BUKTI KORESPONDENSI
ARTIKEL JURNAL INTERNASIONAL BEREPUTASI

Judul artikel : First report of entomopathogenic fungi from South Sumatra (Indonesia): pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*
Jurnal : BIODIVERSITAS
Penulis: Indri Ramayanti, Siti Herlinda, Ahmad Muslim, Hamzah Hasyim

Bukti korespondensi

No.	Perihal	Tanggal
1.	Bukti Konfirmasi submit paper dan full paper yang disubmit	22 September 2022
2.	Bukti konfirmasi review pertama dan hasil revisi pertama	22 Oktober 2022
3.	Bukti konfirmasi paper accepted, uncorrected Proof dan hasil koreksi penulis	3 November 2022
4	Bukti tagihan untuk penerbitan artikel	3 November 2022

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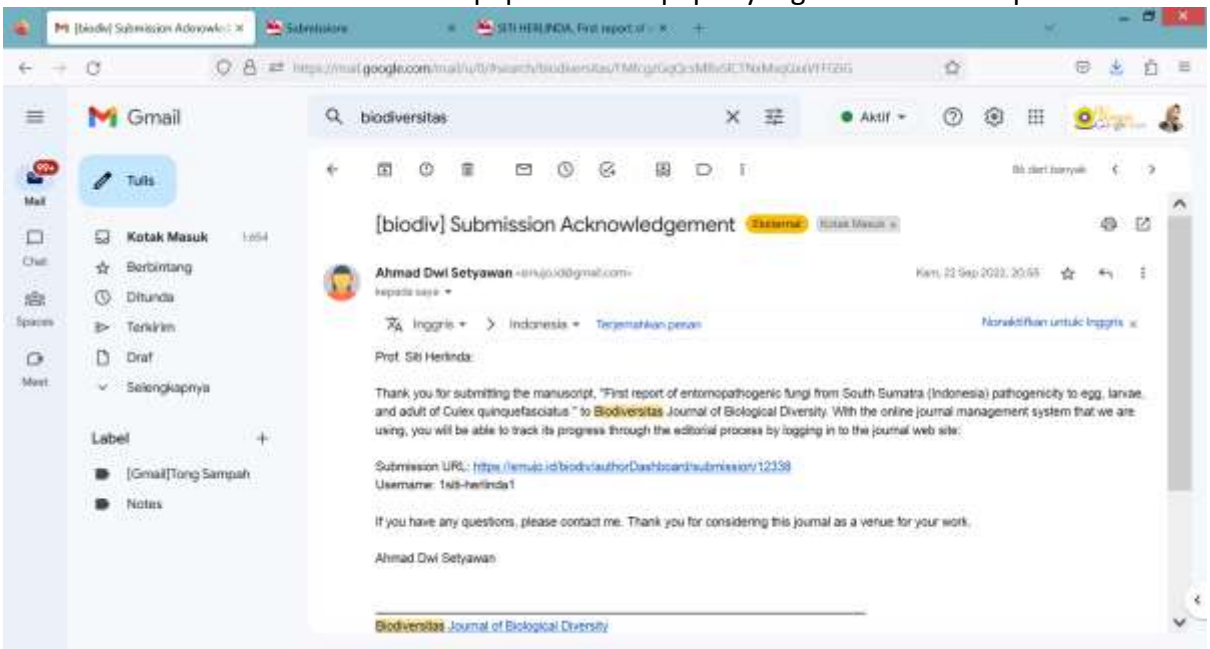

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First report of entomopathogenic fungi from South Sumatra (Indonesia) pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

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BIODIVERSITAS ISSN: 1412-033X

Volume 23, Number 11, November 2022 E-ISSN: 2085-4722

Pages: xxxx DOI: 10.13057/biodiv/d2311xx

First report of entomopathogenic fungi from South Sumatra (Indonesia) pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

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Manuscript received: 22 September 2022. Revision accepted: .....

Abstract. This study aimed to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Culex quinquefasciatus*. The fungal isolates used were eight isolates from South Sumatra and have been identified molecularly. The fungal species that were the most pathogenic to the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *Penicillium citrinum* (BKbTp isolate), and *Talaromyces diversus* (MSwTp1 isolate). The *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The most pathogenic fungal species to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). Finally, The entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as the ovicide, larvicide, and adulticide.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, lymphatic filariasis, *Penicillium citrinum*, *Talaromyces diversus*, *Purpureocillium lilacinum*

Abbreviations (if any): -

Running title: First report of entomopathogenic fungi from South Sumatra

INTRODUCTION

Filariasis or elephantiasis or lymphatic filariasis is an infectious disease caused by the parasitic filarial worms, such as *Wuchereria bancrofti* (Pratiwi et al., 2019) and *Brugia* sp. (Intarapuk and Bhumiratana, 2021). This worm is transmitted by vector insects of the mosquitoes, especially *Culex* (Blut, 2013). There are more than 38 species of mosquitoes that act as vectors of filariasis transmission (Famakinde, 2018), including *Culex quinquefasciatus* (Simonsen and Mwakitalu, 2013; Susilowati, 2018), *Culex vishnui* (Nchoutpouen *et al.*, 2019), *Mansonia africana* and *Mansonia unifo* (Ughasi et al., 2012). Indonesia is an endemic area for lymphatic filariasis (Ginandjar et al., 2018), especially in South Sumatra (Nurjazuli and Santjaka, 2020). The consequences of this elephantiasis disease can cause physical disability, mental, social, and financial losses (Enciso et al., 2021). The higher population density of vector insects tends to be positively correlated with the higher transmission rate of this filarial worms and the higher the number of elephantiasis sufferers (Gordon et al., 2018; Ridha et al., 2020; Santoso et al., 2021).

To suppress and break the transmission of the lymphatic filariasis, the vector insects of transmission needs to be controlled and the population suppressed as low as possible so that the chain of transmission is broken. Some methods have been carried out to decline the population density of filariasis vector insects. For example, *Cx. quinquefasciatus* has been controlled using a repellent insecticide (Aguiar et al., 2015). Control with botanical insecticides has also been carried

out, for example the use of rosmarin leaf oil to kill the larvae of *Cx. quinquefasciatus* (Susilowati, 2018). These vector insects are generally controlled with synthetic insecticides (Nchoutpouen et al., 2019). However, routinely spraying of synthetic insecticides causes the new problems due to the higher level of *Cx. quinquefasciatus* resistance and it has been reported that this mosquito is resistant to permethrin, deltamethrin, DDT (dichloro-diphenyl-trichloroethane) (Nchoutpouen *et al.*, 2019), and bendiocarb (Talipouo et al., 2021). Besides, residues of the synthetic insecticides may cause the non-target animals killed, the human health problems, and the water, air, and soil pollution (Hamid et al., 2017). The use of synthetic insecticides also causes the high operational costs for application or spraying (Chowański et al., 2014).

Currently, mosquito control has used many biocontrol agents, for example the use of entomopathogenic fungi (pathogens that cause insect disease or insect pathology). The use of entomopathogenic fungi have occured, for example in Thailand, conidia of *Penicillium citrinum* has been tested to be effective in killing the larvae of *Cx. quinquefasciatus* (Maketon et al., 2014). In India, the mycelia extract of *Beauveria bassiana* has been tested to be effective in killing larvae. of *Cx. quinquefasciatus* (Vivekanandhan et al., 2018). The entomopathogenic fungi found in Indonesia, especially in South Sumatra have been tested and effectively killed some species of insect pests attacking plants (Herlinda et al., 2020a, 2020b; Gustianingtyas *et al.*, 2021; Herlinda et al., 2021). Although many species of entomopathogenic fungi have been found in South Sumatra, there is no information on the effectiveness of these entomopathogenic fungi to kill the filariasis vector mosquito, *Cx. quinquefasciatus*. The previous study is only the pathogenicity of the entomopathogenic fungi to kill the egg, larvae, and adult of *Aedes aegypti* (Ramayanti et al., 2022). The novelty of this research is that the entomopathogenic fungi from South Sumatra and was first tested to kill eggs, larvae, and adults of *Cx. quinquefasciatus.* This research is feasible because the fungi as potential biological control agents are specific strains from South Sumatra so that they do not disturb the natural balance of existing microorganisms and *Cx. quinquefasciatus* is the main vector of filariasis which needs to be controlled. The purpose of this study was to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. quinquefasciatus.*

MATERIALS AND METHODS

Fungal preparation

The fungal isolates used for this current research were from the Laboratory of Entomology collection and were identified molecularly. The fungal species identified were *B. bassiana* TaAlPA isolate (GenBank acc. no. OM791688), *B. bassiana* LtKrLH isolate (GenBank acc. no. OM791680), *B. bassiana* TaLmME isolate (GenBank acc. no. OM791687), and *B. bassiana* TaPsBA isolate (GenBank acc. no. OM791689) (Ramayanti et al., 2022), *P. citrinum* BKbTp isolate (GenBank acc. no. MT448730), *Talaromyces diversus* MSwTp1 isolate (GenBank acc. no. MT448731), *B. bassiana* BSwTd4 isolate (GenBank acc. no. MT448732), and *Metarhizium anisopliae* MT488733 isolate (GenBank acc. no. MT488733) (Herlinda et al., 2020a) (Table 1). The fungi were originated from South Sumatra, Indonesia with location, Alang-alang Lebar, Palembang (2°56′32″S 104°42′16″E), Kota Raya, Lahat (3°46′38″S 103°35′25″E), Lebak, Muara Enim (3°23′51″S 104°19′41″E), Purwosari, Banyuasin (2°52′19″S 104°33′14″E), Talang Patai, Pagar Alam (4°00′45″S 103°12′21″E) and Talang Dabok, Ogan Komering Ilir (3°00′18″S 104°40′05″E). All fungal isolates were cultured on the agar medium, Sabouraud Dextrose Agar (SDA), and for bioassay they were also re-cultured on the liquid medium, SDB (Sabouraud Dextrose Broth).

Table 1. Origin of the isolates of entomophatogenic fungi from South Sumatra, Indonesia, used in this research

Location, village or district/city	Isolate origin	Altitude (m)	Fungal spesies	Fungal isolate code	GenBank acc no.
Alang-alang Lebar, Palembang	Soil	23.0	*Beauveria bassiana* *	TaAlPA	OM791688
Kota Raya, Lahat	Insect	369.9	*Beauveria bassiana* *	LtKrLH	OM791680
Lebak, Muara Enim	Soil	33.5	*Beauveria bassiana* *	TaLmME	OM791687
Purwosari, Banyuasin	Soil	19.0	*Beauveria bassiana* *	TaPsBA	OM791689
Talang Patai, Pagar Alam	Soil	175.0	*Penicillium citrinum***	BKbTp	MT448730
Talang Dabok, Ogan Komering Ilir	Soil	24.0	*Talaromyces diversus***	MSwTp1	MT448731
Talang Patai, Pagar Alam	Soil	193.0	*Beauveria bassiana***	BSwTd4	MT448732
Talang Patai, Pagar Alam	Soil	193.0	*Metarhizium anisopliae***	MSwTp3	MT488733

Sources: *(Ramayanti et al., 2022), **(Herlinda et al., 2020a)

Mass-rearing of *Culex quinquefasciatus*

Eggs of *Cx. quinquefasciatus* were gained from P2B2 Research and Development Loka, the Health Research and Development Center (the Balitbangkes), the Ministry of Health of Indonesia in Baturaja, South Sumatra, and *Cx. quinquefasciatus* mass-rearing have been carried out since June 2013. The *Cx. quinquefasciatus* mass-rearing for bioassay were carried out at the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. The room temperature and relative humidity during the mass-rearing were 29 ± 1°C and 84 ± 1%, respectively. The room lighting was set to photoperiod with 12 hours of light and 12 hours of dark (Kauffman et al., 2017). The emerging larvae were kept into a

transparent plastic cup (Ø 7 cm, height 9 cm) that has been disinfected and the cup was filled in 50 ml of water (Ramayanti *et al.*, 2022). The larvae were fed with dog biscuits (Vivekanandhan et al., 2018). The larvae within the plastic cup were put into a disinfected transparent plastic cage (50 x 50 x 50 cm) in order to keep the emerging adults remaining in the cage. The 10% sucrose solution infused on cotton wool for adult diet was hanged on the top of the cage. Then, an ovitrap was put in the plastic cage where the adult mosquitoes were emerged from the pupae. The ovitrap was designed as a disinfected transparent plastic cup (Ø 9 cm, height 13 cm) that had dark wall and was filled with water as much as 10 cm of a depth (Wu et al., 2013).

The bioassay of fungal pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. quinquefasciatus* was carried out at the laboratory with the average temperature and the relative humidity, 29.79 °C and 84.11%, respectively. After the fungal isolates were cultured on the SDA medium, then the fungal cultures were re-grown on the SDB medium in order to increase the fungal conidial density (Gustianingtyas *et al.*, 2020). During the process of growing the fungal culture on the SDB medium (the liquid medium) for 14 days, the culture was shaken for 7 days and then not shaken for 7 days. The conidia harvested from the liquid medium was calculated for getting conidial density used for bioassay below.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs of *Cx. quinquefasciatus* was carried out following the the method of Luz et al. (2011). The liquid fungal culture with a concentration of 1 x 10^{10} conidia mL^{-1} was poured 10 mL into the ovitrap containing 100 ml of water. At the same time, the control was only poured 10 mL of sterile distilled water. This experiment was designed using a completely randomized design with 8 isolates and control as treatments. The experiment were repeated three times. Then, the ovitrap was put in the disinfected plastic cage, and 30 gravid female adults were also put in the cage so that the female adults could lay their eggs in the ovitrap. The duration for the female adults laying their eggs were 4 x 24 hours (Blanford *et al.*, 2012). The female adults were also provided with 10% sucrose solution for their diet. The ovitrap containing the eggs laid of 24 hours' duration was replaced from the cage, and then the number of the eggs laid were counted. The viable eggs (the hatched eggs) were monitored and recorded every 2 hours for 48 hours. The egg morphology changing were also recorded daily. The abortion or unhatched eggs were cultured in SDA medium in order to detect and to identify the microorganism that caused abortion or unhatched. The larvae emerging were observed daily and the dead larvae were counted every day. The dead pupae were also monitored every day until adult emerging.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the larvae of *Cx. quinquefasciatus* was carried out following the the method of Alkhaibari et al. (2017). The 30 third-instar larvae were treated with 10 ml suspension of the entomopathogenic fungal isolate, the fungal suspension was put in a disinfected transparent plastic cups (Ø 7 cm, height 9 cm) with 100 ml of water inside. The 30 control larvae were only exposed to 10 mL of sterile water. All treatments were replicated three times and the the experiment was designed using a completely randomized design with 8 isolates and control as treatments. The duration of fungal exposure to the larvae were 1 x 24 hours, and then the dead larvae were monitored and recorded daily for 8 days. The variables observed were the number of larval deaths and the morfology changes of larvae after being treated with the fungi. The time of larval death and the behavior of unhealthy larvae were also observed every day. The time of larval death were used to determine of LT_{50} (the Lethal Time) and LT_{95}. The cadaver or dead larvae were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the adults of *Cx. quinquefasciatus* was carried out following the the method of Blanford et al. (2012) and Shoukat et al. (2020). Fifteen female and 15 male adults (total of 30 adults) per isolate were treated with the fungal suspension for 24 hours. The adults used in this experiment were 3 days old. Ten mL of the fungal suspension (1 x 10^{10} conidia mL^{-1}) were sprayed on the inner wall of disinfected transparent plastic cage (50 × 50 × 50 cm). Then, the cage was air-dried for 2 hours (Mnyone et al., 2011). Then, 30 adults were put in the plastic cage. For the plastic cage of control, ten mL of water were sprayed on the inner wall of the cage. All treatments were repeated three times using a completely randomized design. The adults of fungal treatment and control were given the diet of 10% sucrose solution hang on the top of the cage. After fungal exposure for 24 hours, The number of dead adults were started to be recorded after 24 hours of fungal exposure until 7 days after exposure. The dead adults were characterized with no movement occured (Shoukat et al., 2020). The time of adult death and the behavior of unhealthy adults were also observed daily. The time of adult death were used to determine of LT_{50} and LT_{95}. The cadaver or dead adult were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

Data analysis

The data of egg, larval, and pupal mortality of *Cx. quinquefasciatus*, LT_{50} and LT_{95} of the larvae; , adult mortality, LT_{50} and LT_{95} of *Cx. quinquefasciatus* of each treatment were analyzed using ANOVA (analysis of variance). If there were differences among data of treatments, the data were statistically compared with HSD (Tukey's Honestly Significant) at a 5% level of significance. LT_{50} and LT_{95} value were subjected to probit analysis. Differences in LT_{50} and LT_{95} value were compared by ANOVA and were statistically compared with HSD at a 5% level of significance. All statistical analyses

were calculated using software of SAS University Edition 2.7 9.4 M5. The malformation of eggs, larvae, pupae, and adults of of *Cx. quinquefasciatus* infected by the fungus were presented in photograph.

RESULTS AND DISCUSSION

The bioassay of fungal pathogenicity to egg of *Culex quinquefasciatus*

Obtained findings reported that eggs laid on the ovitrap by the gravid *Cx. quinquefasciatus* female of control (untreated fungal) were the least (1469.67 eggs/female) among those of fungal treatments. Egg mortality of *Cx. quinquefasciatus* of control was the lowest (16.76%) and significantly different from those of of fungal treatments (Table 2). All isolates used were pathogenic to the eggs of *Cx. quinquefasciatus*. Egg mortality of *Cx. quinquefasciatus* caused by *B. bassiana* isolate BSwTd4 was the highest (39.94%) and was not significantly different from those caused by *B. bassiana* isolate TaLmME (38.86%), and *M. anisopliae* isolate MSwTp3 (38.75%), *B. bassiana* isolate TaPsBA (36.91%), *P.citrinum* isolate BKbTp (37.04%), and *T. diversus* isolate MSwTp1(35.66%). Thus, the most pathogenic fungal species against eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). The effect of eggs treated with the fungus still affected the emerging larvae, the larvae resulted higher mortality than those of controls, as well as the pupae from treated eggs produced higher mortality than those of controls.

The morphology of *Cx. quinquefasciatus* eggs infected with the entomopathogenic fungi showed differences from the healthy eggs of control. The body liquid of *Cx. quinquefasciatus* infected eggs had feculent color without embrio inside, while the healthy eggs of control had clearly visible color with embrio inside. The color of anterior and posterior infected eggs was darker than those of the healthy eggs.

Table 2. Effect of eggs treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on egg, larval, and pupal mortality of *Culex quinquefasciatus*

Species	Isolate code	Eggs laid per female[a)]	Egg mortality (%)[b)]	Larval mortality (%)[b)]	Pupal mortality (%)[b)]
Control	-	1469.67b	16.76d	17.59d	0.99d
Beauveria bassiana	TaAlPA	1511.00ab	32.70bc	33.33c	2.86c
Beauveria bassiana	LtKrLH	1482.67b	31.06c	30.58d	2.60c
Beauveria bassiana	TaLmME	1616.67a	38.86a	40.02a	5.06ab
Beauveria bassiana	TaPsBA	1574.33ab	36.91ab	35.23c	3.37c
Penicillum citrinum	BKbTp	1556.33ab	37.04ab	37.72b	3.75bc
Talaromyces diversus	MSwTp1	1563.67ab	35.66abc	34.41c	3.17c
Beauveria bassiana	BSwTd4	1637.33a	39.94a	41.20a	6.31a
Metarhizium anisopliae	MSwTp3	1613.33a	38.75a	40.15a	5.49a
F-value		6.20*	63.5*	345.2*	45.41*
P-value		6.50×10^{-4}	1.29×10^{-11}	2.0×10^{-16}	2.25×10^{-10}
HSD value		0.04	2.98	1.94	1.96

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using logarithmic transformation, [b)]Original data were transformed using Arcsin transformation prior to statistical analysis.

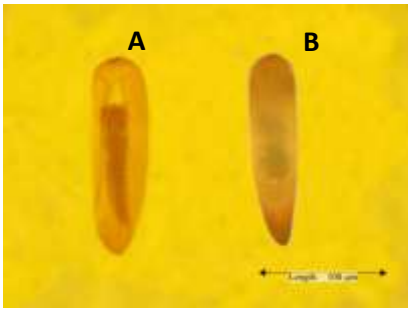

Figure 1. Morphology of the *Culex quinquefasciatus* eggs: a healthy egg of control (A) and an infected treated egg (B)

The bioassay of fungal pathogenicity to larvae of *Culex quinquefasciatus*

The third-instar larvae of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) had significantly higher mortality compared to the control larvae (untreated larvae) (Table 3). All fungal isolates used the current study were pathogenic to the third-instar larvae of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The larval mortality caused by *M. anisopliae* isolate MSwTp3 (100% with LT_{50} 2.02 days and LT_{95} 7.15 days) was the highest and not significantly different from mortality caused by *B. bassiana* isolate BSwTd4 (98.89% with LT_{50} 2.51 days and LT_{95} 7.61 days) and *B. bassiana* isolate TaLmME (97.78% with LT_{50} 2.75 days and LT_{95} 7.85

days). The most pathogenic fungal species to the third-instar larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Obtained findings highlighted that *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) possessed larvicidal activity.

The fungal infected larvae of *Cx. quinquefasciatus* showed typical morphology, particularly the morphology of sick and dead larvae. The sick larvae underwent a lysis gut lumen with milky color and the larvae abdomen had no distinct segment. The epithelial lining possessed milky color with a ruptured anal segment (Figure 2). The healthy larvae of control had a transparent gut lumen with a visible or recognizably different segment of abdomen. The healthy larvae also had a visible epithelial lining and an undamaged anal segment. The larval cadavers of fungal treatment cultured on SDA medium produced conidia and mycellia covering the cadaver body, while the healthy larvae were not infected or covered by fungal conidia or mycellia. The pupae emerging from the infected larvae generally underwent sick and died. The sick pupae body were thinner, hardened, and straight shaped and had the black head. The healthy pupae had fatter, round, flexible and soft body, bent like a comma shape and their head had dark-brown in color (Figure 3).

Table 3. Effect of larvae treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on larval mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Larvae mortality[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]
Control	-	0.00f	14.98a	20.21a
Beauveria bassiana	TaAlPA	84.44cd	3.97b	9.08bc
Beauveria bassiana	LtKrLH	78.89de	4.21b	9.31bc
Beauveria bassiana	TaLmME	97.78ab	2.75cd	7.85cd
Beauveria bassiana	TaPsBA	80.00cde	4.05b	9.15bc
Penicillum citrinum	BKbTp	92.22bc	3.78bc	8.88bcd
Talaromyces diversus	MSwTp1	64.44e	5.04b	10.14b
Beauveria bassiana	BSwTd4	98.89a	2.51d	7.61cd
Metarhizium anisopliae	MSwTp3	100.00a	2.02d	7.15d
F-value		155.00*	116.60*	79.77*
P-value		5.31 x 10^{-15}	6.52 x 10^{-14}	1.80 x 10^{-12}
HSD value		10.62	0.33	0.30

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)] Original data were transformed using square root (sqrt) transformation.

Figure 2. Morphology of the *Culex quinquefasciatus* larvae: a healthy larvae of control (A) and an infected treated larvae (B)

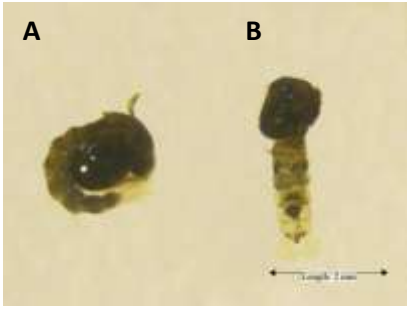

Figure 3. Morphology of the *Culex quinquefasciatus* pupae: a healthy pupae of control (A) and an infected treated pupae (B)

The bioassay of fungal pathogenicity to adult of *Culex quinquefasciatus*

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia ml^{-1}) had significantly higher mortality compared to the control adults (untreated adults) (Table 4). The adult mortality caused by *M. anisopliae*

isolate MSwTp3 (100% with LT_{50} 3.25 days and LT_{95} 6.70 days) was highest and not significantly different from those caused by *B. bassiana* isolate BSwTd4 (100% with LT_{50} 3.46 days and LT_{95} 6.76 days) and *B. bassiana* isolate TaLmME (98.89% with LT_{50} 3.70 days and LT_{95} 7.15 days), and *P.citrinum* isolate BKbTp (98.89% with LT_{50} 3.96 days and LT_{95} 7.41 days). Nevertheless, all fungal isolates in this study were pathogenic to the adults of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The most pathogenic fungal species to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). This research findings highlighted that *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate) had adulticidal activity.

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi underwent sick and finally dead. The typical symptoms of sick and dead infected adults were malformation. The unhealthy adults had the asymmetrical wing shapes, the dried and mycosis body, and a spiral shape proboscis (Figure 4). If the unhealthy adults died, their cadavers grown in SDA medium could be covered with the fungal conidia and mycellia. On the contrary, the healthy adults of control possessed the symmetrical wing shapes, and a straight shaped black proboscis, and no mycosis on the cadaver body. The healthy adults had the elongate abdomen. The cadavers from the healthy adults grown in SDA medium were not covered with the fungal conidia and mycellia.

Table 4. Effect of adults treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on adult mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Adult mortality (%)[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]
Control	-	0.00^{d}	11.99^{a}	15.44^{a}
Beauveria bassiana	TaAlPA	88.89^{b}	4.64^{c}	8.09^{bc}
Beauveria bassiana	LtKrLH	82.22^{b}	4.84^{bc}	8.29^{b}
Beauveria bassiana	TaLmME	98.89^{a}	3.70^{de}	7.15^{d}
Beauveria bassiana	TaPsBA	87.78^{b}	4.63^{c}	8.08^{bc}
Penicillum citrinum	BKbTp	98.89^{a}	3.96^{d}	7.41^{cd}
Talaromyces diversus	MSwTp1	63.33^{c}	5.37^{b}	8.82^{b}
Beauveria bassiana	BSwTd4	100.00^{a}	3.46^{de}	6.76^{d}
Metarhizium anisopliae	MSwTp3	100.00^{a}	3.25^{e}	6.70^{d}
F-value		23.11^{*}	229.30^{*}	183.60^{*}
P-value		5.85×10^{-8}	2.00×10^{-16}	1.19×10^{-15}
HSD value		24.55	0.15	0.15

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)]Original data were transformed using square root (sqrt) transformation.

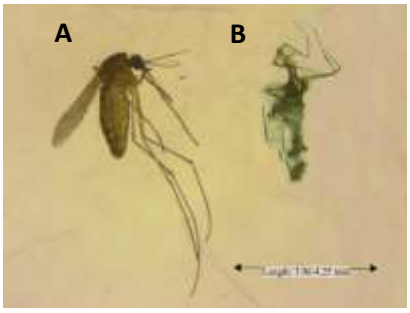

Figure 4. Morphology of the *Cx. quinquefasciatus* adults: a healthy adult of control (A) and an infected treated adult (B)

Discussion

The eggs laid by the gravid *Cx. quinquefasciatus* female of control (untreated) were the least among those of fungal treatments. However, the eggs laid by the fungal treated female were more than those of control because the ovitrap where they were laying eggs had water with dyed color due to added with the fungal suspension. The gravid female *Culex* mosquitoes preferred to lay eggs in dyed water (Day, 2016; Perea and Callaghan, 2017). Although the treated eggs laid by female *Cx. quinquefasciatus* in this study were less than 50%, but the treated eggs induced the sick larvae and produced the mortality of emerging larvae and pupae. Besides, the mycosis on the dead larvae and pupae that failed to emerge from the eggs occured. In this study, the ovitrap used to expose the fungi to the eggs of *Cx. quinquefasciatus* could effectively infected its eggs, larvae, pupae, and adults. The finding highlighted that the *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. The most pathogenic fungal species against the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The entomopathogenic fungi have reported caused the eggs

of mosquito unhatched and abortion and the emerging larvae and pupae could not carry on their life (Leles et al., 2012; Ramayanti et al., 2022). The obtained data also reported the embrio of eggs treated with the fungi were lysis and egg liquid becoming feculent color and no embrio inside the eggs.

The egg mortality of *Cx. quinquefasciatus* caused by the entomopathogenic fungi were lower than the larvae mortality caused by the fungi. The third-instar larvae of *Cx. quinquefasciatus* could be immediately killed by *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) (LT_{50} < 3 days). The high mortality of the larvae treated with the entomopathogenic fungi in short time due to higher fungal suspension used (1 x 10^{10} conidia ml^{-1}) and the fungi cultured in the broth medium (SDB). The broth culture is able to grow the fungus and produce blastospores, and the blastospores are more effective compared to aerial conidia in killing the mosquitoes (Alkhaibari et al., 2017). The findings highlighted that both species of the fungi could be develop to be a larvicide for *Cx. quinquefasciatus* because they have highest level of larvicidal activity (97.78−100% of larvae mortality). The larvae mortality caused by the entomopathogenic fungi was higher than the egg mortality caused by the entomopathogenic fungi because the cuticle of integument of the larvae is thinner than those of the eggs (Farnesi et al., 2015). The thinner was the cuticle of the insect integument, the easier the fungal conidia are penetrated into insect body (Ortiz-Urquiza and Keyhani, 2013).

The results obtained that the larvae infected by the fungi could be sick or dead because their gut lumen ruptured or lysis by the fungi. The fungi also damaged the anal and abdomen segments so that the larva body became malformation. The larvae died due to the conidia of entomopthogenic fungi germinating and their hyphae penetrating into the insect integument, after that the hyphae entered to the body cavity (Boomsma et al., 2014). The hyphae developed to become blastospores in the larvae hemolymph (Mancillas-Paredes et al., 2019). The blastospores of the entomopthogenic fungi could produce secondary metabolites, such as bassiacridin (Quesada-moraga and Vey, 2004) and beauvericin (Safavi, 2012) secreted by *B. bassiana* and destruxin produced by *M. anisopliae* (Borisade et al., 2016). The secondary metabolites or toxin could disrupt normal cell metabolism of the insect (Mancillas-Paredes et al., 2019).

Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). This research findings highlighted that besides *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates), *P.citrinum* (BKbTp isolate) was also pathogenic to the adults of *Cx. quinquefasciatus*. The results obtained that the fungal species that were pathogenic to adults were different from the species that were pathogenic to eggs and larvae of *Cx. quinquefasciatus*. The fungal species that were pathogenic to the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate), while The fungal species that were pathogenic to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). The entomopathogenic fungi could induce the adults of *Cx. quinquefasciatus* becoming malformation (e.g. asymmetrical wing, curled proboscis) and mycosis (after growing onto the SDA medium). The dead adults were caused by conidia germinating to be mycellia and penetrating into the adult body cavity producing secondary metabolites (via blastospores) disrupting normal cell metabolism of the insect (Mancillas-Paredes *et al.*, 2019). After the adults died, the fungi still grow saprophytically on the cadavers of adults and the fungi induce the cadaver body becoming mycosis (Gabarty et al., 2014).

Finally, the fungal species that were the most pathogenic to the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). The finding highlighted that the *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The most pathogenic fungal species to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). So, the entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as ovicide, larvicide, and adulticide.

ACKNOWLEDGEMENTS

This research was funded by the Directorate General of Higher Education, Ministry of Education, Culture, Research, and Technology, Republic of Indonesia, Fiscal Year 2022, in accordance with the Doctoral Dissertation Scheme (*Penelitian Disertasi Doktor*) with Contract Number: 142/E5/PG.02.00.PT/2022 on 10 May 2022, chaired by Siti Herlinda.

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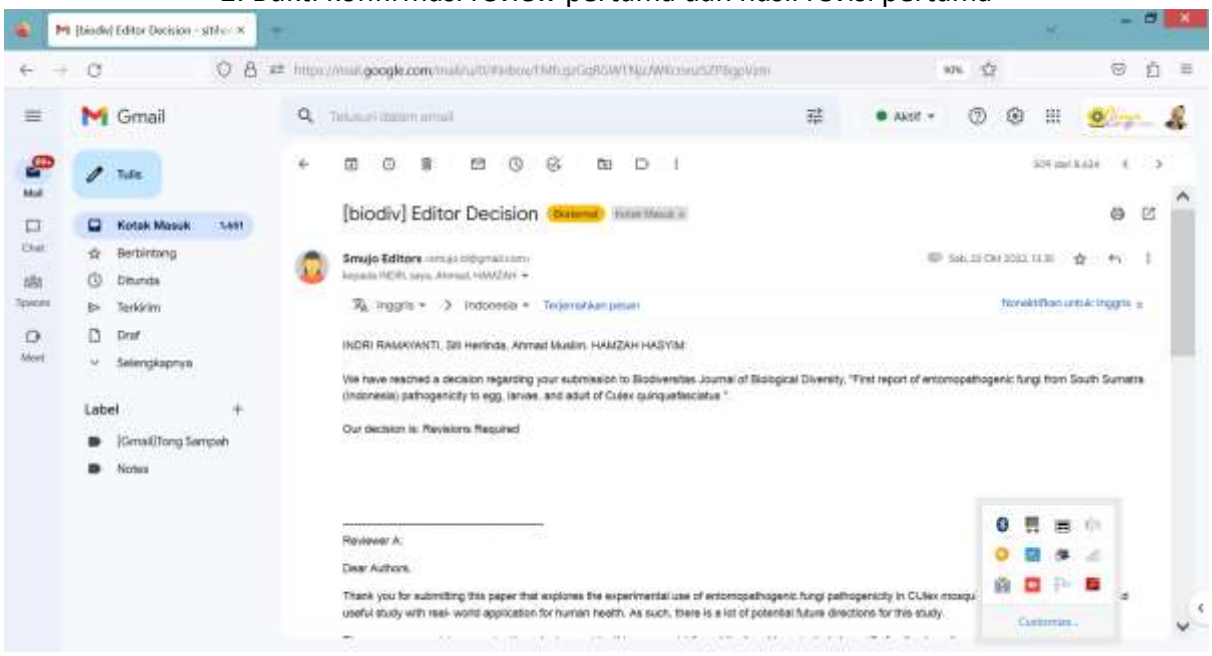
2. Bukti konfirmasi review pertama dan hasil revisi pertama



Bukti konfirmasi review pertama

First report of entomopathogenic fungi from South Sumatra (Indonesia) pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

Abstract. This[JB1] study aimed to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Culex quinquefasciatus*. The fungal isolates used were eight isolates from South Sumatra and have been identified molecularly. The fungal species that were the most pathogenic to the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* [JB2](MSwTp3 isolate), *Penicillium citrinum* (BKbTp isolate), and *Talaromyces diversus* (MSwTp1 isolate). The *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The most pathogenic fungal species to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). Finally, The entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as the ovicide, larvicide, and adulticide[JB3].

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, lymphatic filariasis, *Penicillium citrinum*, *Talaromyces diversus*, *Purpureocillium lilacinum*

Abbreviations (if any): -

Running title: First report of entomopathogenic fungi from South Sumatra

INTRODUCTION

Filariasis or elephantiasis or lymphatic filariasis is an infectious disease caused by the parasitic filarial worms, such as *Wuchereria bancrofti* (Pratiwi et al., 2019)[JB4] and *Brugia* sp. (Intarapuk and Bhumiratana, 2021). This worm is transmitted by vector insects of the mosquitoes, especially *Culex* (Blut, 2013)[JB5]. There are more than 38 species of mosquitoes that act as vectors of filariasis transmission (Famakinde, 2018), including *Culex quinquefasciatus* (Simonsen and Mwakitalu, 2013; Susilowati, 2018), *Culex vishnui* (Nchoutpouen *et al.*, 2019)[JB6], *Mansonia africana* and *Mansonia unifo* (Ughasi et al., 2012). Indonesia is an endemic area for lymphatic filariasis (Ginandjar et al., 2018), especially in South Sumatra (Nurjazuli and Santjaka, 2020). The consequences of this elephantiasis disease can cause physical disability, mental, social, and financial losses (Enciso et al., 2021). The higher population density of vector insects tends to be positively correlated with the higher transmission rate of this filarial worms and the higher the number of elephantiasis sufferers (Gordon et al., 2018; Ridha et al., 2020; Santoso et al., 2021).

To suppress and break the transmission of the lymphatic filariasis, the vector insects of transmission needs to be controlled and the population suppressed as low as possible so that the chain of transmission is broken. Some methods have been carried out to decline[JB7] the population density of filariasis vector insects. For example, *Cx. quinquefasciatus* has been controlled using a repellent insecticide (Aguiar et al., 2015). Control with botanical insecticides has also been carried out, for example the use of rosmarin[JB8] leaf oil to kill the larvae of *Cx. quinquefasciatus* (Susilowati, 2018). These vector insects are generally controlled with synthetic insecticides (Nchoutpouen et al., 2019). However, routinely spraying of synthetic insecticides causes the new problems due to the higher level of *Cx. quinquefasciatus* resistance and it has been reported that this mosquito is resistant to permethrin, deltamethrin, DDT (dichloro-diphenyl-trichloroethane) (Nchoutpouen *et al.*, 2019), and bendiocarb (Talipouo et al., 2021). Besides, residues of the synthetic insecticides may

cause the non-target animals killed, t[JB9]he human health problems, and the water, air, and soil pollution (Hamid et al., 2017). The use of synthetic insecticides also causes the high operational costs for application or spraying (Chowański et al., 2014).

Currently, mosquito control has used many biocontrol agents, for example the use of entomopathogenic fungi (pathogens that cause insect disease or insect pathology). The use of entomopathogenic fungi have occured, for example in Thailand, conidia of *Penicillium citrinum* has been tested to be effective [JB10]in killing the larvae of *Cx. quinquefasciatus* (Maketon et al., 2014). In India, the mycelia extract of *Beauveria bassiana* has been tested[JB11] to be effective in killing larvae. of *Cx. quinquefasciatus* (Vivekanandhan et al., 2018). The entomopathogenic fungi found in Indonesia, especially in South Sumatra have been tested and effectively killed some species of insect pests attacking plants (Herlinda et al., 2020a, 2020b; Gustianingtyas *et al.*, 2021; Herlinda et al., 2021). Although many species of entomopathogenic fungi have been found in South Sumatra, there is no information on the effectiveness of these entomopathogenic fungi to kill the filariasis vector mosquito, *Cx. quinquefasciatus*. The previous study is only the pathogenicity of the entomopathogenic fungi to kill the egg, larvae, and adult of *Aedes aegypti* (Ramayanti et al., 2022). The novelty of this research is that the entomopathogenic fungi from South Sumatra and was first tested to kill eggs, larvae, and adults of *Cx. quinquefasciatus.* This research is feasible because the fungi as potential biological control agents are specific strains from South Sumatra so that they do not disturb the natural balance of existing microorganisms and *Cx. quinquefasciatus* is the main vector of filariasis which needs to be controlled. The purpose of this study was to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. quinquefasciatus.*

MATERIALS AND METHODS

Fungal preparation

The fungal isolates used for this current research were from the Laboratory of Entomology[JB12] collection and were identified molecularly. The fungal species identified were *B. bassiana* TaAlPA isolate (GenBank acc. no. OM791688), *B. bassiana* LtKrLH isolate (GenBank acc. no. OM791680), *B. bassiana* TaLmME isolate (GenBank acc. no. OM791687), and *B. bassiana* TaPsBA isolate (GenBank acc. no. OM791689) (Ramayanti et al., 2022), *P. citrinum* BKbTp isolate (GenBank acc. no. MT448730), *Talaromyces diversus* MSwTp1 isolate (GenBank acc. no. MT448731), *B. bassiana* BSwTd4 isolate (GenBank acc. no. MT448732), and *Metarhizium anisopliae* MT488733 isolate (GenBank acc. no. MT488733) (Herlinda et al., 2020a) (Table 1). The fungi were originated from South Sumatra, Indonesia with location, Alang-alang Lebar, Palembang (2°56′32″S 104°42′16″E), Kota Raya, Lahat (3°46′38″S 103°35′25″E), Lebak, Muara Enim (3°23′51″S 104°19′41″E), Purwosari, Banyuasin (2°52′19″S 104°33′14″E), Talang Patai, Pagar Alam (4°00′45″S 103°12′21″E) and Talang Dabok, Ogan Komering Ilir (3°00′18″S 104°40′05″E). All fungal isolates were cultured on the agar medium, Sabouraud Dextrose Agar (SDA), and for bioassay they were also re-cultured on the liquid medium, SDB (Sabouraud Dextrose Broth).

Table 1. Origin of the isolates of entomophatogenic fungi from South Sumatra, Indonesia, used in this research

Location, village or district/city	Isolate origin	Altitude (m)	Fungal spesies	Fungal isolate code	GenBank acc no.
Alang-alang Lebar, Palembang	Soil	23.0	*Beauveria bassiana* *	TaAlPA	OM791688
Kota Raya, Lahat	Insect	369.9	*Beauveria bassiana* *	LtKrLH	OM791680
Lebak, Muara Enim	Soil	33.5	*Beauveria bassiana* *	TaLmME	OM791687
Purwosari, Banyuasin	Soil	19.0	*Beauveria bassiana* *	TaPsBA	OM791689
Talang Patai, Pagar Alam	Soil	175.0	*Penicillium citrinum***	BKbTp	MT448730
Talang Dabok, Ogan Komering Ilir	Soil	24.0	*Talaromyces diversus***	MSwTp1	MT448731
Talang Patai, Pagar Alam	Soil	193.0	*Beauveria bassiana***	BSwTd4	MT448732
Talang Patai, Pagar Alam	Soil	193.0	*Metarhizium anisopliae***	MSwTp3	MT488733

Sources: *(Ramayanti et al., 2022), **(Herlinda et al., 2020a)

Mass-rearing of *Culex quinquefasciatus*

Eggs of *Cx. quinquefasciatus* were gained from P2B2 Research and Development Loka, the Health Research and Development Center (the Balitbangkes), the Ministry of Health of Indonesia in Baturaja, South Sumatra, and *Cx. quinquefasciatus* mass-rearing have been carried out since June 2013. The *Cx. quinquefasciatus* mass-rearing for bioassay were carried out at the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. The room temperature and relative humidity during the mass-rearing were 29 ± 1°C and 84 ± 1%, respectively. The room lighting was set to photoperiod with 12 hours of light and 12 hours of dark (Kauffman et al., 2017). The emerging larvae were kept into a transparent plastic cup (Ø 7 cm, height 9 cm) that has been disinfected and the cup was filled in 50 ml of water (Ramayanti *et al.*, 2022). The larvae were fed with dog biscuits (Vivekanandhan et al., 2018). The larvae within the plastic cup were put into a disinfected transparent plastic cage (50 x 50 x 50 cm) in order to keep the emerging adults remaining in the cage. The 10% sucrose solution infused on cotton wool for adult diet was hanged[JB13] on the top of the cage. Then, an ovitrap was put in the plastic cage where the adult mosquitoes were emerged from the pupae. The ovitrap was designed as a

disinfected transparent plastic cup (Ø 9 cm, height 13 cm) that had dark wall and was filled with water as much as 10 cm of a depth [JB14](Wu et al., 2013).

The bioassay of fungal pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. quinquefasciatus* was carried out at the laboratory with the average temperature and the relative humidity, 29.79 °C and 84.11%, respectively. After the fungal isolates were cultured on the SDA medium, then the fungal cultures were re-grown on the SDB medium in order to increase the fungal conidial density (Gustianingtyas *et al.*, 2020). During the process of growing the fungal culture on the SDB medium (the liquid medium) for 14 days, the culture was shaken for 7 days and then not shaken for 7 days[JB15]. The conidia harvested from the liquid medium was calculated for getting conidial density used for bioassay below.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs of *Cx. quinquefasciatus* was carried out following the the method of Luz et al. (2011). The liquid fungal culture with a concentration of 1 x 10^{10} conidia mL^{-1} was poured 10 mL into the ovitrap containing 100 ml of water. At the same time, the control was only poured 10 mL of sterile distilled water. This experiment was designed using a completely randomized design with 8 isolates and control as treatments. The experiment were repeated three times. Then, the ovitrap was put in the disinfected plastic cage, and 30 gravid female adults were also put in the cage so that the female adults could lay their eggs in the ovitrap. The duration for the female adults laying their eggs were 4 x 24 hours (Blanford *et al.*, 2012). The female adults were also provided with 10% sucrose solution for their diet. The ovitrap containing the eggs laid of 24 hours' duration was replaced from the cage, and then the number of the eggs laid were counted[JB16]. The viable eggs (the hatched eggs) were monitored and recorded every 2 hours for 48 hours. The egg morphology changing were also recorded daily. The abortion or unhatched eggs were cultured in SDA medium in order to detect and to identify the microorganism that caused abortion or unhatched. The larvae emerging were observed daily and the dead larvae were counted every day. The dead pupae were also monitored every day until adult emerging.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the larvae of *Cx. quinquefasciatus* was carried out following the the method of Alkhaibari et al. (2017). The 30 third-instar larvae were treated with 10 ml suspension of the entomopathogenic fungal isolate, the fungal suspension was put in a disinfected transparent plastic cups (Ø 7 cm, height 9 cm) with 100 ml of water inside. The 30 control larvae were only exposed to 10 mL of sterile water. All treatments were replicated three times and the the experiment was designed using a completely randomized design with 8 isolates and control as treatments. The duration of fungal exposure to the larvae were 1 x 24 hours, and then the dead larvae were monitored and recorded daily for 8 days. The variables observed were the number of larval deaths and the morfology[JB17] changes of larvae after being treated with the fungi. The time of larval death and the behavior of unhealthy larvae [JB18]were also observed every day. The time of larval death were used to determine of LT_{50} (the Lethal Time) and LT_{95}. The cadaver or dead larvae were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the adults of *Cx. quinquefasciatus* was carried out following the the method of Blanford et al. (2012) and Shoukat et al. (2020). Fifteen female and 15 male adults (total of 30 adults) per isolate were treated with the fungal suspension for 24 hours. The adults used in this experiment were 3 days old. Ten mL of the fungal suspension (1 x 10^{10} conidia mL^{-1}) were sprayed on the inner wall of disinfected transparent plastic cage (50 × 50 × 50 cm). Then, the cage was air-dried for 2 hours (Mnyone et al., 2011). Then, 30 adults were put in the plastic cage. For the plastic cage of control, ten mL of water were[JB19] sprayed on the inner wall of the cage. All treatments were repeated three times using a completely randomized design. The adults of fungal treatment and control were given the diet of 10% sucrose solution hang on the top of the cage. After fungal exposure for 24 hours, The number of dead adults were started to be recorded after 24 hours of fungal exposure until 7 days after exposure. The dead adults were characterized with no movement occured (Shoukat et al., 2020). The time of adult death and the behavior of unhealthy adults were also observed daily. The time of adult death were used to determine of LT_{50} and LT_{95}. The cadaver or dead adult were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

Data analysis

The data of egg, larval, and pupal mortality of *Cx. quinquefasciatus*, LT_{50} and LT_{95} of the larvae; , adult mortality, LT_{50} and LT_{95} of *Cx. quinquefasciatus* of each treatment were analyzed using ANOVA[JB20] (analysis of variance). If there were differences among data of treatments, the data were statistically compared with HSD (Tukey's[JB21] Honestly Significant) at a 5% level of significance. LT_{50} and LT_{95} value were subjected to probit analysis. Differences in LT_{50} and LT_{95} value were compared by ANOVA and were statistically compared with HSD at a 5% level of significance. All statistical analyses were calculated using software of SAS University Edition 2.7 9.4 M5. The malformation of eggs, larvae, pupae, and adults of of *Cx. quinquefasciatus* infected by the fungus were presented in photograph.

RESULTS AND DISCUSSION

The bioassay of fungal pathogenicity to egg of *Culex quinquefasciatus*

Obtained findings reported that eggs laid on the ovitrap by the gravid *Cx. quinquefasciatus* female of control (untreated fungal) were the least (1469.67[JB22] eggs/female) among those of fungal treatments. Egg mortality of *Cx. quinquefasciatus* of control was the lowest (16.76%) and significantly different from those of of fungal treatments (Table 2). All isolates used were pathogenic to the eggs of *Cx. quinquefasciatus*. Egg mortality of *Cx. quinquefasciatus* caused by *B. bassiana* isolate BSwTd4 was the highest (39.94%) and was not significantly different from those caused by *B. bassiana* isolate TaLmME (38.86%), and *M. anisopliae* isolate MSwTp3 (38.75%), *B. bassiana* isolate TaPsBA (36.91%), *P.citrinum*[JB23] isolate BKbTp (37.04%), and *T. diversus* isolate MSwTp1(35.66%). Thus, the most pathogenic fungal species against eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). The effect of eggs treated with the fungus still affected the emerging larvae, the larvae resulted higher mortality than those of controls, as well as the pupae from treated eggs produced higher mortality than those of controls.

The morphology of *Cx. quinquefasciatus* eggs infected with the entomopathogenic fungi showed differences from the healthy eggs[JB24] of control. The body liquid of *Cx. quinquefasciatus* infected eggs had feculent color without embrio[JB25] inside, while the healthy eggs of control had [JB26]clearly visible color with embrio[JB27] inside. The color of anterior and posterior infected eggs was darker than those of the healthy eggs.

Table 2. Effect of eggs treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on egg, larval, and pupal mortality of *Culex quinquefasciatus*

Species	Isolate code	Eggs laid per female[a)]	Egg mortality (%)[b)]	Larval mortality (%)[b)]	Pupal mortality (%)[b)]
Control	-	1469.67b	16.76d	17.59d	0.99d
Beauveria bassiana	TaAlPA	1511.00ab	32.70bc	33.33c	2.86c
Beauveria bassiana	LtKrLH	1482.67b	31.06c	30.58d	2.60c
Beauveria bassiana	TaLmME	1616.67a	38.86a	40.02a	5.06ab
Beauveria bassiana	TaPsBA	1574.33ab	36.91ab	35.23c	3.37c
Penicillum citrinum	BKbTp	1556.33ab	37.04ab	37.72b	3.75bc
Talaromyces diversus	MSwTp1	1563.67ab	35.66abc	34.41c	3.17c
Beauveria bassiana	BSwTd4	1637.33a	39.94a	41.20a	6.31a
Metarhizium anisopliae	MSwTp3	1613.33a	38.75a	40.15a	5.49a
F-value		6.20*	63.5*	345.2*	45.41*
P-value		6.50×10^{-4}	1.29×10^{-11}	2.0×10^{-16}	2.25×10^{-10}
HSD value		0.04	2.98	1.94	1.96

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using logarithmic transformation, [b)]Original data were transformed using Arcsin transformation prior to statistical analysis.

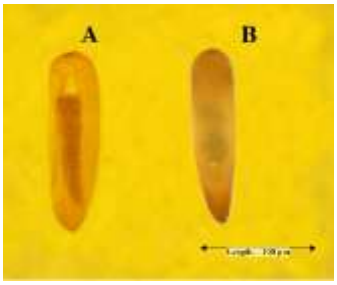


Figure 1. Morphology of the *Culex quinquefasciatus* eggs: a healthy egg of control (A) and an infected treated egg (B)

The bioassay of fungal pathogenicity to larvae of *Culex quinquefasciatus*

The third-instar larvae of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) had significantly higher mortality compared to the control larvae (untreated larvae) (Table 3). All fungal isolates used the current study were pathogenic to the third-instar larvae of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The larval mortality caused by *M. anisopliae* isolate MSwTp3 (100% with LT_{50} 2.02 days and LT_{95} 7.15 days) was the highest and not significantly different from mortality caused by *B. bassiana* isolate BSwTd4 (98.89% with LT_{50} 2.51 days and LT_{95} 7.61 days) and *B. bassiana* isolate TaLmME (97.78% with LT_{50} 2.75 days and LT_{95} 7.85 days). The most pathogenic fungal species to the third-instar larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Obtained findings highlighted that *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) possessed larvicidal activity.

The fungal infected larvae of *Cx. quinquefasciatus* showed typical morphology, particularly the morphology of sick and dead larvae. The sick larvae underwent a lysis g[JB28]ut lumen with milky[JB29] color and the larvae abdomen had no

distinct segment. The epithelial lining possessed milky color with a ruptured anal segment (Figure 2). The healthy larvae of control had a transparent gut lumen with a visible or recognizably different segment of abdomen. The healthy larvae also had a visible epithelial lining and an undamaged anal segment. The larval cadavers of fungal treatment cultured on SDA medium produced conidia and mycellia covering the cadaver body, while the healthy larvae were not infected or covered by fungal conidia or mycellia. The pupae emerging from the infected larvae generally underwent sick and died. The sick pupae body were thinner, hardened, and straight shaped and had the black head. The healthy pupae had fatter, round, flexible and soft body, bent like a comma shape and their head had dark-brown in color (Figure 3).

Table 3. Effect of larvae treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on larval mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Larvae mortality[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]
Control	-	0.00f	14.98a	20.21a
Beauveria bassiana	TaAlPA	84.44cd	3.97b	9.08bc
Beauveria bassiana	LtKrLH	78.89de	4.21b	9.31bc
Beauveria bassiana	TaLmME	97.78ab	2.75cd	7.85cd
Beauveria bassiana	TaPsBA	80.00cde	4.05b	9.15bc
Penicillum citrinum	BKbTp	92.22bc	3.78bc	8.88bcd
Talaromyces diversus	MSwTp1	64.44e	5.04b	10.14b
Beauveria bassiana	BSwTd4	98.89a	2.51d	7.61cd
Metarhizium anisopliae	MSwTp3	100.00a	2.02d	7.15d
F-value		155.00*	116.60*	79.77*
P-value		5.31 x 10^{-15}	6.52 x 10^{-14}	1.80 x 10^{-12}
HSD value		10.62	0.33	0.30

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)] Original data were transformed using square root (sqrt) transformation.

Figure 2. Morphology of the *Culex quinquefasciatus* larvae: a healthy larvae of control (A) and an infected treated larvae (B)

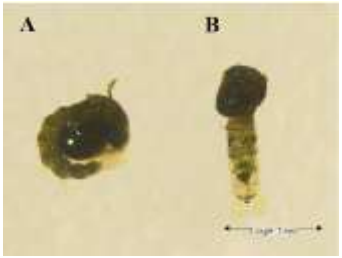


Figure 3. Morphology of the *Culex quinquefasciatus* pupae: a healthy pupae of control (A) and an infected treated pupae (B)

The bioassay of fungal pathogenicity to adult of *Culex quinquefasciatus*

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia ml^{-1}) had significantly higher mortality compared to the control adults (untreated adults) (Table 4). The adult mortality caused by *M. anisopliae* isolate MSwTp3 (100% with LT_{50} 3.25 days and LT_{95} 6.70 days) was highest and not significantly different from those caused by *B. bassiana* isolate BSwTd4 (100% with LT_{50} 3.46 days and LT_{95} 6.76 days) and *B. bassiana* isolate TaLmME (98.89% with LT_{50} 3.70 days and LT_{95} 7.15 days), and *P.citrinum* isolate BKbTp (98.89% with LT_{50} 3.96 days and LT_{95} 7.41 days). Nevertheless, all fungal isolates in this study were pathogenic to the adults of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The most pathogenic fungal species to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum*

(BKbTp isolate). This research findings highlighted that *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate) had adulticidal activity.

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi underwent s[JB32]ick and finally deadd[JB33]. The typical symptoms of sick and dead infected adults were malformation. The unhealthy adults had the asymmetrical wing shapes, the dried and mycosis body, and a spiral shape proboscis (Figure 4). If the unhealthy adults died, their cadavers grown in SDA medium could be covered with the fungal conidia and mycellia. On the contrary, the healthy adults of control possessed the symmetrical wing shapes, and a straight shaped black proboscis, and no mycosis on the cadaver body. The healthy adults had the elongate abdomen. The cadavers from the healthy adults grown in SDA medium were not covered with the fungal conidia and mycellia.

Table 4. Effect of adults treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on adult mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Adult mortality (%)[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]
Control	-	0.00^{d}	11.99^{a}	15.44^{a}
Beauveria bassiana	TaAlPA	88.89^{b}	4.64^{c}	8.09^{bc}
Beauveria bassiana	LtKrLH	82.22^{b}	4.84^{bc}	8.29^{b}
Beauveria bassiana	TaLmME	98.89^{a}	3.70^{de}	7.15^{d}
Beauveria bassiana	TaPsBA	87.78^{b}	4.63^{c}	8.08^{bc}
Penicillum citrinum	BKbTp	98.89^{a}	3.96^{d}	7.41^{cd}
Talaromyces diversus	MSwTp1	63.33^{c}	5.37^{b}	8.82^{b}
Beauveria bassiana	BSwTd4	100.00^{a}	3.46^{de}	6.76^{d}
Metarhizium anisopliae	MSwTp3	100.00^{a}	3.25^{e}	6.70^{d}
F-value		23.11^{*}	229.30^{*}	183.60^{*}
P-value		5.85×10^{-8}	2.00×10^{-16}	1.19×10^{-15}
HSD value		24.55	0.15	0.15

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)]Original data were transformed using square root (sqrt) transformation.

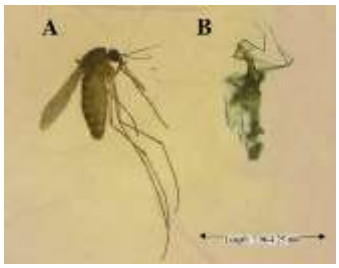


Figure 4. Morphology of the *Cx. quinquefasciatus* adults: a healthy adult of control (A) and an infected treated adult (B)

Discussion

The eggs laid by the gravid *Cx. quinquefasciatus* female of control (untreated) were the least among those of fungal treatments. However, the eggs laid by the fungal treated female were more than those of control because the ovitrap where they were laying eggs had water with dyed color due to added with the fungal suspension. The gravid female *Culex* mosquitoes preferred to lay eggs in dyed water (Day, 2016; Perea and Callaghan, 2017). Although the treated eggs laid by female *Cx. quinquefasciatus* in this study were less than 50%, but the treated eggs induced the sick larvae and produced the mortality of emerging larvae and pupae. Besides, the mycosis on the dead larvae and pupae that failed to emerge from the eggs occured. In this study, the ovitrap used to expose the fungi to the eggs of *Cx. quinquefasciatus* could effectively infected its eggs, larvae, pupae, and adults. The finding highlighted that the *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. The most pathogenic fungal species against the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The entomopathogenic fungi have reported caused the eggs of mosquito unhatched and abortion and the emerging larvae and pupae could not carry on their life (Leles et al., 2012; Ramayanti et al., 2022). The obtained data also reported the embrio[JB34] of eggs treated with the fungi were lysis and egg liquid becoming feculent color and no embrio[JB35] inside the eggs.

The egg mortality of *Cx. quinquefasciatus* caused by the entomopathogenic fungi were lower than the larvae mortality caused by the fungi. The third-instar larvae of *Cx. quinquefasciatus* could be immediately killed by *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) ($LT_{50} < 3$ days). The high mortality of the larvae treated with the entomopathogenic fungi in short time due to higher fungal suspension used (1 x 10^{10} conidia ml^{-1}) and the fungi cultured in the broth medium (SDB). The broth culture is able to grow the fungus and produce blastospores, and the blastospores are more effective compared to aerial conidia in killing the mosquitoes (Alkhaibari et al., 2017). The findings

highlighted that both species of the fungi could be develop to be a larvicide for *Cx. quinquefasciatus* because they have highest level of larvicidal activity (97.78−100% of larvae mortality). The larvae mortality caused by the entomopathogenic fungi was higher than the egg mortality caused by the entomopathogenic fungi because the cuticle of integument of the larvae is thinner than those of the eggs (Farnesi et al., 2015). The thinner was the cuticle of the insect integument, the easier the fungal conidia are penetrated into insect body (Ortiz-Urquiza and Keyhani, 2013).

The results obtained that the larvae infected by the fungi could be sick or dead because their gut lumen ruptured or lysis by the fungi. The fungi also damaged the anal and abdomen segments so that the larva body became malformation. The larvae died due to the conidia of entomopthogenic fungi germinating and their hyphae penetrating into the insect integument, after that the hyphae entered to the body cavity (Boomsma et al., 2014). The hyphae developed to become blastospores in the larvae hemolymph (Mancillas-Paredes et al., 2019). The blastospores of the entomopthogenic fungi could produce secondary metabolites, such as bassiacridin (Quesada-moraga and Vey, 2004) and beauvericin (Safavi, 2012) secreted by *B. bassiana* and destruxin produced by *M. anisopliae* (Borisade et al., 2016). The secondary metabolites or toxin could disrupt normal cell metabolism of the insect (Mancillas-Paredes et al., 2019).

Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). This research findings highlighted that besides *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates), *P.citrinum* (BKbTp isolate) was also pathogenic to the adults of *Cx. quinquefasciatus*. The results obtained that the fungal species that were pathogenic to adults were different from the species that were pathogenic to eggs and larvae of *Cx. quinquefasciatus*. The fungal species that were pathogenic to the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate), while The fungal species that were pathogenic to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). The entomopathogenic fungi could induce the adults of *Cx. quinquefasciatus* becoming malformation (e.g. asymmetrical wing, curled proboscis) and mycosis (after growing onto the SDA medium). The dead adults were caused by conidia germinating to be mycellia and penetrating into the adult body cavity producing secondary metabolites (via blastospores) disrupting normal cell metabolism of the insect (Mancillas-Paredes *et al.*, 2019). After the adults died, the fungi still grow saprophytically on the cadavers of adults and the fungi induce the cadaver body becoming mycosis (Gabarty et al., 2014)[JB36].

Finally, the fungal species that were the most pathogenic to the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). The finding highlighted that the *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The most pathogenic fungal species to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). So, the entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as ovicide, larvicide, and adulticide.

ACKNOWLEDGEMENTS[JB37]

This research was funded by the Directorate General of Higher Education, Ministry of Education, Culture, Research, and Technology, Republic of Indonesia, Fiscal Year 2022, in accordance with the Doctoral Dissertation Scheme (*Penelitian Disertasi Doktor*) with Contract Number: 142/E5/PG.02.00.PT/2022 on 10 May 2022, chaired by Siti Herlinda.

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[JB38]

Hasil revisi pertama

First report of entomopathogenic fungi from South Sumatra (Indonesia) pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

Abstract. Mosquito control has currently used many biocontrol agents, such as the entomopathogenic fungi. So, the study aimed to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Culex quinquefasciatus.* The fungal isolates used were eight isolates from South Sumatra and have been identified molecularly. The fungal species that were the most pathogenic to the eggs of *Cx. quinquefasciatus* were *Beauveria bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *Metarhizium anisopliae* (MSwTp3 isolate), *Penicillium citrinum* (BKbTp isolate), and *Talaromyces diversus* (MSwTp1 isolate). The *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The most pathogenic fungal species to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P. citrinum* (BKbTp isolate). Finally, The entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as the ovicide, larvicide, and adulticide. The future application of the fungi against the mosquito eggs, larvae, and adults can be used an ovitrap, fungal spores formulated in a synthetic oil, and fungus-impregnated black cloths, respectively.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, lymphatic filariasis, *Penicillium citrinum*, *Talaromyces diversus*, *Purpureocillium lilacinum*

Abbreviations (if any): -

Running title: First report of entomopathogenic fungi from South Sumatra

INTRODUCTION

Filariasis or elephantiasis or lymphatic filariasis is an infectious disease caused by the parasitic filarial worms, such as *Wuchereria bancrofti* (Pratiwi et al. 2019) and *Brugia* sp. (Intarapuk and Bhumiratana, 2021). This worm is transmitted by vector insects of the mosquitoes, especially *Culex* (Blut 2013). There are more than 38 species of mosquitoes that act as vectors of filariasis transmission (Famakinde 2018), including *Culex quinquefasciatus* (Simonsen and Mwakitalu, 2013; Susilowati, 2018), *Culex vishnui* (Nchoutpouen et al. 2019), *Mansonia africana* and *Mansonia unifo* (Ughasi et al., 2012). Indonesia is an endemic area for lymphatic filariasis (Ginandjar et al., 2018), especially in South Sumatra (Nurjazuli and Santjaka, 2020). The consequences of this elephantiasis disease can cause physical disability, mental, social, and financial losses (Enciso et al., 2021). The higher population density of vector insects tends to be positively correlated with the higher transmission rate of this filarial worms and the higher the number of elephantiasis sufferers (Gordon et al. 2018; Ridha et al. 2020; Santoso et al. 2021).

To suppress and break the transmission of the lymphatic filariasis, the vector insects of transmission needs to be controlled and the population suppressed as low as possible so that the chain of transmission is broken. Some methods have been carried out to reduce the population density of filariasis vector insects. For example, *Cx. quinquefasciatus* has been controlled using a repellent insecticide (Aguiar et al. 2015). Control with botanical insecticides has also been carried

out, for example the use of rosmarin leaf oil (*Rosmarinus officinalis* L) to kill the larvae of *Cx. quinquefasciatus* (Susilowati 2018). These vector insects are generally controlled with synthetic insecticides (Nchoutpouen et al. 2019). However, routinely spraying of synthetic insecticides causes the new problems due to the higher level of *Cx. quinquefasciatus* resistance and it has been reported that this mosquito is resistant to permethrin, deltamethrin, DDT (dichloro-diphenyl-trichloroethane) (Nchoutpouen et al. 2019), and bendiocarb (Talipouo et al. 2021). Residues of the synthetic insecticides may cause the non-target animals killed, and the insecticides induce the human health problems and the pollution on water, air, and soil (Hamid et al. 2017). The use of synthetic insecticides also causes the high operational costs for application or spraying (Chowański et al. 2014).

Currently, mosquito control has used many biocontrol agents, for example the use of entomopathogenic fungi (pathogens that cause insect disease or insect pathology). The use of entomopathogenic fungi have occured, for example in Thailand, conidia of *Penicillium citrinum* has been found to be effective in killing the larvae of *Cx. quinquefasciatus* (Maketon et al. 2014). In India, the mycelia extract of *Beauveria bassiana* has been found to be effective in killing larvae. of *Cx. quinquefasciatus* (Vivekanandhan et al. 2018). The entomopathogenic fungi found in Indonesia, especially in South Sumatra have been tested and effectively killed some species of insect pests attacking plants (Herlinda et al. 2020a, 2020b; Gustianingtyas et al. 2021; Herlinda et al. 2021). Although many species of entomopathogenic fungi have been found in South Sumatra, there is no information on the effectiveness of these entomopathogenic fungi to kill the filariasis vector mosquito, *Cx. quinquefasciatus*. The previous study is only the pathogenicity of the entomopathogenic fungi to kill the egg, larvae, and adult of *Aedes aegypti* (Ramayanti et al. 2022). The novelty of this research is that the entomopathogenic fungi from South Sumatra and was first tested to kill eggs, larvae, and adults of *Cx. quinquefasciatus.* This research is feasible because the fungi as potential biological control agents are specific strains from South Sumatra so that they do not disturb the natural balance of existing microorganisms and *Cx. quinquefasciatus* is the main vector of filariasis which needs to be controlled. The purpose of this study was to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. quinquefasciatus.*

MATERIALS AND METHODS

Fungal preparation

The fungal isolates used for this current research were from the collection of Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya and they were identified molecularly. The fungal species identified were *B. bassiana* TaAlPA isolate (GenBank acc. no. OM791688), *B. bassiana* LtKrLH isolate (GenBank acc. no. OM791680), *B. bassiana* TaLmME isolate (GenBank acc. no. OM791687), and *B. bassiana* TaPsBA isolate (GenBank acc. no. OM791689) (Ramayanti et al., 2022), *P. citrinum* BKbTp isolate (GenBank acc. no. MT448730), *Talaromyces diversus* MSwTp1 isolate (GenBank acc. no. MT448731), *B. bassiana* BSwTd4 isolate (GenBank acc. no. MT448732), and *Metarhizium anisopliae* MT488733 isolate (GenBank acc. no. MT488733) (Herlinda et al., 2020a) (Table 1). The fungi were originated from South Sumatra, Indonesia with location, Alang-alang Lebar, Palembang (2°56′32″S 104°42′16″E), Kota Raya, Lahat (3°46′38″S 103°35′25″E), Lebak, Muara Enim (3°23′51″S 104°19′41″E), Purwosari, Banyuasin (2°52′19″S 104°33′14″E), Talang Patai, Pagar Alam (4°00′45″S 103°12′21″E) and Talang Dabok, Ogan Komering Ilir (3°00′18″S 104°40′05″E). All fungal isolates were cultured on the agar medium, Sabouraud Dextrose Agar (SDA), and for bioassay they were also re-cultured on the liquid medium, SDB (Sabouraud Dextrose Broth).

Table 1. Origin of the isolates of entomophatogenic fungi from South Sumatra, Indonesia, used in this research

Location, village or district/city	Isolate origin	Altitude (m)	Fungal spesies	Fungal isolate code	GenBank acc no.
Alang-alang Lebar, Palembang	Soil	23.0	*Beauveria bassiana*	TaAlPA	OM791688*
Kota Raya, Lahat	Insect	369.9	*Beauveria bassiana*	LtKrLH	OM791680*
Lebak, Muara Enim	Soil	33.5	*Beauveria bassiana*	TaLmME	OM791687*
Purwosari, Banyuasin	Soil	19.0	*Beauveria bassiana*	TaPsBA	OM791689*
Talang Patai, Pagar Alam	Soil	175.0	*Penicillium citrinum*	BKbTp	MT448730**
Talang Dabok, Ogan Komering Ilir	Soil	24.0	*Talaromyces diversus*	MSwTp1	MT448731**
Talang Patai, Pagar Alam	Soil	193.0	*Beauveria bassiana*	BSwTd4	MT448732**
Talang Patai, Pagar Alam	Soil	193.0	*Metarhizium anisopliae*	MSwTp3	MT488733**

Sources: *(Ramayanti et al. 2022), **(Herlinda et al. 2020a)

Mass-rearing of *Culex quinquefasciatus*

Eggs of *Cx. quinquefasciatus* were gained from P2B2 Research and Development Loka, the Health Research and Development Center (the Balitbangkes), the Ministry of Health of Indonesia in Baturaja, South Sumatra, and *Cx. quinquefasciatus* mass-rearing have been carried out since June 2013. The *Cx. quinquefasciatus* mass-rearing for bioassay were carried out at the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. The room temperature and relative humidity during the mass-rearing were 29 ± 1°C and 84 ± 1%, respectively. The room lighting was set to photoperiod with 12 hours of light and 12 hours of dark (Kauffman et al., 2017). The emerging larvae were kept into a

transparent plastic cup (Ø 7 cm, height 9 cm) that has been disinfected and the cup was filled in 50 ml of water (Ramayanti *et al.*, 2022). The larvae were fed with dog biscuits (Vivekanandhan et al., 2018). The larvae within the plastic cup were put into a disinfected transparent plastic cage (50 x 50 x 50 cm) in order to keep the emerging adults remaining in the cage. The 10% sucrose solution infused on cotton wool for adult diet was hung on the top of the cage. Then, an ovitrap was put in the plastic cage where the adult mosquitoes were emerged from the pupae. The ovitrap was designed as a disinfected transparent plastic cup (Ø 9 cm, height 13 cm) that had dark wall and was filled with water to a depth of 10 cm (Wu et al. 2013).

The bioassay of fungal pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. quinquefasciatus* was carried out at the laboratory with the average temperature and the relative humidity, 29.79 °C and 84.11%, respectively. After the fungal isolates were cultured on the SDA medium, then the fungal cultures were re-grown on the SDB medium in order to increase the fungal conidial density (Gustianingtyas *et al.*, 2020). During the process of growing the fungal culture on the SDB medium (the liquid medium) for 14 days, the culture was shaken continuously for 7 days and then not shaken for 7 days. The conidia harvested from the liquid medium was calculated for getting conidial density used for bioassay below.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs of *Cx. quinquefasciatus* was carried out following the the method of Luz et al. (2011). The liquid fungal culture with a concentration of 1 x 10^{10} conidia mL^{-1} was poured 10 mL into the ovitrap containing 100 ml of water. At the same time, the control was only poured 10 mL of sterile distilled water. This experiment was designed using a completely randomized design with 8 isolates and control as treatments. The experiment were repeated three times. Then, the ovitrap was put in the disinfected plastic cage, and 30 gravid female adults were also put in the cage so that the female adults could lay their eggs in the ovitrap. The duration for the female adults laying their eggs were 4 x 24 hours (Blanford *et al.*, 2012). The female adults were also provided with 10% sucrose solution for their diet. The ovitrap containing the eggs laid was replaced daily from the cage, and then the number of the eggs laid were also counted every day. The viable eggs (the hatched eggs) were monitored and recorded every 2 hours for 48 hours. The egg morphology changing were also recorded daily. The abortion or unhatched eggs were cultured in SDA medium in order to detect and to identify the microorganism that caused abortion or unhatched. The larvae emerging were observed daily and the dead larvae were counted every day. The dead pupae were also monitored every day until adult emerging.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the larvae of *Cx. quinquefasciatus* was carried out following the the method of Alkhaibari et al. (2017). The 30 third-instar larvae were treated with 10 ml suspension of the entomopathogenic fungal isolate, the fungal suspension was put in a disinfected transparent plastic cups (Ø 7 cm, height 9 cm) with 100 ml of water inside. The 30 control larvae were only exposed to 10 mL of sterile water. All treatments were replicated three times and the the experiment was designed using a completely randomized design with 8 isolates and control as treatments. The duration of fungal exposure to the larvae were 1 x 24 hours, and then the dead larvae were monitored and recorded daily for 8 days. The variables observed were the number of larval deaths and the morphology changes of larvae after being treated with the fungi. The time of larval death and the behavior of unhealthy larvae were also observed every day. The health of the larvae identified by observing the changes of the larvae behavior and morphology. The time of larval death were used to determine of LT_{50} (the Lethal Time) and LT_{95}. The cadaver or dead larvae were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the adults of *Cx. quinquefasciatus* was carried out following the the method of Blanford et al. (2012) and Shoukat et al. (2020). Fifteen female and 15 male adults (total of 30 adults) per isolate were treated with the fungal suspension for 24 hours. The adults used in this experiment were 3 days old. Ten mL of the fungal suspension (1 x 10^{10} conidia mL^{-1}) were sprayed on the inner wall of disinfected transparent plastic cage (50 × 50 × 50 cm). Then, the cage was air-dried for 2 hours (Mnyone et al., 2011). Then, 30 adults were put in the plastic cage. For the plastic cage of control, ten mL of water was sprayed on the inner wall of the cage. All treatments were repeated three times using a completely randomized design. The adults of fungal treatment and control were given the diet of 10% sucrose solution hang on the top of the cage. After fungal exposure for 24 hours, The number of dead adults were started to be recorded after 24 hours of fungal exposure until 7 days after exposure. The dead adults were characterized with no movement occured (Shoukat et al., 2020). The time of adult death and the behavior of unhealthy adults were also observed daily. The time of adult death were used to determine of LT_{50} and LT_{95}. The cadaver or dead adult were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

Data analysis

The data of egg, larval, and pupal mortality of *Cx. quinquefasciatus*, LT_{50} and LT_{95} of the larvae; , adult mortality, LT_{50} and LT_{95} of *Cx. quinquefasciatus* of each treatment were analyzed using ANOVA (analysis of variance). We implemented the parametric statistical analysis, and therefore all data were tested for normal distribution using the Shapiro–Wilk test and for variance homogeneity by Levene's test. Logarithmic transformation was performed to homogenous variance for

the eggs laid before being subjected to one-way analyses of variance. Arcsin transformation was performed to homogenous variance for the egg, larval, pupal, adult mortality. The mean of the data were compared using Tukey's Honestly Significant (HSD) at a 5% level of significance. To make a clear understanding that the statements under results section based on a statistical procedure, P values have been added to the result description. All statistical analyses were calculated using software of SAS University Edition 2.7 9.4 M5. The malformation of eggs, larvae, pupae, and adults of of *Cx. quinquefasciatus* infected by the fungus were presented in photograph.

RESULTS AND DISCUSSION

The bioassay of fungal pathogenicity to egg of *Culex quinquefasciatus*

Obtained findings reported that eggs laid on the ovitrap by the gravid *Cx. quinquefasciatus* female of control (untreated fungal) were the least (1469.67 eggs/female per 96 hours) among those of fungal treatments. Egg mortality of *Cx. quinquefasciatus* of control was the lowest (16.76%) and significantly different from those of of fungal treatments (Table 2). All isolates used were pathogenic to the eggs of *Cx. quinquefasciatus*. Egg mortality of *Cx. quinquefasciatus* caused by *B. bassiana* isolate BSwTd4 was the highest (39.94%) and was not significantly different from those caused by *B. bassiana* isolate TaLmME (38.86%), and *M. anisopliae* isolate MSwTp3 (38.75%), *B. bassiana* isolate TaPsBA (36.91%), *P. citrinum* isolate BKbTp (37.04%), and *T. diversus* isolate MSwTp1(35.66%). Thus, the most pathogenic fungal species against eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). The effect of eggs treated with the fungus still affected the emerging larvae, the larvae resulted higher mortality than those of controls, as well as the pupae from treated eggs produced higher mortality than those of controls.

The morphology of *Cx. quinquefasciatus* eggs infected with the entomopathogenic fungi showed differences from the healthy eggs from the control group. The body liquid of *Cx. quinquefasciatus* infected eggs had feculent color without embryo inside, while the healthy eggs from the control group had clearly visible color with embryo inside. The color of anterior and posterior infected eggs was darker than those of the healthy eggs.

Table 2. Effect of eggs treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on the egg laid, the egg, larval, and pupal mortality of *Culex quinquefasciatus*

Species	Isolate code	Eggs laid per female per 96 hours [a)]	Egg mortality (%)[b)]	Larval mortality (%)[b)]	Pupal mortality (%)[b)]
Control	-	1469.67±14.46^{b}	16.76±0.82^{d}	17.59±0.11^{d}	0.99±0.15^{d}
Beauveria bassiana	TaAlPA	1511.00±11.09^{ab}	32.70±0.71^{bc}	33.33±0.45^{c}	2.86±0.14^{c}
Beauveria bassiana	LtKrLH	1482.67±13.74^{b}	31.06±0.42^{c}	30.58±0.55^{d}	2.60±0.06^{c}
Beauveria bassiana	TaLmME	1616.67±9.48^{a}	38.86±0.23^{a}	40.02±0.12^{a}	5.06±0.12^{ab}
Beauveria bassiana	TaPsBA	1574.33±15.59^{ab}	36.91±0.25^{ab}	35.23±0.37^{c}	3.37±0.06^{c}
Penicillum citrinum	BKbTp	1556.33±26.22^{ab}	37.04±1.17^{ab}	37.72±0.31^{b}	3.75±0.32^{bc}
Talaromyces diversus	MSwTp1	1563.67±26.02^{ab}	35.66±0.95^{abc}	34.41±0.28^{c}	3.17±0.12^{c}
Beauveria bassiana	BSwTd4	1637.33±3.60^{a}	39.94±0.08^{a}	41.20±0.28^{a}	6.31±0.49^{a}
Metarhizium anisopliae	MSwTp3	1613.33±29.58^{a}	38.75±1.26^{a}	40.15±0.02^{a}	5.49±0.12^{a}
F-value		6.20*	63.5*	345.2*	45.41*
P-value		6.50 x 10^{-4}	1.29 x 10^{-11}	2.0 x 10^{-16}	2.25 x 10^{-10}
HSD value		0.04	2.98	1.94	1.96

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using logarithmic transformation, [b)]Original data were transformed using Arcsin transformation prior to statistical analysis.

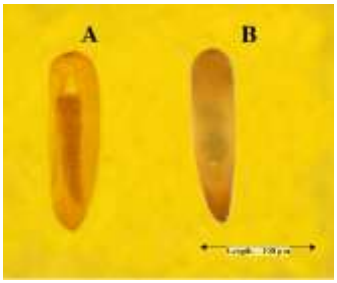

Figure 1. Morphology of the *Culex quinquefasciatus* eggs: a healthy egg of control (A) and an infected treated egg (B)

The bioassay of fungal pathogenicity to larvae of *Culex quinquefasciatus*

The third-instar larvae of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) had significantly higher mortality compared to the control larvae (untreated larvae) (Table 3). All fungal isolates used the

current study were pathogenic to the third-instar larvae of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The larval mortality caused by *M. anisopliae* isolate MSwTp3 (100% with LT_{50} 2.02 days and LT_{95} 7.15 days) was the highest and not significantly different from mortality caused by *B. bassiana* isolate BSwTd4 (98.89% with LT_{50} 2.51 days and LT_{95} 7.61 days) and *B. bassiana* isolate TaLmME (97.78% with LT_{50} 2.75 days and LT_{95} 7.85 days). The most pathogenic fungal species to the third-instar larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Obtained findings highlighted that *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) possessed larvicidal activity.

The fungal infected larvae of *Cx. quinquefasciatus* showed typical morphology, particularly the morphology of sick and dead larvae. The sick larvae underwent a lysis of the gut lumen with white color and the larvae abdomen had no distinct segment. The epithelial lining possessed milky color with a ruptured anal segment (Figure 2). The healthy larvae of control had a transparent gut lumen with a visible or recognizably different segment of abdomen. The healthy larvae also had a visible epithelial lining and an undamaged anal segment. The larval cadavers of fungal treatment cultured on SDA medium produced conidia and mycellia covering the cadaver body, while the healthy larvae were not infected or covered by fungal conidia or mycellia. The pupae emerging from the infected larvae generally became sick and died. The sick pupae body were thinner, hardened, and straight shaped and had the black head. The healthy pupae had fatter, round, flexible and soft body, bent like a comma shape and their head had dark-brown in color (Figure 3).

Table 3. Effect of larvae treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on larval mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Larvae mortality (%)[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]
Control	-	0.00±0.00^{f}	14.98±0.43^{a}	20.21±0.51^{a}
Beauveria bassiana	TaAlPA	84.44±2.40^{cd}	3.97±0.16^{b}	9.08±0.28^{bc}
Beauveria bassiana	LtKrLH	78.89±2.40^{de}	4.21±0.17^{b}	9.31±0.28^{bc}
Beauveria bassiana	TaLmME	97.78±0.91^{ab}	2.75±0.07^{cd}	7.85±0.15^{cd}
Beauveria bassiana	TaPsBA	80.00±3.14^{cde}	4.05±0.11^{b}	9.15±0.12^{bc}
Penicillum citrinum	BKbTp	92.22±0.91^{bc}	3.78±0.51^{bc}	8.88±0.62^{bcd}
Talaromyces diversus	MSwTp1	64.44±0.91^{e}	5.04±0.15^{b}	10.14±0.26^{b}
Beauveria bassiana	BSwTd4	98.89±0.91^{a}	2.51±0.10^{d}	7.61±0.21^{cd}
Metarhizium anisopliae	MSwTp3	100.00±0.00^{a}	2.02±0.07^{d}	7.15±0.07^{d}
F-value		155.00*	116.60*	79.77*
P-value		5.31 x 10^{-15}	6.52 x 10^{-14}	1.80 x 10^{-12}
HSD value		10.62	0.33	0.30

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)] Original data were transformed using square root (sqrt) transformation.

Figure 2. Morphology of the *Culex quinquefasciatus* larvae: a healthy larvae of control (A) and an infected treated larvae (B)

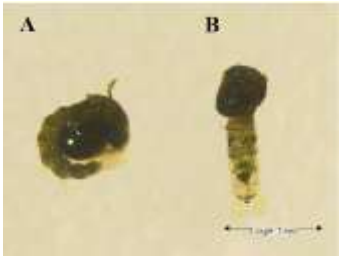

Figure 3. Morphology of the *Culex quinquefasciatus* pupae: a healthy pupae of control (A) and an infected treated pupae (B)

The bioassay of fungal pathogenicity to adult of *Culex quinquefasciatus*

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia ml^{-1}) had significantly higher mortality compared to the control adults (untreated adults) (Table 4). The adult mortality caused by *M. anisopliae* isolate MSwTp3 (100% with LT_{50} 3.25 days and LT_{95} 6.70 days) was highest and not significantly different from those caused by *B. bassiana* isolate BSwTd4 (100% with LT_{50} 3.46 days and LT_{95} 6.76 days) and *B. bassiana* isolate TaLmME (98.89% with LT_{50} 3.70 days and LT_{95} 7.15 days), and *P.citrinum* isolate BKbTp (98.89% with LT_{50} 3.96 days and LT_{95} 7.41 days). Nevertheless, all fungal isolates in this study were pathogenic to the adults of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The most pathogenic fungal species to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). This research findings highlighted that *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate) had adulticidal activity.

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi became sick and finally died. The typical symptoms of sick and dead infected adults were malformation. The unhealthy adults had the asymmetrical wing shapes, the dried and mycosis body, and a spiral shape proboscis (Figure 4). If the unhealthy adults died, their cadavers grown in SDA medium could be covered with the fungal conidia and mycellia. On the contrary, the healthy adults of control possessed the symmetrical wing shapes, and a straight shaped black proboscis, and no mycosis on the cadaver body. The healthy adults had the elongate abdomen. The cadavers from the healthy adults grown in SDA medium were not covered with the fungal conidia and mycellia.

Table 4. Effect of adults treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on adult mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Adult mortality (%)[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]
Control	-	0.00±0.00^{d}	11.99±0.40^{a}	15.44±0.42^{a}
Beauveria bassiana	TaAlPA	88.89±1.81^{b}	4.64±0.03^{c}	8.09±0.06^{bc}
Beauveria bassiana	LtKrLH	82.22±0.91^{b}	4.84±0.02^{bc}	8.29±0.02^{b}
Beauveria bassiana	TaLmME	98.89±0.91^{a}	3.70±0.04^{de}	7.15±0.06^{d}
Beauveria bassiana	TaPsBA	87.78±0.91^{b}	4.63±0.01^{c}	8.08±0.03^{bc}
Penicillum citrinum	BKbTp	98.89±0.91^{a}	3.96±0.05^{d}	7.41±0.06^{cd}
Talaromyces diversus	MSwTp1	63.33±1.57^{c}	5.37±0.06^{b}	8.82±0.09^{b}
Beauveria bassiana	BSwTd4	100.00±0.00^{a}	3.46±0.10^{de}	6.76±0.21^{d}
Metarhizium anisopliae	MSwTp3	100.00±0.00^{a}	3.25±0.10^{e}	6.70±0.10^{d}
F-value		23.11*	229.30*	183.60*
P-value		5.85 x 10^{-8}	2.00 x 10^{-16}	1.19 x 10^{-15}
HSD value		24.55	0.15	0.15

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)]Original data were transformed using square root (sqrt) transformation.

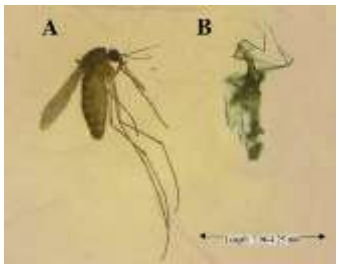

Figure 4. Morphology of the *Cx. quinquefasciatus* adults: a healthy adult of control (A) and an infected treated adult (B)

Discussion

The eggs laid by the gravid *Cx. quinquefasciatus* female of control (untreated) were the least among those of fungal treatments. However, the eggs laid by the fungal treated female were more than those of control because the ovitrap where they were laying eggs had water with dyed color due to added with the fungal suspension. The gravid female *Culex* mosquitoes preferred to lay eggs in dyed water (Day 2016; Perea and Callaghan 2017). Although the treated eggs laid by female *Cx. quinquefasciatus* in this study were less than 50%, but the treated eggs induced the sick larvae and produced the mortality of emerging larvae and pupae. Besides, the mycosis on the dead larvae and pupae that failed to emerge from the eggs occured. In this study, the ovitrap used to expose the fungi to the eggs of *Cx. quinquefasciatus* could effectively infected its eggs, larvae, pupae, and adults. The finding highlighted that the *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. The most pathogenic fungal species against the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The entomopathogenic fungi have reported caused the eggs of

mosquito unhatched and abortion and the emerging larvae and pupae could not carry on their life (Leles et al. 2012; Ramayanti et al. 2022). The obtained data also reported the embryo of eggs treated with the fungi were lysis and egg liquid becoming feculent color and no embryo inside the eggs.

The egg mortality of *Cx. quinquefasciatus* caused by the entomopathogenic fungi were lower than the larvae mortality caused by the fungi. The third-instar larvae of *Cx. quinquefasciatus* could be immediately killed by *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) (LT_{50} < 3 days). The high mortality of the larvae treated with the entomopathogenic fungi in short time due to higher fungal suspension used (1 x 10^{10} conidia ml^{-1}) and the fungi cultured in the broth medium (SDB). The broth culture is able to grow the fungus and produce blastospores, and the blastospores are more effective compared to aerial conidia in killing the mosquitoes (Alkhaibari et al. 2017). The findings highlighted that both species of the fungi could be develop to be a larvicide for *Cx. quinquefasciatus* because they have highest level of larvicidal activity (97.78−100% of larvae mortality). The larvae mortality caused by the entomopathogenic fungi was higher than the egg mortality caused by the entomopathogenic fungi because the cuticle of integument of the larvae is thinner than those of the eggs (Farnesi et al. 2015). The thinner was the cuticle of the insect integument, the easier the fungal conidia are penetrated into insect body (Ortiz-Urquiza and Keyhani 2013).

The results obtained that the larvae infected by the fungi could be sick or dead because their gut lumen ruptured or lysis by the fungi. The fungi also damaged the anal and abdomen segments so that the larva body became malformation. The larvae died due to the conidia of entomopthogenic fungi germinating and their hyphae penetrating into the insect integument, after that the hyphae entered to the body cavity (Boomsma et al. 2014). The hyphae developed to become blastospores in the larvae hemolymph (Mancillas-Paredes et al. 2019). The blastospores of the entomopthogenic fungi could produce secondary metabolites, such as bassiacridin (Quesada-moraga and Vey 2004) and beauvericin (Safavi 2012) secreted by *B. bassiana* and destruxin produced by *M. anisopliae* (Borisade et al. 2016). The secondary metabolites or toxin could disrupt normal cell metabolism of the insect (Mancillas-Paredes et al. 2019).

Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). This research findings highlighted that besides *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates), *P.citrinum* (BKbTp isolate) was also pathogenic to the adults of *Cx. quinquefasciatus*. The results obtained that the fungal species that were pathogenic to adults were different from the species that were pathogenic to eggs and larvae of *Cx. quinquefasciatus*. The fungal species that were pathogenic to the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate), while The fungal species that were pathogenic to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). The entomopathogenic fungi could induce the adults of *Cx. quinquefasciatus* becoming malformation (e.g. asymmetrical wing, curled proboscis) and mycosis (after growing onto the SDA medium). The dead adults were caused by conidia germinating to be mycellia and penetrating into the adult body cavity producing secondary metabolites (via blastospores) disrupting normal cell metabolism of the insect (Mancillas-Paredes et al. 2019). After the adults died, the fungi still grow saprophytically on the cadavers of adults and the fungi induce the cadaver body becoming mycosis (Gabarty et al. 2014). The future application of the fungi against the mosquito eggs, larvae, and adults can be used an ovitrap, fungal spores formulated in a synthetic oil, and fungus-impregnated black cloths, respectively.

The fungal species that were the most pathogenic to the eggs, larvae, and adults of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4 isolate), *M. anisopliae* (MSwTp3 isolate), and *P. citrinum* (BKbTp isolate), however *T. diversus* was also the most pathogenic to the eggs. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to *Cx. quinquefasciatus*. So, the entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as the ovicide, larvicide, and adulticide.

ACKNOWLEDGEMENTS

This research was funded by the Directorate General of Higher Education, Ministry of Education, Culture, Research, and Technology, Republic of Indonesia, Fiscal Year 2022, in accordance with the Doctoral Dissertation Scheme (*Penelitian Disertasi Doktor*) with Contract Number: 142/E5/PG.02.00.PT/2022 on 10 May 2022, chaired by Siti Herlinda.

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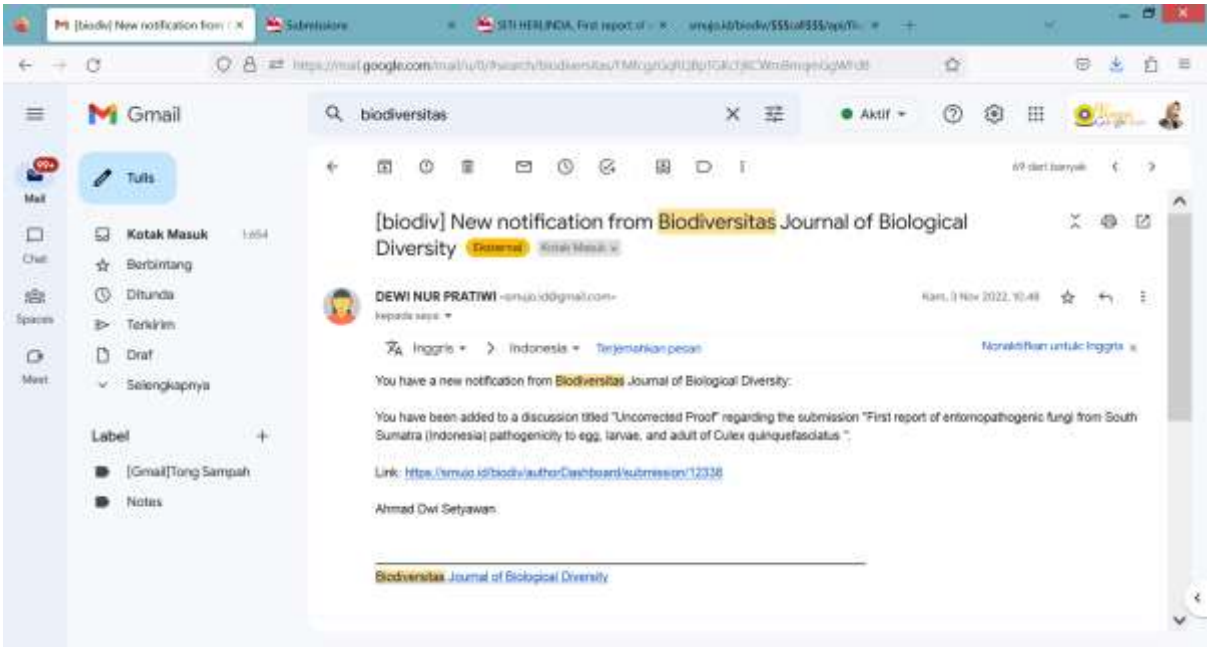
3. Bukti konfirmasi paper accepted, uncorrected Proof dan hasil koreksi penulis



First report of entomopathogenic fungi from South Sumatra (Indonesia): pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

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Manuscript received: xxx 2022. Revision accepted: xxx November 2022.

Abstract. *Ramayanti I, Herlinda S, Muslim A, Hasyim H. 2022. First report of entomopathogenic fungi from South Sumatra (Indonesia): pathogenicity to egg, larvae, and adult of* Culex quinquefasciatus. *Biodiversitas 23: xxxx.* Mosquito control has currently used many biocontrol agents, such as the entomopathogenic fungi. So, the study aimed to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Culex quinquefasciatus.* The fungal isolates used were eight isolates from South Sumatra and have been identified molecularly. The fungal species that were the most pathogenic to the eggs of *Cx. quinquefasciatus* were *Beauveria bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *Metarhizium anisopliae* (MSwTp3 isolate), *Penicillium citrinum* (BKbTp isolate), and *Talaromyces diversus* (MSwTp1 isolate). The *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The most pathogenic fungal species to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P. citrinum* (BKbTp isolate). Finally, The entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as the ovicide, larvicide, and adulticide. The future application of the fungi against the mosquito eggs, larvae, and adults can be used an ovitrap, fungal spores formulated in a synthetic oil, and fungus-impregnated black cloths, respectively.

Keywords: *Beauveria bassiana*, *Metarhizium anisopliae*, lymphatic filariasis, *Penicillium citrinum*, *Talaromyces diversus*, *Purpureocillium lilacinum*

INTRODUCTION

Filariasis or elephantiasis or lymphatic filariasis is an infectious disease caused by the parasitic filarial worms, such as *Wuchereria bancrofti* (Pratiwi et al. 2019) and *Brugia* sp. (Intarapuk and Bhumiratana, 2021). This worm is transmitted by vector insects of the mosquitoes, especially *Culex* (Blut 2013). There are more than 38 species of mosquitoes that act as vectors of filariasis transmission (Famakinde 2018), including *Culex quinquefasciatus* (Simonsen and Mwakitalu, 2013; Susilowati, 2018), *Culex vishnui* (Nchoutpouen et al. 2019), *Mansonia africana* and *Mansonia unifo* (Ughasi et al., 2012). Indonesia is an endemic area for lymphatic filariasis (Ginandjar et al., 2018), especially in South Sumatra (Nurjazuli and Santjaka, 2020). The consequences of this elephantiasis disease can cause physical disability, mental, social, and financial losses (Enciso et al., 2021). The higher population density of vector insects tends to be positively correlated with the higher transmission rate of this filarial worms and the higher the number of elephantiasis sufferers (Gordon et al. 2018; Ridha et al. 2020; Santoso et al. 2021).

To suppress and break the transmission of the lymphatic filariasis, the vector insects of transmission needs to be controlled and the population suppressed as low as possible so that the chain of transmission is broken. Some methods have been carried out to reduce the population density of filariasis vector insects. For example, *Cx. quinquefasciatus* has been controlled using a repellent insecticide (Aguiar et al. 2015). Control with botanical insecticides has also been carried out, for example the use of rosmarin leaf oil (*Rosmarinus officinalis* L) to kill the larvae of *Cx. quinquefasciatus* (Susilowati 2018). These vector insects are generally controlled with synthetic insecticides (Nchoutpouen et al. 2019). However, routinely spraying of synthetic insecticides causes the new problems due to the higher level of *Cx. quinquefasciatus* resistance and it has been reported that this mosquito is resistant to permethrin, deltamethrin, DDT (dichloro-diphenyl-trichloroethane) (Nchoutpouen et al. 2019), and bendiocarb (Talipouo et al. 2021). Residues of the synthetic insecticides may cause the non-target animals killed, and the insecticides induce the human health problems and the pollution on water, air, and soil (Hamid et al. 2017). The use of synthetic insecticides also causes the high

operational costs for application or spraying (Chowański et al. 2014).

Currently, mosquito control has used many biocontrol agents, for example the use of entomopathogenic fungi (pathogens that cause insect disease or insect pathology). The use of entomopathogenic fungi have occured, for example in Thailand, conidia of *Penicillium citrinum* has been found to be effective in killing the larvae of *Cx. quinquefasciatus* (Maketon et al. 2014). In India, the mycelia extract of *Beauveria bassiana* has been found to be effective in killing larvae. of *Cx. quinquefasciatus* (Vivekanandhan et al. 2018). The entomopathogenic fungi found in Indonesia, especially in South Sumatra have been tested and effectively killed some species of insect pests attacking plants (Herlinda et al. 2020a, 2020b; Gustianingtyas et al. 2021; Herlinda et al. 2021). Although many species of entomopathogenic fungi have been found in South Sumatra, there is no information on the effectiveness of these entomopathogenic fungi to kill the filariasis vector mosquito, *Cx. quinquefasciatus*. The previous study is only the pathogenicity of the entomopathogenic fungi to kill the egg, larvae, and adult of *Aedes aegypti* (Ramayanti et al. 2022). The novelty of this research is that the entomopathogenic fungi from South Sumatra and was first tested to kill eggs, larvae, and adults of *Cx. quinquefasciatus.* This research is feasible because the fungi as potential biological control agents are specific strains from South Sumatra so that they do not disturb the natural balance of existing microorganisms and *Cx. quinquefasciatus* is the main vector of filariasis which needs to be controlled. The purpose of this study was to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. Quinquefasciatus.*

MATERIALS AND METHODS

Fungal preparation

The fungal isolates used for this current research were from the collection of Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya and they were identified molecularly. The fungal species identified were *B. bassiana* TaAlPA isolate (GenBank acc. no. OM791688), *B. bassiana* LtKrLH isolate (GenBank acc. no. OM791680), *B. bassiana* TaLmME isolate (GenBank acc. no. OM791687), and *B. bassiana* TaPsBA isolate (GenBank acc. no. OM791689) (Ramayanti et al., 2022), *P. citrinum* BKbTp isolate (GenBank acc. no. MT448730), *Talaromyces diversus* MSwTp1 isolate (GenBank acc. no. MT448731), *B. bassiana* BSwTd4 isolate (GenBank acc. no. MT448732), and *Metarhizium anisopliae* MT488733 isolate (GenBank acc. no. MT488733) (Herlinda et al., 2020a) (Table 1). The fungi were originated from South Sumatra, Indonesia with location, Alang-alang Lebar, Palembang (2°56′32″S 104°42′16″E), Kota Raya, Lahat (3°46′38″S 103°35′25″E), Lebak, Muara Enim (3°23′51″S 104°19′41″E), Purwosari, Banyuasin (2°52′19″S 104°33′14″E), Talang Patai, Pagar Alam (4°00′45″S 103°12′21″E) and Talang Dabok, Ogan Komering Ilir (3°00′18″S 104°40′05″E). All fungal isolates were cultured on the agar medium, Sabouraud Dextrose Agar (SDA), and for bioassay they were also re-cultured on the liquid medium, SDB (Sabouraud Dextrose Broth).

Mass-rearing of Culex quinquefasciatus

Eggs of *Cx. quinquefasciatus* were gained from P2B2 Research and Development Loka, the Health Research and Development Center (the Balitbangkes), the Ministry of Health of Indonesia in Baturaja, South Sumatra, and *Cx. quinquefasciatus* mass-rearing have been carried out since June 2013. The *Cx. quinquefasciatus* mass-rearing for bioassay were carried out at the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. The room temperature and relative humidity during the mass-rearing were 29 ± 1°C and 84 ± 1%, respectively. The room lighting was set to photoperiod with 12 hours of light and 12 hours of dark (Kauffman et al., 2017). The emerging larvae were kept into a transparent plastic cup (Ø 7 cm, height 9 cm) that has been disinfected and the cup was filled in 50 ml of water (Ramayanti *et al.,* 2022). The larvae were fed with dog biscuits (Vivekanandhan et al., 2018).

Table 1. Origin of the isolates of entomophatogenic fungi from South Sumatra, Indonesia, used in this research

Location, village or district/city	Isolate origin	Altitude (m)	Fungal spesies	Fungal isolate code	GenBank acc no.
Alang-alang Lebar, Palembang	Soil	23.0	*Beauveria bassiana*	TaAlPA	OM791688*
Kota Raya, Lahat	Insect	369.9	*Beauveria bassiana*	LtKrLH	OM791680*
Lebak, Muara Enim	Soil	33.5	*Beauveria bassiana*	TaLmME	OM791687*
Purwosari, Banyuasin	Soil	19.0	*Beauveria bassiana*	TaPsBA	OM791689*
Talang Patai, Pagar Alam	Soil	175.0	*Penicillium citrinum*	BKbTp	MT448730**
Talang Dabok, Ogan Komering Ilir	Soil	24.0	*Talaromyces diversus*	MSwTp1	MT448731**
Talang Patai, Pagar Alam	Soil	193.0	*Beauveria bassiana*	BSwTd4	MT448732**
Talang Patai, Pagar Alam	Soil	193.0	*Metarhizium anisopliae*	MSwTp3	MT488733**

Sources: *(Ramayanti et al. 2022), **(Herlinda et al. 2020a)

The larvae within the plastic cup were put into a disinfected transparent plastic cage (50 x 50 x 50 cm) in order to keep the emerging adults remaining in the cage. The 10% sucrose solution infused on cotton wool for adult diet was hung on the top of the cage. Then, an ovitrap was put in the plastic cage where the adult mosquitoes were

emerged from the pupae. The ovitrap was designed as a disinfected transparent plastic cup (Ø 9 cm, height 13 cm) that had dark wall and was filled with water to a depth of 10 cm (Wu et al. 2013).

The bioassay of fungal pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. quinquefasciatus* was carried out at the laboratory with the average temperature and the relative humidity, 29.79 °C and 84.11%, respectively. After the fungal isolates were cultured on the SDA medium, then the fungal cultures were re-grown on the SDB medium in order to increase the fungal conidial density (Gustianingtyas *et al.*, 2020). During the process of growing the fungal culture on the SDB medium (the liquid medium) for 14 days, the culture was shaken continuously for 7 days and then not shaken for 7 days. The conidia harvested from the liquid medium was calculated for getting conidial density used for bioassay below.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs of *Cx. quinquefasciatus* was carried out following the the method of Luz et al. (2011). The liquid fungal culture with a concentration of 1 x 10^{10} conidia mL^{-1} was poured 10 mL into the ovitrap containing 100 ml of water. At the same time, the control was only poured 10 mL of sterile distilled water. This experiment was designed using a completely randomized design with 8 isolates and control as treatments. The experiment were repeated three times. Then, the ovitrap was put in the disinfected plastic cage, and 30 gravid female adults were also put in the cage so that the female adults could lay their eggs in the ovitrap. The duration for the female adults laying their eggs were 4 x 24 hours (Blanford *et al.*, 2012). The female adults were also provided with 10% sucrose solution for their diet. The ovitrap containing the eggs laid was replaced daily from the cage, and then the number of the eggs laid were also counted every day. The viable eggs (the hatched eggs) were monitored and recorded every 2 hours for 48 hours. The egg morphology changing were also recorded daily. The abortion or unhatched eggs were cultured in SDA medium in order to detect and to identify the microorganism that caused abortion or unhatched. The larvae emerging were observed daily and the dead larvae were counted every day. The dead pupae were also monitored every day until adult emerging.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the larvae of *Cx. quinquefasciatus* was carried out following the the method of Alkhaibari et al. (2017). The 30 third-instar larvae were treated with 10 ml suspension of the entomopathogenic fungal isolate, the fungal suspension was put in a disinfected transparent plastic cups (Ø 7 cm, height 9 cm) with 100 ml of water inside. The 30 control larvae were only exposed to 10 mL of sterile water. All treatments were replicated three times and the the experiment was designed using a completely randomized design with 8 isolates and control as treatments. The duration of fungal exposure to the larvae were 1 x 24 hours, and then the dead larvae were monitored and recorded daily for 8 days. The variables observed were the number of larval deaths and the morphology changes of larvae after being treated with the fungi. The time of larval death and the behavior of unhealthy larvae were also observed every day. The health of the larvae identified by observing the changes of the larvae behavior and morphology. The time of larval death were used to determine of LT_{50} (the Lethal Time) and $LT_{95.}$ The cadaver or dead larvae were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the adults of *Cx. quinquefasciatus* was carried out following the the method of Blanford et al. (2012) and Shoukat et al. (2020). Fifteen female and 15 male adults (total of 30 adults) per isolate were treated with the fungal suspension for 24 hours. The adults used in this experiment were 3 days old. Ten mL of the fungal suspension (1 x 10^{10} conidia mL^{-1}) were sprayed on the inner wall of disinfected transparent plastic cage (50 × 50 × 50 cm). Then, the cage was air-dried for 2 hours (Mnyone et al., 2011). Then, 30 adults were put in the plastic cage. For the plastic cage of control, ten mL of water was sprayed on the inner wall of the cage. All treatments were repeated three times using a completely randomized design. The adults of fungal treatment and control were given the diet of 10% sucrose solution hang on the top of the cage. After fungal exposure for 24 hours, The number of dead adults were started to be recorded after 24 hours of fungal exposure until 7 days after exposure. The dead adults were characterized with no movement occured (Shoukat et al., 2020). The time of adult death and the behavior of unhealthy adults were also observed daily. The time of adult death were used to determine of LT_{50} and $LT_{95.}$ The cadaver or dead adult were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

Data analysis

The data of egg, larval, and pupal mortality of *Cx. quinquefasciatus*, LT_{50} and LT_{95} of the larvae; , adult mortality, LT_{50} and LT_{95} of *Cx. quinquefasciatus* of each treatment were analyzed using ANOVA (analysis of variance). We implemented the parametric statistical analysis, and therefore all data were tested for normal distribution using the Shapiro-Wilk test and for variance homogeneity by Levene's test. Logarithmic transformation was performed to homogenous variance for the eggs laid before being subjected to one-way analyses of variance. Arcsin transformation was performed to homogenous variance for the egg, larval, pupal, adult mortality. The mean of the data were compared using Tukey's Honestly Significant (HSD) at a 5% level of significance. To make a clear understanding that the statements under results section based on a statistical procedure, P values have been added to the result description. All statistical analyses were calculated using software of SAS University Edition 2.7 9.4 M5. The malformation of eggs, larvae, pupae, and

adults of of *Cx. quinquefasciatus* infected by the fungus were presented in photograph.

RESULTS AND DISCUSSION

The bioassay of fungal pathogenicity to egg of *Culex quinquefasciatus*

Obtained findings reported that eggs laid on the ovitrap by the gravid *Cx. quinquefasciatus* female of control (untreated fungal) were the least (1469.67 eggs/female per 96 hours) among those of fungal treatments. Egg mortality of *Cx. quinquefasciatus* of control was the lowest (16.76%) and significantly different from those of of fungal treatments (Table 2). All isolates used were pathogenic to the eggs of *Cx. quinquefasciatus*. Egg mortality of *Cx. quinquefasciatus* caused by *B. bassiana* isolate BSwTd4 was the highest (39.94%) and was not significantly different from those caused by *B. bassiana* isolate TaLmME (38.86%), and *M. anisopliae* isolate MSwTp3 (38.75%), *B. bassiana* isolate TaPsBA (36.91%), *P. citrinum* isolate BKbTp (37.04%), and *T. diversus* isolate MSwTp1(35.66%). Thus, the most pathogenic fungal species against eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). The effect of eggs treated with the fungus still affected the emerging larvae, the larvae resulted higher mortality than those of controls, as well as the pupae from treated eggs produced higher mortality than those of controls.

The morphology of *Cx. quinquefasciatus* eggs infected with the entomopathogenic fungi showed differences from the healthy eggs from the control group. The body liquid of *Cx. quinquefasciatus* infected eggs had feculent color without embryo inside, while the healthy eggs from the control group had clearly visible color with embryo inside. The color of anterior and posterior infected eggs was darker than those of the healthy eggs.

The bioassay of fungal pathogenicity to larvae of *Culex quinquefasciatus*

The third-instar larvae of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) had significantly higher mortality compared to the control larvae (untreated larvae) (Table 3). All fungal isolates used the current study were pathogenic to the third-instar larvae of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The larval mortality caused by *M. anisopliae* isolate MSwTp3 (100% with LT_{50} 2.02 days and LT_{95} 7.15 days) was the highest and not significantly different from mortality caused by *B. bassiana* isolate BSwTd4 (98.89% with LT_{50} 2.51 days and LT_{95} 7.61 days) and *B. bassiana* isolate TaLmME (97.78% with LT_{50} 2.75 days and LT_{95} 7.85 days).

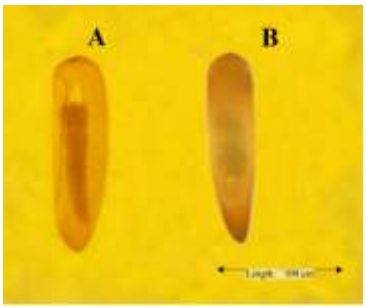

Figure 1. Morphology of the *Culex quinquefasciatus* eggs: a healthy egg of control (A) and an infected treated egg (B)

Table 2. Effect of eggs treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on the egg laid, the egg, larval, and pupal mortality of *Culex quinquefasciatus*

Species	Isolate code	Eggs laid per female per 96 hours [a)]	Egg mortality (%)[b)]	Larval mortality (%)[b)]	Pupal mortality (%)[b)]
Control	-	1469.67±14.46^{b}	16.76±0.82^{d}	17.59±0.11^{d}	0.99±0.15^{d}
Beauveria bassiana	TaAlPA	1511.00±11.09^{ab}	32.70±0.71^{bc}	33.33±0.45^{c}	2.86±0.14^{c}
Beauveria bassiana	LtKrLH	1482.67±13.74^{b}	31.06±0.42^{c}	30.58±0.55^{d}	2.60±0.06^{c}
Beauveria bassiana	TaLmME	1616.67±9.48^{a}	38.86±0.23^{a}	40.02±0.12^{a}	5.06±0.12^{ab}
Beauveria bassiana	TaPsBA	1574.33±15.59^{ab}	36.91±0.25^{ab}	35.23±0.37^{c}	3.37±0.06^{c}
Penicillum citrinum	BKbTp	1556.33±26.22^{ab}	37.04±1.17^{ab}	37.72±0.31^{b}	3.75±0.32^{bc}
Talaromyces diversus	MSwTp1	1563.67±26.02^{ab}	35.66±0.95^{abc}	34.41±0.28^{c}	3.17±0.12^{c}
Beauveria bassiana	BSwTd4	1637.33±3.60^{a}	39.94±0.08^{a}	41.20±0.28^{a}	6.31±0.49^{a}
Metarhizium anisopliae	MSwTp3	1613.33±29.58^{a}	38.75±1.26^{a}	40.15±0.02^{a}	5.49±0.12^{a}
F-value		6.20*	63.5*	345.2*	45.41*
P-value		6.50 x 10^{-4}	1.29 x 10^{-11}	2.0 x 10^{-16}	2.25 x 10^{-10}
HSD value		0.04	2.98	1.94	1.96

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using logarithmic transformation, [b)]Original data were transformed using Arcsin transformation prior to statistical analysis

Table 3. Effect of larvae treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on larval mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Larvae mortality (%)[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]

Control	-	0.00±0.00^{f}	14.98±0.43^{a}	20.21±0.51^{a}
Beauveria bassiana	TaAlPA	84.44±2.40^{cd}	3.97±0.16^{b}	9.08±0.28^{bc}
Beauveria bassiana	LtKrLH	78.89±2.40^{de}	4.21±0.17^{b}	9.31±0.28^{bc}
Beauveria bassiana	TaLmME	97.78±0.91^{ab}	2.75±0.07^{cd}	7.85±0.15^{cd}
Beauveria bassiana	TaPsBA	80.00±3.14^{cde}	4.05±0.11^{b}	9.15±0.12^{bc}
Penicillum citrinum	BKbTp	92.22±0.91^{bc}	3.78±0.51^{bc}	8.88±0.62^{bcd}
Talaromyces diversus	MSwTp1	64.44±0.91^{e}	5.04±0.15^{b}	10.14±0.26^{b}
Beauveria bassiana	BSwTd4	98.89±0.91^{a}	2.51±0.10^{d}	7.61±0.21^{cd}
Metarhizium anisopliae	MSwTp3	100.00±0.00^{a}	2.02±0.07^{d}	7.15±0.07^{d}
F-value		155.00*	116.60*	79.77*
P-value		5.31 x 10^{-15}	6.52 x 10^{-14}	1.80 x 10^{-12}
HSD value		10.62	0.33	0.30

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)] Original data were transformed using square root (sqrt) transformation

The most pathogenic fungal species to the third-instar larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Obtained findings highlighted that *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) possessed larvicidal activity.

The fungal infected larvae of *Cx. quinquefasciatus* showed typical morphology, particularly the morphology of sick and dead larvae. The sick larvae underwent a lysis of the gut lumen with white color and the larvae abdomen had no distinct segment. The epithelial lining possessed milky color with a ruptured anal segment (Figure 2). The healthy larvae of control had a transparent gut lumen with a visible or recognizably different segment of abdomen. The healthy larvae also had a visible epithelial lining and an undamaged anal segment. The larval cadavers of fungal treatment cultured on SDA medium produced conidia and mycellia covering the cadaver body, while the healthy larvae were not infected or covered by fungal conidia or mycellia. The pupae emerging from the infected larvae generally became sick and died. The sick pupae body were thinner, hardened, and straight shaped and had the black head. The healthy pupae had fatter, round, flexible and soft body, bent like a comma shape and their head had dark-brown in color (Figure 3).

The bioassay of fungal pathogenicity to adult of *Culex quinquefasciatus*

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia ml^{-1}) had significantly higher mortality compared to the control adults (untreated adults) (Table 4). The adult mortality caused by *M. anisopliae* isolate MSwTp3 (100% with LT_{50} 3.25 days and LT_{95} 6.70 days) was highest and not significantly different from those caused by *B. bassiana* isolate BSwTd4 (100% with LT_{50} 3.46 days and LT_{95} 6.76 days) and *B. bassiana* isolate TaLmME (98.89% with LT_{50} 3.70 days and LT_{95} 7.15 days), and *P.citrinum* isolate BKbTp (98.89% with LT_{50} 3.96 days and LT_{95} 7.41 days). Nevertheless, all fungal isolates in this study were pathogenic to the adults of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The most pathogenic fungal species to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). This research findings highlighted that *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate) had adulticidal activity.

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi became sick and finally died. The typical symptoms of sick and dead infected adults were malformation. The unhealthy adults had the asymmetrical wing shapes, the dried and mycosis body, and a spiral shape proboscis (Figure 4).

Figure 2. Morphology of the *Culex quinquefasciatus* larvae: a healthy larvae of control (A) and an infected treated larvae (B)

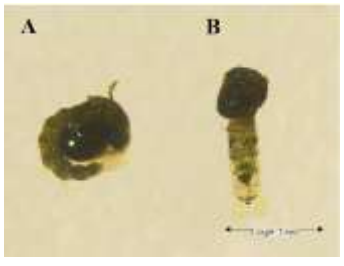

Figure 3. Morphology of the *Culex quinquefasciatus* pupae: a healthy pupae of control (A) and an infected treated pupae (B)

Table 4. Effect of adults treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on adult mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Adult mortality (%)[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]

Control	-	0.00±0.00[d]	11.99±0.40[a]	15.44±0.42[a]
Beauveria bassiana	TaAlPA	88.89±1.81[b]	4.64±0.03[c]	8.09±0.06[bc]
Beauveria bassiana	LtKrLH	82.22±0.91[b]	4.84±0.02[bc]	8.29±0.02[b]
Beauveria bassiana	TaLmME	98.89±0.91[a]	3.70±0.04[de]	7.15±0.06[d]
Beauveria bassiana	TaPsBA	87.78±0.91[b]	4.63±0.01[c]	8.08±0.03[bc]
Penicillum citrinum	BKbTp	98.89±0.91[a]	3.96±0.05[d]	7.41±0.06[cd]
Talaromyces diversus	MSwTp1	63.33±1.57[c]	5.37±0.06[b]	8.82±0.09[b]
Beauveria bassiana	BSwTd4	100.00±0.00[a]	3.46±0.10[de]	6.76±0.21[d]
Metarhizium anisopliae	MSwTp3	100.00±0.00[a]	3.25±0.10[e]	6.70±0.10[d]
F-value		23.11[*]	229.30[*]	183.60[*]
P-value		5.85 x 10^{-8}	2.00 x 10^{-16}	1.19 x 10^{-15}
HSD value		24.55	0.15	0.15

Note: * = significantly different; values within a column followed by the same letters were not significantly different at P < 0.05 according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)]Original data were transformed using square root (sqrt) transformation

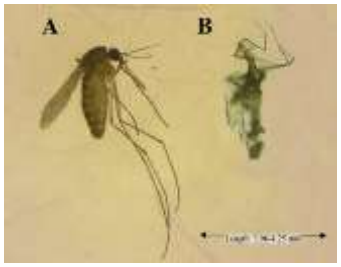

Figure 4. Morphology of the *Cx. quinquefasciatus* adults: a healthy adult of control (A) and an infected treated adult (B)

If the unhealthy adults died, their cadavers grown in SDA medium could be covered with the fungal conidia and mycellia. On the contrary, the healthy adults of control possessed the symmetrical wing shapes, and a straight shaped black proboscis, and no mycosis on the cadaver body. The healthy adults had the elongate abdomen. The cadavers from the healthy adults grown in SDA medium were not covered with the fungal conidia and mycellia.

Discussion

The eggs laid by the gravid *Cx. quinquefasciatus* female of control (untreated) were the least among those of fungal treatments. However, the eggs laid by the fungal treated female were more than those of control because the ovitrap where they were laying eggs had water with dyed color due to added with the fungal suspension. The gravid female *Culex* mosquitoes preferred to lay eggs in dyed water (Day 2016; Perea and Callaghan 2017). Although the treated eggs laid by female *Cx. quinquefasciatus* in this study were less than 50%, but the treated eggs induced the sick larvae and produced the mortality of emerging larvae and pupae. Besides, the mycosis on the dead larvae and pupae that failed to emerge from the eggs occured. In this study, the ovitrap used to expose the fungi to the eggs of *Cx. quinquefasciatus* could effectively infected its eggs, larvae, pupae, and adults. The finding highlighted that the *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. The most pathogenic fungal species against the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The entomopathogenic fungi have reported caused the eggs of mosquito unhatched and abortion and the emerging larvae and pupae could not carry on their life (Leles et al. 2012; Ramayanti et al. 2022). The obtained data also reported the embryo of eggs treated with the fungi were lysis and egg liquid becoming feculent color and no embryo inside the eggs.

The egg mortality of *Cx. quinquefasciatus* caused by the entomopathogenic fungi were lower than the larvae mortality caused by the fungi. The third-instar larvae of *Cx. quinquefasciatus* could be immediately killed by *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) (LT_{50} < 3 days). The high mortality of the larvae treated with the entomopathogenic fungi in short time due to higher fungal suspension used (1 x 10^{10} conidia ml^{-1}) and the fungi cultured in the broth medium (SDB). The broth culture is able to grow the fungus and produce blastospores, and the blastospores are more effective compared to aerial conidia in killing the mosquitoes (Alkhaibari et al. 2017). The findings highlighted that both species of the fungi could be develop to be a larvicide for *Cx. quinquefasciatus* because they have highest level of larvicidal activity (97.78−100% of larvae mortality). The larvae mortality caused by the entomopathogenic fungi was higher than the egg mortality caused by the entomopathogenic fungi because the cuticle of integument of the larvae is thinner than those of the eggs (Farnesi et al. 2015). The thinner was the cuticle of the insect integument, the easier the fungal conidia are penetrated into insect body (Ortiz-Urquiza and Keyhani 2013).

The results obtained that the larvae infected by the fungi could be sick or dead because their gut lumen ruptured or lysis by the fungi. The fungi also damaged the anal and abdomen segments so that the larva body became malformation. The larvae died due to the conidia of entomopthogenic fungi germinating and their hyphae penetrating into the insect integument, after that the hyphae entered to the body cavity (Boomsma et al. 2014). The hyphae developed to become blastospores in the larvae hemolymph (Mancillas-Paredes et al. 2019). The blastospores of the entomopthogenic fungi could produce

secondary metabolites, such as bassiacridin (Quesada-moraga and Vey 2004) and beauvericin (Safavi 2012) secreted by *B. bassiana* and destruxin produced by *M. anisopliae* (Borisade et al. 2016). The secondary metabolites or toxin could disrupt normal cell metabolism of the insect (Mancillas-Paredes et al. 2019).

Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). This research findings highlighted that besides *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates), *P.citrinum* (BKbTp isolate) was also pathogenic to the adults of *Cx. quinquefasciatus*. The results obtained that the fungal species that were pathogenic to adults were different from the species that were pathogenic to eggs and larvae of *Cx. quinquefasciatus*. The fungal species that were pathogenic to the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate), while The fungal species that were pathogenic to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). The entomopathogenic fungi could induce the adults of *Cx. quinquefasciatus* becoming malformation (e.g. asymmetrical wing, curled proboscis) and mycosis (after growing onto the SDA medium). The dead adults were caused by conidia germinating to be mycellia and penetrating into the adult body cavity producing secondary metabolites (via blastospores) disrupting normal cell metabolism of the insect (Mancillas-Paredes et al. 2019). After the adults died, the fungi still grow saprophytically on the cadavers of adults and the fungi induce the cadaver body becoming mycosis (Gabarty et al. 2014). The future application of the fungi against the mosquito eggs, larvae, and adults can be used an ovitrap, fungal spores formulated in a synthetic oil, and fungus-impregnated black cloths, respectively.

The fungal species that were the most pathogenic to the eggs, larvae, and adults of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4 isolate), *M. anisopliae* (MSwTp3 isolate), and *P. citrinum* (BKbTp isolate), however *T. diversus* was also the most pathogenic to the eggs. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to *Cx. quinquefasciatus*. So, the entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as the ovicide, larvicide, and adulticide.

ACKNOWLEDGEMENTS

This research was funded by the Directorate General of Higher Education, Ministry of Education, Culture, Research, and Technology, Republic of Indonesia, Fiscal Year 2022, in accordance with the Doctoral Dissertation Scheme (*Penelitian Disertasi Doktor*) with Contract Number: 142/E5/PG.02.00.PT/2022 on 10 May 2022, chaired by Siti Herlinda.

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BIODIVERSITAS ISSN: 1412-033X

Volume 23, Number 11, November 2022 E-ISSN: 2085-4722

Pages: xxxx DOI: 10.13057/biodiv/d2311xx

Hasil koreksi oleh penulis

First report of entomopathogenic fungi from South Sumatra (Indonesia): pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

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Manuscript received: xxx 2022. Revision accepted: xxx November 2022.

Abstract. *Ramayanti I, Herlinda S, Muslim A, Hasyim H. 2022. First report of entomopathogenic fungi from South Sumatra (Indonesia): pathogenicity to egg, larvae, and adult of* Culex quinquefasciatus. *Biodiversitas 23: xxxx.* Mosquito control has currently used many biocontrol agents, such as the entomopathogenic fungi. So, the study aimed to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Culex quinquefasciatus.* The fungal isolates used were eight isolates from South Sumatra and have been identified molecularly. The fungal species that were the most pathogenic to the eggs of *Cx. quinquefasciatus* were *Beauveria bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *Metarhizium anisopliae* (MSwTp3 isolate), *Penicillium citrinum* (BKbTp isolate), and *Talaromyces diversus* (MSwTp1 isolate). The *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The most pathogenic fungal species to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P. citrinum* (BKbTp isolate). Finally, The entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as the ovicide, larvicide, and adulticide. The future application of the fungi against the mosquito eggs, larvae, and adults can be used an ovitrap, fungal spores formulated in a synthetic oil, and fungus-impregnated black cloths, respectively.

Keywords: *Beauveria bassiana*, *Metarhizium anisopliae*, lymphatic filariasis, *Penicillium citrinum*, *Talaromyces diversus*, *Purpureocillium lilacinum*

INTRODUCTION

Filariasis or elephantiasis or lymphatic filariasis is an infectious disease caused by the parasitic filarial worms, such as *Wuchereria bancrofti* (Pratiwi et al. 2019) and *Brugia* sp. (Intarapuk and Bhumiratana, 2021). This worm is transmitted by vector insects of the mosquitoes, especially *Culex* (Blut 2013). There are more than 38 species of mosquitoes that act as vectors of filariasis transmission (Famakinde 2018), including *Culex quinquefasciatus* (Simonsen and Mwakitalu, 2013; Susilowati, 2018), *Culex vishnui* (Nchoutpouen et al. 2019), *Mansonia africana* and *Mansonia unifo* (Ughasi et al., 2012). Indonesia is an endemic area for lymphatic filariasis (Ginandjar et al., 2018), especially in South Sumatra (Nurjazuli and Santjaka, 2020). The consequences of this elephantiasis disease can cause physical disability, mental, social, and financial losses (Enciso et al., 2021). The higher population density of vector insects tends to be positively correlated with the higher transmission rate of this filarial worms and the higher the number of elephantiasis sufferers (Gordon et al. 2018; Ridha et al. 2020; Santoso et al. 2021).

To suppress and break the transmission of the lymphatic filariasis, the vector insects of transmission needs to be controlled and the population suppressed as low as possible so that the chain of transmission is broken. Some methods have been carried out to reduce the population density of filariasis vector insects. For example, *Cx. quinquefasciatus* has been controlled using a repellent

insecticide (Aguiar et al. 2015). Control with botanical insecticides has also been carried out, for example the use of rosmarin leaf oil (*Rosmarinus officinalis* L) to kill the larvae of *Cx. quinquefasciatus* (Susilowati 2018). These vector insects are generally controlled with synthetic insecticides (Nchoutpouen et al. 2019). However, routinely spraying of synthetic insecticides causes the new problems due to the higher level of *Cx. quinquefasciatus* resistance and it has been reported that this mosquito is resistant to permethrin, deltamethrin, DDT (dichloro-diphenyl-trichloroethane) (Nchoutpouen et al. 2019), and bendiocarb (Talipouo et al. 2021). Residues of the synthetic insecticides may cause the non-target animals killed, and the insecticides induce the human health problems and the pollution on water, air, and soil (Hamid et al. 2017). The use of synthetic insecticides also causes the high operational costs for application or spraying (Chowański et al. 2014).

Currently, mosquito control has used many biocontrol agents, for example the use of entomopathogenic fungi (pathogens that cause insect disease or insect pathology). The use of entomopathogenic fungi have occured, for example in Thailand, conidia of *Penicillium citrinum* has been found to be effective in killing the larvae of *Cx. quinquefasciatus* (Maketon et al. 2014). In India, the mycelia extract of *Beauveria bassiana* has been found to be effective in killing larvae. of *Cx. quinquefasciatus* (Vivekanandhan et al. 2018). The entomopathogenic fungi found in Indonesia, especially in South Sumatra have been tested and effectively killed some species of insect pests attacking plants (Herlinda et al. 2020a, 2020b; Gustianingtyas et al. 2021; Herlinda et al. 2021). Although many species of entomopathogenic fungi have been found in South Sumatra, there is no information on the effectiveness of these entomopathogenic fungi to kill the filariasis vector mosquito, *Cx. quinquefasciatus*. The previous study is only the pathogenicity of the entomopathogenic fungi to kill the egg, larvae, and adult of *Aedes aegypti* (Ramayanti et al. 2022). The novelty of this research is that the entomopathogenic fungi from South Sumatra and was first tested to kill eggs, larvae, and adults of *Cx. quinquefasciatus*. This research is feasible because the fungi as potential biological control agents are specific strains from South Sumatra so that they do not disturb the natural balance of existing microorganisms and *Cx. quinquefasciatus* is the main vector of filariasis which needs to be controlled. The purpose of this study was to determine the pathogenicity of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. Quinquefasciatus.*

MATERIALS AND METHODS

Fungal preparation

The fungal isolates used for this current research were from the collection of Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya and they were identified molecularly. The fungal species identified were *B. bassiana* TaAlPA isolate (GenBank acc. no. OM791688), *B. bassiana* LtKrLH isolate (GenBank acc. no. OM791680), *B. bassiana* TaLmME isolate (GenBank acc. no. OM791687), and *B. bassiana* TaPsBA isolate (GenBank acc. no. OM791689) (Ramayanti et al., 2022), *P. citrinum* BKbTp isolate (GenBank acc. no. MT448730), *Talaromyces diversus* MSwTp1 isolate (GenBank acc. no. MT448731), *B. bassiana* BSwTd4 isolate (GenBank acc. no. MT448732), and *Metarhizium anisopliae* MT488733 isolate (GenBank acc. no. MT488733) (Herlinda et al., 2020a) (Table 1). The fungi were originated from South Sumatra, Indonesia with location, Alang-alang Lebar, Palembang (2°56′32″S 104°42′16″E), Kota Raya, Lahat (3°46′38″S 103°35′25″E), Lebak, Muara Enim (3°23′51″S 104°19′41″E), Purwosari, Banyuasin (2°52′19″S 104°33′14″E), Talang Patai, Pagar Alam (4°00′45″S 103°12′21″E) and Talang Dabok, Ogan Komering Ilir (3°00′18″S 104°40′05″E). All fungal isolates were cultured on the agar medium, Sabouraud Dextrose Agar (SDA), and for bioassay they were also re-cultured on the liquid medium, SDB (Sabouraud Dextrose Broth).

Mass-rearing of Culex quinquefasciatus

Eggs of *Cx. quinquefasciatus* were gained from P2B2 Research and Development Loka, the Health Research and Development Center (the Balitbangkes), the Ministry of Health of Indonesia in Baturaja, South Sumatra, and *Cx. quinquefasciatus* mass-rearing have been carried out since June 2013. The *Cx. quinquefasciatus* mass-rearing for bioassay were carried out at the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. The room temperature and relative humidity during the mass-rearing were 29 ± 1°C and 84 ± 1%, respectively. The room lighting was set to photoperiod with 12 hours of light and 12 hours of dark (Kauffman et al., 2017). The emerging larvae were kept into a transparent plastic cup (Ø 7 cm, height 9 cm) that has been disinfected and the cup was filled in 50 ml of water (Ramayanti *et al.*, 2022). The larvae were fed with dog biscuits (Vivekanandhan et al., 2018).

Table 1. Origin of the isolates of entomophatogenic fungi from South Sumatra, Indonesia, used in this research

Location, village or district/city	Isolate origin	Altitude (m)	Fungal spesies	Fungal isolate code	GenBank acc no.
Alang-alang Lebar, Palembang	Soil	23.0	*Beauveria bassiana*	TaAlPA	OM791688*
Kota Raya, Lahat	Insect	369.9	*Beauveria bassiana*	LtKrLH	OM791680*
Lebak, Muara Enim	Soil	33.5	*Beauveria bassiana*	TaLmME	OM791687*
Purwosari, Banyuasin	Soil	19.0	*Beauveria bassiana*	TaPsBA	OM791689*
Talang Patai, Pagar Alam	Soil	175.0	*Penicillium citrinum*	BKbTp	MT448730**

Talang Dabok, Ogan Komering Ilir	Soil	24.0	*Talaromyces diversus*	MSwTp1	MT448731**
Talang Patai, Pagar Alam	Soil	193.0	*Beauveria bassiana*	BSwTd4	MT448732**
Talang Patai, Pagar Alam	Soil	193.0	*Metarhizium anisopliae*	MSwTp3	MT488733**

Sources: *(Ramayanti et al. 2022), **(Herlinda et al. 2020a)

The larvae within the plastic cup were put into a disinfected transparent plastic cage (50 x 50 x 50 cm) in order to keep the emerging adults remaining in the cage. The 10% sucrose solution infused on cotton wool for adult diet was hung on the top of the cage. Then, an ovitrap was put in the plastic cage where the adult mosquitoes were emerged from the pupae. The ovitrap was designed as a disinfected transparent plastic cup (Ø 9 cm, height 13 cm) that had dark wall and was filled with water to a depth of 10 cm (Wu et al. 2013).

The bioassay of fungal pathogenicity to egg, larvae, and adult of *Culex quinquefasciatus*

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs, larvae, and adults of *Cx. quinquefasciatus* was carried out at the laboratory with the average temperature and the relative humidity, 29.79 °C and 84.11%, respectively. After the fungal isolates were cultured on the SDA medium, then the fungal cultures were re-grown on the SDB medium in order to increase the fungal conidial density (Gustianingtyas et al. 2020). During the process of growing the fungal culture on the SDB medium (the liquid medium) for 14 days, the culture was shaken continuously for 7 days and then not shaken for 7 days. The conidia harvested from the liquid medium was calculated for getting conidial density used for bioassay below.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the eggs of *Cx. quinquefasciatus* was carried out following the the method of Luz et al. (2011). The liquid fungal culture with a concentration of 1 x 10^{10} conidia mL^{-1} was poured 10 mL into the ovitrap containing 100 mL of water. At the same time, the control was only poured 10 mL of sterile distilled water. This experiment was designed using a completely randomized design with 8 isolates and control as treatments. The experiment were repeated three times. Then, the ovitrap was put in the disinfected plastic cage, and 30 gravid female adults were also put in the cage so that the female adults could lay their eggs in the ovitrap. The duration for the female adults laying their eggs were 4 x 24 hours (Blanford et al. 2012). The female adults were also provided with 10% sucrose solution for their diet. The ovitrap containing the eggs laid was replaced daily from the cage, and then the number of the eggs laid were also counted every day. The viable eggs (the hatched eggs) were monitored and recorded every 2 hours for 48 hours. The egg morphology changing were also recorded daily. The abortion or unhatched eggs were cultured in SDA medium in order to detect and to identify the microorganism that caused abortion or unhatched. The larvae emerging were observed daily and the dead larvae were counted every day. The dead pupae were also monitored every day until adult emerging.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the larvae of *Cx. quinquefasciatus* was carried out following the the method of Alkhaibari et al. (2017). The 30 third-instar larvae were treated with 10 ml suspension of the entomopathogenic fungal isolate, the fungal suspension was put in a disinfected transparent plastic cups (Ø 7 cm, height 9 cm) with 100 mL of water inside. The 30 control larvae were only exposed to 10 mL of sterile water. All treatments were replicated three times and the the experiment was designed using a completely randomized design with 8 isolates and control as treatments. The duration of fungal exposure to the larvae were 1 x 24 hours, and then the dead larvae were monitored and recorded daily for 8 days. The variables observed were the number of larval deaths and the morphology changes of larvae after being treated with the fungi. The time of larval death and the behavior of unhealthy larvae were also observed every day. The health of the larvae identified by observing the changes of the larvae behavior and morphology. The time of larval death were used to determine of LT_{50} (the Lethal Time) and LT_{95}. The cadaver or dead larvae were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

The bioassay to determine the pathogenicity of isolates of the entomopathogenic fungi from South Sumatra to the adults of *Cx. quinquefasciatus* was carried out following the the method of Blanford et al. (2012) and Shoukat et al. (2020). Fifteen female and 15 male adults (total of 30 adults) per isolate were treated with the fungal suspension for 24 hours. The adults used in this experiment were 3 days old. Ten mL of the fungal suspension (1 x 10^{10} conidia mL^{-1}) were sprayed on the inner wall of disinfected transparent plastic cage (50 × 50 × 50 cm). Then, the cage was air-dried for 2 hours (Mnyone et al. 2011). Then, 30 adults were put in the plastic cage. For the plastic cage of control, ten mL of water was sprayed on the inner wall of the cage. All treatments were repeated three times using a completely randomized design. The adults of fungal treatment and control were given the diet of 10% sucrose solution hang on the top of the cage. After fungal exposure for 24 hours, The number of dead adults were started to be recorded after 24 hours of fungal exposure until 7 days after exposure. The dead adults were characterized with no movement occured (Shoukat et al., 2020). The time of adult death and the behavior of unhealthy adults were also observed daily. The time of adult death were used to determine of LT_{50} and LT_{95}. The cadaver or dead adult were cultured in SDA medium to determine the fungal infection and to confirm whether the fungus emerged from the cadavers.

Data analysis

The data of egg, larval, and pupal mortality of *Cx. quinquefasciatus*, LT_{50} and LT_{95} of the larvae; , adult mortality, LT_{50} and LT_{95} of *Cx. quinquefasciatus* of each treatment were analyzed using ANOVA (analysis of variance). We implemented the parametric statistical

analysis, and therefore all data were tested for normal distribution using the Shapiro-Wilk test and for variance homogeneity by Levene's test. Logarithmic transformation was performed to homogenous variance for the eggs laid before being subjected to one-way analyses of variance. Arcsin transformation was performed to homogenous variance for the egg, larval, pupal, adult mortality. The mean of the data were compared using Tukey's Honestly Significant (HSD) at a 5% level of significance. To make a clear understanding that the statements under results section based on a statistical procedure, P values have been added to the result description. All statistical analyses were calculated using software of SAS University Edition 2.7 9.4 M5. The malformation of eggs, larvae, pupae, and adults of of *Cx. quinquefasciatus* infected by the fungus were presented in photograph.

RESULTS AND DISCUSSION

The bioassay of fungal pathogenicity to egg of *Culex quinquefasciatus*

Obtained findings reported that eggs laid on the ovitrap by the gravid *Cx. quinquefasciatus* female of control (untreated fungal) were the least (1469.67 eggs/female per 96 hours) among those of fungal treatments. Egg mortality of *Cx. quinquefasciatus* of control was the lowest (16.76%) and significantly different from those of of fungal treatments (Table 2). All isolates used were pathogenic to the eggs of *Cx. quinquefasciatus*. Egg mortality of *Cx. quinquefasciatus* caused by *B. bassiana* isolate BSwTd4 was the highest (39.94%) and was not significantly different from those caused by *B. bassiana* isolate TaLmME (38.86%), and *M. anisopliae* isolate MSwTp3 (38.75%), *B. bassiana* isolate TaPsBA (36.91%), *P. citrinum* isolate BKbTp (37.04%), and *T. diversus* isolate MSwTp1(35.66%). Thus, the most pathogenic fungal species against eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). The effect of eggs treated with the fungus still affected the emerging larvae, the larvae resulted higher mortality than those of controls, as well as the pupae from treated eggs produced higher mortality than those of controls.

The morphology of *Cx. quinquefasciatus* eggs infected with the entomopathogenic fungi showed differences from the healthy eggs from the control group. The body liquid of *Cx. quinquefasciatus* infected eggs had feculent color without embryo inside, while the healthy eggs from the control group had clearly visible color with embryo inside. The color of anterior and posterior infected eggs was darker than those of the healthy eggs.

The bioassay of fungal pathogenicity to larvae of *Culex quinquefasciatus*

The third-instar larvae of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) had significantly higher mortality compared to the control larvae (untreated larvae) (Table 3). All fungal isolates used the current study were pathogenic to the third-instar larvae of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The larval mortality caused by *M. anisopliae* isolate MSwTp3 (100% with LT_{50} 2.02 days and LT_{95} 7.15 days) was the highest and not significantly different from mortality caused by *B. bassiana* isolate BSwTd4 (98.89% with LT_{50} 2.51 days and LT_{95} 7.61 days) and *B. bassiana* isolate TaLmME (97.78% with LT_{50} 2.75 days and LT_{95} 7.85 days).

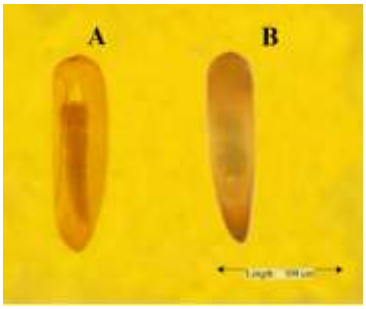

Figure 1. Morphology of the *Culex quinquefasciatus* eggs: a healthy egg of control (A) and an infected treated egg (B)

Table 2. Effect of eggs treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on the egg laid, the egg, larval, and pupal mortality of *Culex quinquefasciatus*

Species	Isolate code	Eggs laid per female per 96 hours [a)]	Egg mortality (%)[b)]	Larval mortality (%)[b)]	Pupal mortality (%)[b)]
Control	-	1469.67±14.46^{b}	16.76±0.82^{d}	17.59±0.11^{d}	0.99±0.15^{d}
Beauveria bassiana	TaAlPA	1511.00±11.09^{ab}	32.70±0.71^{bc}	33.33±0.45^{c}	2.86±0.14^{c}
Beauveria bassiana	LtKrLH	1482.67±13.74^{b}	31.06±0.42^{c}	30.58±0.55^{d}	2.60±0.06^{c}
Beauveria bassiana	TaLmME	1616.67±9.48^{a}	38.86±0.23^{a}	40.02±0.12^{a}	5.06±0.12^{ab}
Beauveria bassiana	TaPsBA	1574.33±15.59^{ab}	36.91±0.25^{ab}	35.23±0.37^{c}	3.37±0.06^{c}
Penicillum citrinum	BKbTp	1556.33±26.22^{ab}	37.04±1.17^{ab}	37.72±0.31^{b}	3.75±0.32^{bc}
Talaromyces diversus	MSwTp1	1563.67±26.02^{ab}	35.66±0.95^{abc}	34.41±0.28^{c}	3.17±0.12^{c}
Beauveria bassiana	BSwTd4	1637.33±3.60^{a}	39.94±0.08^{a}	41.20±0.28^{a}	6.31±0.49^{a}
Metarhizium anisopliae	MSwTp3	1613.33±29.58^{a}	38.75±1.26^{a}	40.15±0.02^{a}	5.49±0.12^{a}
F-value		6.20*	63.5*	345.2*	45.41*
P-value		6.50 x 10^{-4}	1.29 x 10^{-11}	2.0 x 10^{-16}	2.25 x 10^{-10}
HSD value		0.04	2.98	1.94	1.96

Note: * = significantly different; values within a column followed by the same letters were not significantly different at P < 0.05 according to Tukey's HSD test, [a)]Original data were transformed using logarithmic transformation, [b)]Original data were transformed using Arcsin transformation prior to statistical analysis

Table 3. Effect of larvae treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on larval mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Larvae mortality (%)[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]
Control	-	0.00±0.00^{f}	14.98±0.43^{a}	20.21±0.51^{a}
Beauveria bassiana	TaAlPA	84.44±2.40^{cd}	3.97±0.16^{b}	9.08±0.28^{bc}
Beauveria bassiana	LtKrLH	78.89±2.40^{de}	4.21±0.17^{b}	9.31±0.28^{bc}
Beauveria bassiana	TaLmME	97.78±0.91^{ab}	2.75±0.07^{cd}	7.85±0.15^{cd}
Beauveria bassiana	TaPsBA	80.00±3.14^{cde}	4.05±0.11^{b}	9.15±0.12^{bc}
Penicillum citrinum	BKbTp	92.22±0.91^{bc}	3.78±0.51^{bc}	8.88±0.62^{bcd}
Talaromyces diversus	MSwTp1	64.44±0.91^{e}	5.04±0.15^{b}	10.14±0.26^{b}
Beauveria bassiana	BSwTd4	98.89±0.91^{a}	2.51±0.10^{d}	7.61±0.21^{cd}
Metarhizium anisopliae	MSwTp3	100.00±0.00^{a}	2.02±0.07^{d}	7.15±0.07^{d}
F-value		155.00*	116.60*	79.77*
P-value		5.31 x 10^{-15}	6.52 x 10^{-14}	1.80 x 10^{-12}
HSD value		10.62	0.33	0.30

Note: * = significantly different; values within a column followed by the same letters were not significantly different at P < 0.05 according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)] Original data were transformed using square root (sqrt) transformation

The most pathogenic fungal species to the third-instar larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). Obtained findings highlighted that *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) possessed larvicidal activity.

The fungal infected larvae of *Cx. quinquefasciatus* showed typical morphology, particularly the morphology of sick and dead larvae. The sick larvae underwent a lysis of the gut lumen with white color and the larvae abdomen had no distinct segment. The epithelial lining possessed milky color with a ruptured anal segment (Figure 2). The healthy larvae of control had a transparent gut lumen with a visible or recognizably different segment of abdomen. The healthy larvae also had a visible epithelial lining and an undamaged anal segment. The larval cadavers of fungal treatment cultured on SDA medium produced conidia and mycellia covering the cadaver body, while the healthy larvae were not infected or covered by fungal conidia or mycellia. The pupae emerging from the infected larvae generally became sick and died. The sick pupae body were thinner, hardened, and straight shaped and had the black head. The healthy pupae had fatter, round, flexible and soft body, bent like a comma shape and their head had dark-brown in color (Figure 3).

The bioassay of fungal pathogenicity to adult of *Culex quinquefasciatus*

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi (1 x 10^{10} conidia ml^{-1}) had significantly higher mortality compared to the control adults (untreated adults) (Table 4). The adult mortality caused by *M. anisopliae* isolate MSwTp3 (100% with LT_{50} 3.25 days and LT_{95} 6.70 days) was highest and not significantly different from those caused by *B. bassiana* isolate BSwTd4 (100% with LT_{50} 3.46 days and LT_{95} 6.76 days) and *B. bassiana* isolate TaLmME (98.89% with LT_{50} 3.70 days and LT_{95} 7.15 days), and *P.citrinum* isolate BKbTp (98.89% with LT_{50} 3.96 days and LT_{95} 7.41 days). Nevertheless, all fungal isolates in this study were pathogenic to the adults of *Cx. quinquefasciatus* due to the mortality caused by the fungi was more than 60%. The most pathogenic fungal species to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). This research findings highlighted that *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate) had adulticidal activity.

The adults of *Cx. quinquefasciatus* treated with the entomopathogenic fungi became sick and finally died. The typical symptoms of sick and dead infected adults were malformation. The unhealthy adults had the asymmetrical wing shapes, the dried and mycosis body, and a spiral shape proboscis (Figure 4).

Figure 2. Morphology of the *Culex quinquefasciatus* larvae: a healthy larvae of control (A) and an infected treated larvae (B)

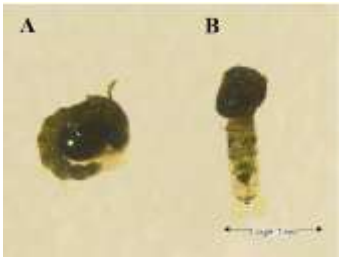

Figure 3. Morphology of the *Culex quinquefasciatus* pupae: a healthy pupae of control (A) and an infected treated pupae (B)

Table 4. Effect of adults treated with entomopathogenic fungi (1 x 10^{10} conidia mL^{-1}) on adult mortality, LT_{50} and LT_{95} of *Culex quinquefasciatus*

Species	Isolate code	Adult mortality (%)[a)]	LT_{50} (days)[b)]	LT_{95} (days)[b)]
Control	-	0.00±0.00^{d}	11.99±0.40^{a}	15.44±0.42^{a}
Beauveria bassiana	TaAlPA	88.89±1.81^{b}	4.64±0.03^{c}	8.09±0.06^{bc}
Beauveria bassiana	LtKrLH	82.22±0.91^{b}	4.84±0.02^{bc}	8.29±0.02^{b}
Beauveria bassiana	TaLmME	98.89±0.91^{a}	3.70±0.04^{de}	7.15±0.06^{d}
Beauveria bassiana	TaPsBA	87.78±0.91^{b}	4.63±0.01^{c}	8.08±0.03^{bc}
Penicillum citrinum	BKbTp	98.89±0.91^{a}	3.96±0.05^{d}	7.41±0.06^{cd}
Talaromyces diversus	MSwTp1	63.33±1.57^{c}	5.37±0.06^{b}	8.82±0.09^{b}
Beauveria bassiana	BSwTd4	100.00±0.00^{a}	3.46±0.10^{de}	6.76±0.21^{d}
Metarhizium anisopliae	MSwTp3	100.00±0.00^{a}	3.25±0.10^{e}	6.70±0.10^{d}
F-value		23.11*	229.30*	183.60*
P-value		5.85 x 10^{-8}	2.00 x 10^{-16}	1.19 x 10^{-15}
HSD value		24.55	0.15	0.15

Note: * = significantly different; values within a column followed by the same letters were not significantly different at $P < 0.05$ according to Tukey's HSD test, [a)]Original data were transformed using Arcsin transformation prior to statistical analysis, [b)]Original data were transformed using square root (sqrt) transformation

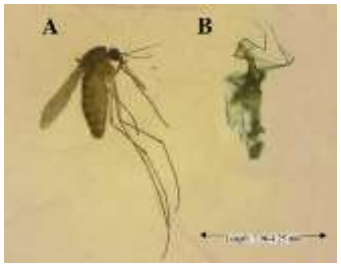

Figure 4. Morphology of the *Cx. quinquefasciatus* adults: a healthy adult of control (A) and an infected treated adult (B)

If the unhealthy adults died, their cadavers grown in SDA medium could be covered with the fungal conidia and mycellia. On the contrary, the healthy adults of control possessed the symmetrical wing shapes, and a straight shaped black proboscis, and no mycosis on the cadaver body. The healthy adults had the elongate abdomen. The cadavers from the healthy adults grown in SDA medium were not covered with the fungal conidia and mycellia.

Discussion

The eggs laid by the gravid *Cx. quinquefasciatus* female of control (untreated) were the least among those of fungal treatments. However, the eggs laid by the fungal treated female were more than those of control because the ovitrap where they were laying eggs had water with dyed color due to added with the fungal suspension. The gravid female *Culex* mosquitoes preferred to lay eggs in dyed water (Day 2016; Perea and Callaghan 2017). Although the treated eggs laid by female *Cx. quinquefasciatus* in this study were less than 50%, but the treated eggs induced the sick larvae and produced the mortality of emerging larvae and pupae. Besides, the mycosis on the dead larvae and pupae that failed to emerge from the eggs occured. In this study, the ovitrap used to expose the fungi to the eggs of *Cx. quinquefasciatus* could effectively infected its eggs, larvae, pupae, and adults. The finding highlighted that the *Cx. quinquefasciatus* eggs infected with the fungus not only could kill the eggs but also could continue to kill the emerging larvae, pupae, and adult. The most pathogenic fungal species against the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate). This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to the eggs of *Cx. quinquefasciatus* and had ovicidal activity. The entomopathogenic fungi have reported caused the eggs of mosquito unhatched and abortion and the emerging larvae and pupae could not carry on their life (Leles et al. 2012; Ramayanti et al. 2022). The obtained data also reported the embryo of eggs treated with the fungi were lysis and egg liquid becoming feculent color and no embryo inside the eggs.

The egg mortality of *Cx. quinquefasciatus* caused by the entomopathogenic fungi were lower than the larvae mortality caused by the fungi. The third-instar larvae of *Cx. quinquefasciatus* could be immediately killed by *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates) ($LT_{50} < 3$ days). The high mortality of the larvae treated with the entomopathogenic fungi in short time due to higher fungal suspension used (1 x 10^{10} conidia ml^{-1}) and the fungi cultured in the broth medium (SDB). The broth culture is able to grow the fungus and produce blastospores, and the blastospores are more

effective compared to aerial conidia in killing the mosquitoes (Alkhaibari et al. 2017). The findings highlighted that both species of the fungi could be develop to be a larvicide for *Cx. quinquefasciatus* because they have highest level of larvicidal activity (97.78−100% of larvae mortality). The larvae mortality caused by the entomopathogenic fungi was higher than the egg mortality caused by the entomopathogenic fungi because the cuticle of integument of the larvae is thinner than those of the eggs (Farnesi et al. 2015). The thinner was the cuticle of the insect integument, the easier the fungal conidia are penetrated into insect body (Ortiz-Urquiza and Keyhani 2013).

The results obtained that the larvae infected by the fungi could be sick or dead because their gut lumen ruptured or lysis by the fungi. The fungi also damaged the anal and abdomen segments so that the larva body became malformation. The larvae died due to the conidia of entomopthogenic fungi germinating and their hyphae penetrating into the insect integument, after that the hyphae entered to the body cavity (Boomsma et al. 2014). The hyphae developed to become blastospores in the larvae hemolymph (Mancillas-Paredes et al. 2019). The blastospores of the entomopthogenic fungi could produce secondary metabolites, such as bassiacridin (Quesada-moraga and Vey 2004) and beauvericin (Safavi 2012) secreted by *B. bassiana* and destruxin produced by *M. anisopliae* (Borisade et al. 2016). The secondary metabolites or toxin could disrupt normal cell metabolism of the insect (Mancillas-Paredes et al. 2019).

Three species of the entomopathogenic fungi that were the most pathogenic to the adults of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (BSwTd4 and TaLmME isolates), and *P.citrinum* (BKbTp isolate). This research findings highlighted that besides *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates), *P.citrinum* (BKbTp isolate) was also pathogenic to the adults of *Cx. quinquefasciatus*. The results obtained that the fungal species that were pathogenic to adults were different from the species that were pathogenic to eggs and larvae of *Cx. quinquefasciatus*. The fungal species that were pathogenic to the eggs of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4, TaLmME, TaPsBA isolates), *M. anisopliae* (MSwTp3 isolate), *P.citrinum* (BKbTp isolate), and *T. diversus* (MSwTp1 isolate), while The fungal species that were pathogenic to the larvae of *Cx. quinquefasciatus* were *M. anisopliae* (MSwTp3 isolate) and *B. bassiana* (BSwTd4 and TaLmME isolates). The entomopathogenic fungi could induce the adults of *Cx. quinquefasciatus* becoming malformation (e.g. asymmetrical wing, curled proboscis) and mycosis (after growing onto the SDA medium). The dead adults were caused by conidia germinating to be mycellia and penetrating into the adult body cavity producing secondary metabolites (via blastospores) disrupting normal cell metabolism of the insect (Mancillas-Paredes et al. 2019). After the adults died, the fungi still grow saprophytically on the cadavers of adults and the fungi induce the cadaver body becoming mycosis (Gabarty et al. 2014). The future application of the fungi against the mosquito eggs, larvae, and adults can be used an ovitrap, fungal spores formulated in a synthetic oil, and fungus-impregnated black cloths, respectively.

The fungal species that were the most pathogenic to the eggs, larvae, and adults of *Cx. quinquefasciatus* were *B. bassiana* (BSwTd4 isolate), *M. anisopliae* (MSwTp3 isolate), and *P. citrinum* (BKbTp isolate), however *T. diversus* was also the most pathogenic to the eggs. This is the first record that *B. bassiana*, *M. anisopliae*, *P.citrinum*, and *T. diversus* from South Sumatera Indonesia were pathogenic to *Cx. quinquefasciatus*. So, the entomopathogenic fungi from South Sumatra have the negative effect on *Cx. quinquefasciatus* growth. The entomopathogenic fungi from South Sumatra have potential to be developed as the ovicide, larvicide, and adulticide.

ACKNOWLEDGEMENTS

This research was funded by the Directorate General of Higher Education, Ministry of Education, Culture, Research, and Technology, Republic of Indonesia, Fiscal Year 2022, in accordance with the Doctoral Dissertation Scheme (*Penelitian Disertasi Doktor*) with Contract Number: 142/E5/PG.02.00.PT/2022 on 10 May 2022, chaired by Siti Herlinda.

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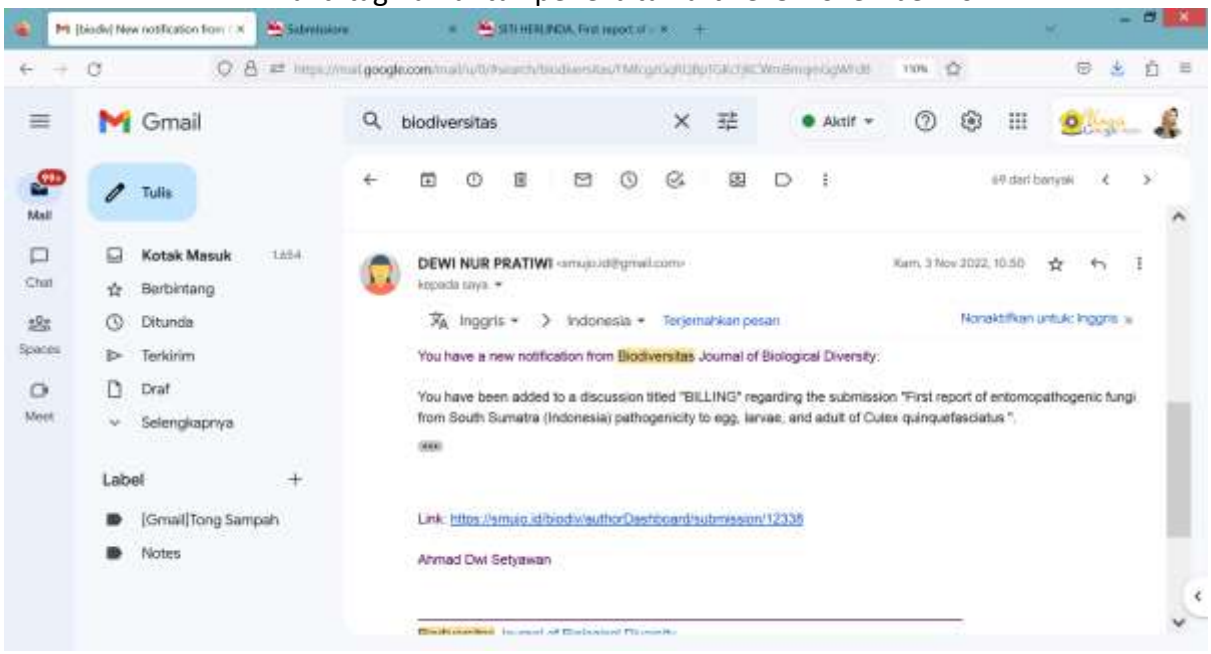

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