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The Effect of Water Content of Medium Containing *Oryctes rhinoceros* Larvae on *Metarhizium anisopliae* Pathogenicity

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Abstract

The entomopathogenic fungus, *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales) would effectively infect the target host on the appropriate medium water content. The aim of this study was to analyze the influence of water content of medium on the effectiveness of *M. anisopliae* fungus infection on *O. rhinoceros* larvae in the laboratory. Fifty healthy third instar larvae of *O. rhinoceros* were obtained from field. The *M. anisopliae* obtained from Estate Crop Protection Board in Salatiga. The conidia density and viability of *M. anisopliae* were examined before used. The medium for maintaining the larva was the sawdust that had been sterilized. A total of 50 plastic cups were prepared to place 50 larvae (1 larva/cup). Each cup was filled with 100 g medium of sawdust plus 2 g of *M. anisopliae* which was then stirred until mixed, with different water content: P1 (20%), P2 (40%), P3 (60%), P4 (80%) and P5 (98%). The result indicated that the water content of the medium affected the effectiveness of *M. anisopliae* fungus infection on *O. rhinoceros* larvae. The water content influenced the duration of larval mortality at each treatment. An important finding in this study is that controlling *O. rhineceros* larvae with *M. anisopliae* can be done by manipulating the water content of medium. The benefit of this study may be used for the recommendation of *O. rhinoceros* pest control using *M. anisopliae* with an effective water media content.

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INTRODUCTION

Coconut is one of the important plantation commodities in the Indonesian (Mulyono, 2007). In coconut cultivation, pest and disease are factors that limiting the coconut production. One of the most commonly found destructive pest of coconut plants is *Oryctes rhinoceros*. The imago of *O. rhinoceros* attacks the coconut shoots with the unopened young leaves, while the larval phase lives in the soil containing organic matter or on rotten coconut trunk (Indriyanti et al., 2016; Abidin et al., 2014). *Oryctes rhinoceros*, commonly known as the rhinoceros beetle is an important agricultural pest that is known to inflict serious damage on young oil palm trees (Manjeri et al., 2014).

There are different methods to control the insect pests. This includes chemical, mechanical, physical and biological control of pest. Chemical control can be done with the use of pesticides (Tarwotjo & Rahardian, 2017) and pheromones (Chakravarthy et al., 2014). Mechanical and physical control can be done by searching and destroying the existing larvae in the soil. Biological control can be done with the use of biological controlling agents. One of the insect pathogens that has been utilized for the control of *O. rhinoceros* is the fungus *Metarhizium anisopliae* (Harjaka, 2011; Manurung et al., 2012). *M. anisopliae* is a soil borne entomopathogen, found worldwide. It is an interesting fungus for biological control (Chen et al., 2014; Simamora et al., 2013). It can infected larvae and adult *Oryctes agamemnon arabicus* (Coleoptera: Scarabaeidae) (Ibrahim, 2017).

Many factors that influence the success of parasitic fungi in infecting insects, including temperature, humidity (Athanassiou et al., 2017; Subhathma et al., 2013). Efficacy *M. anisopliae* in the fields is significantly affected by enviromental conditions, particularly moisture (Bidochka, 2000; Chen et al., 2014; Moslim & Kamarudin, 2014). Water content media where fungus grow is very important. The fungus *M. anisopliae* will effectively infect the host if the water content of the media is suitable for conidia germination.

Water is the main component needed by the fungus in order to keep growing. The water content is an indicator of the presence of water in the environment. Entomopathogenic fungi require a high water content for growth. The optimum growth of fungi increase the possibility of infecting the insect pests effectively (Gupta & Gopal, 2002).

One of the places used as a habitat of *O. rhinoceros* larva is a pile of sawdust. Sawdust media has a hollow structure so that there is more oxygen in there. In controlling *O. rhinoceros* using *M. anisopliae*, it is important to note the conformity of abiotic factors of medium where larvae live including the water content and humidity of the media. The pathogenicity of *M. anisopliae* against *O. rhinoceros* larvae on sawdust media was 86.24%, higher than in manure media (68.27%) (Mulyono, 2007), but it was not known how much water content in these media. Therefore, it is necessary to examine the appropriate water content in media for the optimum fungi germination. The aim of this study was to analyze the influence of water content of medium on the effectiveness of *M. anisopliae* fungus infection on *O. rhinoceros* larvae in the laboratory. This study provides an information the effect of water content of medium *against M. anisopliae* pathogenicity on *O. rhinoceros* larvae. The benefit of this study was used for the manipulation of environmental factors in control *O. rhinoceros* in the field.

METHODS

Larvae *O. rhinoceros*

Fifty healthy third instar larvae of *O. rhinoceros* with length of 7-12 cm and weight of 9-13 g were obtained from field, in Demak Central Java Indonesia. This study was carried out in the laboratory during the dry season with the temperature ranges from 29.8 - 35°C, pH media of 6.9, room light intensity of 70-77 lux and RH of 58-83%.

Fungi *M. anisopliae*

The fungi *M. anisopliae* obtained from Estate Crop Protection Board in Salatiga, Central Java Province, Indonesia. It was cultured on the corn medium. The conidia density and viability of *M. anisopliae* was examined before the use. Conidia density was calculated under microscope using *Haemocytometer*. The viability of conidia was observed after 10 hours incubation on PDA (potato dextrose agar) media. Density and density calculations are performed four repetitions. The formula for calculating the density and viability of conidia were based on the formula recommended by BPTBUN (2012).

Treatment

The medium for maintaining the larva was the sawdust that had been sterilized for 8 hours.

During the treatment, the larvae were placed in plastic cups (diameter = 8.4 cm, height = 11 cm) with the cap section given a small hole for air ventilation. A total of 50 plastic cups are prepared to place 50 larvae (1 larva/cup). Each cup was filled with a medium of 100 g of sawdust

with different water content P1 (20%), P2 (40%), P3 (60%), P4 (80%) and P5 (98%) plus 2 g of *M. anisopliae* which was then stirred until mixed. The *O. rhinoceros* larvae were then fed into prepared media. The observations were conducted every two days for 28 days (estimated death of all larvae). This study used a Randomized Block Design technique with 5 water treatments (P1-P5) and 10 repetitions.

Data analysis

The data obtained including: 1) density and viability of *M. anisopliae* conidia, 2) morphology of *O. rhinoceros* due to *M. anisopliae* application, 3) Mean survival time of *O. rhinoceros* larvae treated with *M. Anisopliae*, data were analyzed statistically using SPSS version 16.0 and further testing by Tukey's test.

RESULTS AND DISCUSSION

The density of conidia greatly affects the speed of the *M. anisopliae* in killing *O. rhinoceros* larvae . The calculation of the conidial density of *M. anisopliae* used is presented in Table 1.

Table 1. The Density of *Metarhizium anisopliae* Conidia

Repetition	Result on the counting area of *Haemocytometer*					Total	Conidia density per gram of sample
	a	b	c	d	e		
1	10	15	9	14	10	58	2.90×10^8
2	10	14	12	10	9	55	2.75×10^8
3	11	12	14	9	11	57	2.85×10^8
4	10	14	11	12	10	57	2.85×10^8
Mean							2.84×10^8

The mean conidial density of 2.84×10^8 includes in a good category according to biological agent quality standard, because the number of conidia was more than 10^6 (BPTBUN, 2012). The result of viability calculation of *M. anisopliae* Conidia is presented in Table 2.

The viability of *M. anisopliae* was 94.6% which included in a very good category (in range of 86-100%) according to the biological agent quality standard from BPTBUN (2012). Conidia growth is listed in the Figure 1.

The Survival Time of *O. rhinoceros* Larvae

Figure 2 shows that there is a difference in the survival time of *O. rhinoceros* larvae in 5 different treatments.

Table 2. The Viability of *M. anisopliae* Conidia

Repetition	Amount of Observed Conidia			Viability (%)
	Not Growing	Growing	Total	
1	3	61	64	95.3
2	5	64	69	92.7
3	2	58	60	96.6
4	4	60	64	93.8
Mean				94.6

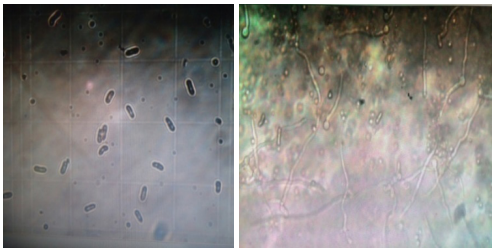

Figure 1. Conidia of *M. anisopliae* (A). Conidia germination (B), at magnification of 400x.

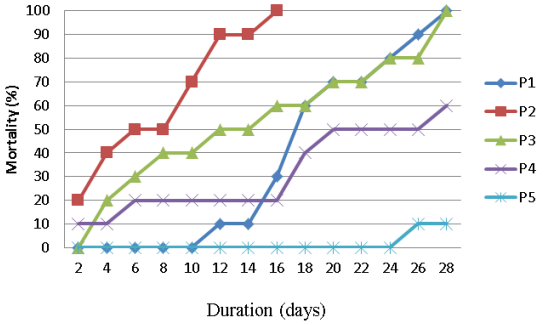

Figure 2. The Percentage of *O. rhinoceros* Larvae Mortality treated by *M. anisopliae* on the Sawdust Medium with Different Water Content (P1= 20%; P2= 40%; P3= 60%; P4= 80%; P5= 98%)

The mean survival time of *O. rhinoceros larvae* treated with *M. anisopliae* is presented in Table 3.

Table 3. Mean survival time of *O. rhinoceros* larvae treated with *M. anisopliae*

Treatment	Mean survival time
P1	19.6 [b]
P2	7.8 [a]
P3	15.0 [ab]
P4	23.6 [b]
P5	34.8 [c]

Note: Numbers in the same column, followed by the same letter, are not significantly different (Tukey's test , $\alpha = 0.05$)

In P2 treatment, the *M. Anisopliae*-treated

larvae had a shortest mean survival time (7.8 days) among all treatments, while larvae in P5 treatment had a longest mean survival time (34.8 days) (Table 3). The result of ANOVA test (F = 15.119; df = 4; P = 0.000; P <0.005), indicated that there is a difference of larvae survival time at each treatment (Table 3). Water content in P1 (20%) treatment was considerably less than the ideal one. It caused the fungus could not germinate and the mortality of treated larvae due to the lack of water occurred (Figure 2). Therefore, the water content greatly affects the effectiveness of fungi *M. anisopliae* in infecting *O. rhinoceros* larvae.

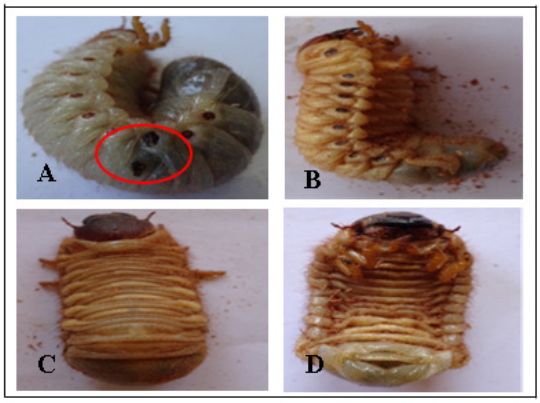

Figure 3. The Morphological Changes of *O. rhinoceros* Larvae in P1 (20%) Treatment

Note:
A: The symptom of *M. anisopliae* infection, indicated by the appearance of brown spot on the cuticle (day 2)
B: The larvae begin to dwarf (day 8)
C: The body of the larvae becomes more shrinkage (day 10)
D: The dead larval with the absence of the symptoms of *M. anisopliae's* infection (day 12)

At the P1 (20%) treatment, there were no larvae died due to *M. anisopliae* infection. Initially, all the larvae in this treatment showed the symptoms of infection such as melanization and the slower movement on the 2nd day of treatment, however, on the 8th and 10th days the larvae morphology became dwarf and the larvae were on the surface of medium. The death of the larvae begins on the 12th day with the condition of dwarf and shrink. This is because the larvae were lost a lot of water or dehydrated (Figure 3).

Insects mortality will occur when the water content drops below the tolerance limit. The reduced water content results in the dwarf growth and low metabolic rate. The content of water in the insect body varies, generally ranging from 50-90% of body weight. In thick skinned insects, the body content of the water is lower. Insects should try to get the right water balance in order to maintain their life (Sodiq, 2009).

The larvae maintained on sawdust media containing *M. anisopliae* fungi with a moisture content of P2 (40%) had the shortest survival time compared to four other media. It was indicated that treatment P2 is the most effective treatment to control the *O. rhinoceros* population. In this treatment, conidia of *M. anisopliae* can grow well so that it can infect large numbers of larvae. Mortality in the treated larvae began on day 2, and reached 100% mortality by day 16 (P2, Figure 2), The average survival time is 7.8 days (Table 3). Statistically, the result of P2 and P3 treatment did not significantly different, so it can be said that the effective water content to maintain the *O. rhinoceros* larvae and *M. anisopliae* growth ranges from 40-60%.

In the treatment of P4 (80%) and P5 (98%), conidia did not grow well due to the high water content resulting in many conidia to die from long waterlogged. In these treatments, the fungi failed to infect the larvae. Treatment of P5 (98%) showed significantly different results compared with all other treatments. This is due to the very high amount of water contained in the P5 treatment. In this treatment, the *M. anisopliae* fungus will not be able to infect the larvae, because the conidia were drowned and eventually dead. The larvae began to die on day 26 and reached 100% mortality by the day 40. The dead of larvae was because they could not last long in water. The interesting phenomenon on high water content of the media was that the larvae tend to be uncomfortable, as evidenced by the larvae tried to get out of the place of maintenance.

In nature, the phenomenon of high water content occurs during the high rainfall for a long time. According to Susanti et al. (2013) rainfall greatly affects the effectiveness of *M. anisopliae* infection because it is related to water content in a medium. In a high rainfall, *M. anisopliae* cannot infect the larvae because it drifts with water or rottenness of germination fungus, because it was submerged in water for a long time.

The higher the water content of the media, the longer mean survival time of the treated larvae. It can be explained that in the medium with moisture content of 80-98%, *M. anisopliae* conidia is submerged in water for a long time, the possible condition of conidia was rotten and die after the germination, so that the larvae were failed to be infected by the fungus. As a result, the mean survival time of the larvae became 23.6 - 34.8

days (P4 & P5, Table 3).

Temperature, humidity, light intensity and pH are factors that also affect the effectiveness of *M. anisopliae* in infecting the *O. rhinoceros* larvae. The appropriate temperature and humidity will reduce the dehydration process in the fungus' body (Prayogo et al., 2005). The study was conducted during the dry season, temperatures ranging from 29.8 - 35°C, so that the temperature was relatively higher.

The development of *M. anisopliae* conidia causes the larvae's body to become weak and end with the larvae mortality (Simamora et al., 2013). According to Susanti et al. (2013), conidia germinate in air humidity above 90% and achieve the optimum germination at a high air humidity (100%). In this study, in air humidity of 58-83%, *M. anisopliae* was able to infect larvae effectively. It is proven with the results of P2 treatment (mean survival time of 7.8 days). *M. anisopliae* can grow and proliferate optimally in places with relatively low light intensity.

In this study, the pH obtained at the time of observation was in the range of 6.9 (relatively neutral). The appropriate pH range supports the activity of enzymes contained in *M. anisopliae* (lipase, chitinase, amylase, proteinase, phosphatase, and esterase). According to Prayogo et al. (2005), chitinase and proteinase are two enzymes that have a very important role. Chitinase serves to assist the activity at the beginning of growth of the fungus, conidia formation, and conidiospore sporulation while protease enzymes play a role in degradation of the cuticle and chitinase stimulation.

The larva undergoes numerous sequential morphological changes since the first until the last stage of infection which was indicated by the appearance of green fungi in the body's surface of the larvae (Figure 4).

The melanization process was first seen in P2 (40%) treatment larvae, indicated by the appearance of a dark brown spot on the larval cuticle. The brown spots mostly seen on the folds between segments adjacent to the spiracles. *M. anisopliae* attach more strongly to the folds of the larval cuticle. It is more easier to penetrate into the body of the *O. rhinoceros* larvae. It is in accordance with the opinion of Manurung et al. (2012) that the infection of *M. anisopliae* on the larvae are characterized by larval cuticles that turn into dark brown color. The cuticle was covered by the hyphae from the fungus which then turns into green which means that the conidia has grown. According to Prayogo et al. (2005), hypha from the conidia of *M. anisopliae* get into the cavities within the host's body due to enzyme aids and mechanical stresses. The infected larvae will undergo the mummification phase because all the tissues and body fluids of the larvae are used up by the fungus for its reproduction (Permadi, 2012). Finally, the whole body of the host insect is full of propagules and the soft part of its body will be pierced out. It shows the growth of hyphae outside the body of the host insect. The growth of external hyphae will produce conidia which then being disseminated to the environment and infect the other healthy insect pests.

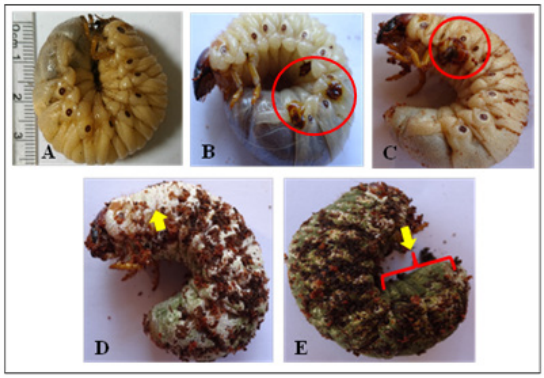

Figure 4. The Morphological Changes of *O. rhinoceros* Larvae due to the Infection of *M. anisopliae* on P2 Treatment.
Note:
A: A healthy *O. rhinoceros* larva (day 1)
B: The infection of *M. anisopliae* indicated by the appearance of brown spot on the cuticle (melanization) (day 2)
C: Dead larvae infected with *M. anisoliae* with the stiff and pale body (day 4)
D: The appearance of white hyphae in the surface of larva's body (day 6)
E: The sporulation of fungi outside the larva's body indicated by the hyphae that turns to green (day 10).

The symptoms of *M. anisopliae*-infected larvae are loss of appetite, slow movement, and then the mortality followed by stiff and pale dead larvae. The green hyphae in the body of the larvae will appear later. According to Herlinda et al. (2005), the length of time required by entomopathogenic fungi isolates ranging from fungal infections to dead larvae can range from 2-10 days. In this study, the mean of survival time of larvae range from 7.8 - 34.8 days (Table 3.)

The results of this study can be suggested that, controlling of the *O. rhineceros* larvae with the *M. anisopliae* fungus, will be optimum if carried out in during the transition season (the beginning of the rainy season) the field. At the be-

ginning of rainy season, usually the intensity of the rain is not too high, thus allowing the fungus to grow and infect the larvae well. *O. rhineceros* larvae controlling will not be optimal if it is conducted in dry season, because conidia will not be able to germinate. The controlling also will not be optimal if it is conducted in the rainy season, because too much water conidia will die, so the infection will not happen.

An important finding in this research is that controlling *O. rhineceros* larvae with *M. anisopliae* can be done by manipulated the environmental, such as water content of the media where the *O. rhineceros* larvae live. The benefit of this study may be used for the recommendation of *O. rhinoceros* pest control using *M. anisopliae* in an effective water media content (40-60%).

CONCLUSION

The water content of the medium affects the effectiveness of the fungus *M. anisopliae* in infecting the *O. rhinoceros* larvae. Medium with 40-60% water content is the suitable medium for *M. anisopliae* growth to infect *O. rhinoceros* larvae. The very low water content (20%) causes the fungus of *M. anisopliae* unable to germinate. The very high water content (98%) causes the conidia died from being submerged in water.

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