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# Selection of Isolates of Entomopathogenic Fungi and the Bioefficacy of Their Liquid Production against *Leptocorisa oratorius* Nymphs

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Entomopathogenic fungi are fungi pathogenic to insects and are widely used as biocontrol agents for insect pests. The aim of this research was to study the virulence of *Beauveria bassiana* and *Metarhizium* sp. isolates and to evaluate the efficacy of liquid production of those fungi against *Leptocorisa oratorius* (rice bug). Twelve isolates of *B. bassiana* and five isolates of *Metarhizium* sp. were used in this research. Selection result of *B. bassiana* isolates on third-instar rice bug nymphs showed that the isolate KBC caused the highest mortality rate (93%), while the lowest (46%) was caused by the isolate BBY 725. The shortest time needed to produce 50% mortality (Lethal time, $LT_{50}$) was 3.52 days (isolate KBC). The longest time (10.36 days) was produced by isolate SLSS. The mortality of rice bug nymphs caused by *Metarhizium* isolates was only 50-62%. The shortest $LT_{50}$ of *Metarhizium* (5.75 days) was produced by isolate Mtm, while the longest (7.46 days) was produced by isolate Mpx. Bioefficacy tests on six kinds of liquid formations of entomopathogenic fungi indicated that all were effective, mostly with $LT_{50}$ d" two days. The mortality rates of rice bug nymphs caused by bioefficacy of fungus liquid production was generally above 85% up to 100%. The liquid media for entomopathogenic fungi performed better compared with solid media (SDA), as indicated by the greater mortality rate and shorter $LT_{50}$.

Key words: Entomopathogenic fungi, isolate, liquid production, *Leptocorisa oratorius*

Entomopathogenic fungi are lethal to insects, and at present these fungi are used as biocontrol agents for insects. Known entomopathogenic fungi, such as *Beauveria*, can kill insect pests of the order Lepidoptera (Soetopo 2004), Coleoptera (Lord 2001; Wraight and Ramos 2002) and Homoptera (Wraight *et al*. 1998), whereas *Metarhizium* is effective in killing insects of the orders Orthoptera (Santiago *et al*. 2001), Diptera (Moraga *et al.* 2006), and Hemiptera (Liu *et al*. 2002). The effectiveness of toxins of either fungus against insects from the order Hemiptera, especially the rice bug (*Leptocorisa oratorius*), has not been reported. *L. oratorius* feeds on developing rice to reduce grain size.

Fungi from both genera have also been developed as granular bioinsecticide formulations (Knudsen *et al*. 1990; Geden and Steinkraus 2003), but the possibilities for developing a liquid bioinsecticide containing *Beauveria* and *Metarhizium* as active agents to kill rice bugs has not yet been investigated. In constructing liquid bioinsecticide, factors that influence the virulence of fungi isolates during the process need to be considered so as to obtain effective products. The virulence of entomopathogenic fungi isolates is affected by a number of factors, such as the medium used in spore germination and production of the bioinsecticides (Alves *et al.* 2002; Geden and Steinkraus 2003; Thompson *et al.* 2007). During the process of bioinsecticide production with pathogenic fungi as the active agents, culture media such as maize, rice, and Sabouraud dextrose-broth (SDB) are believed to modify the effectiveness of bioinsecticide production because of the different chemical compounds they contain. In addition to the cultivation medium, medium used in production is also thought to have an influence on the effectiveness of the bioinsecticide. In this research, the media added to the liquid composition of entomopathogenic fungi were combinations of shrimp shell compost extract (SSCE) and sucrose. The goal of this research was to study the virulence of *B. bassiana* and *Metarhizium* sp. isolates and to evaluate the efficacy of those liquid production on *L. oratorius*.

## MATERIALS AND METHODS

**Isolate Preparation.** Insects infected by *B. bassiana* and *Metarhizium* were collected from various locations in South Sumatera and other provinces in Indonesia. Each fungus was then cultured and sub-cultured to obtain specific isolates (Table 1). The isolation method used for these soil-borne fungi followed the method of Liu *et al.* (2002). *B. bassiana* and *Metarhizium* were collected from infected insects, and then grown on Sabouraud dextrose-agar (SDA) containing 100 ppm streptomycin.

**Isolate Selection.** This was conducted by growing the spores from each isolate on SDA and suspending these at a concentration of $10^6$ spores $ml^{-1}$. The concentration of the spores was measured by the method of Moraga *et al*. (2006). Spores were inoculated topically; each isolate was inoculated on 10 third-instar rice bug nymphs with a dose of 10 ml inoculum per nymph, according to the procedure of Thalib *et al*. (2005), with three replications. The best isolates were then selected based on lowest $LT_{50}$ and highest mortality produced by *B. bassiana* and *Metarhizium.* This gave one

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Table 1 The entomopathogenic fungi isolates collected and used

| Isolate code | Source insect | Insect location |
|---|---|---|
| *Beauveria bassiana* | | |
| TB | *Thrips tabaci* | Bogor |
| Bby 715 | *Hypothenemus hampei* | Jember |
| KBC | *Chrysodeixis chalcites* | Curup (Bengkulu) |
| CPJW8 | *Chrysodeixis chalcites* | Cipanas |
| PD2 | *Chrysodeixis chalcites* | Pagaralam |
| PD1 | *Plutella xylostella* | Pagaralam |
| BTS3 | *Tenebrio molitor* | Palembang |
| BTSS7 | *Tenebrio molitor* | Inderalaya |
| La | *Leptocorisa acuta* | Jember |
| Bby 725 | *Conopomorpha cramerella* | Jember |
| Ua | *Setora nitens* | Jember |
| SLSS | *Spodoptera litura* | Inderalaya |
| *Metarhizium* sp. | | |
| MLa | *Leptocorisa acuta* | Inderalaya |
| Mbl | *Bronstispa longissima* | Manado |
| Mtm | *Tenebrio molitor* | Palembang |
| Mtmt | *Tenebrio molitor* | Inderalaya |
| Mpx | *Plutella xylostella* | Palembang |

best isolate for *B. bassiana* and another for *Metarhizium*. They were then cultivated on maize, rice and SDB media for producing liquid bioinsecticide.

**Spore Cultivation on Maize Media and Production Process.** The best isolates of *B. bassiana* and *Metarhizium* from the previous evaluation were cultivated separately on media of broken maize mixed with 20% (v/v) SSCE and 30% (v/v) sterilized water per 250 g medium. SCCE was prepared according to Suwandi (2004). The mixed medium was sterilized and then inoculated with 10 pieces (each measuring 0.5 x 0.5 cm) of pure fungus culture of the fungi for every 250 g cultivation medium, followed by incubation at room temperature for 10 days.

Each culture of *B. bassiana* (code A) and *Metarhizium* (code D) cultivated on maize media were separately mixed with SCCE solution that had been maintaned at 60°C for two hours. SCCE was poured over the culture to obtain a final spore concentration of $10^9$ spores $ml^{-1}$. This mixture was then crushed in a blender and then filtered using a strainer of 1 mm hole diameter. Each suspension was then added with 30% sugar as preservative to prevent spore germination. This liquid bioinsecticide was then placed in a heat-proof clear glass jar (5 cm diameter and 500 ml volume) that had been sterilized and covered with aluminum foil to be ready for application or storing. From here on, bioinsecticide with *B. bassiana* as the active agent will be referred to as bioinsecticide A, while those with *Metarhizium* as active agent will be referred to as bioinsecticide D. The bioinsecticides were stored a month before testing with storage under ambient condition (23-25°C and 90% humidity).

**Spore Cultivation on Rice Media and Production Process.** The spores of *B. bassiana* and *Metarhizium* were cultivated separately on media of broken rice mixed with 20% SCCE (v/v) and 30% (v/v) sterilized water per 250 g medium as previously described for the maize media.

Each culture of *B. bassiana* (code B) and *Metarhizium* (code E) cultivated on rice media were separately mixed with SCCE solution that had been kept at 60°C for two hours. SCCE was poured into the culture to obtain a spore concentration of $10^9$ spores $ml^{-1}$. The mixture of rice medium, SCCE and fungus was then macerated in a blender, and then filtered using a strainer with 1 mm hole diameter. Each suspension was then combined with 30% (w/v) sugar as spore preservative. This liquid bioinsecticide was placed in clear heat-proof glass jars (5 cm diameter and 500 ml volume) that had been sterilized, covered with aluminum foil to be ready for application or storing. Solution with *B. bassiana* as active agent will be referred to as bioinsecticide B, while solution with *Metarhizium* as the active agent will be referred to as bioinsecticide E. The solution were stored a month before testing with storage under ambient condition (23-25°C and 90% humidity).

**Spore Cultivation on SDB Media and Production Process.** The spores of *B. bassiana* and *Metarhizium* were cultivated separately on SDB media (30 g $l^{-1}$ medium). The medium was inoculated with 10 pieces (each measuring 0.5 x 0.5 cm ) of pure *B. bassiana* culture for every 1.0 liter of cultivating medium. This procedure was repeated for *Metarhizium* sp. Incubation was at room temperature for 10 days using shaking incubator to obtain the optimal number of spores.

Each culture of *B. bassiana* (code C) and *Metarhizium* (code F) grown on SDB media was mixed and blended separately and filtered through a strainer with 1 mm hole diameter. Each suspension was then made to 30% (w/v) sugar as spore preservative. From here on, the mixture of SDB and sugar with *B. bassiana* as active agent will be referred to as bioinsecticide C, while the mixture with *Metarhizium* will be referred to as bioinsecticide F. The bioinsecticide were stored a month before testing with storage under ambient condition (23-25°C and 90% humidity).

**Preparation of Test Insects.** Rice bugs, adults and nymphs were collected from rice fields in various rice production centers, such as Ogan Komering Ulu Timur (OKUT), Musi Banyuasin (MUBA), Ogan Ilir (OI), Ogan Komering Ilir (OKI), and Banyuasin. Nymphs were then transferred to and maintained in the laboratory. They were placed inside a mesh cage (30 x 30 x 100 cm). Inside the cage, some rice plants in the generative state (milky rice grains) were also placed as feed and to provide a surface for egg-laying. Every day, the first instar nymphs produced were moved into a plastic cage (30 x 50 x 50 cm) that contained fresh feed. For the bioefficacy tests, second (F2) or later generations of rice bug nymphs were used.

**Bioefficacy Tests of Bioinsecticide Against Rice Bug.** Liquid solutions with the active ingredient from *B. bassiana* (code A, B, C) , and the solution with the active ingredient from *Metarhizium* (code D, E, F) were tested for their effectiveness by conducting bioefficacy tests at three different concentrations ($10^3$, $10^5$, $10^7$ spores $ml^{-1}$), and a control (sterilized water). Bioefficacy tests were conducted by applying 10 ml bioinsecticide topically to third-instar rice bugs. Each level of concentration was applied to 10 test nymphs with three replications of each. After application of a bioinsecticide, the third-instar nymphs were placed into plastic cylinders (8.5 cm diameter and 15 cm high) covered with muslin and a stem of a rice plant with milky grains was placed inside the cylinder. During the nymph stage, the number of dead nymphs was recorded every three hours, while the number of the nymphs that transformed into imagos was recorded every day until each nymph had transformed.

**Data Analysis.** Mortality data and death time of rice bug nymphs were analyzed using $LT_{50}$, calculated by means of probit analysis employing the program SAS-STAT in SAS 6.12.

## RESULTS

Results of the selection for isolates of *B. bassiana* on third-instar rice bug nymphs showed wide variation in mortality, with a range of 46-93.33% (Table 2). The highest rice bug mortality (93%) was produced by isolate KBC, while the lowest was produced by isolate BBY 725. The mean of ($LT_{50}$) values indicated that the shortest (3.52 days) was produced by isolate KBC, while the longest (10.36 days) was produced by isolate SLSS. Isolate KBC was obtained from *C. chalcites*, isolate BBY 725 from *Conopomorpha cramerella*, and isolate SLSS from *Spodoptera litura*.

The mortality of rice-bug nymphs produced by *Metarhizium* isolates showed different tendencies compared with that of *B. bassiana* isolates. There was little variation in mortality, this ranging only between 50 to 62% (Table 3). The shortest mean $LT_{50}$ values (5.75 days) resulted with isolate Mtm, while the longest value (7.46 days) was associated with isolate Mpx. Isolate Mtm was collected from *T. molitor* while Mpx was collected from *P. xylostella*.

Isolate KBC of *B. bassiana* and isolate Mtm of *Metarhizium* were selected for processing into solutions of entomopathogenic fungus. Results from the bioefficacy testing of six kinds of solutions on rice bug nymphs indicated that all were effective, mostly with $LT_{50}$ values d" 2 days, except for bioinsecticide A at concentration of $10^3$ conidia $ml^{-1}$ and bioinsecticide B at $10^7$ conidia.$ml^{-1}$ (Table 4). $LC_{50}$ values in this research could not be calculated because there was no significant effect of spore concentration on the mortality or $LT_{50}$ for nymphs. The $LT_{50}$ for nymphs with either *B. bassiana* or *Metarhizium* sp. in the liquid bioinsecticides were shorter than values for the same fungi isolates on SDA (Table 2, 3).

In the bioefficacy tests, no bioinsecticide of the fungi cultured on maize media (code A and D), rice media (code B and E), or SDB (code C and F) produced a mortality rate or $LT_{50}$ values better than any other (Table 4, 5). Therefore, those three media are all suitable for the cultivation of

Table 2 Selection results of entomopathogenic fungal isolates (*Beauveria bassiana*) with rice-bug as test insect

| Isolate code | Mortality (%) | $LT_{50}$ (days) | | |
|---|---|---|---|---|
| | | Mean | Lowest | Highest |
| TB | 46.67 | 10.07 | 8.30 | 13.12 |
| Bby 715 | 53.33 | 8.80 | 7.30 | 11.20 |
| KBC | 93.33 | 3.52 | 3.11 | 3.91 |
| CPJW8 | 56.67 | 7.60 | 6.51 | 9.04 |
| PD2 | 46.67 | 9.80 | 7.90 | 13.60 |
| PD1 | 50.00 | 8.90 | 7.40 | 11.20 |
| BTS3 | 65.00 | 6.80 | 4.70 | 11.85 |
| BTSS7 | 50.00 | 9.70 | 6.97 | 11.63 |
| La | 63.33 | 6.20 | 5.30 | 7.20 |
| Bby 725 | 46.00 | 9.96 | 9.18 | 11.04 |
| Ua | 64.00 | 8.05 | 7.23 | 9.11 |
| SLSS | 52.00 | 10.36 | 9.64 | 11.33 |

Table 3 Selection results of entomopathogenic fungus isolates (*Metarhizium*) with rice bug as test insect

| Isolate code | Mortality (%) | $LT_{50}$ (days) | | |
|---|---|---|---|---|
| | | Mean | Lowest | Highest |
| MLa | 62.00 | 7.05 | 6.29 | 8.00 |
| Mbl | 60.00 | 6.96 | 6.28 | 7.78 |
| Mtm | 62.00 | 5.75 | 5.06 | 6.56 |
| Mtmt | 54.00 | 6.93 | 5.82 | 5.60 |
| Mpx | 50.00 | 7.46 | 6.38 | 9.20 |

Table 4 Bioefficacy test results of liquid bioinsecticide (with active ingredient *Beauveria bassiana*) on rice bugs at three different concentrations

| Bioinsecticide source | Spore concentration (spores $ml^{-1}$) | Mean mortality (%) | Mean $LT_{50}$ (days) | 95% confidence level (days) | |
|---|---|---|---|---|---|
| | | | | Lowest | Highest |
| A | 1 x $10^3$ | 100.00 | 2.68 | 2.31 | 3.09 |
| | 1 x $10^5$ | 100.00 | 1.69 | 1.44 | 1.94 |
| | 1 x $10^7$ | 96.67 | 1.14 | 0.92 | 1.35 |
| B | 1 x $10^3$ | 90.00 | 1.75 | 1.47 | 2.02 |
| | 1 x $10^5$ | 100.00 | 1.41 | 1.18 | 1.62 |
| | 1 x $10^7$ | 86.67 | 2.04 | 1.73 | 2.35 |
| C | 1 x $10^3$ | 90.00 | 1.62 | 1.25 | 1.99 |
| | 1 x $10^5$ | 90.00 | 1.52 | 1.24 | 1.79 |
| | 1 x $10^7$ | 80.00 | 1.44 | 1.14 | 1.71 |
| Control | 0 | 0 | - | - | - |

Table 5 Bioefficacy test results of liquid bioinsecticide (with active ingredient *Metarizium*) on rice bugs at three different concentrations

| Bioinsecticide source | Spore concentration (spores $ml^{-1}$) | Mean mortality (%) | Mean $LT_{50}$ (days) | 95% confidence level (days) | |
|---|---|---|---|---|---|
| | | | | Lowest | Highest |
| D | 1 x $10^3$ | 90.00 | 1.41 | 1.13 | 1.67 |
| | 1 x $10^5$ | 90.00 | 1.39 | 1.12 | 1.64 |
| | 1 x $10^7$ | 100.00 | 1.60 | 1.30 | 1.89 |
| E | 1 x $10^3$ | 90.00 | 1.31 | 1.03 | 1.57 |
| | 1 x $10^5$ | 90.00 | 1.26 | 0.98 | 1.53 |
| | 1 x $10^7$ | 93.33 | 1.38 | 1.10 | 1.65 |
| F | 1 x $10^3$ | 86.67 | 1.46 | 1.17 | 1.73 |
| | 1 x $10^5$ | 90.00 | 1.58 | 1.30 | 1.85 |
| | 1 x $10^7$ | 90.00 | 1.30 | 1.02 | 1.56 |
| Control | 0 | | - | - | - |

entomopathogenic fungi. Mortality rates of rice-bug nymphs caused by liquid bioinsecticides were also higher compared to those of isolates grown on SDA. Mortality rates of rice-bug nymphs resulting from the fungal-derived suspension was mostly above 85%, with the highest at 100% (Table 4, 5). As shown by the $LT_{50}$ values, mortality rates of rice bug nymphs resulting from the fungal-derived suspension was not affected by spore concentration treatment.

## DISCUSSION

Results of *B. bassiana* isolates on rice-bug nymphs showed that isolate KBC (from *C. chalcites*) produced the best bioinsecticide, while of the isolates from *Metarhizium* sp. Mtm was the best. Therefore, isolates KBC and Mtm were selected as active agents for the liquid bioinsecticide preparations of entomopathogenic fungi. Those two isolates were selected because they had the highest capabilities and were the quickest to kill rice bug nymphs, i.e. the shortest $LT_{50}$.

The ability of those entomopathogenic fungal isolates to produce the highest mortality rates and lowest $LT_{50}$ values might be caused either by their genetic characteristics, or by their the viability of the spores. According to Soetopo (2004), a high viability of fungal spores tended to cause a high mortality rate on host insects, but it was not the main cause. The main cause here was genetic characteristics of the strains.

*B. bassiana* and *Metarhizium* sp. isolates needed 3.83 days and 3.52 days, respectively, as the shortest time to kill their insect hosts. The time needed was relatively long because the spores attached to the integuments had to germinate first. Fuxa and Richter (2004) stated that hyphae from *Metarhizium* sp. spores entered the host's body with the help of enzymes or mechanical pressure. In the end, the host was covered all over with propagules and the soft parts of the body were penetrated so hyphal growth could be observed outside the host insect's body. External hyphal growth would produce conidia which would be spread spores into the environment upon reaching maturity. These then infect other healthy insects.

In this research, host insects infected by *B. bassiana* showed symptoms such as loss of appetite, slow movement and finally died. After death, white colored fungal hyphae appeared from their stiff and dry bodies. Dead insect hosts infected by *Metarhizium* sp. showed the same symptoms as those infected by *B. bassiana*, except for the color of the hyphae, which was greenish white.

During the inoculation process of fungal spores, the humidity under the cage cover was kept above 90% and room temperature was adjusted to 23-25°C. This was to prevent poor spore germination. Bidochka *et al*. (2000) stated that the optimum temperature needed for entomopathogenic fungal spores to germinate was 22-27°C with optimum humidity above 90%, and that the greater the humidity the more virulent the fungi would be. At under 86% humidity, the virulence would decrease continously.

In bioefficacy tests of the liquid solution from entomopathogenic fungi, the mortality of the insect host was greater and occurred faster when the fungus was in liquid state compared to isolates grown on solid media (SDA). In other words, the liquid state of the fungus was able to increase their effectiveness. Akbar *et al*. (2005) reported that entomopathogenic fungi in liquid fungus tended to have higher viability compared to those on solid media. Consequently, they are also more virulent.

Three kinds of media (maize + SSCE, rice + SSCE and SDB) were used in the processing of liquid bioinsecticide from entomopathogenic fungi were better for fungal culturing than were the solid media used for culturing the same isolates. The liquid fungus caused higher mortality and faster killing rate compared to SDA. The better the media for fungi culturing were, the more mycotoxin would be produced by entomopathogenic fungi (Klinger *et al*. 2006). According to Akbar *et al.* (2005) entomopathogenic fungi grown on liquid media produced mycotoxins and spores with greater viability and virulence compared with those established on solid media. In short, liquid processing of entomopathogenic fungi kill through two processes, firstly by enhanced growth of fungal spores, and secondly by the mycotoxin contained in the resulting suspension resulted in greater mortality (shorter $LT_{50}$).

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