillium* sp., the higher the mortality percentage for *Globitermes sulphureus* termites. Interestingly, the results demonstrate that the application of *Penicillium* sp. led to a higher mortality rate compared to *Metarhizium anisopliae* and *Beauveria bassiana* against dry wood termite (49.55% and 37.01%, respectively) at a conidial density of 10^7 [41]. Furthermore, these findings are consistent with those of Herlinda et al. [42], which reported the highest mortality from culture filtrate treatment with *P. citrinum* (98.67%), a rate not significantly different from that of *T. diversus* (96%) but considerably higher than the mortality rates observed with *B. bassiana* (86.67%) and *M. anisopliae* (82.67%).

The lowest percentage of termite mortality was 44.52% at a conidia density of 10^8/mL. The highest mortality was obtained at 90% at the weakest conidia density, 10^4 conidia/mL, and the lowest at the highest conidia density, 10^6 conidia/mL [43]. Differences in conidia concentration caused differences in the mortality values produced by each fungus isolate. In addition, the virulence of fungus isolates in infecting the host depends on the species and target insect. The differences in fungal strains, formulations, target insects, or arthropods will influence the results, causing varying results between studies of mycoparasite fungi [44].

Termites themselves certainly possess defences in anticipation of entomopathogens. They have adopted effective defensive strategies, such as allogrooming, to overcome fungal infections [34]. Hence, this behaviour is very effective in removing conidia from the cuticles of fellow individuals and protecting termite colonies from entomopathogenic infections [45]. The worker of *Coptotermes formosanus* removes conidia from the bodies of other individuals using their glossae and expels them after ingestion. Termites can differentiate between individuals exposed to pathogenic conidia and those not attacking; cannibalistic and burying behaviour occurs more often in termites exposed to conidia [35, 46].

3.4 Symptomatology of termite

Based on observations after spraying the fungus suspension, the movement of termites will slow down and not be as active as before spraying or at the beginning of spraying the suspension. Initially, infected termites showed a slow locomotive response and eventually died [47], and the living termites then showed more grooming activity than the dead termites. Then, the body of a dead termite changes colour from brown to black [48], which explains that the termite's body colour would turn dark sometime after death, especially on the abdomen. Besides changing colour, termites' bodies become soft, and some even crumble. After the colour changes and the body becomes smooth, the conidia will generally start to come out of the termite's body the next day. As the days go by, the fungus colonies will fill the surface of the termites' bodies (Figure 1).

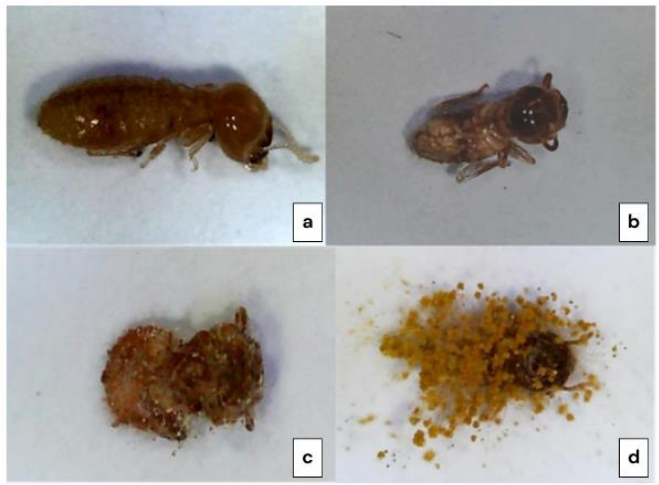

Figure 1. Body surface morphology of *C. curvignathus* after treatment with *Penicillium* sp. conidia (Dino-Lite magnification 60x). (a) Control; (b) 1st day; (c) 3rd day; (d) 7th day

Conidia generally enters the termite's body through the digestive and respiratory systems but primarily through the outer surface layer [49]. Entomopathogenic fungi can kill termites by attaching to their surface and producing enzymes and toxins that enable sprout tubes to mechanically and chemically penetrate the integument [50]. The conidia are generally tightly bound to the cuticle area with few spines; the spores will germinate and penetrate the host after the conidia attach to the cuticle [51]. After penetration, the fungus releases toxins that attack the insect's immune system. Generally, there are five stages to the fungus in killing its host, namely: 1) maximising the distribution of hyphae in the animal's body while the termite is still alive; 2) killing bacteria in the intestine using toxins; 3) intestinal extrusion, aimed at reducing the

possibility of secondary bacteriosis; 4) utilisation of host tissue for conidia production; 5) colonisation with other organisms in the formation of the resting stage [51].

Macroscopic observations of isolates of *Penicillium* sp. that grew on PDA media showed a unique appearance. Using PDA as a growth medium ensures that *Penicillium* can develop optimally. The addition of the antibiotic chloramphenicol to PDA media aims to prevent the growth of bacteria that could inhibit fungal growth [52]. Fungi that grew on the PDA medium showed a dark green colony colour at the beginning of the incubation period. As time passed, the colony turned grey with white edges and a velvety surface structure.

Penicillium commune colonies on PDA media are characteristic of an olive-grey colour that transitions to white at the periphery and a reverse side that varies from white to yellow [53]. Based on research by Ragavendran et al. [54] showed that macroscopic characteristics of isolated *P. daleae* on a petri dish with PDA medium revealed rapid growth, with a diameter of 25 to 35 mm. The colony appeared flat or slightly radially furrowed, velvety in texture, with white mycelium and very light to dark green conidiogenesis, giving the colony a granular, powdery appearance on the front and a pale yellow colour on the reverse side.

Microscopic observation showed that the conidiophores were septate and had a mono-verticillate phialide shape with no branches (Figure 2). The shape of the conidiospores is not perfectly round. Some entomopathogens of *Penicillium* produce cylindrical phialides and oval or spherical conidia (which are round like the earth). Micromorphology of *Penicillium* revealed the presence of hyaline and septate hyphae [55]. The conidiophores were smooth-walled, measuring 96.52-300 µm in length, and were bi-verticillate, attached to the septate. Metulae were found in whorls of 3-5 divergent structures. The phialides were ampulliform, and the conidia were produced in relatively long chains with a globose to sub-globose shape.

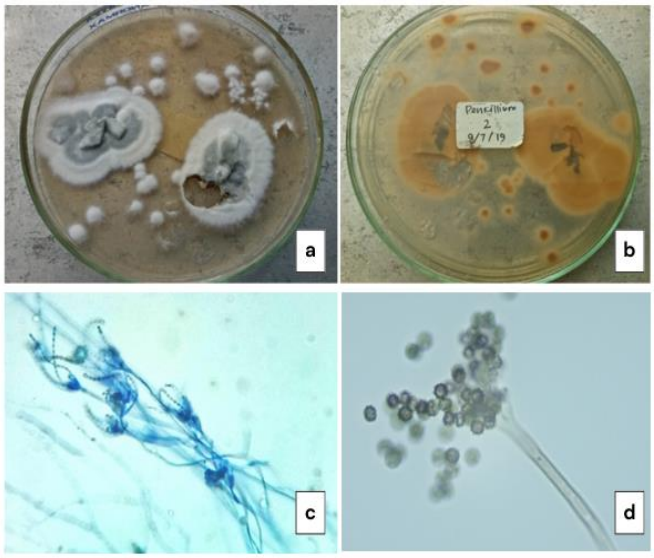

Figure 2. Macroscopic and microscopic morphology of the fungus *Penicillium* sp. a. colony surface (obverse); b. colony base (reverse); c. conidia-producing structure (400x magnification); d. conidiospore structure (1000x magnification)

Not all entomopathogenic microorganisms cause infection after entering the insect's hemocoel, which is probably correlated with the host's resistant characteristics or the entomopathogen's inability to survive and reproduce within the host's body. Many entomopathogens only infect one or a few specific insects, while some are generalists that can infect several insects across different orders. Infectious microorganisms can be categorised into four major categories: opportunistic pathogens, potential pathogens, facultative pathogens, and obligate pathogens [56].

The efficacy of *Penicillium* sp. in killing termites is influenced mainly by the chemical compounds it releases. The successful invasion and growth of entomopathogenic fungi requires the production of extracellular hydrolytic enzymes, such as protease, chitinase, lipase, and adhesive substances, which are soluble in water, allowing the hyphae to spread throughout the host's body [57]. Specifically, the colonisation process of *Penicillium* sp. begins with the attachment of conidiospores to the termite exoskeleton. If environmental conditions are favourable, the fungal spore forms a germination tube that penetrates the epicuticle using oil and protein-degrading enzymes, ultimately reaching the hypodermis, where the hyphae multiply and disseminate throughout the body cavity [34, 50]. Additionally, the production of mycotoxins from *Penicillium* sp., such as peptides/proteins and secondary metabolites, especially patulin and ochratoxins, plays a crucial role in infecting the host. *Penicillium* sp. produces secondary metabolites, including *Brevianamide*, which acted as a feeding inhibitor in *Spodoptera littoralis*, while penicillin acid was lethal to the insect [34, 58]. Ochratoxin is another toxic compound that can be produced by the genus *Penicillium,* and those various toxic secondary metabolites (mycotoxins) have severe negative impacts on consumer health; some are carcinogenic or potentially carcinogenic, while others can trigger allergies, infections, or even result in death [59].

The findings of this study demonstrate that directly applying a conidial suspension of *Penicillium* sp. through spraying resulted in a significant mortality rate of 77.71% at a conidial density of 10^9. This indicates its potential as an environmentally friendly method for termite control [42]. Similarly, Nguyen et al. [60] reported that isolates of *P. citrinum* led to high mortality and sporulation across all tested media, suggesting that *P. citrinum* could be developed into a virulent mycopesticide for managing *S. litura* and *P. xylostella*. *Penicillium* sp. efficient and cost-effective application makes it suitable for industrial use, which reassures its practicality. Traditionally, insect control, including termite management, has relied on *Metarhizium sp*. and *Beauveria* sp., which may result in termite resistance [34]. Therefore, using *Penicillium* sp. offers a promising alternative to mitigate this issue.

4. CONCLUSIONS

This study assessed the effectiveness of *Penicillium* sp. in inducing termite mortality through direct spraying of conidial suspension. The findings revealed a significant mortality rate and a rapid onset of death among the termites. Mortality rates attributed to *Penicillium* sp. varied considerably across different treatments, with the highest percentage (77.71%) observed at a conidial density of 10^9/mL. Furthermore, the lethal concentration (LC50) for *Penicillium* sp. was established at 1×10^7 conidia/mL, and the lethal time (LT50) at this density was determined to be 5.4 days. Notably, the

symptom observed in *C. curvignathus* was mycelial growth covering the insect's body. These results suggest that *Penicillium* sp. holds promise as a biological control alternative for termites, potentially providing an adequate substitute for synthetic pesticides.

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