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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 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., 2018). It has reportedly attacked the coconut plants in Africa, especially in Pacific Island (Jackson, 2009), in Malaysia (Manjeri et al., 2014), Arab (Khudhair et al. 2015). Jepara, located on the Shore of Java Sea, Indonesia (Indriyanti et al., 2018). We have studied *O. rhinoceros* in Jepara, Indonesia since 2015. The beetle destroy the coconut shoot, attacks on growing point of stem while the larvae live in the soil or dead coconut trunk (Moslim et al., 2011). Control larvae with pesticides causes various negative impacts on the environment such as soil, water, and air (Amalia & Yusa, 2018). One of the biological agent to control the larvae of *O.rhinoceros* is *Metarhizium anisopliae* (Latifian & Rad, 2012). It is a parasitic fungus 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 (Hajek & Delalibera, 2010; Divya & Sankar, 2009).

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The semi-field study was conducted in Jepara 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 the MET (2-7 weeks) or the 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 at al., 2017). Ecological research has never been done in Jepara, therefore it is necessary to do.

The 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 a 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.

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 collected 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.

The Application of *M. Anisopliae* (MET)

The 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 10^8 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.

The Application 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 10^6 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.

The Application of the Mixture of MET and NEP

The same procedure as the MET and NEP treatments were 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.

RESULTS AND DISCUSSION

The 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 Jackson & Jaronski (2009), mummification occurs in the larval body because all tissues and body fluids of larvae used up for the proliferation of MET. The observation results on larval infection symptom are presented in Figure 1.

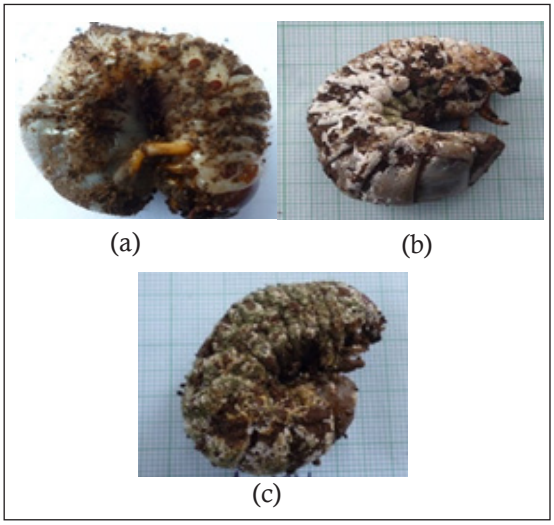

Figure 1. The Symptom of MET Infection on *O. rhinoceros* larva. (a) Dark Brown Necrotic Spot; (b) White MET Hyphae; and (c) MET Hyphae that Turned into Dark Green. The Hardened Body of the Larva.

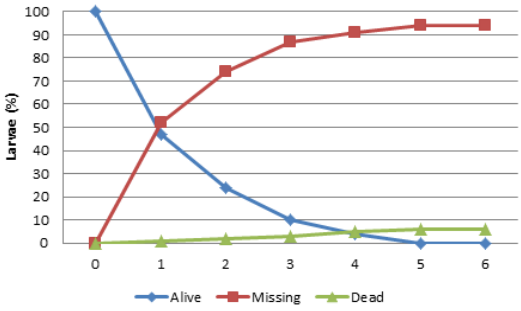

Figure 2. The Percentage of Alive, Missing and MET-Infected Dead Larvae of *O. rhinoceros* with the treatment Using 100gr/m^2 MET on 6 Weeks of Treatment Period.

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% in the 7th week (Latifian & Rad, 2012). 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 was related to the many-disappeared larvae from the treated nest. The death of larvae was caused by the fungal infection. According to Bedford (2013), the larval stages of *O. rhinoceros* actively looked for substrates that contained 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 the 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 moved 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 showed that the speed of MET infection was not comparable with the active movement of the larvae. The fungal conidia took at least 18 hours to germinate (viability), then stick to the skin, infect and breed in the body of larvae. It took at least two days for the MET to infect the larvae. This was 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 was ineffective. The larvae tended to move actively to avoid the passive mushrooms in the nest. In addition, the 3rd instar larvae have a thick cuticle, so, the MET required 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 Moslim et al. (2011), conidia viability is strongly influenced by environmental factors such as temperature, pH, moisture and water content of the media. The temperature in the Jerukwangi Village was 30-33^{0}C. According to Pracaya (2004), the ideal temperature for the growth of MET ranges from 22-27^{o}C. The presence of dead larvae infected by MET proved that the temperature ranging from 30-33^{o}C is still possible for the growth of MET. The soil pH in the study site ranged from 6.7 to 7, which was a proper pH for the growth of the MET. The soil pH at the time of MET application was very important because it was related to the work of MET enzymes. According to Paula et al. (2011), 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 was suspected to be the cause of low MET infection rate. Jackson & Jaronski (2009) reported that the pathogenicity of the MET decreased when the air humidity was below 86%. The intensity of light in this study ranged from 360-860 Lux because the location of the treated nest was in a place shaded by trees, so it was not too exposed to sunlight.

The MET-treated nest would be effective for early instar larvae. It would be more effective when the MET initially applied in the nest and the female put the eggs in that nest, then the eggs hatched releasing the 1st instar larvae. Those larvae would 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 were infected and died, then they could be made into an inoculum at the nest site.

The 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 became soft and so the tissue became aqueous. Changes in body color of *O. rhinoceros* larvae were due to the bacterial reaction of the *Photorhabdus* sp symbionts in the digestive tract of nematodes. The enzymatic activity of *Photorhabdus* sp bacteria caused the destruction of the larval tissue resulting in black dead larvae (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.

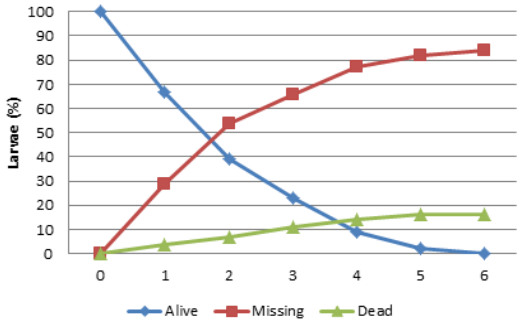

Figure 4. The Percentage of alive, missing and NEP-infected dead larvae of *O. rhinoceros* with the treatment using 3.5 liters dilution of NEP on 6 weeks of treatment period.

Figure 4 shows the same symptom as in the application of MET (Figure 2). The loss of larvae was assumed to be related to the food availability. The larvae were commonly found on the outside of the nest moving toward the new food sources. The percentage of larval mortality showed an increase in the 1st week (4%) to the 6th week (16%). The results were different compared with the previous study which used pots resulting in 100% the larval mortality on the 8th week (Latifian & Rad, 2012).

The results of this study indicated that speed of NEP in infecting the larvae was not comparable to the speed of larval move avoiding the NEP. The NEP needed time to find the natural holes in the body of the larvae, enter the body, and then infect the larvae. The NEP-infected larvae were those that did not move away from the location. Therefore, the addition of NEP to the nest could only control 16% of the larval population. The nematodes have a killing effect on their host because NEP is symbiotic with NEP-induced bacteria. According to Noguez et al. (2012), the NEP of the *Heterorhabditidae* family is symbiotic with the *Photorhabdus* sp. bacterium. The symbiotic bacteria coming out of the NEP after the penetration into the larval body release a toxin causing the O. rhinoceros larvae to die (Kaya, 1993).

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

The 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 allowed the NEP carried by the rain. According to Manan & Suyanto (2009), not all NEP could enter the body of the larvae, this was due to the environmental factors such as excessive rainfall that could wash away the NEP. Therefore, the rainfall was the main factor of the low probability of NEP penetration to the body of the larvae.

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

The observation results showed variety symptoms of larval death, among which the larvae were infected by the MET (Figure 1), the 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 showed a symptom in form of the hyphae growth on the entire larval body except for the abdomen. The texture of the body hardened on the part where the hyphae grew, while the abdomen was mushy with the appearance of blackish brown color. According to Jackson & Jaronski (2009), the occurrence of hardening (mummification) in the body of *O. rhinoceros* larvae was due to the tissue and body fluids of larvae which have been used up by the MET. The abdominal portion of *O. rhinoceros* larvae was suspected to be infected with the NEP. It was in accordance with the statement of Afifah et al. (2013) and Latifian & Rad (2012) that the symptoms of insects infected by the 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.

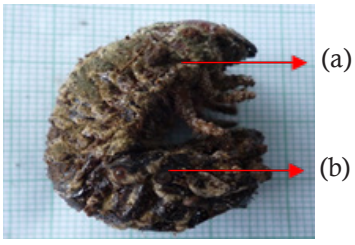

Figure 5. *O. rhinoceros* larvae infected by the mixture of MET and NEP. (a) part of the body that is infected by MET; and (b) part of the body that is infected by NEP

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

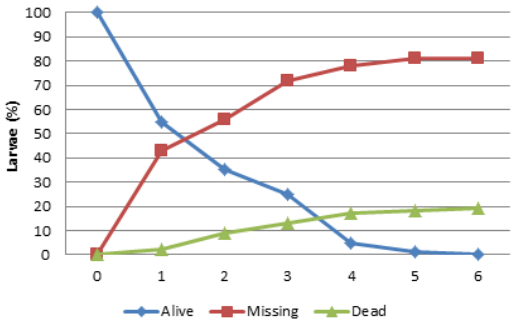

Figure 6. The Percentage of Alive, Missing and MET-infected Dead Larvae of *O. rhinoceros* with the Treatment Using the Mixture of 100 g/m^2 of MET and 3.5 Liters Dilution of NEP on 6 Weeks of Treatment Period.

Figure 6. shows almost the same results as the treatment using the 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%) (Figure 7). In percentage, there was an increase in the number of dead larvae. The mechanism suspected underlying the synergy between MET and NEP was that the MET or NEP-infected larvae condition would 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 corresponded to the decrease in larval activity resulted in a higher probability of NEP infection.

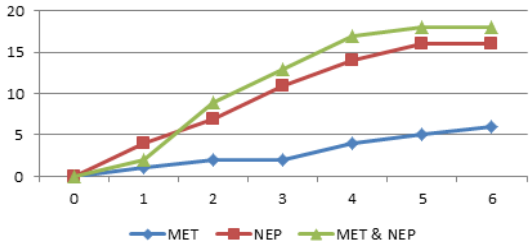

Figure 7. The Percentage of *O. rhinoceros* Dead Larvae with the Treatment Using the MET, NEP and MET + NEP on 6 Weeks of Treatment Period.

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

CONCLUSION

The results of MET, NEP, and the mixture of MET and NEP application on *O. rhinoceros* larvae in the field showed that there were 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. The control using two biological agents (MET and NEP) was better than the MET or NEP only.

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