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MORTALITY AND TISSUE DAMAGE OF ORYCTES RHINOCEROS LARVAE INFECTED BY *Metarhizium anisopliae*

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ABSTRACT

Oryctes rhinoceros is one of the major pest of palm oil in Indonesia. The entomopathogenic fungus, *Metarhizium anisopliae* is a potential biocontrol agent of *O. rhinoceros* larvae. This study aimed to investigate the mortality and tissue damage of *O. rhinoceros* larvae infected by *M. anisopliae* using four different dosages. Forty eight larvae were used in this study. The larval mortality was observed for 12 days. Four samples of infected larvae with different levels of tissue damage were taken and made into a microscopic object. The result showed that various dosages of *M. anisopliae* resulting in different periods of larval mortality. The black spot in the integument of *O. rhinoceros* larvae was an early symptom of *M. anisopliae* infection. Eventually, the mycelia spread out from the larval body to the integument surface and form green conidia which searching for a new host. *M. anisopliae* cause *O. rhinoceros* larvae mortality occurred on 2^{nd} – 12^{th} day (P3), while P1 and P2 treatment took more than 12 days to die. The beneficial dose was P3 (4 g *M. anisopliae* + 100 g manure). The infection of *M. anisopliae* in *O. rhinoceros* larvae was characterized by brown spots (melanization); mummify symptoms (mummification), the appearance of white mycelium (mycoses), and dark green conidia in the final stage.

Keyword: *oryctes rhinoceros,* infection mechanism of *metarhizium anisopliae,* biological pest control, *metarhizium anisopliae,* palm oil.

INTRODUCTION

Oryctes rhinoceros (Coleoptera: Scarabaeidae) is one of the major pest that attacks palm oil in Indonesia [1]. This pest also attacks palm oil in other countries of the world [2]. The attacks of *O. rhinoceros* resulting in decreased production of oil in Indonesia. *O. rhinoceros* destroy plants by eating the palm oil's young leaves and cause damage to the tissues of growing palm oil [3, 4]. One of the symptoms of *O. rhinoceros* infection in palm oil is cutouts leaf shaped like the letter "V" [5].

O. rhinoceros larvae live in the soil containing organic material or the palm trunk that has been weathered. Larvae control can be performed by biological agents such as fungal parasite of insects. One of biological control techniques of *O. rhinoceros* is by using *Metarhizium anisopliae* [6, 7].

M. anisopliae is one of entomopathogenic fungus that can infect 200 insect species from a various different plants [8]. *M. anisopliae* are widely used to control insect pest because they have high conidial production, relatively short life cycle and resistant to environmental influences [9].

Laboratory studies showed that *M. anisopliae* effectively controls some insect pests, for example, *Spodopteralitura* [10]. This insect has thin cuticle and small larvae. *Coptotermes curvignathus* [11], *Stibaropus molginus* [9], *Lepidiota stigma* [12] and *O. rhinoceros* larvae [13, 14]. The results of these studies are evidence of *M. anisopliae*'s pathogenicity.

Histopathological study of tissue damage due to attack from *M. anisopliae* has conducted on *Anastrepha fraterculus* larvae [15]. However, it has never been done on *O. rhinoceros* larvae. The objective of this study was to investigate the tissue damage of *O. rhinoceros* larvae caused by *M. anisopliae* infection. The results of the study are useful to analyze the growing mechanisms of fungus as biological agents in controlling pests.

MATERIALS AND METHODS

This research was conducted at Biology Laboratory, Universitas Negeri Semarang, Indonesia and Diagnostic Alert Laboratory Semarang, Indonesia from December 2015 to April 2016.

O. rhinoceros Larvae

O. rhinoceros larvae were obtained from palm oil plantation in Jepara, Central Java, Indonesia. The larvae used were 3^{rd} healthy instar larvae, weight of 6.8-7.8 g and 5-8 cm length. Healthy larvae were detected by observing their behavior that always actively move and immediately sink into the ground when they get exposed to sunlight and have a clean body with no injuries. Required larvae were 48 (40 for testing pathogenicity, and 8 for analyzed tissue damage due to the fungus infection).

M. anisopliae

Samples of *M. Anisopliae* were obtained from Central Plantation Crop Protection in Salatiga, Indonesia. They were packed in kaolin powder. *M. anisopliae's* conidia were calculated for its density and viability before used.

Media

Organic manure media was used to nourish the larvae during the study. Media contained a mixture of organic manure and *M. anisopliae* with various concentrations. *M. anisopliae* was in the form of dust full of the fungus conidia. Conidia density and viability was 7.32 x 10^8 $g^{-1}$and 90.4% respectively. The media composition used in this study was:

Po = 100 gram manure
P1 = 1 g*M. anisopliae* + 100g manure
P2 = 2 g*M. anisopliae* + 100 g manure
P3 = 4 g*M. anisopliae* + 100 g manure

Each media mixed evenly then put into a plastic cup container with a height of 13.2 cm, upper diameter of 8.9 cm, a bottom diameter of 5.7 cm and volume of 821.4 cm^3. Each cup was given one 3rd instar larvae and covered with perforated plastic for air circulation with water content of 60%. Plastic cups were labeled and then placed on a shelf in the laboratory. Each treatment was repeated 10 times for a total of 40 larvae. The observation was carried out for 12 days.

Data analysis

Data of larval mortality was obtained from observations to a total of 40 larvae. Observations were conducted everyday. The percentage of larval mortality was analyzed descriptively and presented in form of graphic.

Observation of tissue damage in *O. rhinoceros* larvae was started when larvae showed infected symptoms of 1) brown spots, 2) stiff larvae (mummification), 3) overgrown by white fungi (mycoses) and 4) overgrown by dark green fungal colonies. The observation conducted at Diagnostic Alert Laboratory in Semarang, Indonesia.

Larvae that showed those symptoms were immediately preserved with formalin 10%, then tissue damage of two larvae from each symptom were analyzed. The infected larvae were made into a microscopic object in order to observe the damaged tissue and take pictures for analysis. The microscopic object was stained using HE (Hematoxylin-Eosin). Data of the tissue damage was observed through microscopic object images (microscope binocular CX 23 Olympus) and analyzed by comparing healthy tissue and infected one.

Statistical analysis: We used descriptive analysis. The percentage of larval mortality was analyzed descriptively and presented in form of graphic. The damage tissue caused by fungus was presented by microscope slide figure.

RESULTS

***O. rhinoceros* Larvae mortality**

The observation of *O. rhinoceros* mortality for 12 days is presented in Figure-1. In control group (P0) all larvae remained healthy and alive until the end of the study (12 days). P3 treatment (4 g of *M. anisopliae*) caused larval mortality occurred on 2nd - 12th day, while larvae in P1 and P2 treatment took more than 12 days to die. The mortal larvae increased every day. High concentration of conidia, causing the death of larvae becomes faster as shown in Figure-1.

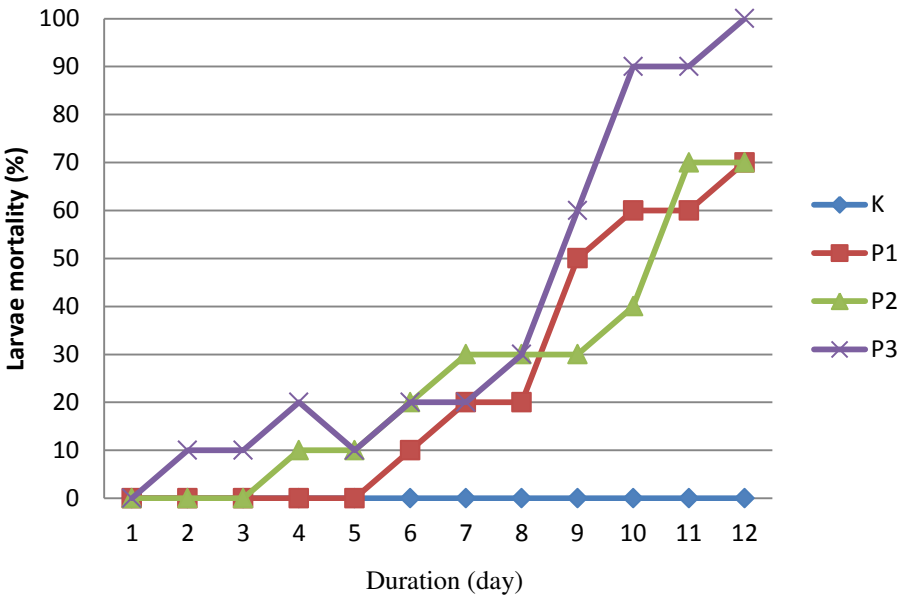
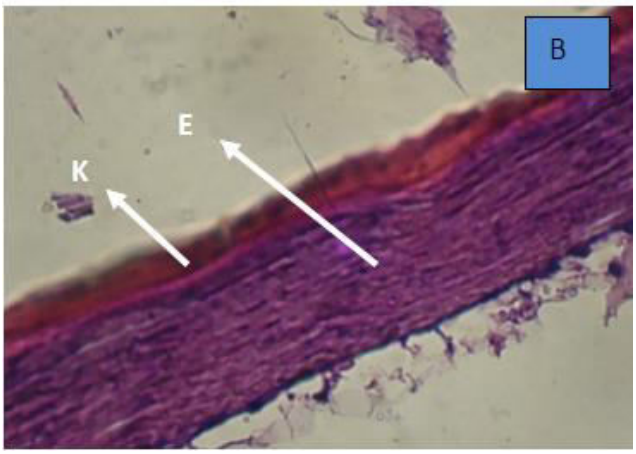


Figure-1. Percentage of *O. rhinoceros* larvae mortality from the 1st to 12thday.

Tissue damage of O. rhinoceros caused by M. anisopliae

Characteristic of O. rhinoceros uninfected larvae (healthy larvae) were: beige color, uninjured body and the clear larvae tissue (no damage) as shown in Figure-2.

Healthy Larvae

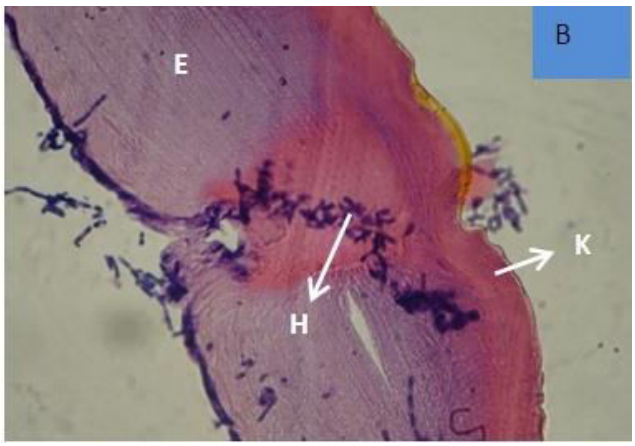


Healthy Larvae Tissue, K= Cuticle, E=Epidermis

Figure-2. Healthy *O. rhinoceros* Larvae (A) and healthy *O. rhinoceros* Larvae microscopic (B) object under 400 × magnification

Healthy *O. rhinoceros* larvae under microscopic observation as shown in Figure-2B. In healthy *O. rhinoceros* larvae, it can be observed that its tissue, cuticle, and epidermis are undamaged.

O. rhinoceros larvae got infected by *M. Anisopliae* on the second day after application

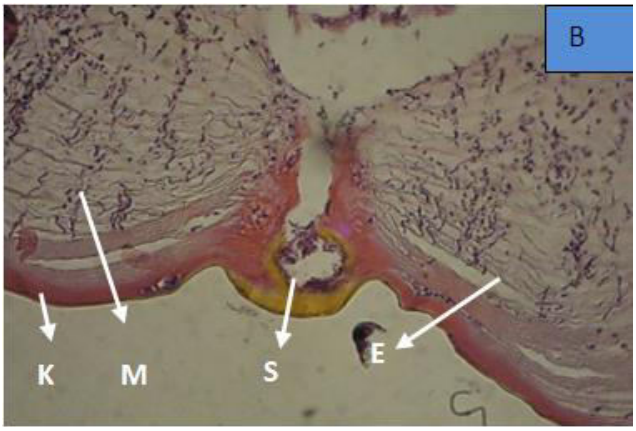


O. rhinoceros larvae tissue infected by *M. Anisopliae* on the second day after application. K= cuticle, E=epidermis dan H= hyphae. Under microscopic magnification at 400×

Figure-3. *O. rhinoceros* Larvae (A) and Larvae tissue (B) Infected by *M. Anisopliae* on the second day after application.

The first infected larvae by M. anisopliae were characterized by brown spots (Figure-3A) appearing on the second day after the application, brown spot was visible around the spiracles hole. Larvae showing a symptom of the brown spot was first obtained from the P3 treatment (4 g of M. anisopliae).

O. rhinoceros larvae got infected by *M. anisopliae*on the sixth day after application

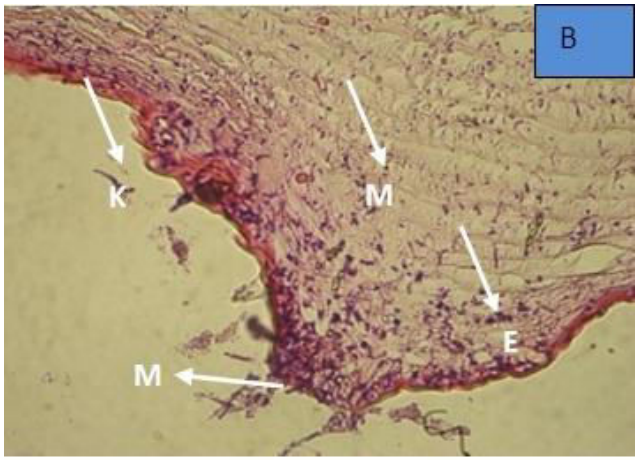


O. rhinoceros larvae tissue infected by*M. Anisopliae* on the sixth day after application.K=Cuticle, E= Epidermis, S= Spiracle dan M= Mycelium. Under microscopic magnification at 400×

Figure-4. *O. rhinoceros* Larvae (A) dan Larvae tissue (B) Infected by *M. Anisopliae* on the sixth day after application.

Further infection ssymptom was determined by observing the larval body that became stiff called mummification (Figure-4A). In this infection symptom, larvae body became stiff because *O. rhinoceros* larvae's body tissue and fluid were absorbed by *M. anisopliae* fungi to their own reproduction.

O. rhinoceros larvae got infected by *M. Anisopliae* on the eighth day after application

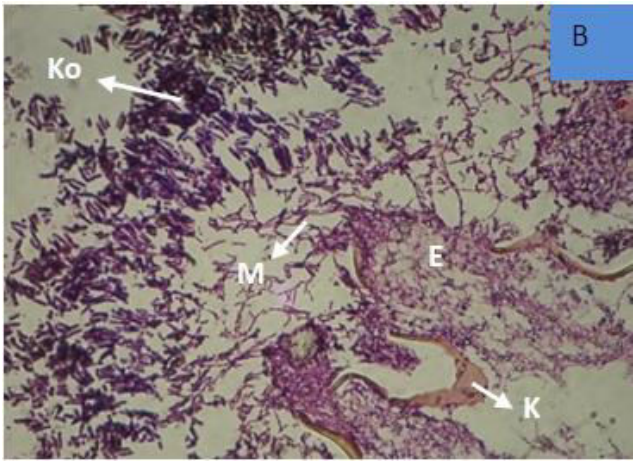


O. rhinoceros larvae tissue infected by *M. Anisopliae* on a ninth day after application. K=Cuticle, E= Epidermis, M=Mycelium. Under microscopic magnification at 400×

Figure-5. *O. rhinoceros* Larvae (A) dan Larvae tissue (B) Infected by*M. Anisopliae* on the eighth day after application.

Three days after larvae was mummified, white mycelium appeared on the body surface (Figure-5A)? The emergence of white mycelium outside the larval body indicates that the nutrients contained in the larval body have been exhausted to be used for the growth and development of *M. anisopliae*. Larval tissue in this stage was severely damaged. Figure-5B shows epidermis tissue is already filled with mycelium, and mycelium comes out from the larvae cuticle.

O. rhinoceros larvae got infected by *M. Anisopliae* on the twelfth day after application



O. rhinoceros larvae tissue infected by *M. Anisopliae* on the twelfth day after application.. K=Cuticle, E= Epidermis, M=Mycelium. Ko= Conidia. zoom 400×.

Figure-6. *O. rhinoceros* Larvae (A) dan Larvae tissue (B) Infected by *M. anisopliae* on the twelfth day after application.

On the twelfth day, the color of white mycelium on the larval body changed to dark green (Figure-6A). The emergence of green conidia on the larval body was a symptom of the latest stage of *M. anisopliae*'s infection on *O. rhinoceros* larvae body. Conidia have spread to the surface of the larval body and been ready to look for another host (Figure 6B).

DISCUSSIONS

M. anisopliae fungi require several stages to infect *O. rhinoceros* larvae [20]. The infection mechanism of *M. anisopliae* can be classified into four stages. The first stage is the inoculation, at this stage, the contact between conidia of fungi the larval body is occurred. The second stage is the process of attachment and germination of fungi propagules (conidia) on insect integument. The third stage is the penetration and fungal invasion by penetrating hyphae in the integument and forming tube sprouts (appressorium). The fourth stage is the destruction at the point of penetration and the formation of blastopore which then circulating inside hemolymph and forming hyphae to invade other tissues [21].

If the conidia contact with the larvae and moist media, the conidia will germinate. So, the first and the second stage (contact, attachment, and germination) can be occurred on the first day. *M. anisopliae* conidia need 18 hours to start germinate. After germination of conidia, the third stage (penetration and invasion) will follow. Evolving hyphae attacked the larvae body and disrupt its metabolism system. The fourth stage is the destruction of larval tissue resulting in larval mortality. In the P3 treatment (4g) the larvae got infected and died faster than P1 (1g) and P2(2 g) because P3 has the higher number of conidia. *M. anisopliae* effectively control *O. rhinoceros* [2]. *M. anisopliae* release toxins that cause paralysis in larvae's limb. Paralysis causes coordination lost in movement system resulting in irregular movement, then gradually weakened and causing total paralysis or death [16].

The pathogenicity (virulence) of fungi is influenced by the number of conidia [17]. Conidia have a major role in the infection process. The higher number of *M. anisopliae*, the more conidia attached to *O. rhinoceros* larvae's integument. Conidia then germinate into hyphae, attack the larvae and disrupt *O. rhinoceros* larvae's metabolic system [18].

Larvae age can also affect the speed of larvae mortality. The young larvae die sooner than older larvae. Young larvae integument is softer than the old larvae. Old larvae can decrease its sensitivity to entomopathogenic fungi [19]. *O. rhinoceros* larvae mortality was faster when given by higher dosage of *M. anisopliae*.

On the first day of treatment, the larvae still look active and beige color (Figure-2). But at the second day, larvae's motion became slower. *O. rhinoceros* larvae got infected by *M. Anisopliae* on the second day after application showed brown spot (Figure-3A).

Brown spots on the *O. rhinoceros* larvae body is melanin. It is the body's defense response characteristics because of *M. anisopliae*'s attack [22]. The formation of melanin is called melanization conducted by phenol oxidase enzyme. Melanization of larvae occurs in the lower body, chest, abdomen, and part of the body between the segments. After the larvae have contact with conidia of fungi when the temperature is low and the humidity is high, the conidia will attach to the insect cuticle and germination of *M. anisopliae* will occur [23]. Hyphae in cuticle tissue showed in figure 3 B. According to Urquiza & Keyhani [24], *M. anisopliae* ability to penetrate the larvae cuticle is done with the help of enzymes. Germination and growth of *M. anisopliae* on the larvae cuticle surface are accelerated by the activity of hydrolytic enzymes (protease, chitinase, and lipase) produced by fungi and other factors to facilitate the entry of hyphae into the larvae tissue. Hyphae that grow on the *O. rhinoceros* larvae tissue will release compounds that disrupt the immune system of insects resulting in larvae mortality. Destruction compound will be continuously be produced by *M. anisopliae* as long as it stays in the larvae's body [25].

Mummify symptoms (mummification) appeared on the 6^{th} day after application (Figure-4A). In this infection symptom, larvae body became stiff because *O. rhinoceros* larvae's body tissue and fluid were absorbed by *M. anisopliae* fungi to their own reproduction [26]. According to Moslim *et al*. [6] larval body hardening is caused by the absorption of whole tissue and fluid of larval body by *M. anisopliae* to support their growth, so the larvae become hard. Figure-4B shows that larvae tissue has been fulfilled by the mycelium of *M. anisopliae* fungi. Epidermis layers (E) on larval tissue were seen to be terribly damaged. This damage is caused by toxic destruction compound. In this stage, infected larvae which were previously aggressive became lethargic, sluggish and then died. The infected insect by *M. anisopliae* showed symptoms of lethargic moves (reduced feeding activity) [18]. Insect mortality infected by *M. Anisopliae* is because

of the production of the toxic compound. Toxic compounds produced by *M. anisopliae* will cause damage to the tissues and the digestive systems of insect. It eventually causes losing of appetite and then died [27]. Hyphae were also seen grouping in spiracle. *M. Anisopliae* penetrates the larvae body through the body pores and spiracles [28].

White mycelium appeared on the surface of the larva body after the larvae got infected by *M. Anisopliae* on the eighth day after application (Figure-5A). The epidermis tissue was already filled with mycelium, and mycelium comes out from the larvae cuticle (Figure-5B). *M. anisopliae* will come out of the insect body through the cuticle if the inside insect's body is no longer able to be used as a source of nutrients [29]

On the twelfth day, the color of white mycelium on the larval body changed to dark green (Figure-6A). The emergence of green conidia on the larval body was a symptom of the latest stage of *M. anisopliae*'s infection on *O. rhinoceros* larvae body. When the air was dry and humid, the hyphae were able to penetrate the cuticle and cover the body of the insect and formed conidia [30]. This is shown in Figure-6B conidia have spread to the surface of the larval body and been ready to look for another host. Some larvae were found dead without any appearance of hyphae on the outside of the body until the fifteenth day. According to Prayogo [26], fungi do not always grow out in the integument. When the condition does not support the development of the saprophyte, fungi will only grow in the larval body without penetrating out the integument. Fungi will establish a special structure to survive; called arthrospores o. larval tissue in this stage looks very damaged. Cuticle and epidermis performance were damaged, mycelium and conidia multiply and were ready to infect a new host (Figure 6B). The formation of conidia fungi indicate that there has been a cycle of *M. anisopliae* [20].

Environmental factors involved in this research were temperature ranged from 25-29^{o}C, pH 7.0 and humidity of 83-99%. According to Dimbi *et al*. [31], the temperature for fungi growth was between 5-35 °C with optimal growth at a temperature of 25°C. Soil pH condition during observations was in the optimum pH range (7.0). According to Matsumoto[31], the optimum pH for fungus growth ranged from 2.5 to 10.5. Good and maximum air humidity for the growth of conidia is ranged between 80-92% [9]. Therefore, the range of temperature, pH, and humidity during the research was in the normal range.

CONCLUSIONS

M. anisopliae cause *O. rhinoceros* larvae mortality occurred on 2nd - 12th day (P3), while P1 and P2 treatment took more than 12 days to die. The infection of *M. anisopliae* in *O. rhinoceros* larvae was characterized by brown spots (melanization), mummify symptoms (mummification), the appearance of white mycelium (mycoses), and dark green conidia in the final stage.

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