Growth and viability of entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin in different alternative media

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**Abstract.** At present, the use of synthetic chemical pesticides become a threat to the biotic and abiotic environment. The use biological agents such as entomopathogen may become an alternative in supporting integrated pest management. One of entomopathogenic fungus that has been used in controlling pests is *Beauveria bassiana*. However, a good alternative media for the growth of *B. bassiana* is required to produce more optimal conidia density and germination. This study was aimed to determine the effect of several media on the growth of *B. bassiana* and its conidial density. The method used in this study was completely randomized design with 5 replications. The experimental treatment consisted of PDA, maize, rice, and mungbean. The result showed that there was no significant differences among maize, mungbean, and PDA media in supporting the growth of *B. bassiana*, with the means of colonies diameter at 8.91 cm, 8.89 cm, and 9.00 cm, respectively. Moreover, the growth rate, conidia density, and viability of the alternative media were not significantly different with PDA. Therefore, further research to determine other alternative media for the growth and viability of *B. bassiana* is needed.

1. **Introduction**

The concept of integrated pest management (IPM) is recommended by the Indonesian Government to regulate pest control method [1]. Biological control is the use of natural enemies (parasitoid, predator, pathogen) in controlling the pests. One of biological agents that widely used is entomopathogenic fungus.

Entomopathogenic fungus become a prospective biological agents due to its high reproductive capacity, short life cycle, and its ability to form resting spores in unfavorable conditions [2, 4]. In addition, entomopathogenic fungi can easily infect the target and have many strains [3]. One of the entomopathogenic fungus that commonly used in biological control is *Beauveria bassiana*, a fungus belongs to Ascomycota phylum. This fungus is pathogenic to various types of insects which have wide host range.

The artificial media (substrate) commonly used for mass propagation of *B. bassiana* is potato dextrose agar (PDA) which is suitable and supports the growth of fungi. For more applicable used of *B. bassiana* by the farmers, alternative media that can support the growth of *B. bassiana* is needed. The growth media should provide source of nutrient [5], support the germination, growth, and sporulation [6], and ideal for mass propagation [7]. In addition, macronutrients such as carbon, nitrogen, oxygen, sulfur, and phosphate should be available in the media since they are the main
components of nutrients needed by fungi. Mycelium growth and spore production in artificial media depended on the character of isolates and nutrient content in the media [8]. Therefore, the nutrient content of media greatly determines the growth rate and virulence of fungi [9, 10]. Rice, mungbean, and maize have a high nutrient content for the growth of entomopathogenic fungi and they have been reported as a good alternative media for multiplication of *B. bassiana* [11]. This study was aimed to obtain alternative media that support the growth and viability of *B. bassiana*.

2. Methods

The experiment was conducted in complete randomized design (CRD), with 4 type of media as the treatments and 5 replications for each treatment. The treatments consisted of PDA, maize, rice, and mungbean. The observed variables were colony diameter and growth rate colonies; conidia density and germination of *B. bassiana*.

2.1. Preparation of alternative media

Rice, maize and mungbean were washed and steamed for 25 min, then dried. Dried rice, maize, and mungbean were put in plastic bags of 30 g each/bag and then sterilized in autoclave. Inoculum of *B. bassiana* was then inoculated to sterilized rice, maize, and mungbean. Inoculation was carried out in laminar air flow (LAF) to avoid contamination. Each bag containing *B. bassiana* that has been isolated was labeled according to the propagation time and media, then placed on a shelf. The culture will be incubated in an incubator for 21 d at 20-23 °C. As check control, PDA medium was used.

2.2. Colony diameter of *B. bassiana*

Colony diameter measurements were carried out every d until the media is 21-d-old. The diameter was measured using a ruler by taking 4 points followed by calculating the average diameter.

\[
\text{Radial diameter} = \frac{\phi W + \phi X + \phi Y + \phi Z}{4}
\]

\(\phi W = \text{Axis Diameter } W\)
\(\phi X = \text{Axis Diameter } X\)
\(\phi Y = \text{Axis Diameter } Y\)
\(\phi Z = \text{Axis Diameter } Z\)

2.3. Colony growth rate of *B. bassiana*

Colony growth rate was measured every day within 21 d. The growth rate was measured in units of mm/d. The method for this calculation is the slope of the line in the distribution of the diameter with the time of observation.

\[\text{The direction of the radial colony} = \frac{L2-L1}{t2-t1}\]

\(L1 = \text{Area of diameter } 1\)
\(L2 = \text{Area of diameter } 2\)
\(t1 = D1\)
\(t2 = D2\)

2.4. Calculation of conidia density and germination rate

After the fungus were 21-d-old, conidia density and germination were observed using a haemocytometer. A conidia suspension was taken as much as 0.2 mL using a type pipette at a dilution of 10\(^1\), a conidia suspension is slowly dropped on the haemocytometer canal. Conidia density contained in the haemocytometer was calculated after 10 hr-incubation, with a magnification of 400x. The measurement was taken twice in each observed field followed by calculating the percentage of germination.
2.5. Data analysis

Data was analyzed using F test at the level of 5%. If the results of the F test are significantly different, a further test with Duncan Multiple Range Test / DMRT was carried out at the level of 5% to determine the highest growth [12].

3. Results and discussion

3.1. The colonies diameter of B. bassiana

The diameter of B. bassiana colonies grew continously day by day. This is relevant with the common phenomenon saying that 1 parameter of growth is an irreversible increase in cell volume, meaning that it cannot return to its original form [13]. The more conidia germinate, the faster the fungi grow [14]. The ability of fungi to form conidia plays major role for fungus dispersion and infection [15]. Overall, the colony diameter has a significant value among the media on 3, 9, 12, 18, 21 d after inoculation and not significantly different at 6 d after inoculation (Table 1).

| Media      | Colonies diameter (mm/d) on d-n^a |
|------------|-----------------------------------|
|            | 3  | 6  | 9  | 12 | 15  | 18  | 21  |
| PDA        | 0.91a | 3.00a | 4.01ab | 5.93ab | 8.62a | 8.88a | 9.00a |
| Maize      | 0.99a | 3.00a | 4.94b  | 6.28a  | 8.19a  | 8.61a  | 8.91a  |
| Rice       | 0.00b | 3.00a | 3.67b  | 4.98b  | 6.85b  | 7.58b  | 8.00b  |
| Mungbean   | 0.00b | 3.00a | 5.14a  | 6.26a  | 8.27a  | 8.69a  | 8.89a  |

^a The values with the same letter are significant difference in the Duncan Multiple Range Test (DMRT) at α=5%.

The diameter of colonies in PDA media increased higher at the age of 15, 18 and 21 d after inoculation compared to other media. The colonies diameter of B. bassiana in 21 d were 8.91 and 8.89 on maize and mungbean, respectively. These values are not significantly different with the colonies growth on PDA. This data indicates that maize and mungbean are favorable for B. bassiana growth because they have suitable nutrients needed by B. bassiana for its bioenergy [13]. Previous research reported that cereals such as maize can be used as substrates because they contain nutrients needed by the fungus for their growth so that they can colonize the substrate easily [16]. However, the difference of colonies characteristic were affected by the different media used. A medium with wide enough surface is needed to produce the maximum number of conidia. Media material which tends to clot has a narrow surface area, so the conidia production is lower. The amount of conidia produced in each substrate is related to the amount of nutrients contained in the medium. Some alternative media have different compositions that show different growth and virulence.
Figure 1. Growth of *B. bassiana* colonies diameter in 4 types of media

Regression analysis was performed based on colony diameter data up to 21 d after inoculation (Fig. 1). The regression equation of the *B. bassiana* colony diameter on PDA media was $y = 1.4525x + 0.0471$, indicated that if the diameter of *B. bassiana* colony increases by 10$^1$, the growth rate will increase by 1.45%. The regression equation of the *B. bassiana* colony diameter on maize was $y = 1.36x + 0.38$, it means that if the diameter of *B. bassiana* colony increases by 10$^1$, the growth rate will increase by 1.36%. The regression equation of the *B. bassiana* colony diameter on rice was $y = 1.30x + 0.32$, it means that if the diameter of *B. bassiana* colony increases by 10$^1$, the growth rate will increase by 1.30%. The regression equation of the *B. bassiana* colony diameter on mungbean was $y = 1.47x + 0.13$, it means that if the diameter of *B. bassiana* colony increases by 10$^1$, the growth rate will increase by 1.47%.

3.2. Growth rate of *B. bassiana*

Each entomopathogenic fungus has different growth and development rate according to the nutrition and environment that the fungus needs. The media used to grow entomopathogenic fungi greatly determines the rate of colony growth and the number of conidia produced during growth [17].

Table 2. Average growth rate of *B. bassiana* in several alternative growing media.

| Media        | Growth rate (cm/d)$^a$ |
|--------------|------------------------|
| PDA          | 0.41a                  |
| Maize        | 0.35b                  |
| Rice         | 0.27c                  |
| Mung beans   | 0.38ab                 |

$^a$ The values with the same letter are not significantly different by Duncan Multiple Range Test (DMRT) at $\alpha=5\%$. 
The type of media is significantly affecting the growth rate of *B. bassiana*. Mungbean media with a growth rate of 0.38 cm/d did not cause different growth rate of the fungus compared to PDA (Table 2). Mungbean has a soft texture that matches the growth conditions of the fungus *B. bassiana*. Although maize and rice have the same steamed treatment with mungbean, the texture of maize and rice remains harder.

Optimal growth of *B. bassiana* was achieved faster in the mungbean and maize than in rice. Mungbean has mineral content needed by fungi to increase the growth and virulence [18]. The difference in the growth rate of *B. bassiana* in various media is thought to be due to differences in nutrition and oxygen content in the growth chamber. Fungi are aerobic which requires oxygen for its growth [19]. The growth of hyphae in the media determines the speed of growth and development of fungi in growing media. The growth and development of fungi showed a proportional increase to the length of incubation time until it showed a stationary point of growth [20].

### 3.3. **Conidia density of** *B. bassiana*

The effectiveness of entomopathogenic fungi for controlling target pests depends on the type of isolate, conidia density and age of pest stages [21]. High and low density of fungus conidia were also influenced not only by genetic factors but also by external factors such as temperature, pH, and the length of the incubation period [22]. Pathogenic fungi tends to have higher conidial density and viability [23].

The effectiveness of entomopathogenic fungi to control target pests depends on the age of the insect, developmental stage, cuticle surface, and conidial density [24]. Three conidia concentrations of *B. bassiana* (1.10 x 10⁸, 3.36 x 10⁷, dan 1.68 x 10⁷) which were applied directly to the body of *Helopeltis antonii* in the laboratory, showing a mortality rate of 94-98 % at 6 d after application [25]. Infection with *B. bassiana* fungi will increase with increasing dose [25].

**Table 3.** Average conidia density of *B. bassiana* in several alternative growing media.

| Media  | Conidia density (conidia/mL)¹  |
|--------|-------------------------------|
| PDA    | 1.39x10⁸ a                    |
| Maize  | 1.06x10⁸ ab                   |
| Rice   | 4.49x10⁷ c                    |
| Mungbean | 6.75x10⁷ bc                |

¹The values with the same letter are not significantly different by Duncan Multiple Range Test (DMRT) α =5%.

The type of media significantly affected the conidia density at 21 d after inoculation. Conidia density on maize media (1.06x10⁸ conidia/mL) was not significantly different with those on PDA media (1.39x10⁸ conidia/mL). The lowest conidia density was on rice media, i.e. 4.49x10⁷. This is in line with previous research in which conidia density of *B. bassiana* on maize media reached 2.7 x 10¹⁰ conidia/mL.[26].

Fat is one of the factors that influences the increase in the number of conidia and % germination [17]. Conidia germination is influenced by humidity, temperature, light, and nutrition. At sufficiently high humidity, the conidia will germinate and form appressoria, then the appressorium penetrates the cuticle by removing cuticle degrading enzymes, such as lipase, protease, and chitinase [27]. In general, the more resistance insects to pathogen infections, the higher concentration of conidia is needed [28]. *Metarhizium anisopliae* fungi were able to cause 50% *Leptidiota stigma* mortality at a density of 1.03 x 10⁸ conidia/mL [29].
Germinated conidia of *B. bassiana* will look like a string and at one time it started to branch out (Fig. 2). The branches that arise will always grow away from the main hyphae or the 1st hyphae. These branches will touch each other. The more conidia germinate, the faster growth of the fungus [30].

![Figure 2](image1.png)

**Figure 2.** (a) The growth of *B. bassiana* colonies on PDA media (a); and its mycelia at 400x magnification (b); conidia (1) and hyphae (2).

3.4. Germination of *B. bassiana* in various media

Germination is the first step of entomopathogenic fungus to infect the host. One of the factors that determine the level of virulence is germination; the higher germination rate, the higher fungal infection to pests [31].

| Media   | Germination rate (%)<sup>a</sup> |
|---------|---------------------------------|
| PDA     | 72.83 a                         |
| Maize   | 68.37 ab                        |
| Rice    | 58.70 c                         |
| Mungbean| 65.76 b                         |

<sup>a</sup>Note: The values with the same letter are not significantly different by Duncan Multiple Range Test (DMRT) α =5%.

The type of media had a significant effect on the germination capacity of *B. bassiana* at 21 d after inoculation. The conidia of *B. bassiana* and its germination stage is shown in Fig 2. The germination capacity of maize media (68.37%) was not significantly different from that of PDA media (72.83%). An optimal entomopathogenic germination rate was more than 80% [32]. Differences in conidia viability can be caused by culture media, temperature and humidity [33]. Maize and mungbean contain nutrients that are suitable for the growth and development of *B. bassiana*. Maize contains a lot of protein and carbohydrates, which is needed by fungus for vegetative growth and spore formation. Research showed that maize containing 1% sugar substrate can increase conidia viability to 96.1% [34]. The percentage of spore germination in maize and rice substrate was > 95% and 70–79%, respectively; while the conidial number was 1.7 x 10<sup>7</sup> conidia/mL and 5.02 x 10<sup>6</sup> conidia/mL with harvesting time at 11 d [35]. Lack of protein intake from media can reduce the ability of spores to germinate so that spore viability decreases. Spore viability is strongly influenced by spore density and food nutrients available in the media. However, a high amount of protein does not guarantee the ability
of spores to germinate. The suitability of the composition between proteins, carbohydrates, starches, glucose also determines the spores to grow.

Previous research reported that growth rate of B. bassiana conidia reached 95%-100% if sufficient protein is available for germination [33]. Conidia is considered viable if the sprout tube has reached twice the diameter of the conidia [36]. The sprout tubes will develop to form an aporium which functions to attach the infective organ to the host surface. The faster the sprout tube is formed, the larger its size were suspected and the greater chance that the host can be penetrated. It was because the surface of the host is hydrolyzed faster by the fungus [34].

Figure 3. The conidia of B. bassiana (b); and the emergence of germination tubes at 10 hr after incubation (b).

4. Conclusions
There is a significant effect of growth media on the diameter and growth rate of B. bassiana colonies; density and germination capacity of the conidia od B. bassiana. B. bassiana grew well in maize and mungbean media.

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