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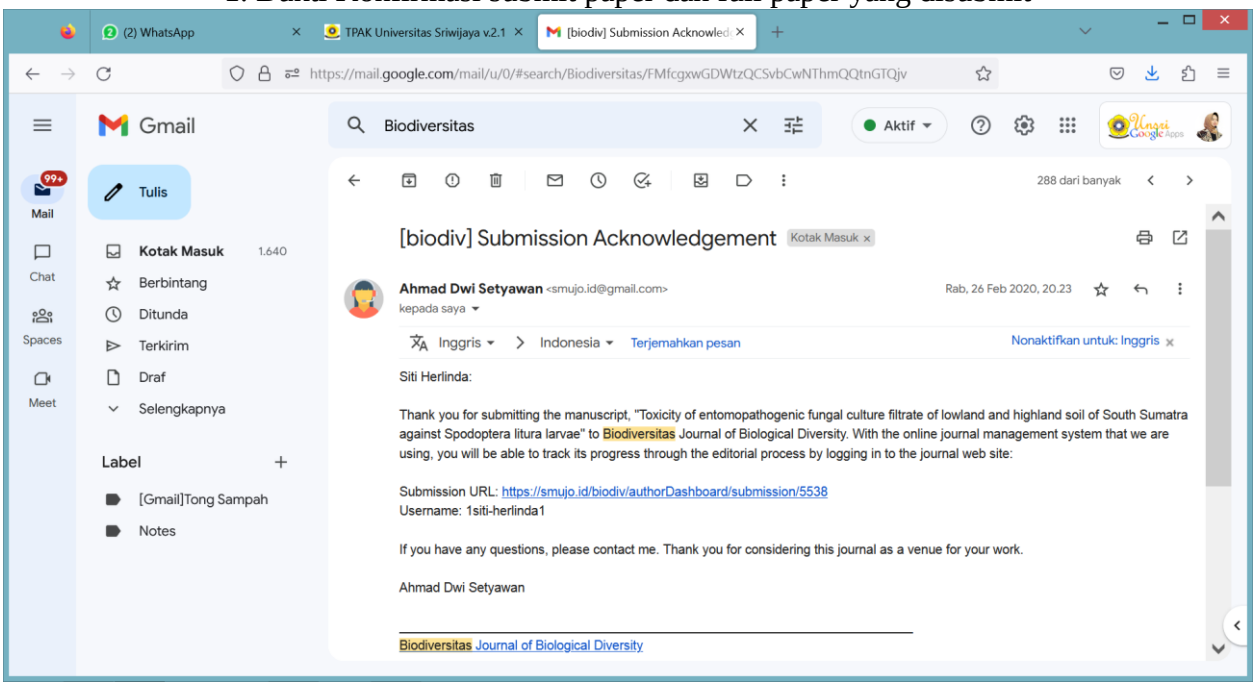

COVERING LETTER

Dear **Editor-in-Chief,**

I herewith enclosed a research article,

Title:

Toxicity of entomopathogenic fungal culture filtrate of lowland and highland soil of South Sumatra against *Spodoptera litura* larvae

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This study highlights several findings, such as entomopathogenic fungal filtrate cultures (*Beauveria bassiana* and *Metarhizium anisopliae*) are toxic and capable of killing *Spodoptera litura* larvae in a short time. The most toxic fungal isolates are BSwTd2 isolate of *B. bassiana* and MKbTp2 isolate of *M. anisopliae* originated from the lowlands and highlands of South Sumatra.

Statements:

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Place and date:

Palembang, 24 February 2020

Sincerely yours,

(fill in your name, no need scanned autograph)

Siti Herlinda

Toxicity of entomopathogenic fungal culture filtrate of lowland and highland soil of South Sumatra against *Spodoptera litura* larvae

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Abstract. The use of secondary fungal metabolites for the active ingredient of mycoinsecticide is more effective and more easily integrated with other pest control techniques. This study aimed to measure the toxicity of the culture filtrate of entomopathogenic fungi originating from South Sumatra against the *Spodoptera litura* larvae. *Beauveria bassiana* (25 isolates) and *Metarhizium anisopliae* (20 isolates) of South Sumatra were cultured in liquid media and filtered to produce culture filtrate. The larvae which were sick due to the filtrate showed the symptoms of decreased appetite and were not actively moving, while the dead larvae were characterized by being wrinkled, dry, black integument, and odorless. Mortality caused by *B. bassiana* filtrate was the highest 98% (BJgTs isolate) and not significantly different from the BSwTd2 isolate (94.67%). Yet, the LT_{50} BSwTd2 isolate was shorter (5.92 days) compared to the LT_{50} BJgTs isolate (6.35 days). The most toxic *M. anisopliae* filtrate produced the mortality of 96% (MKbTp2 isolate) and 85.33% (MPdB isolate) each of which had LT_{50} of 7.36 days and 8.09 days, respectively. So, the most toxic culture filtrate was BSwTd2 isolate of *B. bassiana* and MKbTp2 isolate of *M. anisopliae*. The entomopathogenic fungi producing filtrate which are toxic have the potential to be active ingredients of mycoinsecticides.

Key words: *Beauveria. bassiana*, chili, $LT_{50,}$ *Metarhizium anisopliae*, mortality

Abbreviations (if any): -

Running title: Toxicity of entomopathogenic fungi againts *Spodoptera*

INTRODUCTION

South Sumatra is generally dominated by lowland lands, namely freshwater swamps and tidal lowlands (Kartika et al. 2018; Karenina et al. 2019), and a small portion of medium and highland. Freshwater swamps can be found, among others in the Districts of Ogan Ilir, Ogan Komering Ilir, Musi Banyuasin, and Palembang City; the widest tide exists in Banyuasin District and the medium and highlands occur, among others, in the Districts of Ogan Komering Ulu Selatan, Lahat and Pagaralam City. The varied topographical conditions between districts/cities characterize the differences including the crops and microorganisms cultivated there.

In the lowlands of South Sumatra are generally cultivated annual crops, such as paddy (Herlinda et al. 2018a; Prabawati et al. 2019), corn (Juhriah et al. 2019), chili (Johari et al. 2016; Nasution & Respatijarti, 2019; Sagrim et al. 2017), and palm oil (Darlan et al. 2016). Meanwhile, in the medium and high plains are generally cultivated annual crops such as coffee, tea (Zamhari et al. 2017), vegetables such as potatoes (Maryanto et al. 2018), cabbage, mustard greens, and caisim (Situmorang et al. 2019). Vegetables and seasonal plants of the lowlands and highlands generally have a major pest, namely *Spodoptera litura* (Turnip et al. 2019). *S. litura* is a polyphagic pest that attacks many vegetables in Indonesia, for example chili (Nagal et al. 2016), cotton fruit (Maqsood et al. 2017), soybean (Fattah et al. 2018). To control *S. litura*, it needs natural enemies such as entomopathogenic fungi that can adapt in low to high altitudes.

In the lowlands and highlands, the soil types have different chemical and physical characteristics; acidic pH soils are generally found in the lowlands and neutral or basic pH soils are found in the highlands (Munir & Herman 2019). Soil pH can affect the presence of entomopathogenic fungi and tends to be neutral or high pH soils which more commonly occur in entomopathogenic fungi than low pH soils (Safitri et al. 2018). Besides pH, the texture of sandy soils tends to be low and muddy which is not suitable for fungal propagule life as well as water-saturated soil where fungi are rarely spotted to

survive (Garrido-Jurado et al. 2011). Temperature (Pinnamaneni et al. 2010) and humidity (Oreste et al. 2015; Liu et al. 2017) also affect pathogenicity of entomopathogenic fungi.

Previous study found that more than 30 isolates of entomopathogenic fungi were found in the freshwater swamp, tidal, and highland soil of South Sumatra (Safitri et al. 2018). Apart from the soil, the entomopathogenic fungi were also found from the insect pests of vegetable and food crops of the lowlands to the highlands of South Sumatra (Herlinda et al. 2018b). Various origins of entomopathogenic fungi tend to produce pathogenenicity variations (Sumikarsih et al. 2019).

The pathogenicity of entomopathogenic fungi is influenced by the ability of the fungi to produce secondary metabolites (Zibaee et al. 2009). The fungi that develop in insect haemolymph or in liquid media are able to produce toxic metabolites, such as extracellular enzymes, proteins, and toxins (Bandani 2005). In vitro media, toxic metabolites can be produced by the fungi which are grown in liquid media and produce culture filtrate (Soesanto et al. 2019). The use of secondary entomopathogenic fungal metabolites for the active ingredient of mycoinsecticide is more effective and more easily integrated with other pest control techniques (Zibaee et al. 2009). Entomopathogenic fungal culture filtrate found in low to high altitude of South Sumatra potentially gets the most toxic isolates that can be utilized in the development of mycoinsecticide technology. For this reason, this study aimed to measure the toxicity of the culture filtrate isolates of entomopathogenic fungi from low to high land of South Sumatra to the *Spodoptera litura* larvae.

MATERIALS AND METHODS

The experiments were carried out at the Entomology Laboratory, Department of Pests and Plant Diseases, Faculty of Agriculture, Universitas Sriwijaya from May to November 2018 at relative temperatures and humidity of 29.78 °C and 82.72%, respectively. The entomopathogenic fungi isolates used in this study were explored by Herlinda et al. (2018) and Safitri et al. (2018), (Tables 1 and 2) spread from the lowlands to the highlands of South Sumatra. The isolates were grouped into two: 25 isolates of *Beauveria bassiana* (Figure 1) and 20 isolates of *Metarhizium anisopliae* (Figure 2). All isolates were identified by Dr. Suwandi (a mycologist from Universitas Sriwijaya).

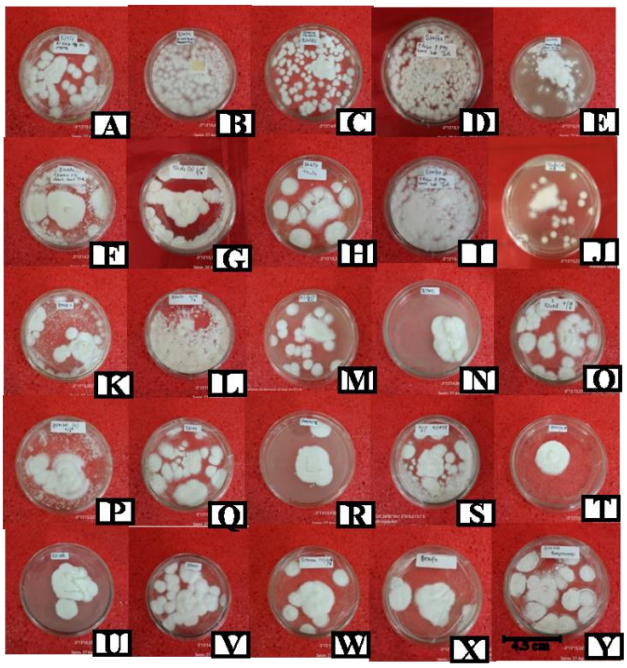

Figure 1. *Beauveria bassiana* isolates cultured in SDA media: BJgTs (A), BSmMs (B), BSwTd1 (C), BSwTd2 (D), BSwTd3 (E), BSwTd4 (F), BPdR (G), BKbTp (H), BKKPp2 (I), Ts1d3 (J), BTmPc (K), BTmTr (L), (M) Ts1d2 (M), BTmTs (N), BlePd2 (O), BTmKt (P), BPCmS (Q), BMkMs (R), BtmGa (S), BTmSr (T), BPcPd2 (U), BtmSo (V), BtmMa (W), BtmPe (X), Blepd (Y)

Test insect preparation

S. litura larvae were collected from the chilli planting area in the experimental field of the Faculty of Agriculture, Sriwijaya University and farmers' vegetable fields which were not applied by synthetic insecticides. Then, the larvae were brought to the laboratory and maintained in a plastic cylinder whose upper part was covered with gauze (Ø 25 cm, height 30 cm) having been washed thoroughly using detergent. Next, water spinach plants were put into it for larvae feed.

Every day the larvae feed was replaced with fresh new feed. The larvae entering the pupae phase were transferred into a plastic cylinder (Ø 10 cm, height 15 cm) with the top open and the bottom of the cylinder sprinkled with sifted soil and

sterilized in an oven for 1 hour at 100 ºC. The thickness of the soil inserted into the cylinder was 3 cm. The plastic cylinder containing the pupae was put into a gauze cage (30x30x30 cm^3) which had been chopped with chilies for laying eggs. Adults arising from the pupae were fed with honey smeared on cotton and hung over a cage. The hatched eggs were transferred into a plastic cylinder and fed with water spinach which was replaced every day. The larvae used for toxicity testing were the second instar of the third offspring or afterwards.

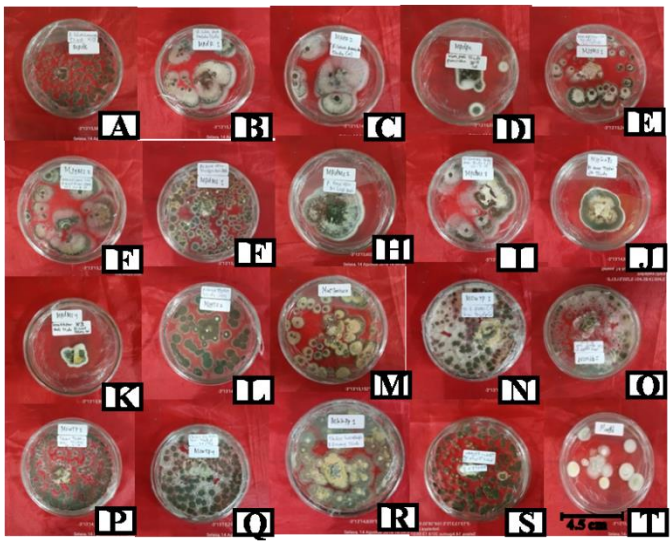

Figure 2. *Metarhizium* isolates cultured in SDA media: MPdB (A), MPdR1 (B), MPdR2 (C), MpdPe (D), MJgMs1 (E), MJgMs2 (F), MPdMs1 (G), MPdMs2 (H), MPdMs3 (I), MjgKeTs (J), MPdMs4 (K), MJgTs2 (L), MKbTp1 (M), MSwTp1 (N), MSwTp2 (O), MSwTp3 (P), MSwTp4 (Q), MKKPp1 (R), MKbTp2 (S), MagPd (T)

Table 1. *Beauveria bassiana* isolates from South Sumatra used in this research

Isolate code	Isolate origin	Geographic origin
BJgTs	Tidal lowlands, corn	Telang Sari
BSmMs	Tidal lowlands, watermelon	Mulya Sari
BSwTd1	Peatlands, oil palm	Talang Dabok
BSwTd2	Peatlands, oil palm	Talang Dabok
BSwTd3	Peatlands, oil palm	Talang Dabok
BSwTd4	Peatlands, oil palm	Talang Dabok
BPdR	Freshwater swamps, paddy	Rambutan
BKbTp	Highlands, cabbage	Talang Patai
BKKPp2	Highlands, rubber and coffee	Pulau Pinang
Ts1d3	Peatlands	Talang Dabok
BTmPc	Freshwater swamps	Indralaya
BTmTr	Freshwater swamps	Telang Rejo
Ts1d2	Peatlands	Talang Dabok
BTmTs	Highlands	Mulia Sari
BlePd2	*Lipaphis erysimi*	Pagardin
BTmkt	Freshwater swamps	Kenten
BPCmS	*Pseudoplusia chalcites*	Muara Siban
BMkMs	Highlands	Muara Siban
BtmGa	Freshwater swamps	Gandus
BTmSr	Tidal lowlands	Srikaton
BPcPd2	*Chrysodeixis chalcites*	Pagardin
BTmSo	Freshwater swamps	Soak
BTmMa	Freshwater swamps	Mariana
BTmPe	Freshwater swamps	Pemulutan
Blepd	*Lipaphis erysimi*	Pagardin

Table 2. *Metarhizium anisopliae* isolates from South Sumatra used in this research

Isolate code	Isolate soil origin	Geographic origin
MPdB	Freshwater swamps, paddy	Banyuasin
MPdR1	Freshwater swamps, paddy	Rambutan
MPdR2	Freshwater swamps, paddy	Rambutan
MPdPe	Freshwater swamps, paddy	Pemulutan
MJgMs1	Tidal lowlands, corn	Mulya Sari
MJgMs2	Tidal lowlands, corn	Mulya Sari
MPdMs1	Tidal lowlands, paddy	Mulya Sari
MPdMs2	Tidal lowlands, paddy	Mulya Sari
MPdMs3	Tidal lowlands, paddy	Mulya Sari
MJgKeTs	Tidal lowlands, corn and oil palm	Telang Sari
MPdMs4	Tidal lowlands, paddy	Mulya Sari
MJgTs2	Tidal lowlands, corn	Telang Sari
MKbTp1	Highlands, cabbage	Talang Patai
MSwTp1	Highlands, mustard	Talang Patai
MSwTp2	Highlands, mustard	Talang Patai
MSwTp3	Highlands, mustard	Talang Patai
MSwTp4	Highlands, mustard	Talang Patai
MKKPp1	Highlands, mustard, rubber, coffee	Pulau Pinang
MKbTp2	Highlands, cabbage	Talang Patai
MagPd	Highlands, *Aphis gossypii*	Pagardin

Culture filtrate production

All isolates before being filtered were first made fit by modifying the method of Herlinda (2010). The media for making fit used Sabouraud Dextrose Agar (SDA, Merck) as much as 16.2 g and added 250 mL aquadest enriched with 1.25 g *Tenebrio molitor* larvae flour (5 g). After that, the ingredients were mixed and stirred evenly and then put in an erlenmeyer (size 250 mL), sterilized in an autoclave for 120 minutes at a pressure of 1 atm. The media was poured into the Petri dish as much as 10 mL (Ø 9 cm) in aseptic laminar flow air conditions. Then, the entomopathogenic fungi isolates were inoculated in the SDA media and incubated for 14 days.

Isolates of entomopathogenic fungi derived from SDA media aged 14 days were then transferred and grown into liquid media (broth media), PDB (*Potato Dextrose Broth*) prepared as follows: the PDB media composition consisting of 20 g dextrose monohydrate, 200 g potatoes, and 1000 mL aquadest. Before the potatoes were extracted, they were cut into cubes with a size of ± 2x2x2 cm^3 and boiled using 1000 mL aquadest for 20 minutes. This PDB medium was sterilized in the autoclave and after it got cold, the entomopathogenic fungus isolates were inoculated into it. This liquid culture was incubated for 6 weeks.

After the fungus liquid culture (fungal broth) was 6 weeks old, the fungus filtration is carried out. The filtration was conducted in two stages, namely filtering using filter paper and syringe filter. In the first stage, 100 mL of 6-week-old fungal broth was filtered using Whatman filter paper no. 42 and coated with a thickness of 1 cm cotton. The culture filtrate obtained from the first stage of filtering was then sucked up to 10 mL using a hypodermic needle (spike). Then the needle was removed and the base of the needle was fitted with a syringe filter (0.45 µm-25 mm). The 10 mL spike was pressed so that it released culture filtrate from the syringe filter. The 100 mL of fungal broth produced ± 70 mL of culture filtrate. The culture filtrate was taken as much as 1 mL to be dripped on a piece of chili leaf which was used as a toxicity test. This culture filtrate is often referred to as raw secondary metabolites (Soesanto et al. 2019). To ensure the culture filtrate did not contain propagules (hyphae, micelia) and conidia, it was grown on agar media (SDA) before it was applied. If it does not grow fungus on the SDA media, the culture filtrate can be applied.

Insecticidal activity test of entomopathogenic fungus culture filtrate

The chili leaves that had been dropped by the culture filtrate were first drained before 25 *S. litura* larvae which were fasted for 2 hours were put into them. The second instar larvae were left to eat leaves that had been dropped with culture filtrate for 6 hours. After 6 hours, the larvae were transferred into a plastic cylinder topped with gauze (Ø 25 cm, height 30 cm) containing 15-30 clean chili leaves which were not dripped with culture filtrate. Every day the chili leaves were replaced with the new ones. Every day the dead larvae were recorded up to 12 days after the application.

Data analysis

The differences in mortality data and lethal time that killed 50% of test insects (LT50) were analyzed using analysis of variance (ANOVA), after that further tests were carried out using the 5% Tukey's Honestly Significant Difference (HSD). All data were calculated using software of SAS University Edition 2.7 9.4 M5.

RESULTS AND DISCUSSION

Insecticidal activities of entomopathogenic fungi

The culture filtrate derived from twenty-five *B. bassiana* isolates tested for the insecticidal activity showed that the most toxic *B. bassiana* isolates were those coded BJgTs (98%) and were not significantly different, among others with BSwTd2 (94.67%) and BTmTs isolates (96%). All *B. bassiana* isolates showed the ability to kill the test larvae (Table 3). The BJgTs, BSwTd2, and BTmTs isolates were the most toxic because they produced the highest mortality and also their ability to kill the shortest as evidenced by the short LT_{50} (6.35, 5.92, and 7.78 days) isolates. The most toxic isolates were shown with the highest mortality and their shortest LT_{50}. Consequently, for *B. bassiana*, the most toxic isolate was BSwTd2 isolate.

The culture filtrate of the twenty *M. anisopliae* isolates which were tested for their insecticidal activity showed that the most toxic isolates were those coded MKbTp2 (96%) and MPdB (85.33%) and the mortality of both isolates was significantly different from the other isolates (Table 4). The BSwTd2 isolate was the most toxic because they produced the highest mortality as well as the ability to kill the shortest as evidenced by the shortest LT_{50} (7.36 days), and then followed by LT_{50} isolate MPdB (8.09 days). The most toxic *M. anisopliae* isolate was proven by the highest mortality and the shortest LT_{50} which was MKbTp2 isolate.

The color of the fungal broth (Figure 3) and culture filtrate (Figure 4) of *B. bassiana* varied among the isolates. All fungal broth isolates were generally transplanted brown, but there were among those isolates dark brown in color, for example isolates BSwTd2, BSwTd3, BPdR, TS1d2. There was a tendency for the darker color of the fungal broth and culture filtrate to cause higher mortality. The color of fungal broth (Figure 5) and culture filtrate (Figure 6) of *M. anisopliae* differed from the colors of fungal broth and culture of *B. bassiana*. The *M. anisopliae* fungal broth was generally dark greenish brown, but the culture filtrate tended to have a more varied color, from greenish dark brown to light brown. The same tendency was also produced in *M. anisopliae* isolates, i.e., the color of the older culture filtrate resulting in higher mortality. The isolates having a darker colored culture filtrate were MPdB, MPdMs1, MPdMs2, MPdMs3, MKbTp1, MKbTp2.

Table 3. Mortality of larval *Spodoptera litura* after being infested with *Beauveria bassiana* culture filtrates

Isolate codes	Mortality ±SE*(%)	LT_{50} (days)± SE
BJgTs	98.00 ± 1.41^{b}	6.35 ± 0.49
BSmMs	50.00 ± 32.53^{ab}	11.57 ± 4.08
BSwTd1	49.33 ± 15.36^{ab}	12.03 ± 3.52
BSwTd2	94.67 ± 4.35^{ab}	5.92 ± 0.46
BSwTd3	73.33 ± 18.51^{ab}	8.86 ± 1.78
BSwTd4	26.00 ± 1.41^{ab}	16.07 ± 0.97
BPdR	86.67 ± 3.93^{ab}	8.17 ± 0.61
BKbTp	62.67 ± 10.39^{ab}	10.71 ± 0.91
BKKPp2	58.00 ± 7.07^{ab}	10.68 ± 0.34
TS1d3	10.67 ± 2.88^{a}	17.45 ± 0.76
BTmPc	46.67 ± 17.01^{ab}	14.32 ± 3.72
BTmTr	29.33 ± 9.68^{ab}	14.31 ± 1.79
TS1d2	88.00 ± 8.22^{ab}	7.87 ± 1.11
BTmTs	96.00 ± 0.00^{ab}	7.78 ± 0.09
BLePd2	70.00 ± 21.21^{ab}	9.59 ± 1.43
BTmKt	80.00 ± 14.73^{ab}	8.80 ± 1.41
BPcMs	81.33 ± 9.49^{ab}	7.87 ± 1.32
BMkMs	72.00 ± 17.99^{ab}	10.82 ± 2.46
BTmGa	66.67 ± 22.34^{ab}	12.27 ± 3.60
BTmSr	21.33 ± 9.30^{ab}	16.80 ± 2.67
BPcPd2	18.67 ± 7.85^{ab}	9.43 ± 3.86
BTmSo	80.00 ± 14.14^{ab}	7.55 ± 1.35
BTmMa	52.00 ± 19.69^{ab}	11.09 ± 1.62
BTmPe	70.00 ± 18.38^{ab}	9.30 ± 1.94
BLePd	94.00 ± 4.24^{ab}	9.30 ± 0.45
ANOVA F-value	2.09*	1.20^{ns}
P value (0.05)	0.02	0.30
Tukey's HSD test	67.09	-

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

Table 4. Mortality of larval *Spodoptera litura* after being infested with *Metarhizium anisopliae* culture filtrates

Isolate codes	Mortality ±SE*(%) (%)	LT_{50} (days)± SE
MPdB	85.33 ± 14.67^{b}	8.09 ± 1.88^{a}
MPdR1	57.33 ± 19.37^{ab}	11.87 ± 2.56^{a}
MPdR2	65.33 ± 13.33^{ab}	10.43 ± 0.51^{a}
MPdPe	42.67 ± 9.61^{ab}	12.67 ± 0.89^{a}
MJgMs1	53.33 ± 23.25^{ab}	13.67 ± 4.52^{a}
MJgMs2	25.33 ± 3.53^{ab}	14.50 ± 0.56^{a}
MPdMs1	73.33 ± 10.41^{ab}	9.90 ± 0.97^{a}
MPdMs2	84.00 ± 9.24^{ab}	8.70 ± 1.26^{a}
MPdMs3	68.00 ± 16.17^{ab}	10.08 ± 0.86^{a}
MJgKeTs	42.67 ± 10.67^{ab}	12.68 ± 1.05^{a}
MPdMs4	16.00 ± 10.07^{ab}	53.70 ± 23.11^{b}
MJgTs2	46.67 ± 13.92^{ab}	14.29 ± 2.23^{a}
MKbTp1	84.00 ± 10.07^{ab}	8.69 ± 0.95^{a}
MSwTp1	56.00 ± 12.22^{ab}	14.64 ± 3.24^{a}
MSwTp2	74.67 ± 13.92^{ab}	10.62 ± 1.39^{a}
MSwTp3	61.33 ± 16.38^{ab}	10.91 ± 1.99^{a}
MSwTp4	61.33 ± 23.13^{ab}	14.60 ± 5.54^{a}
MKKPp1	44.00 ± 10.07^{ab}	12.33 ± 0.90^{a}
MKbTp2	96.00 ± 4.00^{b}	7.36 ± 0.62^{a}
MagPd	33.33 ± 3.53^{ab}	12.98 ± 0.23^{a}
ANOVA F-value	2.39*	50.13*
P value (0.05)	0.01	0.00
Tukey's HSD test	53.35	2.65

Note: ns = not significantly different; * = significantly different; values within a column (the data of each isolate) followed by the same letters were not significantly different at P < 0.05 according to Tukey's HSD test. Original data were transformed using Arcsin transformation prior to statistical analysis

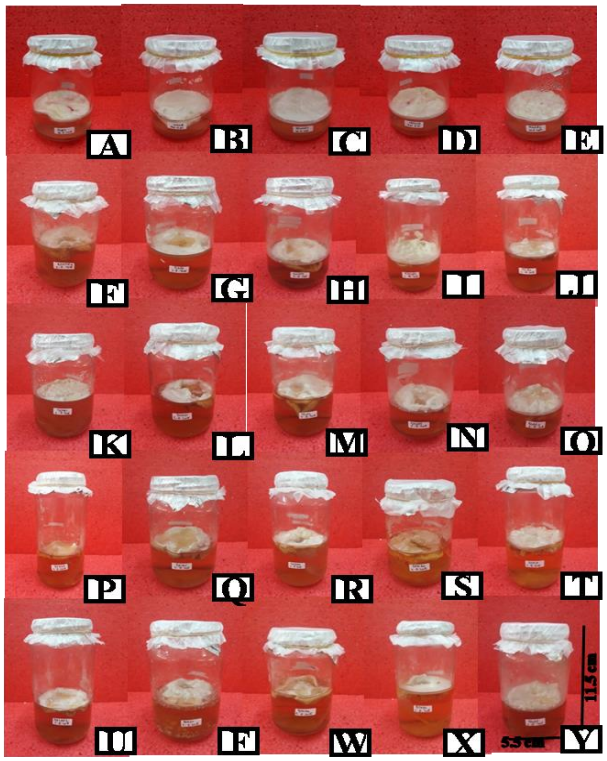

Figure 3. *Beauveria bassiana* isolates cultured in PDB: BJgTs (A), BSmMs (B), BSwTd1 (C), BSwTd2 (D), BSwTd3 (E), BSwTd4 (F), BPdR (G), BKbTp (H), BKKPp2 (I), Ts1d3 (J), BTmPc (K), BTmTr (L), (M) Ts1d2 (M), BTmTs (N), BlePd2 (O), BTmKt (P), BPCmS (Q), BMkMs (R), BtmGa (S), BTmSr (T), BPcPd2 (U), BtmSo (V), BtmMa (W), BtmPe (X), Blepd (Y)

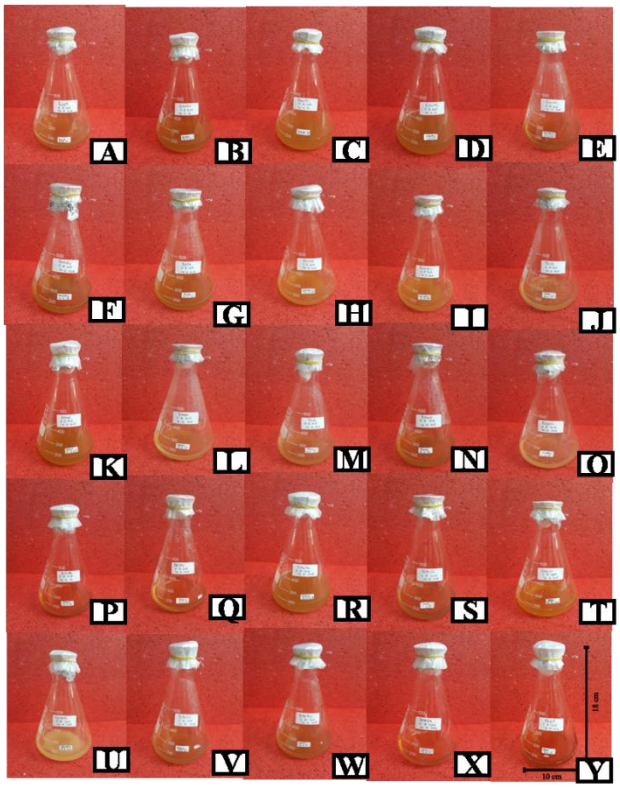

Figure 4. *Beauveria bassiana* culture filtrate: BJgTs (A), BSmMs (B), BSwTd1 (C), BSwTd2 (D), BSwTd3 (E), BSwTd4 (F), BPdR (G), BKbTp (H), BKKPp2 (I), Ts1d3 (J), BTmPc (K), BTmTr (L), (M) Ts1d2 (M), BTmTs (N), BlePd2 (O), BTmKt (P), BPCmS (Q), BMkMs (R), BtmGa (S), BTmSr (T), BPcPd2 (U), BtmSo (V), BtmMa (W), BtmPe (X), Blepd (Y)

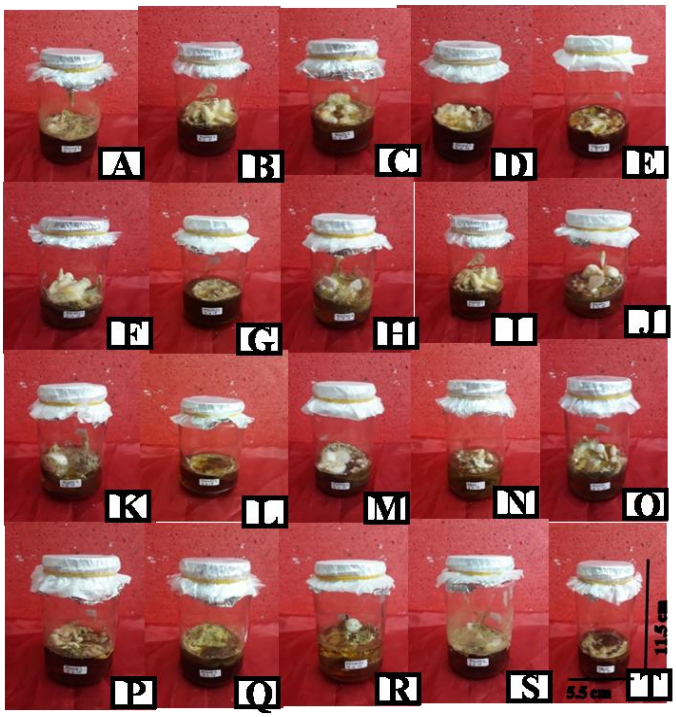

Figure 5. *Metarhizium anisopliae* isolates cultured in PDB: MPdB (A), MPdR1 (B), MPdR2 (C), MpdPe (D), MJgMs1 (E), MJgMs2 (F), MPdMs1 (G), MPdMs2 (H), MPdMs3 (I), MjgKeTs (J), MPdMs4 (K), MJgTs2 (L), MKbTp1 (M), MSwTp1 (N), MSwTp2 (O), MSwTp3 (P), MSwTp4 (Q), MKKPp1 (R), MKbTp2 (S), MagPd (T)

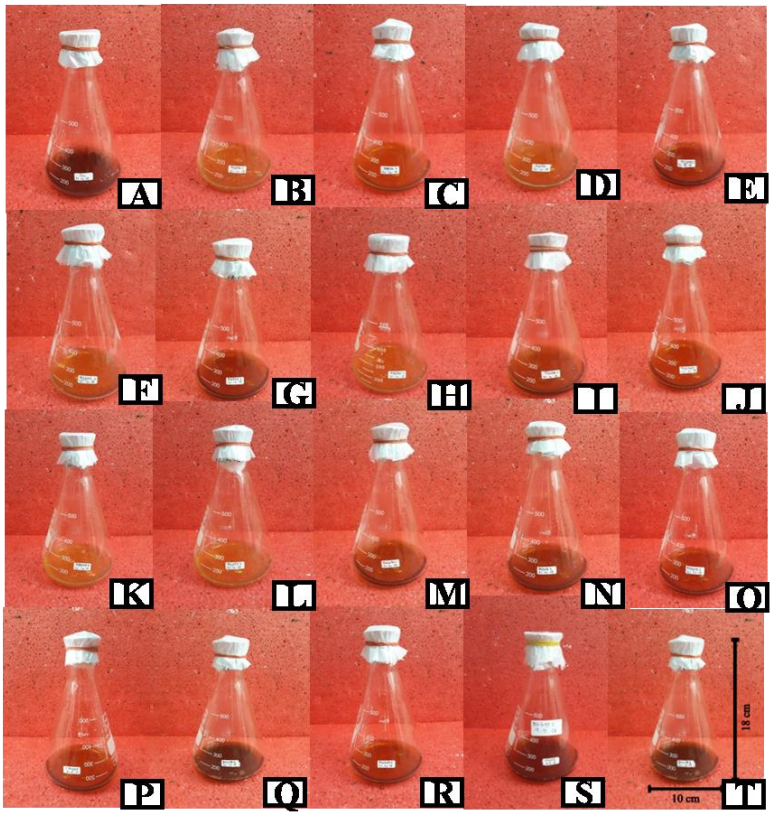

Figure 6. *Metarhizium anisopliae* culture filtrate: MPdB (A), MPdR1 (B), MPdR2 (C), MpdPe (D), MJgMs1 (E), MJgMs2 (F), MPdMs1 (G), MPdMs2 (H), MPdMs3 (I), MjgKeTs (J), MPdMs4 (K), MJgTs2 (L), MKbTp1 (M), MSwTp1 (N), MSwTp2 (O), MSwTp3 (P), MSwTp4 (Q), MKKPp1 (R), MKbTp2 (S), MagPd (T)

Effect of entomopathogenic fungal culture filtrate on test larvae

S. litura larvae fed with chili leaves applied with *B. bassiana* or *M. anisopliae* culture filtrate exhibited the same behavior, i.e., they increasingly ate less, while the controls not given culture filtrate continued to eat greedily. The chili leaves given to the larvae which were not given culture filtrate generally only left a leaf bone, whereas in the larvae given culture filtrate there were still leaves remaining (Figure 7 dan 8). Thus, the culture filtrate could reduce the appetite of *S. litura* larvae.

The larvae *S. litura* given culture filtrate besides being more lazy to eat showed more and more lazy to move. After 2 and 3 days being given the culture filtrate, the larvae began to change the color of integument which was previously bright green to become dull and the larvae were discovered to be dead. Four days after being given the culture filtrate, the larvae increasingly began to shrink and die. The dead larvae were wrinkled, dry, black, and odorless. The larvae not given the culture filtrate had larger body sizes and the integument colors were greener, and brighter (Figure 9 dan 10).

Figure 7. The leaf damage: control (A) and larval *Spodoptera litura* applied with *Beauveria bassiana* culture filtrate (B)

Figure 8. The leaf damage: control (A) and larval *Spodoptera litura* applied with *Metarhizium anisopliae* culture filtrate (B)

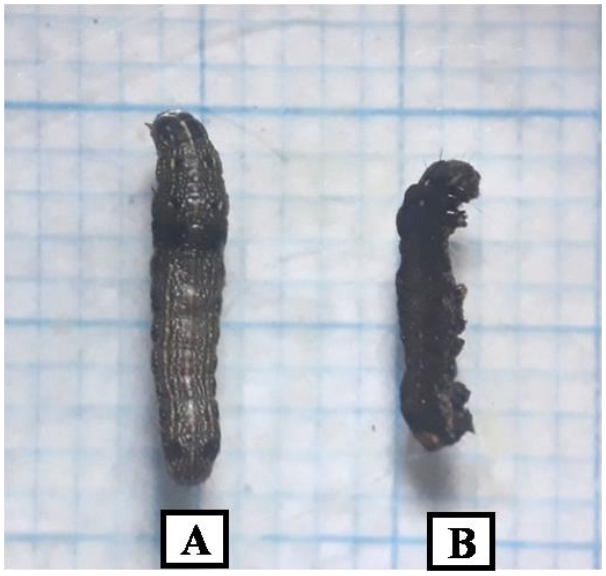

Figure 9. The healthy (A) and dead larvae (B) of *Spodoptera litura* caused by *Beauveria bassiana* culture filtrate

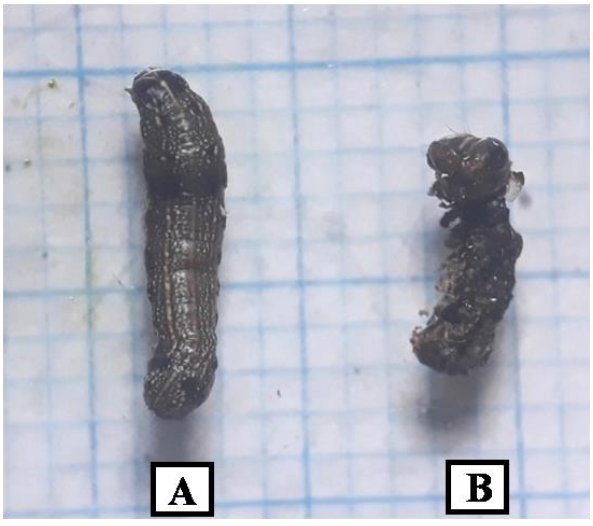

Figure 10. The healthy (A) and dead larvae (B) of *Spodoptera litura* caused by *Metarhizium anisopliae* culture filtrate

Discussion

The *B. bassiana* culture filtrate, particularly the isolates coded BSwTd2, were the most toxic compared to other isolates with LT_{50} of only 5.91 days. The BSwTd2 isolate was from peat soils overgrown with oil palm plants. For *M. anisopliae*, the most toxic MKbTp2 isolate was from the cabbage in highland. The larvae began to die after 3x24 hours after the treatment either due to *B. bassiana* or *M. anisopliae*. The larvae death resulted from the 6 weeks of incubation of

entomopathogenic fungi causing the toxic culture filtrate. According to Pinnamaneni et al. (2010) the culture of filtrates of the toxic *B. bassiana* was caused by the incubation in liquid culture media in which the fungus produced chitinolytic exochitinase enzymes so that when they entered the body the insect was able to degrade the cuticle. In addition, the fungal broth during the incubation could produce protease enzymes (Qazi 2008).

The time of death of the larvae by this culture filtrate was faster than that of by conidia. El Husseini (2019) stated that the death of *Spodoptera* larvae by *M. anis*opliae conidia began to occur at the fourth day (4x24 hours) or the fifth day after the treatment (post-treatment), whereas in this study the *M. anisopliae* culture filtrate began to kill *S. litura* at the third day after the treatment. The lethal time difference between these was due to the mode of action of fungal conidia being different from the culture filtrate. To kill the host insect, the fungus conidia got contact first with it and then produced toxins to kill the host, while the culture filtrate directly killed the host insect. El-Ghany (2015) states that entomopathogenic fungus conidia kills its host insect in two stages: parasitic and saprophytic phases. The parasitic phase began with the fungal conidia attaching to the host insect cuticle (Augustyniuk-Kram & Kram 2012). Then, if the humidity was high, the conidia started to germinate on the host cuticle (El-Ghany 2015). The conidia germinating by forming germ tubes continued to grow sticking out to find a soft integument position to facilitate entry into the cuticle (Fernandes et al. 2007). The infection occurs when the germ tubes are able to penetrate insect cuticles and the ability to infect them is a determining factor for the fungus virulence (Altre & Vandenberg 2001). After the germ tubes penetrated the cuticle and reached the haemocoel, and then they produced specific infection hyphae originating at appressoria (El-Ghany 2015). Furthermore, the hyphae spread to the haemolymph and developed to produce blastospores, and the blastospores produced fungal cytotoxic, for example destruxins by *M. anisopliae* which killed the host insects (Mancillas-Paredes et al. 2019), but the death of these host insects was not caused only by the toxin but also due to the mechanical damage by the penetration of fungi into the body of insects (El-Ghany 2015).

After the host insect died, it entered into the saprophytic phase which was influenced by the favorable environmental conditions (Peña-Peña et al. 2015). In the body of the dead insect, the fungus formed mycelia and hyphae which continued to grow covering the body of the host insect, and then the hyphae formed conidiogenous cells and the conidia was produced by utilizing the nutrients/fluids of the host insect and finally the infection process was complete (El-Ghany 2015).

In contrast to the mode of action of fungal conidia, a fungal culture filtrate directly kills the host insect due to the toxins produced by fungal broth so that the time needed to kill the host insect is shorter. The entomopathogenic fungus cultured in liquid media can produce conidia in the form of blastospores (Mascarin et al. 2015) which can produce toxins (Mascarin & Jaronski 2016). In this study, the result of calculating conidia density after being incubated for 6 weeks was generally more than $1x10^9$ conidia.mL^{-1}. Fungal conidia and propagules were separated from the filtrate through two stages of filtration to produce toxic fungal culture filtrate. The *B. bassiana* and *M. anisopliae* culture filtrates were able to kill up to 94.67% and 96% of the larvae of *S. litura*, respectively. The short time of death by this culture filtrate resulted from the death of the direct host insect by consuming feed (chili leaves) moistened with the culture filtrate. Soesanto et al. (2019) states that entomopathogenic fungal culture filtrate contains secondary metabolites. *B. bassiana* culture filtrate containing the secondary metabolites can weaken the host insect's immune system (Zibaee et al. 2011). The secondary metabolites produce toxins, for example destruxins and efrapeptins (Zibaee et al. 2009). *B. bass*iana also produces a protease enzyme that can kill host insects by dissolving their body proteins (Mancillas-Paredes et al. 2019).

The color of fungal broth and culture of *B. bassiana* and *M. anisopliae* varied between isolates. The darker color of fungal broths and culture filtrates tend to cause higher mortality. In line with the results obtained by Ayudya et al. (2019), the darker culture filtrates tended to be more toxic than the light-colored ones. The darker culture filtrates show higher production of secondary metabolites (Lou et al. 2017) and the activity of extracellular enzymes (Khachatourians et al. 2007) and the enzymes produced such as proteases are able to dissolve integument insects that result in death (Mancillas-Paredes et al. 2019).

The symptoms of sick host insects by culture filtrate in this study differed from the deaths by fungal conidia. The insects getting sick by the culture filtrate were not covered by micelia or conidia on the integument. However, they got sick because of the fungal conidia infection which were generally covered in micelia (Sumikarsih et al. 2019). The body shape of the sick *S. litura* larvae due to the culture filtrate was shrunk, wrinkled, dry, and odorless. Similar to the results of the study of Ayudya et al. (2019), the *S litura* larvae treated with *B. bassiana* culture filtrate decreased their body weight and became dried, odorless, and the integument was not overgrown with fungal micelia because in the culture filtrate it no longer contained conidia but contained toxic compounds for host insects. Therefore, the mortality of *S. litura* larvae in this study was caused by the culture filtrate containing toxic compounds. Zibaee et al. (2011) states that toxic compounds in the culture filtrate are secondary metabolites. Zibaee et al. (2009) states that the secondary metabolites are easier to apply in the field because they can be integrated with other control techniques including synthetic insecticides. Consequently, the culture filtrate which is toxic due to the high content of secondary metabolites has the potential to be further developed for the active ingredient of mycoinsecticide.

The most toxic isolates of the culture filtrate were BSwTd2 of *B. bassiana* and MKbTp2 of *M. anisopliae* resulting in the mortality of above 90%. The two isolates that were found each came from the lowlands and highlands so that in the future there is an opportunity for the isolates to be developed and applied in low and highland ecosystems.

Entomopathogenic fungi that produce toxic filtrate cultures are capable of killing their hosts in a short time, so that they have the potential to be active ingredients in the development of mycoinsecticides technology.

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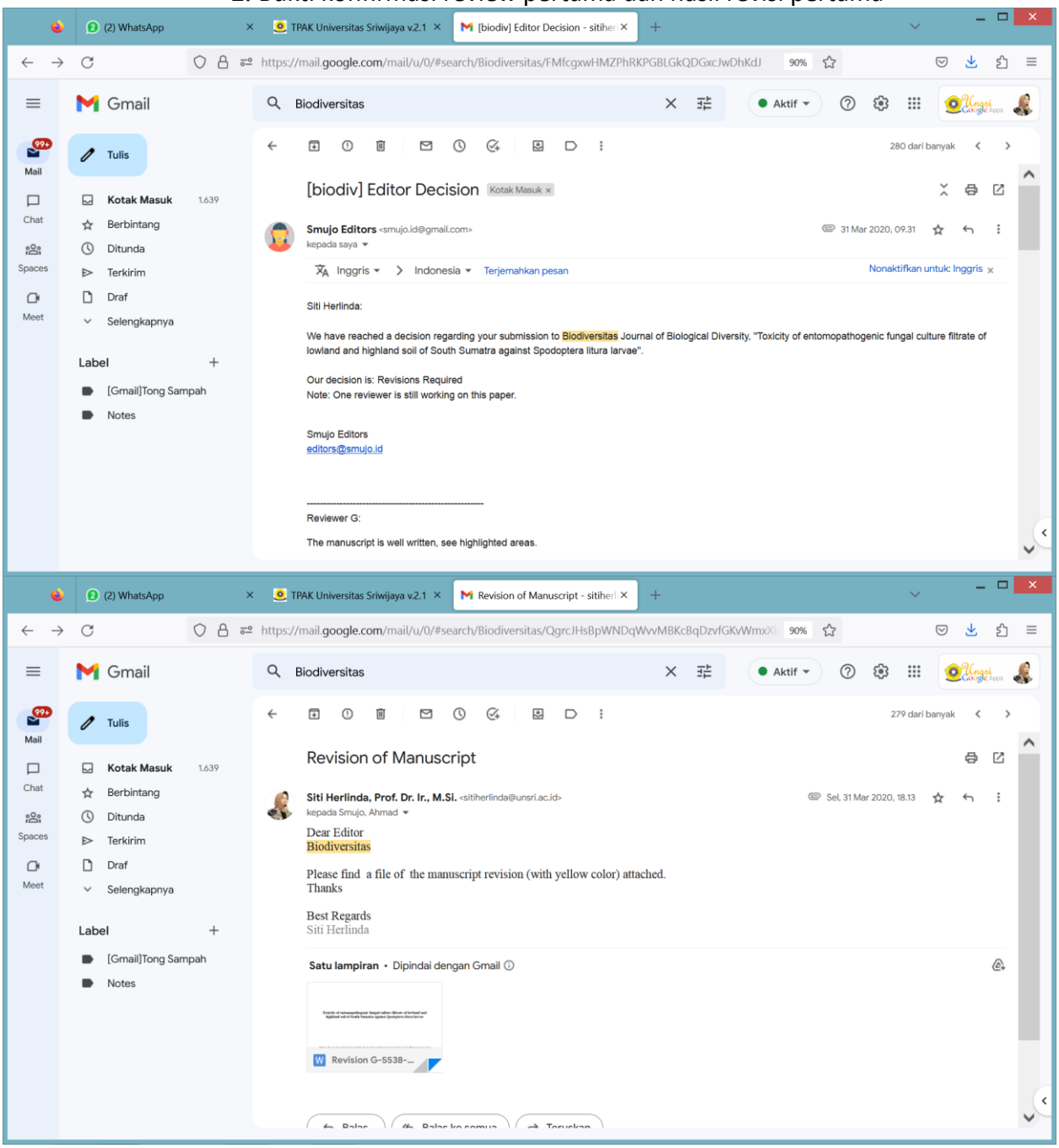

Toxicity of entomopathogenic fungal culture filtrate of lowland and highland soil of South Sumatra against *Spodoptera litura* larvae

Abstract. The use of secondary fungal metabolites for the active ingredient of mycoinsecticide is more effective and more easily integrated with other pest control techniques. This study aimed to measure the toxicity of the culture filtrate of entomopathogenic fungi originating from South Sumatra against the *Spodoptera litura* larvae. *Beauveria bassiana* (25 isolates) and *Metarhizium anisopliae* (20 isolates) of South Sumatra were cultured in liquid media and filtered to produce culture filtrate. The larvae which were sick due to the filtrate showed the symptoms of decreased appetite and were not actively moving, while the dead larvae were characterized by being wrinkled, dry, black integument, and odorless. Mortality caused by *B. bassiana* filtrate was the highest 98% (BJgTs isolate) and not significantly different from the BSwTd2 isolate (94.67%). Yet, the LT_{50} BSwTd2 isolate was shorter (5.92 days) compared to the LT_{50} BJgTs isolate (6.35 days). The most toxic *M. anisopliae* filtrate produced the mortality of 96% (MKbTp2 isolate) and 85.33% (MPdB isolate) each of which had LT_{50} of 7.36 days and 8.09 days, respectively. So, the most toxic culture filtrate was BSwTd2 isolate of *B. bassiana* and MKbTp2 isolate of *M. anisopliae*. The entomopathogenic fungi producing filtrate which are toxic have the potential to be active ingredients of mycoinsecticides.

Key words: *Beauveria bassiana*, chili, $LT_{50,}$ *Metarhizium anisopliae*, mortality

Running title: Toxicity of entomopathogenic fungi againts *Spodoptera*

INTRODUCTION

South Sumatra is generally dominated by lowland lands, namely freshwater swamps and tidal lowlands (Kartika et al. 2018; Karenina et al. 2019), and a small portion of medium and highland. Freshwater swamps can be found, among others in the Districts of Ogan Ilir, Ogan Komering Ilir, Musi Banyuasin, and Palembang City; the widest tide exists in Banyuasin District and the medium and highlands occur, among others, in the Districts of Ogan Komering Ulu Selatan, Lahat and Pagaralam City. The varied topographical conditions between districts/cities characterize the differences including the crops and microorganisms cultivated there.

In the lowlands of South Sumatra are generally cultivated annual crops, such as paddy (Herlinda et al. 2018a; Prabawati et al. 2019), maize (Juhriah et al. 2019), chili (Johari et al. 2016; Nasution & Respatijarti, 2019; Sagrim et al. 2017), and palm oil (Darlan et al. 2016). Meanwhile, in the medium and high plains are generally cultivated annual crops such as coffee, tea (Zamhari et al. 2017), vegetables such as potatoes (Maryanto et al. 2018), cabbage, mustard greens, and caisim (Situmorang et al. 2019). Vegetables and seasonal plants of the lowlands and highlands generally have a major pest, namely *Spodoptera litura* (Turnip et al. 2019). The yield loss caused by its larval stage is more than 35% a year (Bueno et al. 2011). This pest has egg, larval, pupal, and adult stages (Vijaya and Rani 2016). *S. litura* is a polyphagic pest that attacks many vegetables in Indonesia, for example chili (Nagal et al. 2016), cotton fruit (Maqsood et al. 2017), soybean (Fattah et al. 2018). A polyphagic pest is a pest that has a broad range of host species (more than a family) (Nagal et al. 2016). For the control of *S. litura*, it needs natural enemies such as entomopathogenic fungi that can adapt in low to high altitudes.

In the lowlands and highlands, the soil types have different chemical and physical characteristics; acidic pH soils are generally found in the lowlands and neutral or basic pH soils are found in the highlands (Munir & Herman 2019). Soil pH can affect the presence of entomopathogenic fungi and tends to be neutral or high pH soils which more commonly occur in entomopathogenic fungi than low pH soils (Safitri et al. 2018). Besides pH, the texture of sandy soils tends to be low and muddy which is not suitable for fungal propagule life as well as water-saturated soil where fungi are rarely spotted to survive (Garrido-Jurado et al. 2011). Temperature (Pinnamaneni et al. 2010) and humidity (Oreste et al. 2015; Liu et al. 2017) also affect pathogenicity of entomopathogenic fungi.

Previous study found that more than 30 isolates of entomopathogenic fungi were found in the freshwater swamp, tidal, and highland soil of South Sumatra (Safitri et al. 2018). Apart from the soil, the entomopathogenic fungi were also found from the insect pests of vegetable and food crops of the lowlands to the highlands of South Sumatra (Herlinda et al. 2018b). Various origins of entomopathogenic fungi tend to produce pathogenicity variations (Sumikarsih et al. 2019).

The pathogenicity of entomopathogenic fungi is influenced by the ability of the fungi to produce secondary metabolites (Zibaee et al. 2009). The fungi that develop in insect haemolymph or in liquid media are able to produce toxic metabolites, such as extracellular enzymes, proteins, and toxins (Bandani 2005). In vitro media, toxic metabolites can be produced by the fungi which are grown in liquid media and produce culture filtrate (Soesanto et al. 2019). The use of secondary entomopathogenic fungal metabolites for the active ingredient of mycoinsecticide is more effective and more easily integrated with other pest control techniques (Zibaee et al. 2009). Entomopathogenic fungal culture filtrate found in low to high altitude of South Sumatra potentially gets the most toxic isolates that can be utilized in the development of mycoinsecticide technology. For this reason, this study aimed to measure the toxicity of the culture filtrate isolates of entomopathogenic fungi from low to high land of South Sumatra against *Spodoptera litura* larvae.

MATERIALS AND METHODS

The experiments were carried out at the Entomology Laboratory, Department of Pests and Plant Diseases, Faculty of Agriculture, Universitas Sriwijaya from May to November 2018. The room temperatures and relative humidity during the experiment in the laboratory was 29.78°C and 82.72%, respectively. The entomopathogenic fungi isolates used in this study were explored by Herlinda et al. (2018) and Safitri et al. (2018), (Tables 1 and 2) spread from the lowlands to the highlands of South Sumatra. The isolates were grouped into two: 25 isolates of *Beauveria bassiana* (Figure 1) and 20 isolates of *Metarhizium anisopliae* (Figure 2). All isolates were identified by Dr. Suwandi (a mycologist from Universitas Sriwijaya).

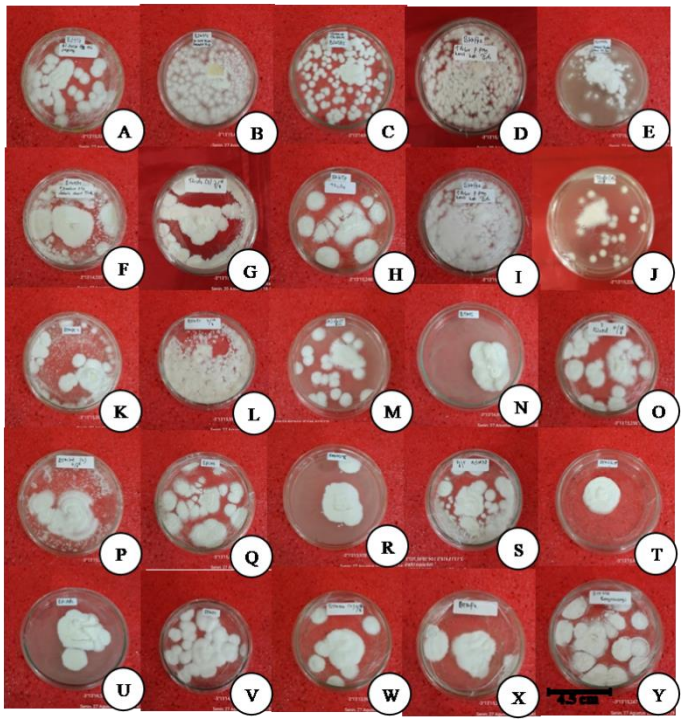

Figure 1. *Beauveria bassiana* isolates cultured in SDA media: BJgTs (A), BSmMs (B), BSwTd1 (C), BSwTd2 (D), BSwTd3 (E), BSwTd4 (F), BPdR (G), BKbTp (H), BKKPp2 (I), Ts1d3 (J), BTmPc (K), BTmTr (L), (M) Ts1d2 (M), BTmTs (N), BlePd2 (O), BTmKt (P), BPCmS (Q), BMkMs (R), BtmGa (S), BTmSr (T), BPcPd2 (U), BtmSo (V), BtmMa (W), BtmPe (X), Blepd (Y)

Test insect preparation

S. litura larvae were collected from the chili planting area in the experimental field of the Faculty of Agriculture, Sriwijaya University and farmers' vegetable fields which were not applied by synthetic insecticides. Then, the larvae were brought to the laboratory and maintained in a plastic cylinder whose upper part was covered with gauze (Ø 25 cm, height 30 cm) having been washed thoroughly using detergent. Next, water spinach plants were put into it for larvae feed.

Every day the larvae feed was replaced with fresh new feed. The larvae entering the pupae phase were transferred into a plastic cylinder (Ø 10 cm, height 15 cm) with the top open and the bottom of the cylinder sprinkled with sifted soil and sterilized in an oven for 1 hour at 100ºC. The thickness of the soil inserted into the cylinder was 3 cm. The plastic cylinder containing the pupae was put into a gauze cage (30x30x30 cm^3) which had been chopped with chilies for laying eggs. Adults arising from the pupae were fed with honey smeared on cotton and hung over a cage. The hatched eggs were transferred into a plastic cylinder and fed with water spinach which was replaced every day. The larvae used for toxicity testing were the second instar of the third offspring or afterwards.

Figure 2. *Metarhizium* isolates cultured in SDA media: MPdB (A), MPdR1 (B), MPdR2 (C), MpdPe (D), MJgMs1 (E), MJgMs2 (F), MPdMs1 (G), MPdMs2 (H), MPdMs3 (I), MjgKeTs (J), MPdMs4 (K), MJgTs2 (L), MKbTp1 (M), MSwTp1 (N), MSwTp2 (O), MSwTp3 (P), MSwTp4 (Q), MKKPp1 (R), MKbTp2 (S), MagPd (T)

Table 1. *Beauveria bassiana* isolates from South Sumatra used in this research

Isolate code	Isolate origin	Geographic origin
BJgTs	Tidal lowlands, maize	Telang Sari
BSmMs	Tidal lowlands, watermelon	Mulya Sari
BSwTd1	Peatlands, oil palm	Talang Dabok
BSwTd2	Peatlands, oil palm	Talang Dabok
BSwTd3	Peatlands, oil palm	Talang Dabok
BSwTd4	Peatlands, oil palm	Talang Dabok
BPdR	Freshwater swamps, paddy	Rambutan
BKbTp	Highlands, cabbage	Talang Patai
BKKPp2	Highlands, rubber and coffee	Pulau Pinang
Ts1d3	Peatlands	Talang Dabok
BTmPc	Freshwater swamps	Indralaya
BTmTr	Freshwater swamps	Telang Rejo
Ts1d2	Peatlands	Talang Dabok
BTmTs	Highlands	Mulia Sari
BlePd2	*Lipaphis erysimi*	Pagardin
BTmkt	Freshwater swamps	Kenten
BPCmS	*Pseudoplusia chalcites*	Muara Siban
BMkMs	Highlands	Muara Siban
BtmGa	Freshwater swamps	Gandus
BTmSr	Tidal lowlands	Srikaton
BPcPd2	*Chrysodeixis chalcites*	Pagardin
BtmSo	Freshwater swamps	Soak
BtmMa	Freshwater swamps	Mariana
BtmPe	Freshwater swamps	Pemulutan
Blepd	*Lipaphis erysimi*	Pagardin

Table 2. *Metarhizium anisopliae* isolates from South Sumatra used in this research

Isolate code	Isolate soil origin	Geographic origin
MPdB	Freshwater swamps, paddy	Banyuasin
MPdR1	Freshwater swamps, paddy	Rambutan

MPdR2	Freshwater swamps, paddy	Rambutan
MpdPe	Freshwater swamps, paddy	Pemulutan
MJgMs1	Tidal lowlands, maize	Mulya Sari
MJgMs2	Tidal lowlands, maize	Mulya Sari
MPdMs1	Tidal lowlands, paddy	Mulya Sari
MPdMs2	Tidal lowlands, paddy	Mulya Sari
MPdMs3	Tidal lowlands, paddy	Mulya Sari
MjgKeTs	Tidal lowlands, maize and oil palm	Telang Sari
MPdMs4	Tidal lowlands, paddy	Mulya Sari
MJgTs2	Tidal lowlands, maize	Telang Sari
MKbTp1	Highlands, cabbage	Talang Patai
MSwTp1	Highlands, mustard	Talang Patai
MSwTp2	Highlands, mustard	Talang Patai
MSwTp3	Highlands, mustard	Talang Patai
MSwTp4	Highlands, mustard	Talang Patai
MKKPp1	Highlands, mustard, rubber, coffee	Pulau Pinang
MKbTp2	Highlands, cabbage	Talang Patai
MagPd	Highlands, *Aphis gossypii*	Pagardin

Culture filtrate production

All isolates before being filtered were first made fit by modifying the method of Herlinda (2010). The media for making fit used Sabouraud Dextrose Agar (SDA, Merck) as much as 16.2 g and added 250 mL aquadest enriched with 1.25 g *Tenebrio molitor* larvae flour (5 g). After that, the ingredients were mixed and stirred evenly and then put in an erlenmeyer (size 250 mL), sterilized in an autoclave for 120 minutes at a pressure of 1 atm. The media was poured into the Petri dish as much as 10 mL (Ø 9 cm) in aseptic laminar flow air conditions. Then, the entomopathogenic fungi isolates were inoculated in the SDA media and incubated for 14 days.

Isolates of entomopathogenic fungi derived from SDA media of aged 14 days were then transferred and grown into liquid media (broth media), PDB (*Potato Dextrose Broth*) prepared as follows: the PDB media composition consisting of 20 g dextrose monohydrate, 200 g potatoes, and 1000 mL aquadest. Before the potatoes were extracted, they were cut into cubes with a size of ± 2x2x2 cm^3 and boiled using 1000 mL aquadest for 20 minutes. This PDB medium was sterilized in the autoclave and after it got cold, the entomopathogenic fungus isolates were inoculated into it. This liquid culture was incubated for 6 weeks.

After the fungus liquid culture (fungal broth) was 6 weeks old, the fungus filtration is carried out. The filtration was conducted in two stages, namely filtering using filter paper and syringe filter. In the first stage, 100 mL of 6-week-old fungal broth was filtered using Whatman filter paper no. 42 and coated with a thickness of 1 cm cotton. The culture filtrate obtained from the first stage of filtering was then sucked up to 10 mL using a hypodermic needle (spike). Then, the needle was removed and the base of the needle was fitted with a syringe filter (0.45 µm-25 mm). The 10 mL spike was pressed so that it released culture filtrate from the syringe filter. The 100 mL of fungal broth produced ± 70 mL of culture filtrate. The culture filtrate was taken as much as 1 mL to be dripped on a piece of chili leaf which was used as a toxicity test. This culture filtrate is often referred to as raw secondary metabolites (Soesanto et al. 2019). To ensure the culture filtrate did not contain propagules (hyphae, micelia) and conidia, it was grown on agar media (SDA) before it was applied. If it does not grow fungus on the SDA media, the culture filtrate can be applied.

Insecticidal activity test of entomopathogenic fungus culture filtrate

The chili leaves that had been dropped by the culture filtrate were first drained; before 25 *S. litura* larvae were fasted for 2 hours, and then put into them. The second instar larvae were left to eat leaves that had been dropped with culture filtrate for 6 hours. After 6 hours, the larvae were transferred into a plastic cylinder topped with gauze (Ø 25 cm, height 30 cm) containing 15-30 clean chili leaves which were not dripped with culture filtrate. Every day the chili leaves were replaced with the new ones. Every day the dead larvae were recorded up to 12 days after the application.

Data analysis

The differences in mortality data and lethal time that killed 50% of test insects (LT50) were analyzed using analysis of variance (ANOVA), after that further tests were carried out using the 5% Tukey's Honestly Significant Difference (HSD). All data were calculated using software of SAS University Edition 2.7 9.4 M5.

RESULTS AND DISCUSSION

Insecticidal activities of entomopathogenic fungi

The culture filtrate derived from twenty-five *B. bassiana* isolates tested for the insecticidal activity showed that the most toxic *B. bassiana* isolates were those coded BJgTs (98%) and were not significantly different, among others with BSwTd2 (94.67%) and BTmTs isolates (96%). All *B. bassiana* isolates showed the ability to kill the test larvae (Table 3). The BJgTs, BSwTd2, and BTmTs isolates were the most toxic because they produced the highest mortality and also their ability to kill the shortest as evidenced by the short LT_{50} (6.35, 5.92, and 7.78 days) isolates. The most toxic isolates were shown with the highest mortality and their shortest LT_{50}. Consequently, for *B. bassiana*, the most toxic isolate was BSwTd2.

The culture filtrate of the twenty *M. anisopliae* isolates which were tested for their insecticidal activity showed that the most toxic isolates were those coded MKbTp2 (96%) and MPdB (85.33%) and the mortality of both isolates was significantly different from the other isolates (Table 4). The BSwTd2 isolate was the most toxic because they produced the highest mortality as well as the ability to kill the shortest as evidenced by the shortest LT_{50} (7.36 days), and then followed by LT_{50} isolate MPdB (8.09 days). The most toxic *M. anisopliae* isolate was proven by the highest mortality and the shortest LT_{50} which was MKbTp2 isolate.

The color of the fungal broth (Figure 3) and culture filtrate (Figure 4) of *B. bassiana* varied among the isolates. All fungal broth isolates were generally transplanted brown, but there were among those isolates dark brown in color, for example isolates BSwTd2, BSwTd3, BPdR, TS1d2. There was a tendency for the darker color of the fungal broth and culture filtrate to cause higher mortality. The color of fungal broth (Figure 5) and culture filtrate (Figure 6) of *M. anisopliae* differed from the colors of fungal broth and culture of *B. bassiana*. The *M. anisopliae* fungal broth was generally dark greenish brown, but the culture filtrate tended to have a more varied color, from greenish dark brown to light brown. The same tendency was also produced in *M. anisopliae* isolates, i.e., the color of the older culture filtrate resulting in higher mortality. The isolates having a darker colored culture filtrate were MPdB, MPdMs1, MPdMs2, MPdMs3, MKbTp1, MKbTp2.

Table 3. Mortality of larval *Spodoptera litura* after being infested with *Beauveria bassiana* culture filtrates

Isolate codes	Mortality ± SE*(%)	LT_{50} (days)± SE
BJgTs	98.00 ± 1.41^{b}	6.35 ± 0.49
BSmMs	50.00 ± 32.53^{ab}	11.57 ± 4.08
BSwTd1	49.33 ± 15.36^{ab}	12.03 ± 3.52
BSwTd2	94.67 ± 4.35^{ab}	5.92 ± 0.46
BSwTd3	73.33 ± 18.51^{ab}	8.86 ± 1.78
BSwTd4	26.00 ± 1.41^{ab}	16.07 ± 0.97
BPdR	86.67 ± 3.93^{ab}	8.17 ± 0.61
BKbTp	62.67 ± 10.39^{ab}	10.71 ± 0.91
BKKPp2	58.00 ± 7.07^{ab}	10.68 ± 0.34
TS1d3	10.67 ± 2.88^{a}	17.45 ± 0.76
BTmPc	46.67 ± 17.01^{ab}	14.32 ± 3.72
BTmTr	29.33 ± 9.68^{ab}	14.31 ± 1.79
TS1d2	88.00 ± 8.22^{ab}	7.87 ± 1.11
BTmTs	96.00 ± 0.00^{ab}	7.78 ± 0.09
BLePd2	70.00 ± 21.21^{ab}	9.59 ± 1.43
BTmKt	80.00 ± 14.73^{ab}	8.80 ± 1.41
BPcMs	81.33 ± 9.49^{ab}	7.87 ± 1.32
BMkMs	72.00 ± 17.99^{ab}	10.82 ± 2.46
BTmGa	66.67 ± 22.34^{ab}	12.27 ± 3.60
BTmSr	21.33 ± 9.30^{ab}	16.80 ± 2.67
BPcPd2	18.67 ± 7.85^{ab}	9.43 ± 3.86
BTmSo	80.00 ± 14.14^{ab}	7.55 ± 1.35
BTmMa	52.00 ± 19.69^{ab}	11.09 ± 1.62
BTmPe	70.00 ± 18.38^{ab}	9.30 ± 1.94
BLePd	94.00 ± 4.24^{ab}	9.30 ± 0.45
ANOVA F-value	2.09*	1.20^{ns}
P value (0.05)	0.02	0.30
Tukey's HSD test	67.09	-

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

Table 4. Mortality of larval *Spodoptera litura* after being infested with *Metarhizium anisopliae* culture filtrates

Isolate codes	Mortality ± SE*(%)	LT_{50} (days) ± SE

MPdB	85.33 ± 14.67[b]	8.09 ± 1.88[a]
MPdR1	57.33 ± 19.37[ab]	11.87 ± 2.56[a]
MPdR2	65.33 ± 13.33[ab]	10.43 ± 0.51[a]
MPdPe	42.67 ± 9.61[ab]	12.67 ± 0.89[a]
MJgMs1	53.33 ± 23.25[ab]	13.67 ± 4.52[a]
MJgMs2	25.33 ± 3.53[ab]	14.50 ± 0.56[a]
MPdMs1	73.33 ± 10.41[ab]	9.90 ± 0.97[a]
MPdMs2	84.00 ± 9.24[ab]	8.70 ± 1.26[a]
MPdMs3	68.00 ± 16.17[ab]	10.08 ± 0.86[a]
MJgKeTs	42.67 ± 10.67[ab]	12.68 ± 1.05[a]
MPdMs4	16.00 ± 10.07[ab]	53.70 ± 23.11[b]
MJgTs2	46.67 ± 13.92[ab]	14.29 ± 2.23[a]
MKbTp1	84.00 ± 10.07[ab]	8.69 ± 0.95[a]
MSwTp1	56.00 ± 12.22[ab]	14.64 ± 3.24[a]
MSwTp2	74.67 ± 13.92[ab]	10.62 ± 1.39[a]
MSwTp3	61.33 ± 16.38[ab]	10.91 ± 1.99[a]
MSwTp4	61.33 ± 23.13[ab]	14.60 ± 5.54[a]
MKKPp1	44.00 ± 10.07[ab]	12.33 ± 0.90[a]
MKbTp2	96.00 ± 4.00[b]	7.36 ± 0.62[a]
MagPd	33.33 ± 3.53[ab]	12.98 ± 0.23[a]
ANOVA F-value	2.39[*]	50.13[*]
P value (0.05)	0.01	0.00
Tukey's HSD test	53.35	2.65

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

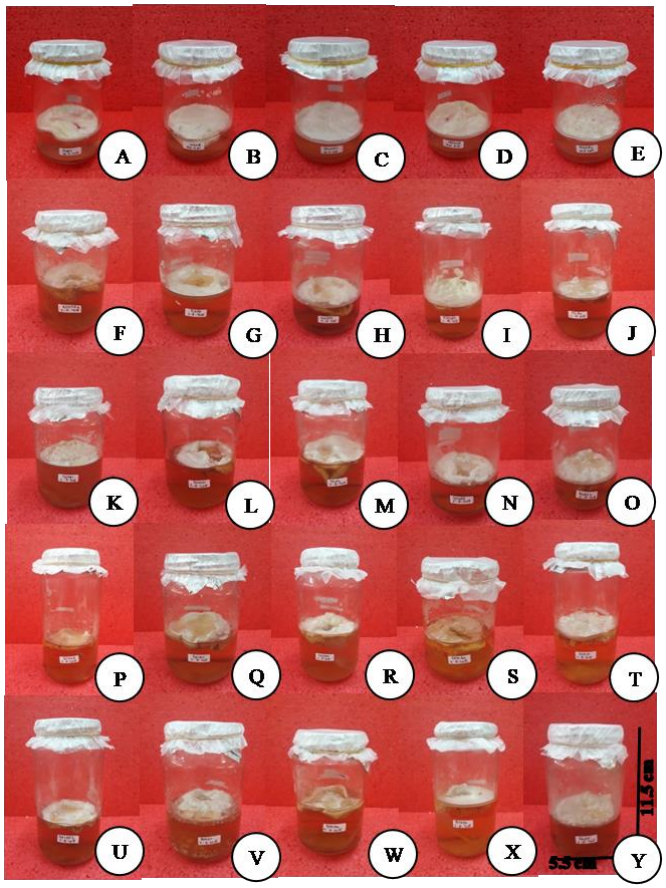

Figure 3. *Beauveria bassiana* isolates cultured in PDB: BJgTs (A), BSmMs (B), BSwTd1 (C), BSwTd2 (D), BSwTd3 (E), BSwTd4 (F), BPdR (G), BKbTp (H), BKKPp2 (I), Ts1d3 (J), BTmPc (K), BTmTr (L), (M) Ts1d2 (M), BTmTs (N), BlePd2 (O), BTmKt (P), BPCmS (Q), BMkMs (R), BtmGa (S), BTmSr (T), BPcPd2 (U), BtmSo (V), BtmMa (W), BtmPe (X), Blepd (Y)

Figure 4. *Beauveria bassiana* culture filtrate: BJgTs (A), BSmMs (B), BSwTd1 (C), BSwTd2 (D), BSwTd3 (E), BSwTd4 (F), BPdR (G), BKbTp (H), BKKPp2 (I), Ts1d3 (J), BTmPc (K), BTmTr (L), (M) Ts1d2 (M), BTmTs (N), BlePd2 (O), BTmKt (P), BPCmS (Q), BMkMs (R), BtmGa (S), BTmSr (T), BPcPd2 (U), BtmSo (V), BtmMa (W), BtmPe (X), Blepd (Y)

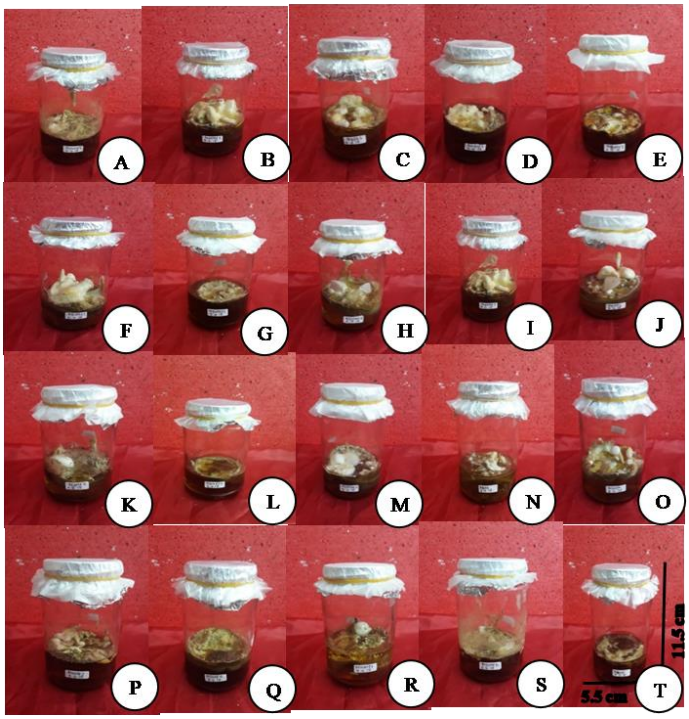

Figure 5. *Metarhizium anisopliae* isolates cultured in PDB: MPdB (A), MPdR1 (B), MPdR2 (C), MpdPe (D), MJgMs1 (E), MJgMs2 (F), MPdMs1 (G), MPdMs2 (H), MPdMs3 (I), MjgKeTs (J), MPdMs4 (K), MJgTs2 (L), MKbTp1 (M), MSwTp1 (N), MSwTp2 (O), MSwTp3 (P), MSwTp4 (Q), MKKPp1 (R), MKbTp2 (S), MagPd (T)

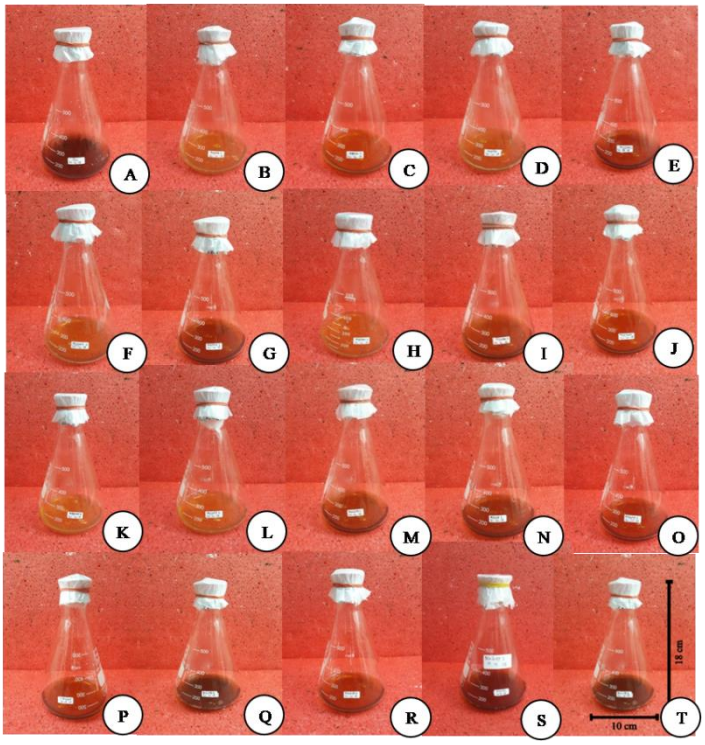

Figure 6. *Metarhizium anisopliae* culture filtrate: MPdB (A), MPdR1 (B), MPdR2 (C), MpdPe (D), MJgMs1 (E), MJgMs2 (F), MPdMs1 (G), MPdMs2 (H), MPdMs3 (I), MjgKeTs (J), MPdMs4 (K), MJgTs2 (L), MKbTp1 (M), MSwTp1 (N), MSwTp2 (O), MSwTp3 (P), MSwTp4 (Q), MKKPp1 (R), MKbTp2 (S), MagPd (T)

Effect of entomopathogenic fungal culture filtrate on test larvae

S. litura larvae fed with chili leaves applied with *B. bassiana* or *M. anisopliae* culture filtrate exhibited the same behavior, i.e., they increasingly ate less, while the controls not given culture filtrate continued to eat greedily. The chili leaves given to the larvae which were not given culture filtrate generally only left a leaf bone, whereas in the larvae given culture filtrate there were still leaves remaining (Figure 7 dan 8). Thus, the culture filtrate could reduce the appetite of *S. litura* larvae.

The larvae *S. litura* given culture filtrate besides being more lazy to eat showed more and more lazy to move. After 2 and 3 days being given the culture filtrate, the larvae began to change the color of integument which was previously bright green to become dull and the larvae were discovered to be dead. Four days after being given the culture filtrate, the larvae increasingly began to shrink and die. The dead larvae were wrinkled, dry, black, and odorless. The larvae not given the culture filtrate had larger body sizes and the integument colors were greener, and brighter (Figure 9 dan 10).

Figure 7. The leaf damage: control (A) and larval *Spodoptera litura* applied with *Beauveria bassiana* culture filtrate (B)

Figure 8. The leaf damage: control (A) and larval *Spodoptera litura* applied with *Metarhizium anisopliae* culture filtrate (B)

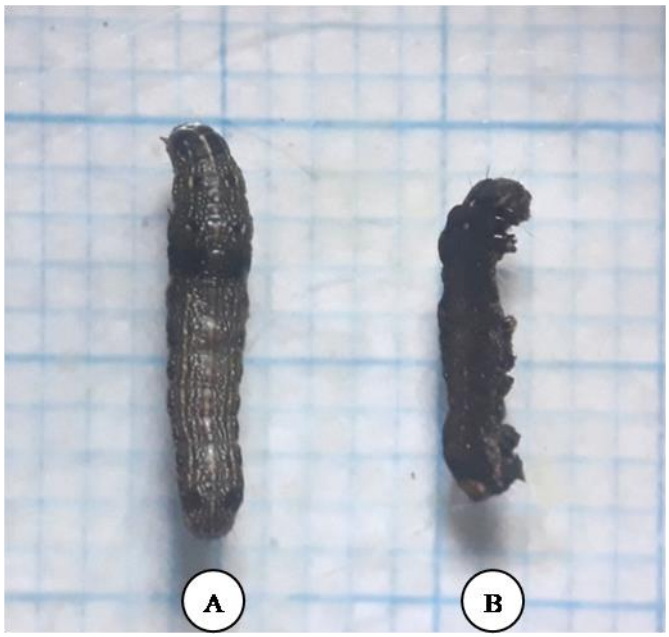

Figure 9. The healthy (A) and dead larvae (B) of *Spodoptera litura* caused by *Beauveria bassiana* culture filtrate

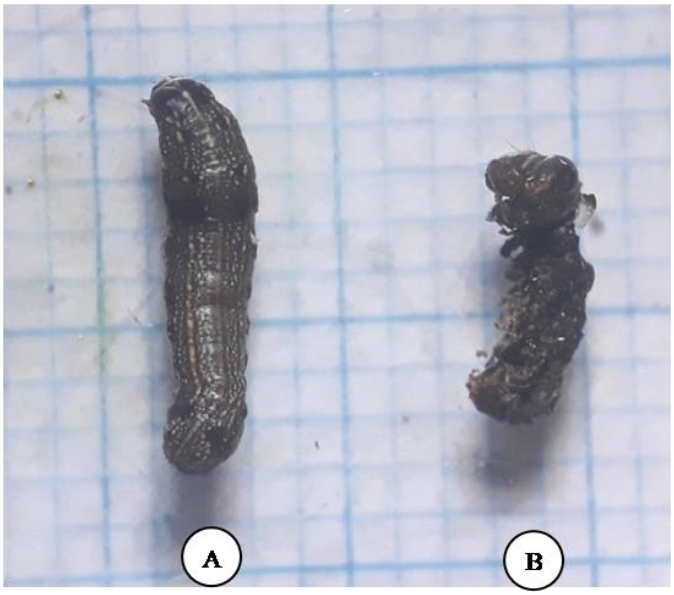

Figure 10. The healthy (A) and dead larvae (B) of *Spodoptera litura* caused by *Metarhizium anisopliae* culture filtrate

Discussion

The *B. bassiana* culture filtrate, particularly the isolates coded BSwTd2, were the most toxic compared to other isolates with LT_{50} of only 5.91 days. The BSwTd2 isolate was from peat soils overgrown with oil palm plants. For *M. anisopliae*, the most toxic MKbTp2 isolate was from the cabbage in highland. The larvae began to die after 3x24 hours after the treatment either due to *B. bassiana* or *M. anisopliae*. The larvae death resulted from the 6 weeks of incubation of entomopathogenic fungi causing the toxic culture filtrate. According to Pinnamaneni et al. (2010) the culture of filtrates of the toxic *B. bassiana* was caused by the incubation in liquid culture media in which the fungus produced chitinolytic exochitinase enzymes so that when they entered the body the insect was able to degrade the cuticle. In addition, the fungal broth during the incubation could produce protease enzymes (Qazi 2008).

The time of death of the larvae by this culture filtrate was faster than that of by conidia. El Husseini (2019) stated that the death of *Spodoptera* larvae by *M. anis*opliae conidia began to occur at the fourth day (4x24 hours) or the fifth day after the treatment (post-treatment), whereas in this study the *M. anisopliae* culture filtrate began to kill *S. litura* at the third day after the treatment. The lethal time difference between these was due to the mode of action of fungal conidia being different from the culture filtrate. To kill the host insect, the fungus conidia got contact first with it and then produced toxins to kill the host, while the culture filtrate directly killed the host insect. El-Ghany (2015) states that entomopathogenic fungus conidia kill its host insect in two stages: parasitic and saprophytic phases. The parasitic phase began with the fungal conidia attaching to the host insect cuticle (Augustyniuk-Kram & Kram 2012). Then, if the humidity was high, the conidia started to germinate on the host cuticle (El-Ghany 2015). The conidia germinating by forming germ tubes continued to grow sticking out to find a soft integument position to facilitate entry into the cuticle (Fernandes et al. 2007). The infection occurs when the germ tubes are able to penetrate insect cuticles and the ability to infect them is a determining factor for the fungus virulence (Altre & Vandenberg 2001). After the germ tubes penetrated the cuticle and reached the haemocoel, and then they produced specific infection hyphae originating at appressoria (El-Ghany 2015). Furthermore, the hyphae spread to the haemolymph and developed to produce blastospores, and the blastospores produced fungal cytotoxic, for example destruxins by *M. anisopliae* which killed the host insects (Mancillas-Paredes et al. 2019), but the death of these host insects was not caused only by the toxin but also due to the mechanical damage by the penetration of fungi into the body of insects (El-Ghany 2015).

After the host insect died, it entered into the saprophytic phase which was influenced by the favorable environmental conditions (Peña-Peña et al. 2015). In the body of the dead insect, the fungus formed mycelia and hyphae which continued to grow covering the body of the host insect, and then the hyphae formed conidiogenous cells and the conidia was produced by utilizing the nutrients/fluids of the host insect and finally the infection process was complete (El-Ghany 2015).

In contrast to the mode of action of fungal conidia, a fungal culture filtrate directly kills the host insect due to the toxins produced by fungal broth so that the time needed to kill the host insect is shorter. The entomopathogenic fungus cultured in liquid media can produce conidia in the form of blastospores (Mascarin et al. 2015) which can produce toxins (Mascarin & Jaronski 2016). In this study, the result of calculating conidia density after being incubated for 6 weeks was generally more than $1x10^9$ conidia mL^{-1}. Fungal conidia and propagules were separated from the filtrate through two stages of filtration to produce toxic fungal culture filtrate. The *B. bassiana* and *M. anisopliae* culture filtrates were able to kill up to 94.67% and 96% of the larvae of *S. litura*, respectively. The short time of death by this culture filtrate resulted from the death of the direct host insect by consuming feed (chili leaves) moistened with the culture filtrate. Soesanto et al. (2019) stated that entomopathogenic fungal culture filtrate contains secondary metabolites. *B. bassiana* culture filtrate containing the secondary metabolites can weaken the host insect's immune system (Zibaee et al. 2011). The secondary metabolites produce toxins, for example destruxins and efrapeptins (Zibaee et al. 2009). *B. bass*iana also produces a protease enzyme that can kill host insects by dissolving their body proteins (Mancillas-Paredes et al. 2019).

The color of fungal broth and culture of *B. bassiana* and *M. anisopliae* varied between isolates. The darker color of fungal broths and culture filtrates tend to cause higher mortality. In line with the results obtained by Ayudya et al. (2019), the darker culture filtrates tended to be more toxic than the light-colored ones. The darker culture filtrates show higher production of secondary metabolites (Lou et al. 2017) and the activity of extracellular enzymes (Khachatourians et al. 2007) and the enzymes produced such as proteases are able to dissolve integument insects that result in death (Mancillas-Paredes et al. 2019).

The symptoms of sick host insects by culture filtrate in this study differed from the deaths by fungal conidia. The insects getting sick by the culture filtrate were not covered by micelia or conidia on the integument. However, they got sick because of the fungal conidia infection which was generally covered in micelia (Sumikarsih et al. 2019). The body shape of the sick *S. litura* larvae due to the culture filtrate was shrunk, wrinkled, dry, and odorless. Similar to the results of the study of Ayudya et al. (2019), the *S litura* larvae treated with *B. bassiana* culture filtrate decreased their body weight and became dried, odorless, and the integument was not overgrown with fungal micelia because in the culture filtrate it no longer contained conidia but contained toxic compounds for host insects. Therefore, the mortality of *S. litura* larvae in this study was caused by the culture filtrate containing toxic compounds. Zibaee et al. (2011) states that toxic compounds in the culture filtrate are secondary metabolites. Zibaee et al. (2009) stated that the secondary metabolites are easier to apply in the field because they can be integrated with other control techniques including synthetic insecticides. Consequently, the

culture filtrate which is toxic due to the high content of secondary metabolites has the potential to be further developed for the active ingredient of mycoinsecticide.

The most toxic isolates of the culture filtrate were BSwTd2 of *B. bassiana* and MKbTp2 of *M. anisopliae* resulting in the mortality of above 90%. The two isolates that were found each came from the lowlands and highlands so that in the future there is an opportunity for the isolates to be developed and applied in low and highland ecosystems. Entomopathogenic fungi that produce toxic filtrate cultures are capable of killing their hosts in a short time, so that they have the potential to be active ingredients in the development of mycoinsecticides technology.

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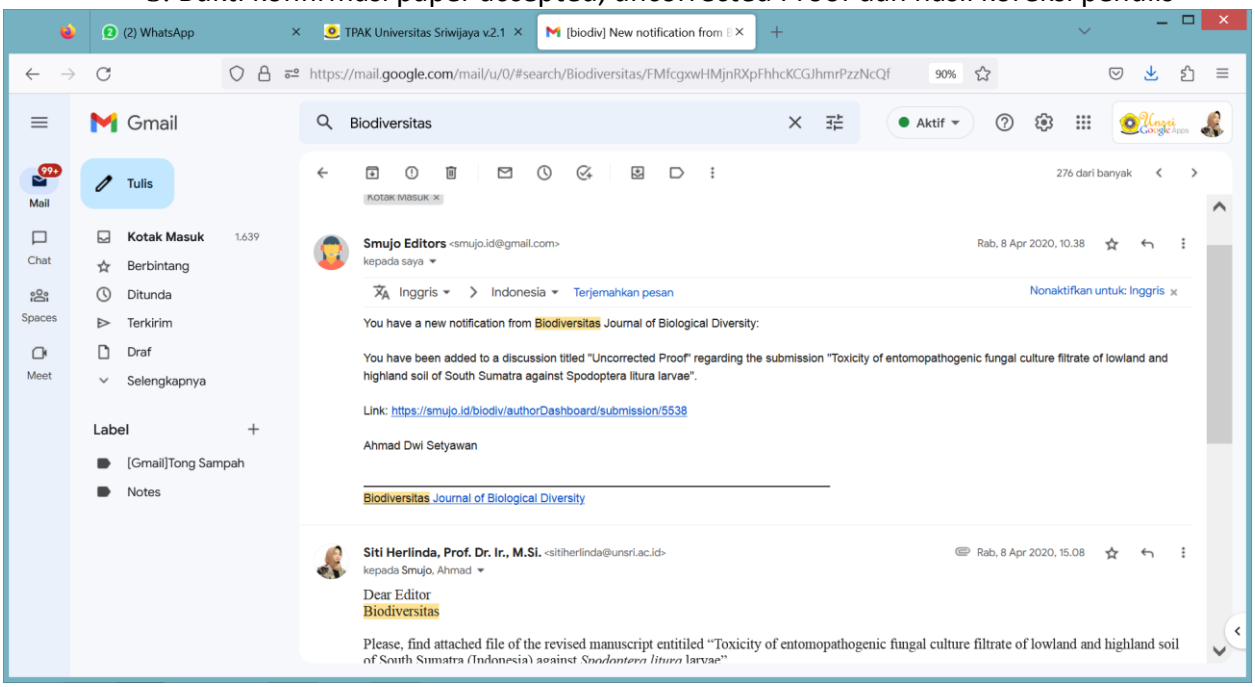

Toxicity of entomopathogenic fungal culture filtrate of lowland and highland soil of South Sumatra (Indonesia) against *Spodoptera litura* larvae

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Abstract. *Gustianingtyas M, Herlinda S, Suwandi, Suparman, Hamidson H, Hasbi, Setiawan A, Verawaty M, Elfita, Arsi. 2020. Toxicity of entomopathogenic fungal culture filtrate of lowland and highland soil of South Sumatra against* Spodoptera litura *larvae. Biodiversitas 21: xxxx.* The use of secondary fungal metabolites for the active ingredient of mycoinsecticide is more effective and more easily integrated with other pest control techniques. This study aimed to measure the toxicity of the culture filtrate of entomopathogenic fungi originating from South Sumatra against the *Spodoptera litura* larvae. *Beauveria bassiana* (25 isolates) and *Metarhizium anisopliae* (20 isolates) of South Sumatra were cultured in liquid media and filtered to produce culture filtrate. The larvae which were sick due to the filtrate showed the symptoms of decreased appetite and were not actively moving, while the dead larvae were characterized by being wrinkled, dry, black integument, and odorless. Mortality caused by *B. bassiana* filtrate was the highest 98% (BJgTs isolate) and not significantly different from the BSwTd2 isolate (94.67%). Yet, the LT_{50} BSwTd2 isolate was shorter (5.92 days) compared to the LT_{50} BJgTs isolate (6.35 days). The most toxic *M. anisopliae* filtrate produced the mortality of 96% (MKbTp2 isolate) and 85.33% (MPdB isolate) each of which had LT_{50} of 7.36 days and 8.09 days, respectively. So, the most toxic culture filtrate was BSwTd2 isolate of *B. bassiana* and MKbTp2 isolate of *M. anisopliae*. The entomopathogenic fungi producing filtrate which are toxic have the potential to be active ingredients of mycoinsecticides.

Keywords: *Beauveria bassiana*, chili, LT_{50}, *Metarhizium anisopliae*, mortality

INTRODUCTION

South Sumatra is generally dominated by lowland lands, namely freshwater swamps and tidal lowlands (Kartika et al. 2018; Karenina et al. 2019), and a small portion of medium and highland. Freshwater swamps can be found, among others in the Districts of Ogan Ilir, Ogan Komering Ilir, Musi Banyuasin, and Palembang City; the widest tide exists in Banyuasin District and the medium and highlands occur, among others, in the Districts of Ogan Komering Ulu Selatan, Lahat and Pagaralam City. The varied topographical conditions between districts/cities characterize the differences including the crops and microorganisms cultivated there.

In the lowlands of South Sumatra are generally cultivated annual crops, such as paddy (Herlinda et al. 2018a; Prabawati et al. 2019), maize (Juhriah et al. 2019), chili (Johari et al. 2016; Nasution and Respatijarti 2019; Sagrim et al. 2017), and palm oil (Darlan et al. 2016). Meanwhile, in the medium and high plains are generally cultivated annual crops such as coffee, tea (Zamhari et al. 2017), vegetables such as potatoes (Maryanto et al. 2018), cabbage, mustard greens, and caisim (Situmorang et al. 2019). Vegetables and seasonal plants of the lowlands and highlands generally have a major pest, namely *Spodoptera litura* (Turnip et al. 2019). The yield loss caused by its larval stage is more than 35% a year (Bueno et al. 2011). This pest has egg, larval, pupal, and adult stages (Vijaya and Rani 2016). *S. litura* is a polyphagic pest that attacks many vegetables in Indonesia, for example chili (Nagal et al. 2016), cotton fruit (Maqsood et al. 2017), soybean (Fattah et al. 2018). A polyphagic pest is a pest that has a broad range of host species (more than a family) (Nagal et al. 2016). For the control of *S. litura*, it needs natural enemies such as entomopathogenic fungi that can adapt in low to high altitudes.

In the lowlands and highlands, the soil types have different chemical and physical characteristics; acidic pH soils are generally found in the lowlands and neutral or basic pH soils are found in the highlands (Munir and Herman 2019). Soil pH can affect the presence of entomopathogenic fungi and tends to be neutral or high pH soils which more commonly occur in entomopathogenic fungi than low pH soils (Safitri et al. 2018). Besides pH,

the texture of sandy soils tends to be low and muddy which is not suitable for fungal propagule life as well as water-saturated soil where fungi are rarely spotted to survive (Garrido-Jurado et al. 2011). Temperature (Pinnamaneni et al. 2010) and humidity (Oreste et al. 2015; Liu et al. 2017) also affect pathogenicity of entomopathogenic fungi.

Previous study found that more than 30 isolates of entomopathogenic fungi were found in the freshwater swamp, tidal, and highland soil of South Sumatra (Safitri et al. 2018). Apart from the soil, the entomopathogenic fungi were also found from the insect pests of vegetable and food crops of the lowlands to the highlands of South Sumatra (Herlinda et al. 2018b). Various origins of entomopathogenic fungi tend to produce pathogenicity variations (Sumikarsih et al. 2019).

The pathogenicity of entomopathogenic fungi is influenced by the ability of the fungi to produce secondary metabolites (Zibaee et al. 2009). The fungi that develop in insect haemolymph or in liquid media are able to produce toxic metabolites, such as extracellular enzymes, proteins, and toxins (Bandani 2005). In vitro media, toxic metabolites can be produced by the fungi which are grown in liquid media and produce culture filtrate (Soesanto et al. 2019). The use of secondary entomopathogenic fungal metabolites for the active ingredient of mycoinsecticide is more effective and more easily integrated with other pest control techniques (Zibaee et al. 2009). Entomopathogenic fungal culture filtrate found in low to high altitude of South Sumatra potentially gets the most toxic isolates that can be utilized in the development of mycoinsecticide technology. For this reason, this study aimed to measure the toxicity of the culture filtrate isolates of entomopathogenic fungi from low to high land of South Sumatra against *Spodoptera litura* larvae.

MATERIALS AND METHODS

The experiments were carried out at the Entomology Laboratory, Department of Pests and Plant Diseases, Faculty of Agriculture, Universitas Sriwijaya from May to November 2018. The room temperatures and relative humidity during the experiment in the laboratory was 29.78°C and 82.72%, respectively. The entomopathogenic fungi isolates used in this study were explored by Herlinda et al. (2018) and Safitri et al. (2018), (Table 1 and 2) spread from the lowlands to the highlands of South Sumatra. The isolates were grouped into two: 25 isolates of *Beauveria bassiana* (Figure 1) and 20 isolates of *Metarhizium anisopliae* (Figure 2). All isolates were identified by Dr. Suwandi (a mycologist from Universitas Sriwijaya).

Test insect preparation

S. litura larvae were collected from the chili planting area in the experimental field of the Faculty of Agriculture, Sriwijaya University and farmers' vegetable fields which were not applied by synthetic insecticides. Then, the larvae were brought to the laboratory and maintained in a plastic cylinder whose upper part was covered with gauze (Ø 25 cm, height 30 cm) having been washed thoroughly using detergent. Next, water spinach plants were put into it for larvae feed.

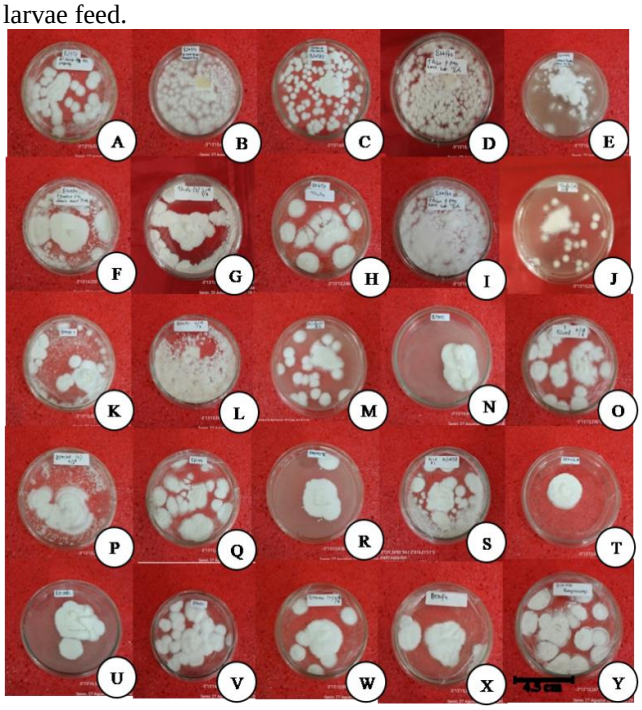

Figure 1. *Beauveria bassiana* isolates cultured in SDA media: BJgTs (A), BSmMs (B), BSwTd1 (C), BSwTd2 (D), BSwTd3 (E), BSwTd4 (F), BPdR (G), BKbTp (H), BKKPp2 (I), Ts1d3 (J), BTmPc (K), BTmTr (L), (M) Ts1d2 (M), BTmTs (N), BlePd2 (O), BTmKt (P), BPCmS (Q), BMkMs (R), BtmGa (S), BTmSr (T), BPcPd2 (U), BtmSo (V), BtmMa (W), BtmPe (X), Blepd (Y)

Figure 2. *Metarhizium* isolates cultured in SDA media: MPdB (A), MPdR1 (B), MPdR2 (C), MpdPe (D), MJgMs1 (E), MJgMs2 (F), MPdMs1 (G), MPdMs2 (H), MPdMs3 (I), MjgKeTs (J), MPdMs4 (K), MJgTs2 (L), MKbTp1 (M), MSwTp1 (N), MSwTp2 (O), MSwTp3 (P), MSwTp4 (Q), MKKPp1 (R), MKbTp2 (S), MagPd (T)

Every day the larvae feed was replaced with fresh new feed. The larvae entering the pupae phase were transferred into a plastic cylinder (Ø 10 cm, height 15 cm) with the top open and the bottom of the cylinder sprinkled with sifted soil and sterilized in an oven for 1 hour at 100ºC. The thickness of the soil inserted into the cylinder was 3 cm. The plastic cylinder containing the pupae was put into a gauze cage (30x30x30 cm^3) which had been chopped with chilies for laying eggs. Adults arising from the pupae were fed with honey smeared on cotton and hung over a cage. The hatched eggs were transferred into a plastic cylinder and fed with water spinach which was replaced every day. The larvae used for toxicity testing were the second instar of the third offspring or afterwards.

Table 1. *Beauveria bassiana* isolates from South Sumatra used in this research

Isolate code	Isolate origin	Geographic origin
BJgTs	Tidal lowlands, maize	Telang Sari
BSmMs	Tidal lowlands, watermelon	Mulya Sari
BSwTd1	Peatlands, oil palm	Talang Dabok
BSwTd2	Peatlands, oil palm	Talang Dabok
BSwTd3	Peatlands, oil palm	Talang Dabok
BSwTd4	Peatlands, oil palm	Talang Dabok
BPdR	Freshwater swamps, paddy	Rambutan
BKbTp	Highlands, cabbage	Talang Patai
BKKPp2	Highlands, rubber and coffee	Pulau Pinang
Ts1d3	Peatlands	Talang Dabok
BTmPc	Freshwater swamps	Indralaya
BTmTr	Freshwater swamps	Telang Rejo
Ts1d2	Peatlands	Talang Dabok
BTmTs	Highlands	Mulia Sari
BlePd2	*Lipaphis erysimi*	Pagardin
BTmkt	Freshwater swamps	Kenten
BPCmS	*Pseudoplusia chalcites*	Muara Siban
BMkMs	Highlands	Muara Siban
BtmGa	Freshwater swamps	Gandus
BTmSr	Tidal lowlands	Srikaton
BPcPd2	*Chrysodeixis chalcites*	Pagardin
BtmSo	Freshwater swamps	Soak
BtmMa	Freshwater swamps	Mariana
BtmPe	Freshwater swamps	Pemulutan
Blepd	*Lipaphis erysimi*	Pagardin

Table 2. *Metarhizium anisopliae* isolates from South Sumatra used in this research

Isolate code	Isolate soil origin	Geographic origin
MPdB	Freshwater swamps, paddy	Banyuasin
MPdR1	Freshwater swamps, paddy	Rambutan
MPdR2	Freshwater swamps, paddy	Rambutan
MpdPe	Freshwater swamps, paddy	Pemulutan
MJgMs1	Tidal lowlands, maize	Mulya Sari
MJgMs2	Tidal lowlands, maize	Mulya Sari
MPdMs1	Tidal lowlands, paddy	Mulya Sari
MPdMs2	Tidal lowlands, paddy	Mulya Sari
MPdMs3	Tidal lowlands, paddy	Mulya Sari
MjgKeTs	Tidal lowlands, maize and oil palm	Telang Sari
MPdMs4	Tidal lowlands, paddy	Mulya Sari
MJgTs2	Tidal lowlands, maize	Telang Sari
MKbTp1	Highlands, cabbage	Talang Patai
MSwTp1	Highlands, mustard	Talang Patai
MSwTp2	Highlands, mustard	Talang Patai
MSwTp3	Highlands, mustard	Talang Patai
MSwTp4	Highlands, mustard	Talang Patai
MKKPp1	Highlands, mustard, rubber, coffee	Pulau Pinang
MKbTp2	Highlands, cabbage	Talang Patai

Culture filtrate production

All isolates before being filtered were first made fit by modifying the method of Herlinda (2010). The media for making fit used Sabouraud Dextrose Agar (SDA, Merck) as much as 16.2 g and added 250 mL aquadest enriched with 1.25 g *Tenebrio molitor* larvae flour (5 g). After that, the ingredients were mixed and stirred evenly and then put in an erlenmeyer (size 250 mL), sterilized in an autoclave for 120 minutes at a pressure of 1 atm. The media was poured into the Petri dish as much as 10 mL (Ø 9 cm) in aseptic laminar flow air conditions. Then, the entomopathogenic fungi isolates were inoculated in the SDA media and incubated for 14 days.

Isolates of entomopathogenic fungi derived from SDA media of aged 14 days were then transferred and grown into liquid media (broth media), PDB (*Potato Dextrose Broth*) prepared as follows: the PDB media composition consisting of 20 g dextrose monohydrate, 200 g potatoes, and 1000 mL aquadest. Before the potatoes were extracted, they were cut into cubes with a size of ± 2x2x2 cm^3 and boiled using 1000 mL aquadest for 20 minutes. This PDB medium was sterilized in the autoclave and after it got cold, the entomopathogenic fungus isolates were inoculated into it. This liquid culture was incubated for 6 weeks.

After the fungus liquid culture (fungal broth) was 6 weeks old, the fungus filtration is carried out. The filtration was conducted in two stages, namely filtering using filter paper and syringe filter. In the first stage, 100 mL of 6-week-old fungal broth was filtered using Whatman filter paper no. 42 and coated with a thickness of 1 cm cotton. The culture filtrate obtained from the first stage of filtering was then sucked up to 10 mL using a hypodermic needle (spike). Then, the needle was removed and the base of the needle was fitted with a syringe filter (0.45 µm-25 mm). The 10 mL spike was pressed so that it released culture filtrate from the syringe filter. The 100 mL of fungal broth produced ± 70 mL of culture filtrate. The culture filtrate was taken as much as 1 mL to be dripped on a piece of chili leaf which was used as a toxicity test. This culture filtrate is often referred to as raw secondary metabolites (Soesanto et al. 2019). To ensure the culture filtrate did not contain propagules (hyphae, micelia) and conidia, it was grown on agar media (SDA) before it was applied. If it does not grow fungus on the SDA media, the culture filtrate can be applied.

Insecticidal activity test of entomopathogenic fungus culture filtrate

The chili leaves that had been dropped by the culture filtrate were first drained; before 25 *S. litura* larvae were fasted for 2 hours, and then put into them. The second instar larvae were left to eat leaves that had been dropped with culture filtrate for 6 hours. After 6 hours, the larvae were transferred into a plastic cylinder topped with gauze (Ø 25 cm, height 30 cm) containing 15-30 clean chili leaves which were not dripped with culture filtrate. Every day the chili leaves were replaced with the new ones. Every day the dead larvae were recorded up to 12 days after the application.

Data analysis

The differences in mortality data and lethal time that killed 50% of test insects (LT_{50}) were analyzed using analysis of variance (ANOVA), after that further tests were carried out using the 5% Tukey's Honestly Significant Difference (HSD). All data were calculated using software of SAS University Edition 2.7 9.4 M5.

RESULTS AND DISCUSSION

Insecticidal activities of entomopathogenic fungi

The culture filtrate derived from twenty-five *B. bassiana* isolates tested for the insecticidal activity showed that the most toxic *B. bassiana* isolates were those coded BJgTs (98%) and were not significantly different, among others with BSwTd2 (94.67%) and BTmTs isolates (96%). All *B. bassiana* isolates showed the ability to kill the test larvae (Table 3). The BJgTs, BSwTd2, and BTmTs isolates were the most toxic because they produced the highest mortality and also their ability to kill the shortest as evidenced by the short LT_{50} (6.35, 5.92, and 7.78 days) isolates. The most toxic isolates were shown with the highest mortality and their shortest LT_{50}. Consequently, for *B. bassiana*, the most toxic isolate was BSwTd2.

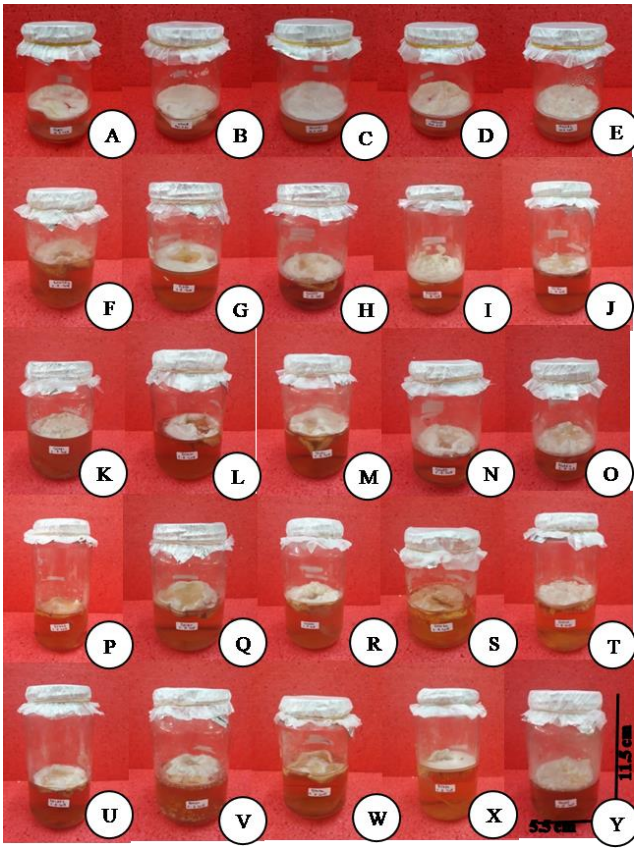

Figure 3. *Beauveria bassiana* isolates cultured in PDB: BJgTs (A), BSmMs (B), BSwTd1 (C), BSwTd2 (D), BSwTd3 (E), BSwTd4 (F), BPdR (G), BKbTp (H), BKKPp2 (I), Ts1d3 (J), BTmPc (K), BTmTr (L), (M) Ts1d2 (M), BTmTs (N), BlePd2 (O), BTmKt (P), BPCmS (Q), BMkMs (R), BtmGa (S), BTmSr (T), BPcPd2 (U), BtmSo (V), BtmMa (W), BtmPe (X), Blepd (Y)

Table 3. Mortality of larval *Spodoptera litura* after being infested with *Beauveria bassiana* culture filtrates

Isolate codes	Mortality ± SE*(%)	LT_{50} (days)± SE
BJgTs	98.00 ± 1.41^{b}	6.35 ± 0.49
BSmMs	50.00 ± 32.53^{ab}	11.57 ± 4.08
BSwTd1	49.33 ± 15.36^{ab}	12.03 ± 3.52
BSwTd2	94.67 ± 4.35^{ab}	5.92 ± 0.46
BSwTd3	73.33 ± 18.51^{ab}	8.86 ± 1.78
BSwTd4	26.00 ± 1.41^{ab}	16.07 ± 0.97
BPdR	86.67 ± 3.93^{ab}	8.17 ± 0.61
BKbTp	62.67 ± 10.39^{ab}	10.71 ± 0.91
BKKPp2	58.00 ± 7.07^{ab}	10.68 ± 0.34
TS1d3	10.67 ± 2.88^{a}	17.45 ± 0.76
BTmPc	46.67 ± 17.01^{ab}	14.32 ± 3.72
BTmTr	29.33 ± 9.68^{ab}	14.31 ± 1.79
TS1d2	88.00 ± 8.22^{ab}	7.87 ± 1.11
BTmTs	96.00 ± 0.00^{ab}	7.78 ± 0.09
BLePd2	70.00 ± 21.21^{ab}	9.59 ± 1.43
BTmKt	80.00 ± 14.73^{ab}	8.80 ± 1.41
BPcMs	81.33 ± 9.49^{ab}	7.87 ± 1.32
BMkMs	72.00 ± 17.99^{ab}	10.82 ± 2.46
BTmGa	66.67 ± 22.34^{ab}	12.27 ± 3.60
BTmSr	21.33 ± 9.30^{ab}	16.80 ± 2.67
BPcPd2	18.67 ± 7.85^{ab}	9.43 ± 3.86
BTmSo	80.00 ± 14.14^{ab}	7.55 ± 1.35
BTmMa	52.00 ± 19.69^{ab}	11.09 ± 1.62
BTmPe	70.00 ± 18.38^{ab}	9.30 ± 1.94
BLePd	94.00 ± 4.24^{ab}	9.30 ± 0.45
ANOVA F-value	2.09*	1.20^{ns}
P value (0.05)	0.02	0.30
Tukey's HSD test	67.09	-

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

Table 4. Mortality of larval *Spodoptera litura* after being infested with *Metarhizium anisopliae* culture filtrates

Isolate codes	Mortality ± SE*(%)	LT_{50} (days) ± SE
MPdB	85.33 ± 14.67^{b}	8.09 ± 1.88^{a}
MPdR1	57.33 ± 19.37^{ab}	11.87 ± 2.56^{a}
MPdR2	65.33 ± 13.33^{ab}	10.43 ± 0.51^{a}
MPdPe	42.67 ± 9.61^{ab}	12.67 ± 0.89^{a}
MJgMs1	53.33 ± 23.25^{ab}	13.67 ± 4.52^{a}
MJgMs2	25.33 ± 3.53^{ab}	14.50 ± 0.56^{a}
MPdMs1	73.33 ± 10.41^{ab}	9.90 ± 0.97^{a}
MPdMs2	84.00 ± 9.24^{ab}	8.70 ± 1.26^{a}
MPdMs3	68.00 ± 16.17^{ab}	10.08 ± 0.86^{a}
MJgKeTs	42.67 ± 10.67^{ab}	12.68 ± 1.05^{a}
MPdMs4	16.00 ± 10.07^{ab}	53.70 ± 23.11^{b}
MJgTs2	46.67 ± 13.92^{ab}	14.29 ± 2.23^{a}
MKbTp1	84.00 ± 10.07^{ab}	8.69 ± 0.95^{a}
MSwTp1	56.00 ± 12.22^{ab}	14.64 ± 3.24^{a}
MSwTp2	74.67 ± 13.92^{ab}	10.62 ± 1.39^{a}
MSwTp3	61.33 ± 16.38^{ab}	10.91 ± 1.99^{a}
MSwTp4	61.33 ± 23.13^{ab}	14.60 ± 5.54^{a}
MKKPp1	44.00 ± 10.07^{ab}	12.33 ± 0.90^{a}
MKbTp2	96.00 ± 4.00^{b}	7.36 ± 0.62^{a}
MagPd	33.33 ± 3.53^{ab}	12.98 ± 0.23^{a}
ANOVA F-value	2.39*	50.13*
P value (0.05)	0.01	0.00
Tukey's HSD test	53.35	2.65

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

The culture filtrate of the twenty *M. anisopliae* isolates which were tested for their insecticidal activity showed that the most toxic isolates were those coded MKbTp2 (96%) and MPdB (85.33%) and the mortality of both isolates was significantly different from the other isolates (Table 4). The BSwTd2 isolate was the most toxic because they produced the highest mortality as well as the ability to kill the shortest as evidenced by the shortest LT_{50} (7.36 days), and then followed by LT_{50} isolate MPdB (8.09 days). The most toxic *M. anisopliae* isolate was proven by the highest mortality and the shortest LT_{50} which was MKbTp2 isolate.

The color of the fungal broth (Figure 3) and culture filtrate (Figure 4) of *B. bassiana* varied among the isolates. All fungal broth isolates were generally transplanted brown, but there were among those isolates dark brown in color, for example isolates BSwTd2, BSwTd3, BPdR, TS1d2. There was a tendency for the darker color of the fungal broth and culture filtrate to cause higher mortality. The color of fungal broth (Figure 5) and culture filtrate (Figure 6) of *M. anisopliae* differed from the colors of fungal broth and culture of *B. bassiana*. The *M. anisopliae* fungal broth was generally dark greenish brown, but the culture filtrate tended to have a more varied color, from greenish dark brown to light brown. The same tendency was also produced in *M. anisopliae* isolates, i.e., the color of the older culture filtrate resulting in higher mortality. The isolates having a darker colored culture filtrate were MPdB, MPdMs1, MPdMs2, MPdMs3, MKbTp1, MKbTp2.

Effect of entomopathogenic fungal culture filtrate on test larvae

S. litura larvae fed with chili leaves applied with *B. bassiana* or *M. anisopliae* culture filtrate exhibited the same behavior, i.e., they increasingly ate less, while the controls not given culture filtrate continued to eat greedily. The chili leaves given to the larvae which were not given culture filtrate generally only left a leaf bone, whereas in the larvae given culture filtrate there were still leaves remaining (Figures 7 and 8). Thus, the culture filtrate could reduce the appetite of *S. litura* larvae.

The larvae *S. litura* given culture filtrate besides being more lazy to eat showed more and more lazy to move. After 2 and 3 days being given the culture filtrate, the larvae began to change the color of integument which was previously bright green to become dull and the larvae were discovered to be dead. Four days after being given the culture filtrate, the larvae increasingly began to shrink and die. The dead larvae were wrinkled, dry, black, and odorless. The larvae not given the culture filtrate had larger

body sizes and the integument colors were greener, and brighter (Figures 9 and 10).

Figure 4. *Beauveria bassiana* culture filtrate: BJgTs (A), BSmMs (B), BSwTd1 (C), BSwTd2 (D), BSwTd3 (E), BSwTd4 (F), BPdR (G), BKbTp (H), BKKPp2 (I), Ts1d3 (J), BTmPc (K), BTmTr (L), (M) Ts1d2 (M), BTmTs (N), BlePd2 (O), BTmKt (P), BPCmS (Q), BMkMs (R), BtmGa (S), BTmSr (T), BPcPd2 (U), BtmSo (V), BtmMa (W), BtmPe (X), Blepd (Y)

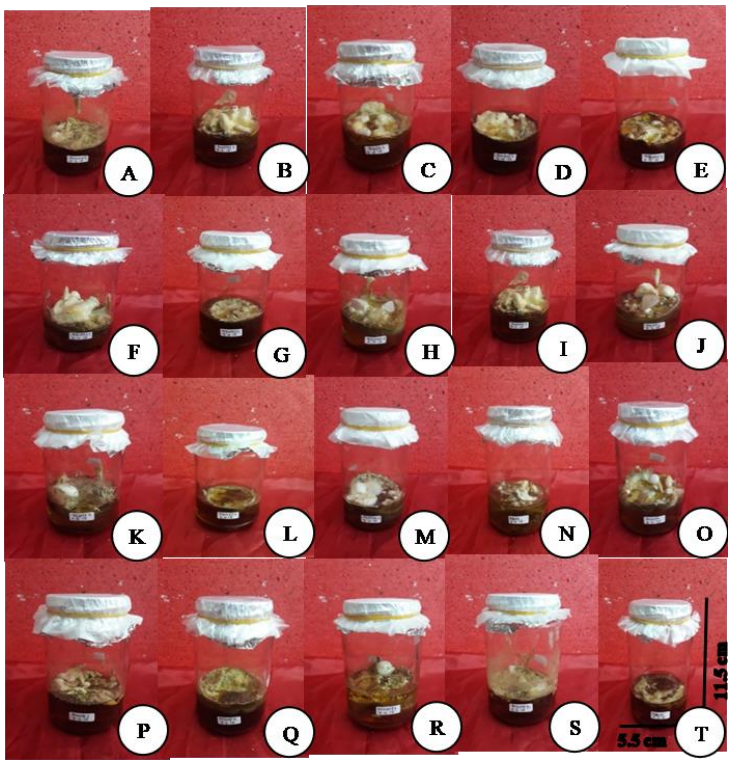

Figure 5. *Metarhizium anisopliae* isolates cultured in PDB: MPdB (A), MPdR1 (B), MPdR2 (C), MpdPe (D), MJgMs1 (E), MJgMs2 (F), MPdMs1 (G), MPdMs2 (H), MPdMs3 (I), MjgKeTs (J), MPdMs4 (K), MJgTs2 (L), MKbTp1 (M), MSwTp1 (N), MSwTp2 (O), MSwTp3 (P), MSwTp4 (Q), MKKPp1 (R), MKbTp2 (S), MagPd (T)

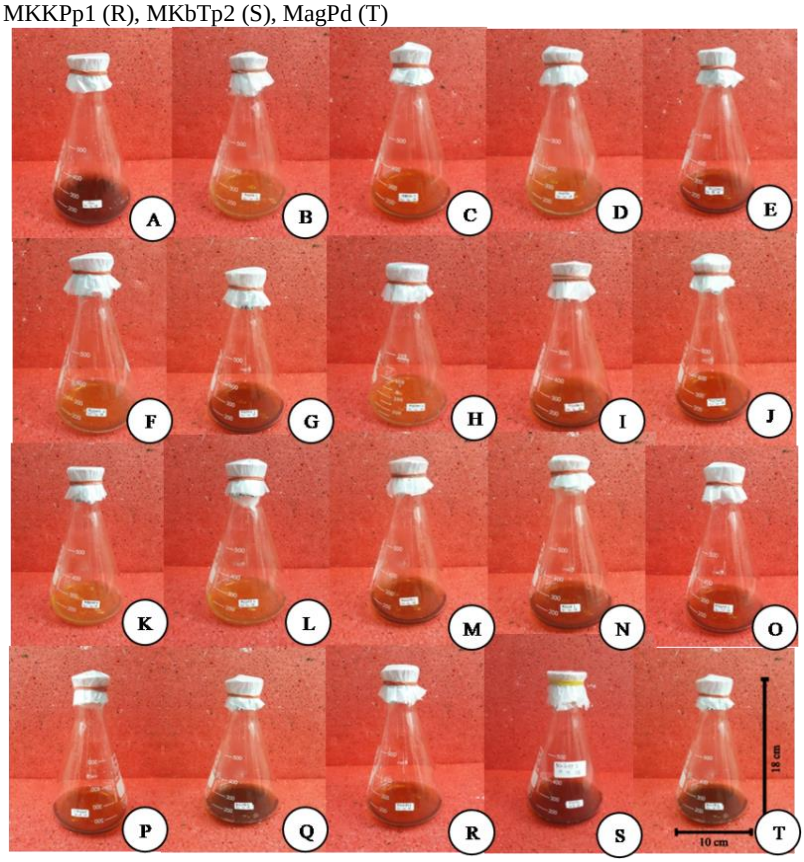

Figure 6. *Metarhizium anisopliae* culture filtrate: MPdB (A), MPdR1 (B), MPdR2 (C), MpdPe (D), MJgMs1 (E), MJgMs2 (F), MPdMs1 (G), MPdMs2 (H), MPdMs3 (I), MjgKeTs (J), MPdMs4 (K), MJgTs2 (L), MKbTp1 (M), MSwTp1 (N), MSwTp2 (O), MSwTp3 (P), MSwTp4 (Q), MKKPp1 (R), MKbTp2 (S), MagPd (T)

Discussion

The *B. bassiana* culture filtrate, particularly the isolates coded BSwTd2, were the most toxic compared to other isolates with LT_{50} of only 5.91 days. The BSwTd2 isolate was from peat soils overgrown with oil palm plants. For *M. anisopliae*, the most toxic MKbTp2 isolate was from the cabbage in highland. The larvae began to die after 3x24 hours after the treatment either due to *B. bassiana* or *M. anisopliae*. The larvae death resulted from the 6 weeks of incubation of entomopathogenic fungi causing the toxic culture filtrate. According to Pinnamaneni et al. (2010) the culture of filtrates of the toxic *B. bassiana* was caused by the incubation in liquid culture media in which the fungus produced chitinolytic exochitinase enzymes so that when they entered the body the insect was able to degrade the cuticle. In addition, the fungal broth during the incubation could produce protease enzymes (Qazi 2008).

The time of death of the larvae by this culture filtrate was faster than that of by conidia. El Husseini (2019) stated that the death of *Spodoptera* larvae by *M. anisopliae* conidia began to occur at the fourth day (4x24 hours) or the fifth day after the treatment (post-treatment), whereas in this study the *M. anisopliae* culture filtrate began to kill *S. litura* at the third day after the treatment. The lethal time difference between these was due to the mode of action of fungal conidia being different from the culture filtrate. To kill the host insect, the fungus conidia got contact first with it and then produced toxins to kill the host, while the culture filtrate directly killed the host insect. El-Ghany (2015) states that entomopathogenic fungus conidia kill its host insect in two stages: parasitic and saprophytic phases. The parasitic phase began with the fungal conidia attaching to the host insect cuticle (Augustyniuk-Kram and Kram 2012). Then, if the humidity was high, the conidia started to germinate on the host cuticle (El-Ghany 2015). The conidia germinating by forming germ tubes continued to grow sticking out to find a soft integument position to facilitate entry into the cuticle (Fernandes et al. 2007). The infection occurs when the germ tubes are able to penetrate insect cuticles and the ability to infect them is a determining factor for the fungus virulence (Altre and Vandenberg 2001). After the germ tubes penetrated the cuticle and reached the haemocoel, and then they produced specific infection hyphae originating at appressoria (El-Ghany 2015). Furthermore, the hyphae spread to the haemolymph and developed to produce blastospores, and the blastospores produced fungal cytotoxic, for example destruxins by *M. anisopliae* which killed the host insects (Mancillas-Paredes et al. 2019), but the death of these host insects was not caused only by the toxin but also due to the mechanical damage by the penetration of fungi into the body of insects (El-Ghany 2015).

After the host insect died, it entered into the saprophytic phase which was influenced by the favorable environmental conditions (Peña-Peña et al. 2015). In the body of the dead insect, the fungus formed mycelia and hyphae which continued to grow covering the body of the host insect, and then the hyphae formed conidiogenous cells and the conidia was produced by utilizing the nutrients/fluids of the host insect and finally the infection process was complete (El-Ghany 2015).

In contrast to the mode of action of fungal conidia, a fungal culture filtrate directly kills the host insect due to the toxins produced by fungal broth so that the time needed to kill the host insect is shorter. The entomopathogenic fungus cultured in liquid media can produce conidia in the form of blastospores (Mascarin et al. 2015) which can produce toxins (Mascarin and Jaronski 2016). In this study, the result of calculating conidia density after being incubated for 6 weeks was generally more than $1x10^9$ conidia mL^{-1}. Fungal conidia and propagules were separated from the filtrate through two stages of filtration to produce toxic fungal culture filtrate. The *B. bassiana* and *M. anisopliae* culture filtrates were able to kill up to 94.67% and 96% of the larvae of *S. litura*, respectively. The short time of death by this culture filtrate resulted from the death of the direct host insect by consuming feed (chili leaves) moistened with the culture filtrate. Soesanto et al. (2019) stated that entomopathogenic fungal culture filtrate contains secondary metabolites. *B. bassiana* culture filtrate containing the secondary metabolites can weaken the host insect's immune system (Zibaee et al. 2011). The secondary metabolites produce toxins, for example destruxins and efrapeptins (Zibaee et al. 2009). *B. bassiana* also produces a protease enzyme that can kill host insects by dissolving their body proteins (Mancillas-Paredes et al. 2019).

Figure 7. The leaf damage: control (A) and larval *Spodoptera litura* applied with *Beauveria bassiana* culture filtrate (B)

Figure 8. The leaf damage: control (A) and larval *Spodoptera litura* applied with *Metarhizium anisopliae* culture filtrate (B)

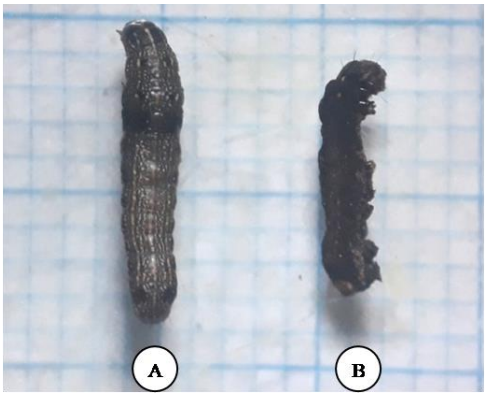

Figure 9. The healthy (A) and dead larvae (B) of *Spodoptera litura* caused by *Beauveria bassiana* culture filtrate

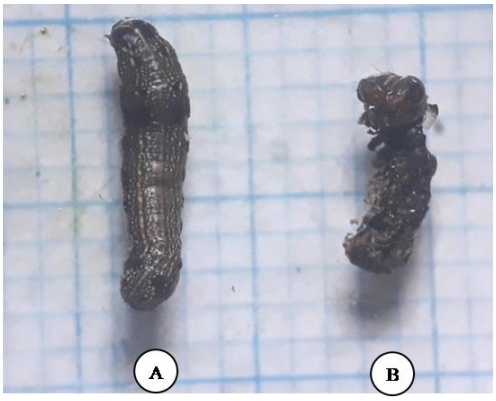

Figure 10. The healthy (A) and dead larvae (B) of *Spodoptera litura* caused by *Metarhizium anisopliae* culture filtrate

The color of fungal broth and culture of *B. bassiana* and *M. anisopliae* varied between isolates. The darker color of fungal broths and culture filtrates tend to cause higher mortality. In line with the results obtained by Ayudya et al. (2019), the darker culture filtrates tended to be more toxic than the light-colored ones. The darker culture filtrates show higher production of secondary metabolites (Luo et al. 2017) and the activity of extracellular enzymes (Khachatourians et al. 2007) and the enzymes produced such as proteases are able to dissolve integument insects that result in death (Mancillas-Paredes et al. 2019).

The symptoms of sick host insects by culture filtrate in this study differed from the deaths by fungal conidia. The insects getting sick by the culture filtrate were not covered by micelia or conidia on the integument. However, they got sick because of the fungal conidia infection which was generally covered in micelia (Sumikarsih et al. 2019). The body shape of the sick *S. litura* larvae due to the culture filtrate was shrunk, wrinkled, dry, and odorless. Similar to the results of the study of Ayudya et al. (2019), the *S litura* larvae treated with *B. bassiana* culture filtrate decreased their body weight and became dried, odorless, and the integument was not overgrown with fungal micelia because in the culture filtrate it no longer contained conidia but contained toxic compounds for host insects. Therefore, the mortality of *S. litura* larvae in this study was caused by the culture filtrate containing toxic compounds. Zibaee et al. (2011) states that toxic compounds in the culture filtrate are secondary metabolites. Zibaee et al. (2009) stated that the secondary metabolites are easier to apply in the field because they can be integrated with other control techniques including synthetic insecticides. Consequently, the culture filtrate which is toxic due to the high content of secondary metabolites has the potential to be further developed for `the active ingredient of mycoinsecticide.

The most toxic isolates of the culture filtrate were BSwTd2 of *B. bassiana* and MKbTp2 of *M. anisopliae* resulting in the mortality of above 90%. The two isolates that were found each came from the lowlands and highlands so that in the future there is an opportunity for the isolates to be developed and applied in low and highland ecosystems. Entomopathogenic fungi that produce toxic filtrate cultures are capable of killing their hosts in a short time, so that they have the potential to be active ingredients in the development of mycoinsecticides technology.

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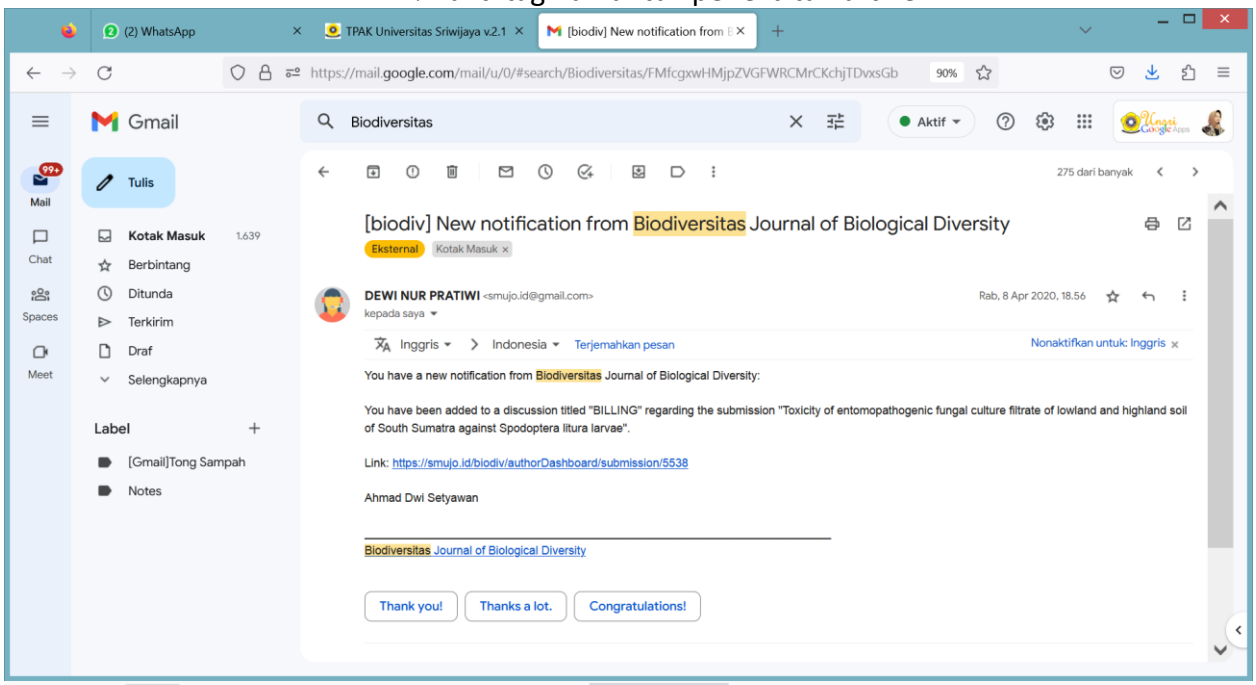

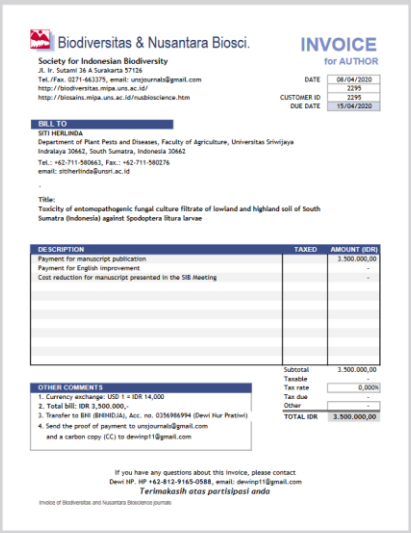

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