**Ecological studies of *Oryctes Rhinoceros* larvae controlled by *Metarhizium anisopliae* and Enthomopatogenic Nematodes**

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**ABSTRACT**

*Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae) is a pest of coconut plant.*Metarhizium* *anisopliae* and Enthomopatogenic Nematodes are biological control agents. The purpose of this study was to determine the ecology of insect pests controlled by fungi *Metarhizium anisopliae* (MET), Enthomopatogenic Nematodes (NEP) and and the mixture of MET and NEP for controlling *O. rhinoceros* larvae on the field. *M. anisopliae* used was in the form of kaolin powder formulation (WP). The nematodes used were *Heterorhabditios* sp. Nematodes formulation was in the form of liquid with sponge medium contained 10 x 106 NEP in every pack. The study used Randomized Block Design with 3 types of treatment and 10 times repetition on 10 larvae. The result of six weeks treatment showed that the highest larval mortality was obtained from the treatment with the mixture of MET and NEP. Biological control using two biological agents (MET and NEP) is better than only MET or NEP. The benefit of research is for the recommendation of *O. rhinoceros* larvae control.

**Keywords**: application of *Metarhizium* *anisopliae,* Enthomopatogenic Nematodes, *Oryctes*

 *rhinoceros*, biological control.

**INTRODUCTION**

*Oryctes rhinoceros* beetle is one of the major pests attacking coconut crops in Indonesia (Indriyanti et al., 2018a). It has reportedly attacked the coconut plants in Africa, especially in Ivory Coast (Allou et al., 2006), in Malaysia (Manjeri et al., 2014), Jepara, located on the Shore of Java Sea (Indriyanti et al., 2018b). Thebeetle destroy the coconut shoot, attacks on growing point of stem while the larvae live in the soil or dead coconut trunk (Indriyanti et al., 2017a). Control larvae with pesticides causes various negative impacts on the environment such as soil, water, and air (Amalia & Yusa, 2018). One of the biological agency to control the larvae of *O.rhinoceros* is *Metarhizium anisopliae* (Indriyanti et al., 2017b).It is a parasitic fungi that infect many insects (Agali et al., 2017). Beside fungus, there is nematode netomopatogen which also attack insect. Both are safe for the environment, because it does not cause environmental pollution and does not cause resistance to insect pests (Indriyanti et al., 2017c; Indriyanti & Widiyaningrum, 2014).

The semi-field study was conducted to analyze the effect of MET, NEP and the mixture of both substances on the *O. rhinoceros* larval mortality. The treatment was conducted in coconut plantation owned by the local people using pots containing soil medium with organic material. The results showed that controlling *O. rhinoceros* larvae with the mixture of MET and NEP resulted in faster larval mortality (2-5 weeks) than the treatment with MET (2-7 weeks) or NEP (2-8 weeks), while larval mortality without the administration of MET or NEP (control) was 13-20%, the larvae that remained alive was 80-87% (Indriyanti et al., 2017a).

 MET and NEP applications on a semi-field scale have been proven effective in controlling the *O. rhinoceros* larvae. However, the results of the application of MET and NEP in field have not been reported yet. The biological control of *O. rhinoceros* larvae with parasitic fungus (MET) and (NEP) offers environmentally friendly pest controls without the emerge of pest resistance. Therefore, it is necessary to conduct a research for the purpose of analyzing the effect of MET and NEP application for controlling *O. rhinoceros* larvae on a field scale.

**MATERIALS AND METHODS**

This study was conducted in Jerukwangi, Jepara, Indonesia. This study used Randomized Block Design with three types of treatment (MET, NEP, and the mixture of MET and NEP). The population in this study was all *O. rhinoceros* larvae in Jerukwangi Village. The samples in this study were 330 *O. rhinoceros* larvae (3rd instar) obtained from nests in the field. The larvae were obtained by digging the suspected place. The places where larva often encounters were in the piles of cattle dung (cows and goats), the livestock fodder, garbage piles, and rotted coconut trunks. The recorded abiotic factors were temperature, air humidity, soil pH, soil moisture, and light intensity. 100 grams of soil was taken to check the soil moisture content.

**Application of *M. Anisopliae* (MET)**

MET used was in the form of conidia with kaolin powder formulation (ZIUM ORWP) obtained from BPTBUN Salatiga. The conidial density was 2.50 x 108 conidia L-1, with the viability of 93%. Treatment places were in the same places where the larvae were found, which were then called nests. The size of the nest was 1 x 1 x 0.3 m. 100 grams of MET was added to the soil (in the nest) and then stirred in depth of 0.3 m. 10 larvae were placed in the nest. The location was covered by a net (hole size of 3x3 mm) and then be marked. This closure aimed to avoid the interference from the outside. The MET treatment was repeated 10 times. For comparison (control), the same procedure was applied in the other nest without the administration of MET. Data collection was conducted once a week for 6 weeks by counting the MET-infected larvae.

**Applications of Entomopathogenic Nematodes (NEP)**

The nematodes used were *Heterorhabditios* sp obtained from the Plant Pest and Disease Department, Faculty of Agriculture, Jember. The nematodes formulation was in the form of liquid with sponge media containing 10 x 106 NEP per pack. The recommended dosage of NEP is one plastic package for 1 spray tank (14 liters) for 500 m2. Based on the preliminary research, the dose of dilution for application in the field scale was needed to be improved, so that the dilution used was 3.5 liters for one package. The application of the nematodes was conducted by spraying the NEP solution to the nest (soil) with the size of 1 x 1 x 0.3 m. Ten larvae were then placed in the nest. As a control, water was splashed to the ground to replace the NEP solution. The NEP treatment was repeated 10 times with one control. Both treatment and control nest were covered by a net and then marked. Data collection was conducted once a week for 6 weeks.

**Application of the Mixture of MET and NEP**

The same procedure as the MET and NEP treatments was applied in this treatment. The dosage used was 100 grams of MET combined with 1 pack of NEP in 3.5 liters of water, while for comparison, the control nest was administered only by the water (without MET and NEP). The treatment was repeated 10 times with one control. Both treatment and control nest (1 x 1m) were covered by a net and then marked. Observations were conducted once a week. The nest was dismantled and the characteristics or symptoms of infected larvae were then observed. The observations were performed until all the larvae in the nest died. The dead larvae were observed in the Biology Laboratory of Universitas Negeri Semarang to ascertain that the death of larvae was caused by the infection of MET or NEP. Data collection was conducted once a week for six weeks.

**RESULT AND DISCUSSION**

**Application of *M. anisopliae* (MET)**

 The results of observation showed that MET-infected larvae were initially characterized by the appearance of dark brown necrotic spots at the location of the hyphae penetration on the body of larvae. In addition, the larvae showed behavioral changes in form of laziness, slow motion, loss of coordination ability, decreased in feeding activity and the change in color (from white into dull). According to Gusmara (2011), the dark brown necrotic spots are a sign of melanization that indicates a fungal infection occurs in the larvae. Melanization is a form of self-defense against fungal infections that serve to inhibit the growth of fungi in the body of the larvae. The dark brown necrotic spots are melanin produced by phenol compounds catalyzed by phenol oxidase enzymes.

 The MET-infected dead Larvae were characterized by a hardened body (mummification) and the growth of fungus MET on the entire body of larvae. The color of hyphae changed from white to green along with the increasing age. According to Prayogo et al. (2005), mummification occurs in the larval body because all tissues and body fluids of larvae used up for the proliferation of MET. The result of observation on larval infection symptom is presented in Figure 1.

  

c

b

a

Figure 1. The symptom of MET infection on *O. rhinoceros* larva. a) Dark brown necrotic spot, b) White MET hyphae, c) MET hyphae that turned into dark green. hardened body of larva.

 The result of observation on the effect of MET application on the nest for 6 weeks is presented in Figure 2.

Figure 2. Percentage of alive, missing and MET-infected dead larvae of *O. rhinoceros* with the treatment using 100gr/m2 MET on 6 weeks of treatment period.

 Based on Figure 2, it is known that MET application on *O. rhinoceros* nest resulted in many missing larvae. The larvae were not found at the observation site (nest). Larval control effort on the nest could only kill 6% of the larval population. The missing larva is an interesting phenomenon, which has not occurred in the previous study (treatment with the pots). In the previous study, the MET-infected larval mortality can reach 100% at the 7th week (Indriyanti et al., 2017b). This might happen because the larvae were isolated in pots. In the field, the result was very different because the larvae can easily and freely move away from the treated place.

 The larval mortality rate as a result of MET treatment only reached 6% until the last day of observation (Figure 2). This is related to the many-disappeared larvae from the treated nest. The death of larvae is caused by the fungal infection. According to Badford (2013) the larval stages of *O. rhinoceros* actively look for substrates that contain lots of organic material as a source of food. Larvae moved out of the treated nest because the source of organic materials used to feed was finished and they looked for a source of more abundant organic material outside the nest.

 Some of missing larvae were found around the nest. They were still alive and some of them were going into the soil deeper. Larvae turned active, looking for food that has not been eaten. This resembles the cultivation of earthworms. When the dirt has been eaten, the worm moves to another place around the media. The larvae move away horizontally from the nest because they want to get food around it. The larvae also move vertically to avoid the light. The movement of larvae into the soil was reported by Pujiastuti (2010) who found that most urethral larvae were at a depth of more than 30 cm, followed by a depth of 20 cm, and 10 cm.

 The results of this study show that the speed of MET infection is not comparable with the active movement of the larvae. The fungal conidia take at least 18 hours to germinate (viability), then stick to the skin, infect and breed in the body of larvae. It takes at least two days to MET infect the larvae. This is in accordance with previous research found that the larvae take 2-7 weeks to die. Therefore, controlling the 3rd instar larvae of *O. rhinoceros* in the nest is ineffective. The larvae tend to move actively to avoid the passive mushrooms in the nest. In addition, the 3rd instar larvae have a thick cuticle, so the MET takes a longer time to cause an infection.

 The larval mortality is influenced by the pathogenicity of MET conidia. The pathogenicity is determined by the number of conidia that are able to germinate (viability). The higher the viability of conidia, the higher pathogenicity they have. The high viability of MET conidia is influenced by environmental factors. According to Indriyanti et al. (2017a), conidia viability is strongly influenced by environmental factors such as temperature, pH, moisture and water content of the media. Temperatures in the Jerukwangi Village was 30-330C. According to Pracaya (2004), the optimum temperature for the growth of MET ranges from 22-27oC. The presence of dead larvae infected by MET proves that the temperature range of 30-33oC is still possible for the growth of MET. Soil pH in the study site ranged from 6.7 to 7, which was a proper PH for the growth of the MET. Soil pH at the time of MET application is very important because it is related to the work of MET enzymes. According to Windarti (2010), an appropriate pH for the growth of MET ranges from 3.3-8.5 and optimum growth occurs at pH 7. The air humidity in this study ranged from 68-87%. It is suspected to be the cause of low MET infection rate. Prayogo et al. (2005) reported that the pathogenicity of the MET will decrease when the air humidity is below 86%. The intensity of light in this study ranged from 360-860 Lux because the location of treated nest was in a place shaded by trees, so it was not too exposed to sunlight.

 A MET-treated nest will be effective for early instar larvae. It will be more effective when the MET initially applied in the nest and the female put the eggs in that nest, then the eggs will hatch releasing the 1st instar larvae. That larvae will be easily infected because their skin is relatively soft and they are not yet actively moving. Therefore, for the MET-treatment of the 3rd instar larvae, they should be placed in a particular container. After the larvae are infected and die then they can be made into an inoculum at the nest site.

**Application of Entomopathogenic Nematodes (NEP)**

 The observation on larvae infected by NEP showed changes in larval body color from clean white to brownish-black and the texture of the larval body became mushy. These results are in accordance with the statement of Sucipto (2008) that the symptoms of NEP attack on host insects are characterized by changes in the color of the body surface to dark brown. The body also becomes soft and so the tissue becomes aqueous. Changes in body color of *O. rhinoceros* larvae due to the bacterial reaction of the *Photorhabdus* sp symbionts in the digestive tract of nematodes. The enzymatic activity of *Photorhabdus* sp bacteria causes the destruction of the larval tissue, so that the color of dead larvae is black (Suyanto et al., 2012). Figure 3 shows the *O. rhinoceros* larvae infected by NEP.

Figure 3. *O. rhinoceros* larva infected by *Heterorhabditios* sp Entomopathogenic Nematodes (NEP).

 The result of observation on the application of NEP on *O. rhinoceros* larval nest for 6 weeks is presented in Figure 4.

Figure 4. Percentage of alive, missing and NEP-infected dead larvae of *O. rhinoceros* with the treatment using 3.5 liters dilution of NEP on 6 weeks of treatment period.

Figure 4 shows the same symptom as in the application of MET (Figure 2). The loss of larvae is thought to be related to the food availability. The larvae are commonly found on the outside of the nest moving toward the new food sources. The percentage of larval mortality showed an increase from the 1st week (4%) to the 6th week (16%). The result is different compared with the result of previous study with the use of pots in which the larval mortality reached 100% on the 8th week (Indriyanti *et al*., 2017b).

 The results of this study indicate that speed of NEP in infecting the larvae is not comparable to the speed of larval move avoiding the NEP. NEP need time to find the natural holes in the body of the larvae, enter the body and then infect the larvae. The larvae infected with NEP are the larvae that do not move away from the location. Therefore, the addition of NEP to the nest can only control 16% of larval population. Nematodes have a killing effect on their host because NEP is a symbiotic with NEP-induced bacteria. According to Boemare (1996), the NEP of the Heterorhabditidae family is a symbiotic with the Photorhabdus sp. bacterium. The symbiotic bacteria that come out of the NEP after the penetration into the larval body release a toxin that causes the *O. rhinoceros* larvae to die (Kaya, 1993).

 The nematode killing ability is not only determined by the symbiosis between NEP and the symbiotic bacteria, but is also determined by the ability of the self-defense of *O.* *rhinoceros* larvae. In this study, the larval movement away from the nest is an effort to defend themselves from being infected with NEP. Environmental conditions such as temperature, sunlight intensity, humidity, and the rainfall are also important related to NEP activity in the soil. Temperature measurements in the study ranged from 30-330C, which is still possible for NEP activity. It is evidenced by the presence of dead larvae infected by NEP. According to Adam & Nguyen (2002), nematodes can increase their activity by 80% at a temperature of 21-30oC and decrease at a temperature of 12-16oC. The soil moisture range during the study was 49-70%. According to Suyanto et al. (2012), moisture is the most important thing that affects the activity of NEP in the soil. An apprpriate soil moisture ranges from 40-90%.

 NEP applications in the field were conducted in April-June with high rainfall ranging from 100-200 mm (Weather & Climate 2016). The continuous intensity of rainfall allows NEP to be carried by the rain. According to Manan & Suyanto (2009), not all NEP can enter the body of the larvae, this can be caused by environmental factors such as excessive rainfall that can wash away the NEP. So, the rainfall is indicated as the main factor of the low probability of NEP penetration to the body of the larvae.

**Application of the Mixture of *M. anisopliae* (MET) and Entomopathogenic Nematodes (NEP)**

 The result of observation showed a variety of symptoms of larval death, among which the larvae were infected by MET (Figure 1), NEP (Figure 3), and the mixture of MET and NEP in a single larval body. The larvae infected by the mixture of MET and NEP showing a symptom in form of the growth of the hyphae on the entire larval body except for the abdomen. The texture of the body hardened on the part where the hyphae grow, while the abdomen was mushy with the appearance of blackish brown color. According to Prayogo et al. (2005), the occurrence of hardening (mummification) in the body of the larvae of *O. rhinoceros* due to the tissue and body fluids of larvae has been used up by MET. The abdominal portion of *O. rhinoceros* larvae is suspected to be infected with NEP. It is in accordance with the statement of Sucipto, 2009: Indriyanti et al., 2017b) that the symptoms of insects infected by NEP are marked by a change of color, the body becomes tender because the tissue in the body becomes liquid. The appearance of larvae infected with MET and NEP is presented in Figure 5.



b

a

Figure 5. *O. rhinoceros* larvae infected by the mixture of MET and NEP. a) part of the body that is infected by MET, b) part of the body that is infected by NEP

 The result of observation on the application of MET and NEP mixture on O. *rhinoceros* larval nest for 6 weeks is presented in Figure 6.

Figure 6. Percentage of alive, missing and MET-infected dead larvae of *O. rhinoceros* with the treatment using the mixture of 100 g/m2 of MET and 3.5 liters dilution of NEP on 6 weeks of treatment period.

 Figure 6. shows almost the same result as the treatment using MET (Figure 2) and NEP (Figure 4). Many larvae are not found in the observed nest. However, the dead larvae found in this treatment (19%) are more than the ones found on the treatment using only MET (6%) or only NEP (16%). In percentage, there is an increase in the number of dead larvae. The mechanism suspected underlying the synergy between MET and NEP is that the condition of larvae that previously infected by MET or NEP will be weaker and more susceptible to the subsequent infections. This is consistent with the study by Ansari et al. (2008) showed that the MET-infected larvae will be weaker and vulnerable to the infection of NEP infection. This fact corresponds to a decrease in larval activity resulted in higher probability of NEP infection.

 Mixing the MET and NEP in this study is expected to accelerate the process of infection so that the process of controlling *O. rhinoceros* will be accelerated too. However, the results show that economically a mixture of MET and NEP is not favorable because the difference of larval mortality is small (3-13%). Therefore, it is suggested to choose only one kind of substances. In this treatment, many larvae show symptoms of MET and NEP infection, but the most found are NEP symptoms (mushy larval body). The result of the field test is very different compared with the semi-field test using pots, in which larval mortality reaches 100% at the 5th week. The benefit of research is for the recommendation of *O. rhinoceros* larvae control in the field.

**Conclusion**

The result of MET, NEP, and the mixture of MET and NEP application on *O. rhinoceros* larvae in field shows that there are many larvae missing from the nest and moving both horizontally and vertically. The larval mortality as a result of the application of MET, NEP, and the mixture of MET and NEP on the 6th week are 6%, 16%, and 19% respectively. Control using two biological agents (MET and NEP) is better than only MET or NEP.

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