Growth of entomopathogenic fungi colonies *Metarhizium anisopliae* (Metchnikoff) Sorokin enriched with termite juice

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**Abstract.** *Coptotermes curvignathus* are termites classified as pest organisms or destructive organisms, because these termites are often found to attack homes, buildings, and plantations. Entomopathogenic fungi of *Metarhizium anisopliae* is an alternative way to control the termite in environmentally friendly approach. This study aimed to determine the combination media of termite juice concentration for the growth of *Metarhizium anisopliae*. The fungi were inoculated on growth media (Potato Dextrose Agar) supplemented with termite juice using various concentration of 0% (control), 50%, 75% and 100%. This study showed that fungi grown on media supplemented with 50% and 75% of termite juice yielded 2.9 x 10\(^7\) spore/mL density. This result was not significantly different from the media supplemented with 25% and 75% of termite juice yielding 2.4 x 10\(^7\) and 2.7 x 10\(^7\) spore/mL density, respectively. However, this result was significantly different compared to the control treatment and media supplemented with 100% of termite juice yielding spore density of 1.1 x 10\(^7\) and 1.4 x 10\(^7\), respectively. The growth media supplemented with termite juice is considered to have more nutritional composition to stimulate spore formation promising its application for promoting media of entomopathogenic fungi.

1. Introduction

*Metarhizium anisopliae* are entomopathogenic fungi infecting more than 200 types of insects from various types of orders [1] including lepidoptera, coleoptera, homoptera, hemiptera and isoptera [2]. *M. anisopliae* produce toxins, called dextruxin [3] or desmethyl dextruxin, cyclopeptide [4] assisting the fungi to penetrate the insect host’s body. In addition, *M. anisopliae* also produce a number of enzymes, such as chitinase, lipase and protease enzymes [5].

*M. anisopliae* often used as an entomopathogenic agent offer some advantages. The fungi produce high number of spores within a short life cycle that are durable even in unfavorable conditions and they are relatively easy to culture in the laboratory [6]. However, the application of entomopathogenic fungi might also have a disadvantage in decreasing virulence due to mass culture in natural growth media. This decrease in virulence could be related to the origin of the isolate and its growth media [7].
The growth media for fungi have to contain nutrient requirements including glucose, glucosamine, chitin, flour, and nitrogen [8]. The growth of fungal sporulation and hyphae is strongly influenced by nitrogen and carbon sources in the media [7]. The addition of compounds such as chitin to the growth medium can also promote the growth of fungal colonies. Chitin is a linear polymer compound of N-acetyl-glucosamine with sub-units linked by β-(1,4) -glucoside bonds. Chitin can be found in crustacean shells, fungal cell walls and insect exoskeletons [9].

The application of chitin in media has been widely used previously, such as the use of chitin from shrimp shells [8], Gryllus assimilis, Scylla serrata and Perna viridis [9]. Termites can also be used as a source of supplementary nutrition for fungal growth media as the termite exoskeletons also contain chitin. Moreover, termites also contain other ingredients such as carbohydrates, protein and fat. Based on a proximate analysis of the subterranean termite Coptotermes curvignathus Holmgren from Irian Jaya, as they contain a crude protein content (48.77%), fat (2.13%), crude fiber (17.48%), calcium (1.30%) phosphorus (0, 64%) and metabolic energy (3806 Kcal) [10].

Therefore, this study will use termites as growth media supplement due to nutritional content of termite including chitin for promoting the fungal growth. The purpose of this study was to determine the influence of termite juice concentration supplemented in fungal growth media (potato dextrose agar) on the fungal spore density as well as morphological features of Metarhizium anisopliae.

2. Methods
2.1. Experimental design
This study used a completely randomized design using five treatments, namely control [potato dextrose agar (PDA) only] and treatments consisting of fungal growth media supplemented with 25% [75% PDA media + 25% termite juice + agar], 50% [50% PDA media + 50% termite juice + agar], 75% [PDA media 25% + termite juice 75% + gelatin], and 100% of termite juice [100% termite juice + gelatin]. The termite juice was prepared by blending termite of Coptotermes curvignathus that had been previously collected from oil palm plantations, rotten wood and other places. All media were sterilized using an autoclave at a temperature of 121 °C for 15-30 minutes.

2.2. Propagation of Metarhizium anisopliae in termite juice media
M. anisopliae were inoculated onto the media using a loop needle and placed in the centre of the media. The inoculated media were then incubated for 14 days at room temperature and the colony morphology was observed.

2.3. Spore Density of Metarhizium anisopliae calculation
The spore density of M. anisopliae was calculated using a haemocytometer. A 100 µl of M. anisopliae suspension of was dropped on a haemocytometer then covered with a cover glass and observed using a stereo optical microscope with a magnification of 400x. The spore density was calculated using the following formula [11]:

\[
C = \frac{t}{(n \times 0.25)} \times 10^6
\]

with C as spore density per ml of suspension; t as the total number of spores in the sample box observed; n as number of sample boxes (5 large boxes x 16 small boxes); and 0.25 as correction factor using a small-scale sample box on the haemocytometer.

3. Results and discussion
The spore density of M. anisopliae grown on control media and media supplemented with termite juice are shown in Table 1. The results indicate that M. anisopliae showed the highest spore density (2.9 x 10^7 spores/mL) when the fungi were grown on media supplemented with 50% of termite juice. The following highest spore density observed in the fungi grown on media supplemented with 75%, 25% of termite juice reaching 2.7 x 10^7 spores / mL, 2.4 x 10^7 spores / mL, respectively. The fungal growth media supplemented with 50% of termite juice showed better spore results might be the optimal a source
of nutrition to stimulate spore formation. In the sporulation process, nutritional factors affect the formation of spores including carbon, nitrogen and other microelements. Some fungi even require specific carbon and nitrogen sources for the sporulation process. Apart from nutritional factors, environmental conditions also affect the spore formation process, such as light, humidity, temperature, and UV radiation [12]. The combination of carbon and nitrogen is essential for spore production and for M. anisopliae it requires a combination of carbon and nitrogen in a ratio of 10:1 (0.6% glucose and 1% peptone) [13].

Based on its effect on entomopathogenic fungi, the treatment of media supplemented with a concentration of 50% termite juice was not different from other treatments of 25% and 75% of termite juice. This suggests that PDA media combined with termite juice provides a suitable source of nutrients for spore growth and development. The composition of PDA media which is rich in glucose and termite juice which contains compounds such as carbohydrates, protein, fat, fiber, and chitin. The presence of sufficient nutrient sources in the growth media leads to increase spore production and the virulence power of entomopathogenic fungi. The presence of protein, chitin, lipid and wax compounds found in the cuticles of insects’ bodies promote entomopathogenic fungi to produce enzymes assisting the entomopathogenic fungi to invade the insect's body more quickly [14]. The carbon source will accelerate the formation of spores, while the nitrogen source is used for the formation of advanced hyphae [15]. The virulence of entomopathogenic fungi might be increased during the growth supplemented with termite juice as chitin source as the fungi adapt to these insects by increasing the activity of entomopathogenic fungi enzymes [16].

Table 1. Spore density of M. anisopliae.

| Treatments | Spore Density (spore/mL) |
|------------|--------------------------|
| Control    | 1.1 x 10⁷ᵃ                |
| 25%        | 2.4 x 10⁷ᵃᵇᶜ             |
| 50%        | 2.9 x 10⁷ᶜ                 |
| 75%        | 2.7 x 10⁷ᵇᶜ               |
| 100%       | 1.4 x 10⁷ᵃᵇ                |

The values followed by the same superscript are not significantly different at α=0.05

The fungal colonies grown in control and treatment media showed differently. The fungal growth media supplemented with 50% of termite juice showed extensive growth compared to other treatments in day-1 (Figure 1) and day 14 (Figure 2). It is assumed that the composition of the energy source in this medium is suitable for the growth of fungal colonies, so that the number of colonies present is more than the others as PDA supplemented with 50% of termite juice contain additional sources of energy such as fat, protein, fiber and chitin which help the growth of hyphae and spores faster. The protein contained in the growth media plays a role in spore formation and allow the fungal cells to synthesis enzymes that play an active role in infecting insect integument [17]. Peptone, potassium nitrate and urea added to growth media are excellent nitrogen sources for the growth of entomopathogenic fungi despite the fact that peptone compounds promote better conidia growth than others [18]. Thus, the provision of carbon and nitrogen sources to the growth medium significantly affects the growth and development of entomopathogenic fungi. The sporulation process is strongly influenced by the growth substrate, with the addition of various carbon sources, chitin and fatty acids, including termite juice might increase the number of spores [19]. Figure 2 shows that the colour of the spores turned green as mostly pigments contained in entomopathogenic fungi composed of melanin and carotenoids influenced by the light received by the fungus during growth and commonly formed to defend fungal cells from unfavourable conditions [20].

Figure 3 shows the different color of the spores a suspension in liquid media. The fungal pigmentation of hyphae and fungal spores might be influenced by the source of nutrients available. Polysaccharide compounds, such as cellulose and hemicellulose help fungi form color pigments in their
spores in addition to external factors such as humidity, temperature, pH, and oxygen also influence the formation of fungal spore pigments [21].

**Figure 1.** Growth characteristics of *Metarhizium anisopliae* fungal colony on the first day (a) control; (b) concentration of 25%, (c) concentration of 50%, (d) concentration of 75%, and (e) concentration 100%.

**Figure 2.** Growth characteristics of *Metarhizium anisopliae* fungal colony on the first day (a) control; (b) concentration of 25%, (c) concentration of 50%, (d) concentration of 75%, and (e) concentration 100%.

**Figure 3.** The difference in the color of the spores produced by *Metarhizium anisopliae* in liquid media supplemented with (a) 100% termite juice; (b) 75% of termite juice; (c) 50% of termite juice; (d) 25% of termite juice; and (e) control.
4. Conclusion

It is concluded the optimal growth media for entomopathogenic fungi was PDA supplemented with 50% of termite juice despite the fact that it did not significantly differ from 25% and 75% of termite juice. The growth media supplemented with termite juice is considered to have more nutritional composition to stimulate spore formation promising its application for promoting media of entomopathogenic fungi.

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Acknowledgment

We thanked the Termite Research Group of Syiah Kuala University for assistance in the field and laboratory. This work was partly supported by funds from Thesis Master 2020 Research Grant of DRPM RISTEKDIKTI.