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Pathogenicity of entomopathogenic fungi isolated from *Periplaneta americana* against *Dolichoderus* sp.

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Abstract. *Rosa E, Damayanti RD, Yuswantoro J, Indra RN, Oktariana P, Mardianto MFF, Arifiyanto A. 2022. Pathogenicity of entomopathogenic fungi isolated from* Periplaneta americana *against* Dolichoderus *sp.. Biodiversitas 23: 5461-5468. Dolichoderus* sp. was found to cause a problem for plants, buildings, and households. Many efforts have been made to regulate such animals, but they have not always been successful. Biological control has gained significance in studies as an alternative to chemical control, in which fungi and nematodes are among the successful microorganisms in managing insects. This study aimed to understand the toxicity impact of cockroach-entomopathogen against *Dolichoderus* sp. Isolated entomopathogens were then administrated to *Dolichoderus* sp. colonies under laboratory conditions to assess their mortality efficiency. The fungal suspension was sprayed on the ant colonies with concentrations of 10^7, 10^8, and 10^9 conidia/mL. *Aspergillus carbonarius* KIF3, and *Penicillium* sp. KIF4 were identified as entomopathogenic fungi. In this investigation, 15 individuals of *Dolichoderus* sp. were employed in each treatment, with three replications. Pure distilled water with 1% molasses and 0.1% Tween 80 was used as a positive control, whereas sterile distilled water was used as a negative control. The result showed that the entomopathogenic fungus *Aspergillus carbonarius* KIF3 significantly affected mortality in black ants when administered 10^9 conidia/mL conidial suspension. It was also observed that the presence of the *Dolichoderus* sp. population in nature is often associated with mealybugs. There is an opportunity to use an ecological control approach in black ant populations by utilizing entomopathogenic fungi.

Keywords: Biocontrol, cacao plant, *Dolichoderus*, entomopathogen, *Periplaneta americana*

INTRODUCTION

Ants are social insects that live in colonies. Its existence is not limited to the natural habitat but also spreads to reach human residences. They can be easily found making nests behind house tools, walls, trees, and mounds of earth (Juhász et al. 2020). Black ants are members of the Formicidae family that live in association with the mealybugs *Planococcus lilacinus* and *P. citri*. The mealybug secretes honeydew, tastes sweet, and is a food source for black ants. The association between black ant and mealybugs causes the movement of black ants to be more active on cocoa and cashew pods (Cocco et al. 2021). The presence of black ants can protect the fruit and shoots of both plants from the sucking ladybug attack, *Helopeltis* sp., and laying cocoa pod borer eggs. On the other hand, mealybugs are considered pests because they cause a decrease in cocoa productivity. This pest causes the development of cocoa pods to be hampered, the fruit shape becomes irregular, and finally causes death (Way and Khoo 1992; Offenberg 2015; Singh et al. 2021).

Insects provide vital ecological benefits to humans, such as biological pest management. Biocontrol for invasive pests is a more eco-friendly and affordable management method. For example, following its invasion of Southeast Asia in 2008, the cassava mealybug *Phenacoccus manihoti* (Hemiptera: Pseudococcidae) was biologically controlled in Thailand by introducing and then releasing the host-specific parasitoid *Anagyrus lopezi* (Hymenoptera: Encyrtidae) (Thancharoen et al. 2018). The use of biocontrols in the control of mealybugs has been widely developed. On the other hand, chemical control is frequently utilized, owing to the poor adaptation of the primary natural enemies to the climatic conditions of the crop habitat. As an alternative, pheromones are promoted to monitor the mealybug in citrus due to reason commercially accessible. Unfortunately, this effort is constrained by many mealybug populations and the wide distribution of plantations that require mass trapping. Another viable method for controlling mealybugs is to improve biological control by managing ant numbers, considering the role of black ants in the ecology of mealybugs as a protector (Franco et al. 2004). Bio-control comprises a variety of frequently utilized alternatives, such as natural predators, antagonism, or other organisms employed for pest management. Biological agents include yeast strains, molds, beneficial insects, and other non-microbial pests such as probiotics and bacteria (Legein et al. 2020). Each use of a bio-control agent has its considerations, especially the suitability factor for the target organism. For example, insects considered problems with rapid colony growth require direct targeting efforts in

the form of eradication (Lahlali et al. 2022). On the other hand, even though the results are adequate, the use of chemical pesticides leaves other environmental problems, especially the safety factor of crop yields for humans (Nicolopoulou-Stamati et al. 2016).

The entomopathogenic approach is another solution that has also been widely promoted because it works specifically on target organisms, is easy to propagate, inexpensive, and environmentally friendly (Bamisile et al. 2021). *Beauveria bassiana*, *Metarhizium anisopliae*, and *Isaria fumosorosea* are a group of fungi that are often used as biological control agents (Robles-acosta et al. 2019). Studies of controlling cockroach using biological agent was reported to be successful using entomopathogenic fungi. Interestingly, it has high mortality to the target (Rosa et al. 2020), but the type of mold has not been identified. In addition, entomopathogenic fungi have not yet been tested for their susceptibility to non-target tissues and other kinds of insects, whether they still have good mortality. Therefore, this study aimed to obtain information about the effect of cockroach-entomopathogen fungal toxicity on *Dolichoderus* sp.

MATERIALS AND METHODS

Isolation and identification of entomopathogenic fungi

Rejuvenating strain

Fungi were isolated from *Periplaneta americana* by a wet chamber technique (Gandarilla-Pacheco et al. 2021). First, cockroach body parts were immersed in 70% ethyl alcohol for a half minute and washed with sterile distilled water twice in a row for 2 minutes. Then, they were placed on wetted tissue in a chamber and incubated at 28°C for 3-5 days. Next, growing fungi were transferred on PDA media (Potato Dextrose Agar). After obtaining the purified culture, morphology was observed by slide culture (72 hours) under a microscope. Finally, the characteristics (the mycelium, conidia, and conidia-producing bodies) of fungi were compared to each other.

DNA amplification

Mycelia from fungal isolates were collected and extracted using Qiagen's DNeasy kit: (i) The mycelia of fungus were crushed till smooth in mortal; (ii) The blended mycelia were supplemented with 400 µL buffer AP1, 40 µL PVP 26%, and 4 µL RNase (100 mg/mL in a stock solution) into a microtube, then homogenized using a vortex; (iii) It was incubated in a water bath at 65°C for a half hour, and then the solution was centrifuged.

Fungal-DNA was amplified using primers ITS1 and ITS4. The amplification process began with creating a PCR mix comprised of HotStar mix PCR (Qiagen), primers ITS 1 and ITS 4, DNA working, and DDH_2O. The PCR mix solution was then placed in the PCR equipment for in vitro DNA amplification. The solution was loaded into the PCR machine, and amplified with an initial denaturation at 95°C for 5 minutes, followed by denaturation at 94°C for 1 minute, annealing at 55.4°C for 1 minute, and a final extension at 72°C for 10 minutes. The DNA amplification steps were repeated 35 times till the final extension stage. The DNA amplification procedure was completed in 2 hours and 16 minutes (Janik et al. 2020).

Separation

DNA amplification product separation was carried out using a horizontal electrophoresis method. This method used 2% agarose and Tris Acetate EDTA (TAE) buffer 1 x. The results of separation were then inserted into a gel documentation-transilluminator machine. Finally, the separation DNA amplification product was fixed to determine whether the amplification process was successful and the size of the amplification product was also selected (Nas et al. 2021).

DNA sequencing

The successfully amplified PCR results from the fungus strains were forwarded to IPB Bogor-Genetic Science in Jakarta for sequencing. The Basic Local Alignment Search Tool (BLAST) was used to assess the sequencing findings. Meanwhile, sequencing data in Fasta format was displayed in a phylogenetic tree analysis of neighbor-joining relationships compiled using the bootstrap method using Mega11 software (Arifiyanto et al. 2020).

Susceptibility host test

The fungal suspension (10^8 conidia/mL) was scratched into the surface of bulb onion slices, corn, rice, mustard, cabbage, Chinese cabbage, curly lettuce, bean, coyote, cucumber, dragon fruit, long bean, tomato, watermelon, and tubers of garlic and carrot. The infected plant sections were placed in a sterile chamber (15x60 mm) and stored for 48 hours. Then, every day for a week, observations were made to record the symptoms (color-changing, necrosis, soft rot) on the infected plant tissues (Aeny et al. 2020).

Hydrolase activity screening

Fungi were screened for their ability to produce hydrolase enzymes, such as hydrolyzing fats, proteins, and chitin. Screening was performed according to Rosa et al. (2020). Screening results were recorded as characteristics of entomopathogenic fungi.

Ants maintenance

The exact size of adults *Dolichoderus* sp. were collected from cacao plants in The Center for Study of Agricultural Technology's (BPTP) experimental garden located in Hajimena Natar, South Lampung. The ants obtained from the field were brought to the laboratory for testing, identified to the genus level by Hashimoto (2003), and acclimatized in maintenance jars for two days. During the acclimatization period, the ants were fed with fresh fish heads.

Preparation of spore suspension

After incubating *Aspergillus* sp. and *Penicillium* sp. isolates for 48 hours, spores were collected and suspended in a test tube with 10 mL sterile distilled water, 1% molasses, and 0.1 percent tween 80. The spore suspension was then homogenized with a vortex mixer. Finally, spore

density in suspension was estimated using a hemocytometer and calculated using a formula (Yunizar 2018) as follows:

$$c = \frac{t}{n\ x\ 0.25} 10^6$$

Where:
C : Spore density per mL of solution
t : Total number of spores in the observed sample box
n : Number of sample boxes used
0.25 : Small-scale sample box correction factor

After obtaining an appropriate amount of spore density, a desire dilution of spore suspension i.e. 10^7, 10^8, and 10^9 was made.

Fungal application to ants

15 ants were used in each container. The fungal suspension was sprayed with a predetermined concentration of 10^7, 10^8 and 10^9 conidia/mL, and each treatment was repeated three times. Observations were made after the application of 1, 3, 6, 12, and 48 hours. The ants were declared dead if they did not move for 15 minutes after being touched. The percentage of *Dolichoderus* sp. death was calculated using the formula:

$$P = (\frac{X}{Y})\ x\ 100\%$$

Where:
P : Death percentages
x : Total number of death ants
y : Total of ants

Analysis of data

This study employed a Randomized Block Design with two parameters: spore density and mortality observation time. The acquired data were analyzed using non-parametric test followed by Kruskal-Wallis test analysis at a significant level of 5%.

RESULTS AND DISCUSSION

Results

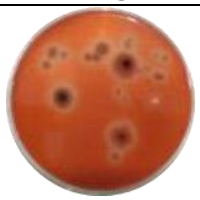
Nuclear ribosomal DNA (rDNA) markers were commonly utilized in fungal phylogenetic and systematic research. The findings of fungal strain identification using rDNA with internal transcribed spacers ITS1 and ITS4 revealed that strain IK3 was *Aspergillus carbonarius*. Then, DNA sequence was sent to GenBank and recorded as *Aspergillus carbonarius* isolate KIF 3, with accession number MW466772. In addition, strain IK4 was identified as *Penicillium* sp., then DNA sequence was deposited in GenBank as *Penicillium* sp. isolate KIF 4 with accession number MW466802 (Figure 1).

The two fungi have the capacity to hydrolyze various essential substrates, including fat, protein, and chitin (Table 1). In addition, strains KIF3 and KIF4 were able to hydrolyze fatty acid and protein chains, which was shown from the clear zone formation in substrate media. Unfortunately, chitinase was absent in KIF4 even though the colony growth was expressed.

Table 2 shows the results of scratching the two fungi on various vegetable and fruit commodities to identify the spectrum of hosts that may be colonized if insects transfer spores to other tissues. Carrying the fungus in the insect body has the possibility to spread to agricultural goods. It was also observed that KIF 3 fungus could grow on most vegetables, fruits, and staple food grains.

The ant colony of *Dolichoderus* sp. had a synergistic relationship with mealybug. In field observation, the black ant protected mealybug from attack by other insects, while the mealybug secreted a sugar that the ant colony likes (Figure 2). Unfortunately for cocoa and fruit plants, mealybug attacks usually damage the appearance generally. Their presence was also often accompanied by the growth of other co-parasites, especially borer pests that damaged fruit tissue and cocoa fruit was no exception.

Table 1. Hydrolase activity of isolates *Aspergillus carbonarius* KIF 3, and *Penicillium* sp. KIF 4

Hydrolase activity	Fungal strains			
	KIF3	Result	KIF4	Result
Lipase		+		+
Protease		+		+
Chitinase		+		-

Note: - : No activity; + : Showing the capability of hydrolase enzyme

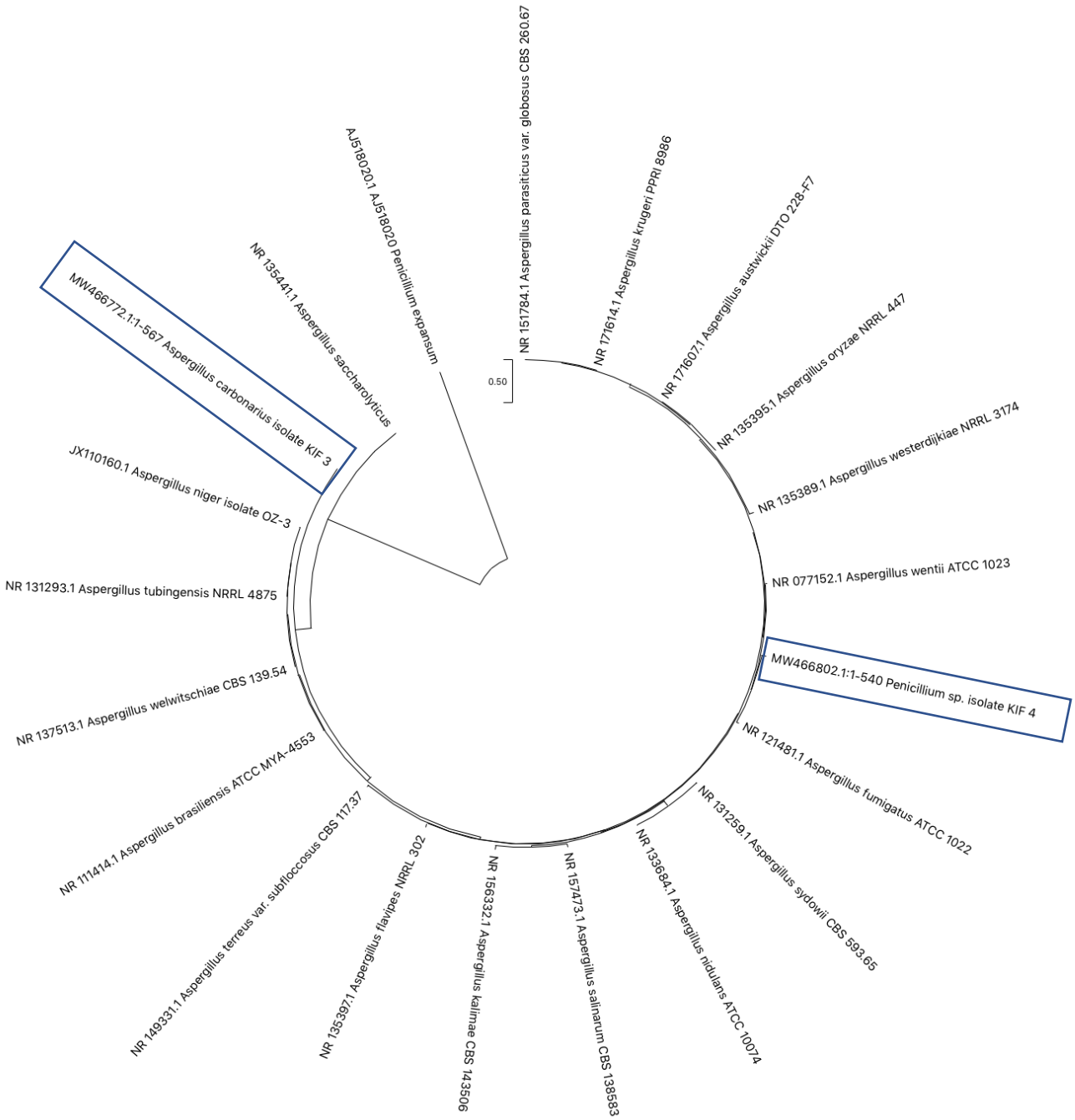


Figure 1. Phylogenetic tree of *Aspergillus carbonarius* isolate KIF 3, and *Penicillium* sp. isolate KIF 4 using the neighbor-joining method

The mortality rate of *Dolichoderus* sp. was evaluated from 3 hours to 48 hours. During 48 hours, the number of ant death rates increased compared to the initial hours of observation. Higher concentrations of suspended spores did not increase the mortality rate of ants. Therefore, the concentration of 10^7 was more lethal than 10^8 and 10^9 conidia/mL. The KIF 3 strain had a more significant impact on ant mortality than KIF 4. According to the result of Levene's test analysis (Table 3), the significance level of the mean on each treatment group was less than 0.05. This indicates that each data group comes from a population with a different variance (not homogeneous)**.** As a result, data analysis employed a non-parametric approach, beginning with the Kolmogorov-Smirnov Test. The following case assumptions were used:

H0: Observation data of fungal spore suspension with an observation period of 3-48 hours with normal distribution.

H1: Observation data of fungal spore suspension with an observation period of 3-48 hours were not normally distributed.

Table 2. Observations of vegetables and fruits inoculated with fungal inoculum after 48 hours

Vegetables/fruits	Fungal strains	
	KIF3	KIF4
Bulb onion slices	**	-
Corn	**	**
Rice	**	**
Mustard	-	-
Cabbage	*	-
Chinese cabbage	*	-
Curly lettuce	-	-
Bean	**	-
Coyote	-	-
Cucumber	*	-
Dragon fruit	*	-
Long bean	*	-
Tomato	*	-
Watermelon	*	-
Carrot	*	-
Tuber garlic	**	-
Orange	**	**
Grapes	**	**
Pears	**	**
Citrus	**	**
-	No changes	
*	More mature and dark color	
**	Mycelia proliferate, and there are black/green spores.	

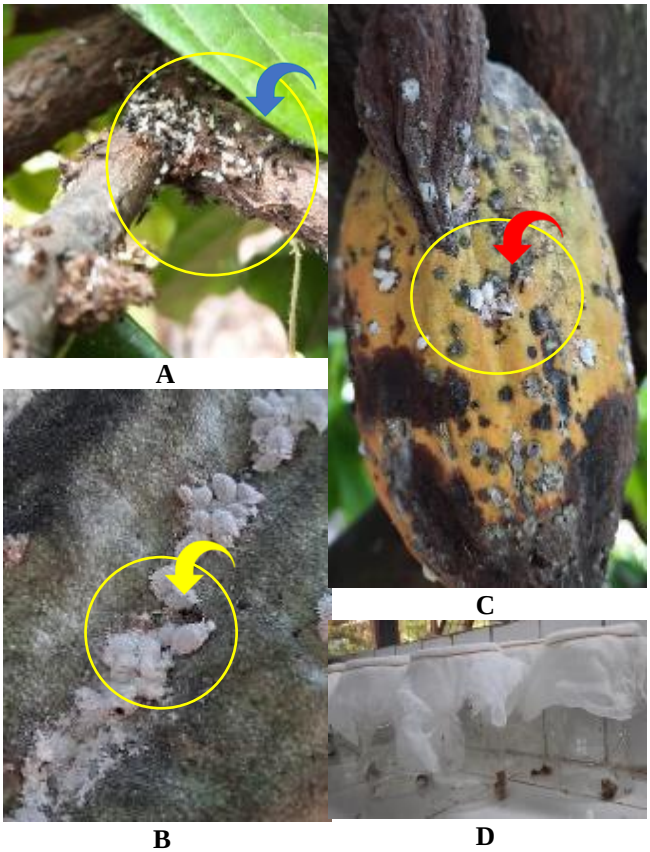


Figure 2. Black ants (*Dolichoderus* sp.) colony on cacao branch tree (A), sucking sugar from mealybugs (B), co-parasites that cause damage to ready-to-harvest fruit in cocoa (C), and black ants rearing in the laboratory (D).

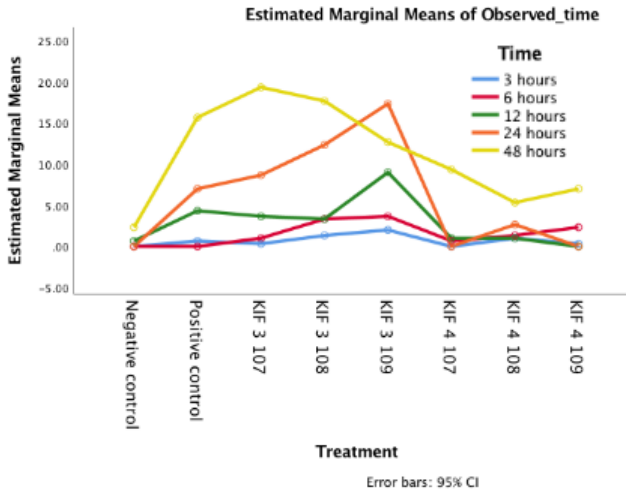


Figure 3. Rate of ant's mortality at fungal spore-administration during 3-48 hours

Table 3. Levene's test of equality of error variances[a,b]

	Levene statistic	df1	df2	Sig.
Observed_time based on mean	5.648	39	80	.000

Note: Test the null hypothesis that the error variance of the dependent variable is equal across groups. a. Dependent variable: Observed_time; b. Design: Intercept + Treatment + Time + Treatment * Time

Table 4. One-sample Kolmogorov-Smirnov Test

		Mortality	Fungal_ treatment	Observed_ time
N		120	120	120
Normal parameters[a,b]	Mean	4.4583	4.5000	3.0000
	Std. Deviation	6.28978	2.30089	1.42014
Most extreme differences	Absolute	.239	.118	.159
	Positive	.227	.118	.159
	Negative	-.239	-.118	-.159
Test statistic		.239	.118	.159
Asymp. Sig. (2-tailed)		.000[c]	.000[c]	.000[c]

Note: a. Test distribution is normal; b. Calculated from data; c. Lilliefors significance correction

According to the initial analytical findings, the value of asymp sig (2-tailed) in the Kolmogorov-Smirnov Test (Table 4) for two-sided testing was 0.000[c]. Because the probability value (p) < 0.05, H0 was rejected, and H1 was accepted, which means that the observation data for the treatment of fungal spore suspension with an observation period of 3-48 hours was not normally distributed. Further tests were conducted using the Kruskal-Wallis test as an alternative, whereas this test is an analysis of variance that does not require normally distributed data. Based on the results, it was noticed that the observation time with the highest number of ant deaths was at 48 hours with an average of 337.08 (Table 5). H0 was rejected because the value of asymp.Sig. was <0.005 (Table 6).

The administration of *Aspergillus* sp. KIF 3 treatment with a spore suspension concentration of 10^7 conidia/mL showed that the minimal spore dose was sufficient to impact the death of black ants. The average data for *Aspergillus* sp. KIF 3 treatment with a spore suspension concentration of 10^7 conidia/mL was ranked first with an average of 334.91. In other words, H0 was rejected because the value of asymp Sig. was <0.005 (Table 8). The results of fungal inoculation showed that there were no injuries, holes or even rot on the fruits and cocoa fruits were ripe after five days of observation (Figure 4).

Table 5. Kruskal-Wallis Test on the correlation between mortality rate of ants toward observed time

	Ranks		
	Observation_time	**N**	**Mean rank**
Mortality	3 hours	17	25.41
	6 hours	37	56.96
	12 hours	69	142.65
	24 hours	144	282.36
	48 hours	268	337.08
	Total	535	

Note: Hypothesis: H0: μ1 = μ2 = μ3 = μ4; H1: At least one observation time shows the difference in the average death rate of black ants with other treatments

Table 6. Correlation test between mortality number of ants toward observed time

	Mortality
Kruskal-Wallis H	211.856
df	4
Asymp. Sig.	.000

Note: a. Kruskal Wallis Test; b. Grouping Variable: Observed time

Table 7. Kruskal-Wallis Test on the correlation between mortality number of ants toward fungal treatment

	Ranks		
	Fungal_treatment	**N**	**Mean rank**
Mortality	Negetive control	9	39.11
	Positive control	83	243.98
	Aspergillus n9	134	314.92
	Aspergillus n8	114	298.92
	Aspergillus n7	99	334.91
	Penicillium n9	29	124.81
	Penicillium n8	34	90.85
	Penicillium n7	33	201.11
	Total	535	

Note: Hypothesis: H0: μ1 = μ2 = μ3 = μ4; H1: At least one fungal treatment shows the difference in the average death rate of black ants with other treatments

Table 8. Correlation test between mortality number of ants toward fungal treatment

	Mortality
Kruskal-Wallis H	133.470
df	7
Asymp. Sig.	.000

Note: a. Kruskal Wallis Test; b. Grouping Variable: Fungal treatment

Discussion

Aspergillus carbonarius is a toxic pathogenic fungus that can infect a wide range of plant hosts and release ochratoxin A (OTA). For effective target colonization, virulence, and toxin release, pathogenic fungi must adapt efficiently to alterations in ambient pH (Barda et al. 2020). In order to survive, this type of fungus tries to adapt to acidity conditions through the mechanism of acidifying its environmental conditions. This mechanism is achieved with the help of the production of gluconic acid (GLA). The presence of OTA toxin can help infect the host in the grape group. The KIF 3 strain grows and infects fruits and seeds. The present finding supports this opinion that post-harvest deterioration of numerous fruits and vegetables is usually caused by *Aspergillus carbonarius*. The acidity of the apple fruit environment as a host increases through the secretion of organic acids assisted by maceration and fruit colonization by *Penicillium expansum* (Maor et al. 2020). Individual ant mortality was assumed to be high in treating the KIF 3 strain due to various factors, namely toxins and chitinase. The *Aspergillus* genus is known for producing toxins. *Aspergillus fumigatus* is even used to help kill several grain-eating fleas in feed mills through its toxins. The toxicity of ochratoxin A (OTA) did not only work on microbes but also had a significant impact on animals, including insects (Latgé and Chamilos 2019; Pfliegler et al. 2020). The results confirmed that *Aspergillus carbonarius* KIF 3 was more dominant in producing the enzyme chitinase. A similar finding was also reported by Rosa et al. (2020).

Chitinase activity is thought to help in destroying the exoskeleton of black ants. The cuticle or exoskeleton of insects is an extracellular matrix composed chiefly of two structural biopolymers, chitin and protein. Through every molt stage, molting liquid proteases such as epidermal chitinases establish a new cuticle while degrading the interior section of the chitinous procuticle of the overlaying old exoskeleton (Zhao et al. 2018). Chitinolytic enzymes, also known as chitinases, are peptides that can hydrolyze the -1,4 bond of N-acetyl glucosamine groups in chitin. Chitin is a moderately resistant insoluble amino polysaccharide and a critical structural component in a variety of species, including worms, arthropods (exoskeleton), fungi (cell wall), crustaceans (shells), and shellfish (radula) (da Silva and do Couto 2019).

Chitinase has long attracted attention, especially as a biocontrol agent in controlling microbes and insects (Arifiyanto et al. 2021; Delfini et al. 2021). Microbial chitinases are also gaining popularity as potential plant pest control agents. These enzymes can be utilized directly as biocontrol agents or in collaboration with chemical pesticides or other biopesticides to reduce their environmental effects and improve their performance (Berini et al. 2019). For example, using chitinase from biological agents to control mosquitoes carrying Plasmodium parasites can reduce the impact of the malaria spread (Arifiyanto et al. 2021).

A **B** **C**

Figure 4. Entomopathogenic fungi (EPF) scratched on cocoa fruits to know the effect of EPF biocontrol treatment. A. Observation in Day 1 after inoculation with EPF: Left control, middle treated with KIF 3, right treated with KIF4; B. Observation in Day 3 after inoculation with EPF: Left control, middle treated with KIF 3, right treated with KIF4; C. Observation in Day 5 after inoculation with EPF: Left control, middle treated with KIF 3, right treated with KIF4

In addition, the ability to kill *Aedes aegypti* mosquito larvae using chitinase-producing agents was also reported to have success (Lestari et al. 2022), which means it can suppress the spread of dengue virus outbreaks. In other words, chitinase also controls the spread of disease outbreaks that threaten human health. Therefore, it is possible that the results of this research can also be developed in that direction.

Penicillium KIF 4, on the other hand, although not as effective as strain KIF 3 in determining the death rate of *Dolichoderus* sp., produced some intriguing results. The genus *Penicillium*, along with the *Streptomyces* group of bacteria, is a primary producer of antibiotics. *Penicillium ochrochloron* exhibited strong antifeedant and growth inhibitory activity against *Helicoverpa armigera* (Patil and Jadhav 2015), and *Penicillium* sp. was also successful in controlling the rust red beetle (*Tribolium castaneum*) (Al-Keridis 2015). *Penicillium* sp. antifungal protein (PAF) has the potential to be further developed and tested, for example, as a protective agent against *Botrytis cinerea* infection in tomato plant leaves (Tóth et al. 2020) and the pine weevil *Hylobius abietis* (Azeem et al. 2013).

Ants are inseparable insects in human life. Since these animals play an essential role in ecosystems as environmental cleaners, unfortunately, their presence often causes harm to humans. In the case of *Dolichoderus* sp., which lives in association with mealybugs, the presence of ants should be a concern. Controlling mealybugs by neglecting ant control achieves obstacles, as research notes given by Franco et al. (2004). The entomopathogenic fungus *Aspergillus carbonarius* KIF3 strongly impacts death rates in black ants when supplemented with spore at a density of 10^9 conidia/mL, this showed that the toxicity impact of cockroach-entomopathogen against *Dolichoderus* sp. information about mealybugs control integrated with the management of *Dolichoderus* sp. ants can play a role in improving and maintaining the quality of cocoa fruit trees. From another perspective, the existence of ants closely related to humans is another consideration that the biocontrol findings of *Dolichoderus* sp. ants can be applied to other species of ants.

Entomopathogenic fungi (EPF) have been found as prospective biocontrol agents in the management of insect pest populations without affecting non-target insects (Sabbahi et al. 2022). In addition, EPF can have a lethal impact not only on the initial insect host but also on other insect organisms but is limited to insect groups only. EPF is extensively used to control a wide variety of arthropod pests. They are effective against various insects from various feeding guilds, including aphids, locusts, thrips, grubs, moths, mites, mosquitoes, whiteflies, ants, and tephritid fruit flies. EPF is also harmful to phytopathogenic nematodes and other soil-borne pest (Bamisile 2021). The results showed that EPF treatment was not able to rot the fruit of cocoa. On the contrary, the fruits ripened day by day after five days of observation. In addition, in a study using *Metarhizium anisopliae*, it was shown to recover spontaneously infected cocoa from adult *Helopeltis theivora*. *Metarhizium anisopliae* decreased *H. theivora* incidence in cocoa in cage and field experiments. The usage of *M. anisopliae* proved more successful in the field by protecting cocoa pods from *H. theivora* invasion (Hausrao et al. 2022). Supporting with another argument came from the finding about the application of *B. bassiana* isolates that also protected cocoa seedlings, not only the fruit body (Ambele et al. 2020). EPF application as a biocontrol proved safe in protecting cocoa fruit and was found to control black ant colonization.

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