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Study of Effectivity *Bacillus thuringiensis* Based Bio-Insecticide Against *Oryctes rhinoceros* Larvae at Shade House

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Abstract

Oryctes rhinoceros is an important pest in oil palm plantations. Adult stage of the beetle causes damage, however larval stage is very important to be controlled to break the cycle of life. An entomopathogenic bacterium Bacillus thuringiensis is one of biological agents to control these insects. Its toxic protein content provides specific insect targets as stomach poison. Objective of the research was to study the impact of B. thuringiensis propagated in bio-urine enriched with 5 per cent molasses towards Oryctes larvae. The research was carried out in the shade house of Plant Protection Study Program, Faculty of Agriculture, Sriwijaya University from August to November 2021. Experiment was designed in a randomized complete block design with 7 treatments and 4 replications. A total of 20 ml of bio-insecticide was dissolved in 280 ml of water, sprayed evenly on the soil mixed with male palm flowers as feed of larvae. The treatments were 6 isolates of B. thuringiensis *isolated from soil in oilpalm plantation, namely with codes: C14, C15, A15, OJ, BK, and LK as well. The results showed density of* B. thuringiensis *spores in bio-urine media was different in each isolate. The highest spore density in isolate code LK was 4.83 x 10^{10} spores/ml and the lowest (in isolate A15) was at 3.5 x 10^{10} spores/ml. Mortality rates were significantly different between isolate treatments starting from day 3 to day 12 of observation. C15 isolate lead the highest mortality rate of 100% on day 12 while other isolates showed mortality data below 100% (88-98%). Body weight and length showed significantly differences on days 0, 6 and 12 after application. Symptoms of infection begin with a change in skin color from white to brown, dark brown and black. Death is characterized by a soft body texture and wet rot.*

Keywords : Bacillus thuringiensis, *Bio-insecticide, oil palm, mortality,* Oryctes rhinoceros

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1. Introduction

Bacillus thuringiensis is an entompathogenic bacterium and has been used to control various species of insect pests. This bacterium is characterized by the presence of spores and proteins causing mortality in target insects. Proteins content of each isolate show different molecular weights and posses different toxicity in different insect species [1]. Because of its uniqueness, the usage of *B. thuringiensis* as a biological agent is increasing. Propagation of *B. thuringiensis* in waste media produces spores and proteins. Waste media contains sufficient organic matter to multiply *B. thuringiensis*. Several researchers have used industrial waste [2], [3], food waste [4] and other agricultural waste [5] as a medium for *B. thuringiensis* propagation. When it was tested to target insects and resulted high mortality of insects [6].

Oryctes beetle as one of important pests in oil palm plantations must be controlled [7]. These pests induce damage to oil palm plants by eating young leaves or coconut leaves causing the death of young plants [8]. Life cycle of oryctes beetle ranges from 6-9 months and adult live up to a year [9]. In the field, it is often found overlapping of generations, which means all life stages of oryctes are found in nature [10]. Thus, controlling larvae is very important. Therefore, the aim of research was to study the impact of *B. thuringiensis*-based bio-insecticide application

towards oryctes larvae in shade house.

2. Materials and Methods

The research was conducted in shade house of Plant Protection Department, Faculty of Agriculture Sriwijaya University, from October until December 2021. Temperature in shade house ranged from 29.58 – 40.72°C and humidity ranged from 58.26 - 82.57%. *B. thuringiensis* isolates were collected from oilpalm plantation in Riau (unpublished data) with their codes : C14, C15, A15, OJ, BK and LK as well. The growth medium of *B. thuringiensis* isolates was bio-urine enriched with 5% molasses [11]. The experiment was designed using a Randomized Block Design with 7 treatments (including control) and 4 replications.

2.1 Preparation of Bio-insecticide with active ingredient *B. thuringiensis*

Production of bio-insecticides was started with refreshing *B. thuringiensis* isolates on NGKG media [11]. One loop of *B. thuringiensis* colony was put into 10 ml of NB and be shaken for 12 hours at 200 rpm at room temperature. After 12 hours, 90 ml of NB was added and continuing be shaken for 12 hours. Seed culture was ready to use.

Media of bio-urine enriched with 5% molasses (100 ml) was prepared in an Erlenmeyer tube with a volume of 250 ml. After sterilization, 10 ml of seed culture was added on that media. Fermentation process to propagated *B. thuringiensis* was carried out for up to 72 hours, hereinafter the spore content was checked. Bio-insecticide was ready to be used for bioassay.

2.2 Preparation of test insects

Oryctes larvae were obtained from personal-owned oil palm plantations in Makarti Jaya village, Banyuasin district, South Sumatera province. Second instar healthy larvae were selected and separated in plastic box (d=20 cm, h=25 cm). They were fed with male oil palm flowers. The top of box was covered with gauze in order aeration was occurred. Breeding medium was sterile soil mixed with male palm flowers. It was checked every day for soil moisture. When soil humidity was low, it was sprayed by water. Every week, soil and feed were changed to ensure oryctes larvae get fresh food.

2.3 Applications *B. thuringiensis*-based bio-insecticide in shade house.

The soil was sifted and sterilized. A total of 2 kg of soil was placed in a box with sizes 35 x 25 x 15 cm. Larval feed was male flowers of oil palm. 15 g of feed was put on soil in that box and mixed well. Furthermore, 20 ml of bio-insecticide was diluted with 280 ml of water and sprayed on soil mixed with male flowers of oil palm. Then 10 individues of 3rd instar larvae were put into the box. Each treatment was repeated 4 times. Prior to application, larvae were fasted for about 1 hour and their body weight and body length were measured. Observations of larval mortality were carried out every day, while larval weight, infection symptoms and larval length were weighed every 6 days simultaneously with soil and feed replacement. Soil temperature observations were carried out twice a day.

Observations were done on spore density of each bio-insecticide with a different isolate code after 72 hours, mortality of larvae, symptoms of larval infection, larval body weight, larval length, soil temperature, temperature and humidity.

2.4 Data analysis

Data on larval mortality, larval body weight, larval length and soil temperature were analyzed by using analysis of variance (ANOVA). Tukey's Honest;y Significant Difference (HSD) Test was employed to test for significant differences among the treatments (isolates) at P=0.05. All data were analyzed using software of SAS University Edition 2.79.4M5.

3. Results and Discussion

Propagation of bio-insecticide with *B. thuringiensis* active ingredient was carried out in bio-urine enriched with 5% molasses. After 72 hours fermentation, spore density of each isolate was calculated. It was found all isolates produced spores in the range of 3.6 - 4.83 x 10^{10} spores/ml. The highest spore density was in isolates of *B. thuringiensis* with LK code at 4.83 x 10^{10} spores/ml and the lowest spore density was in isolate with A15 code of 3.50 x 10^{10} spores/ml (Figure 1.).

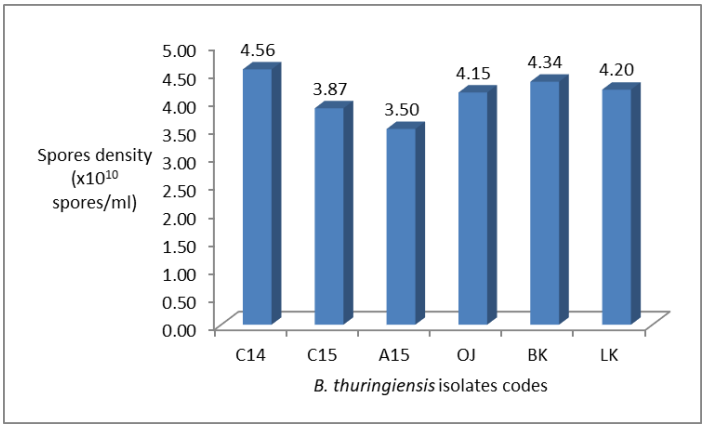

Figure 1. *B. thuringiensis* spore density propagated on bio-urine enriched with 5% molasses.

The choice of propagation media in the form of biourine was in line with the results of previous research which stated bio-urine media enriched with 5% molasses showed the highest level of spore density[11]. Therefore,

in this experiment, these waste materials were used. Six isolates of *B. thuringiensis* isolated from oil palm plantations were then propagated using selected media in the form of biourine enriched with 5% molasses. After calculation of spores density, it was found the highest one was in LK isolate and the lowest was in A15 isolate. Although there was a difference in spore density, in general development and propagation of *B. thuringiensis* into a bio-insecticide in this medium was very good. This is considering that *B. thuringiensis* propagation carried out on suitable media will produce large amounts of spores and even protein [12].

data of BK and C15 isolate codes. Although BK isolate had a higher spore density than C15 isolate (Table 1), mortality rate of Oryctes larvae was lower than that of C15 isolate. Therefore, the level of toxicity of *B. thuringiensis* is also influenced by the characteristics of spores and protein contents of *B. thuringiensis* isolate [15].

Symptoms of infection were starting to appear on the second day by changing in color from white to light brown followed by dark brown. On the 6th day, body color of larvae became increasingly black, followed by the process of changing body texture to become softer and watery.

Table 1. Mortality of Oryctes larvae after application of *B. thuringiensis*-based bio-insecticide

Treatments (isolates code)	Mortality of Oryctes larvae (%) days of (n=50 ind)..											
	1	2	3	4	5	6	7	8	9	10	11	12
C14	2	6	6 ab	26 b	42 b	66 d	86 d	90cd	90d	90cd	92 d	96 bc
C15	4	4	12 bc	54 c	74c	80 d	82d	82c	88d	90 cd	94d	100 c
A15	2	10	12 bc	30 b	46 b	52 cd	52c	56b	58c	62b	76bc	90b
OJ	4	16	28 c	84 d	92d	92 e	92d	94d	96d	98 d	98d	98 bc
BK	0	0	0 a	2 a	10 a	30 ab	30ab	38b	42b	62 b	70 b	88 b
LK	2	2	2 a	10 a	26 a	38bc	38bc	44b	58c	78bc	88 cd	94 b
Control	0	4	4 ab	4 a	18 a	18 a	18a	18 a	18a	18 a	18 a	18 a
F value	0.79 ^{tn}	1.4 ^{tn}	3.5 *	12.7 *	12.3 *	9.8 *	11.0 *	12.1 *	12.1 *	10.0 *	14.5 *	18.5 *
F Table	2.51	2.51	2.51	2.51	2.51	2.51	2.51	2.51	2.51	2.51	2.51	2.51
Tukeys HSD test	-	-	10.35	13.41	12.74	12.6	13.18	12.38	12.45	13.33	11.32	10.48

Notes : * significantly different, tn: not significabtly different. Values within a column followed by the same letters were not signifificantly different at P,0.05 according to Tukey's HSD test.

Larval mortality was observed from the first day after application until 100% mortality data were obtained. The results of statistical analysis were obtained no significantly different between mortality of test larvae on the first and second day observations after application (with a range of 0-16%). Significant difference in all treatments was found from day 3 to day 12 of observation. Oryctes larvae experienced the highest mortality (100%) on day 12 in treatment P2, namely *B. thuringiensis* isolate with code C15 (Table 1).

Mortality of *Oryctes* larvae was observed from the first day to determine the process of *B. thuringiensis* infection. On the third day onwards, significant mortality occurred in various isolate treatments. Among all isolates, mortality at LK isolate code started on the 4th day, while C15 isolate code had reached 54% and OJ isolate code had reached 84%. It indicated significant differences among three isolates. The mortality rate of Oryctes larvae was largely determined by abiotic and biotic conditions [13]. In homogeneous abiotic environmental conditions, internal factors or the nature of *B. thuringiensis* may cause a mortality rate [14]. This can be seen in the treatment

On the 12th day, larvae died with symptoms of the body becoming more fragile and rotting and emitting a foul-smelling liquid as well (Figure 2).

The process of mortality of oryctes larvae was started by the entry of spores into larval body through the food route [1]. The spores grow in hemolymph and midgut of larva. In midgut, protein will be degraded by protease to form smaller protein molecules [16], hereafter a binding process on the intestinal/midgut wall [12]. It will cause formation of pores in intestinal wall followed by the release of mineral elements inside and outside of midgut. These symptoms were followed by a process of reducing appetite and changing in integument color and body texture. All of these symptoms will end in death [17].

Larval length was measured before application and on the 6th and 12th day after application. Measurement of length was related to the growth and development of oryctes larvae. At the day of application (day 0) it can be seen from the measurement results the length of larvae were not significantly different, however on the 6th and 12th days all treatments showed a significant difference. At control (without B. thuringiensis treatment) larval length in-

creased although only slightly during the 12 days of observation (Table 2).

Figure 2. Symptoms of *B. thuringiensis* infected on Oryctes larvae, a). Symptoms of mild attacks change color from white to light brown, b). Color change to brown, c). Physical discoloration is blackened and emits a pungent odor, d) Larvae were blackened and the organs were ruptured and emit fluid and a foul odor and e) healthy larvae

Table 2. Average length of Oryctes larval after application of *B. thuringiensis*-based bio-insecticide

Treatments (isolates code)	Average length of Oryctes larval body (cm) days of …		
	0	6	12
C14	8,57	8,22 b	0,92 ab
C15	8,43	6,82 b	0,00 a
A15	8,36	8,46 b	8,60 cd
OJ	8,37	3,70 a	0,20 a
BK	8,88	8,69 b	5,05 bc
LK	8,49	8,66 b	5,15 c
Control	8,58	8,89 b	9,01 d
F value	1,261^{tn}	3,359*	3,445*
F Table	2,51	2,51	2,51
Tukeys HSD test	0,05	0,56	0,96

Notes : * significantly different, tn: not significantly different. Values within a column followed by the same letters were not signifificantly different at P,0.05 according to Tukey's HSD test.

The results of statistical analysis of larval length on day 0 were not significantly different, Oryctes larvae used were almost uniform, namely 3rd instar larvae. On the 6th and 12th day observations, all data on larval body length showed significant differences in all isolate treatments. At the time of entering 6th day, OJ isolate showed symptoms of larvae'mortality by 92% (Table 1). Number of surviving oryctes larvae was only 8% of all test larvae or as many as 4 individues. The remaining larvae showed symptoms of illness and not healthy which was proved by changing in color (Figure 2). This indicated *B. thuringiensis* has worked well as a stomach poison after being ingested by tested larvae as presented by [17]. In

addition to spores, there was a protein isolated from *B. thuringiensis* which works as a toxin [12]. The presence of both (spores and protein) will cause a faster death time and suitability of target insects [18].

Table 3. Soil temperature in each treatment box in shade house

Treatments (isolates code)	Temperature of soil (°C) day of											
	1	2	3	4	5	6	7	8	9	10	11	12
C14	34.30	31.10	31.90	30.30	28.60	32.00	32.10	30.60	30.60	28.60	30.00	31.90
C15	34.00	31.50	33.50	30.40	28.90	31.90	32.60	31.00	31.00	28.50	30.20	32.30
A15	32.00	30.00	34.80	31.30	28.80	32.10	31.10	31.00	31.00	28.60	30.50	32.30
OJ	32.00	30.50	34.60	30.70	28.70	31.60	31.90	30.40	30.40	29.10	29.80	31.70
BK	34.30	31.50	30.80	31.60	28.60	31.10	30.90	31.80	31.80	31.60	31.90	31.80
LK	34.80	30.20	29.60	31.20	28.40	31.40	31.20	31.90	31.90	32.20	31.50	31.80
Average	33.57	30.80	32.53	30.92	28.67	31.68	31.63	31.12	31.12	29.77	30.65	31.97

Table 4. Temperature (°C) and relative humidity (%) in shade house during observations

Observation day of	Temperature (°C)			Relative humidity (%)		
	Morning	Noon	Evening	Morning	Noon	Evening
1	36.40	40.00	35.00	64.20	57.00	60.20
2	30.40	37.50	28.30	87.20	61.20	79.40
3	30.00	40.00	33.10	83.50	54.40	60.00
4	26.90	44.50	30.10	99.90	64.40	68.20
5	26.50	37.70	30.30	99.90	55.00	65.00
6	27.00	36.80	34.40	91.00	61.90	62.00
7	33.20	43.20	27.70	74.80	57.80	72.00
8	28.10	40.90	28.00	72.20	56.40	74.00
9	29.60	43.00	30.30	83.00	58.10	66.00
10	27.20	41.20	34.70	77.30	59.10	62.00
11	31.50	42.30	34.90	73.40	53.80	65.00
12	28.10	41.50	35.40	84.40	60.00	61.00
Average	29.58	29.58	29.58	82.57	58.26	66.23

Soil temperature in each rearing container was measured every day during observations (12 days). Soil temperature in rearing container was fluctuated. The mortality of test larvae also fluctuated from day 1 to day 12 (Table 1). Soil temperature also fluctuated from 28.6-35.1°C (Table 3). The temperature and humidity of shade house were observed daily with air temperature ranging from 26.5-44.5 and air humidity ranging from 53.8-99.9% (Table 4).

Soil as habitat of Oryctes larvae played a very important role in the survival of Oryctes larvae [8]. If the temperature range is appropriate, microorganisms and insects can live optimally. In this case Oryctes larvae had an optimal temperature range of 27 - 29°C [9],[19]. In this temperature range, *B. thuringiensis* living in bio-insecticide grow optimally and develop in the body of Oryctes larvae. Therefore, *B. thuringiensis* isolates were effective in causing mortality in the test larvae.

4. Conclusion

The level of spore density of *B. thuringiensis* in bio-urine media enriched with 5% molasses varied differently in each isolate. Mortality rate was significantly different between isolate treatments starting from day 3 to day 12. Isolate C15 produced the highest mortality rate, namely 100% on day 12, while other isolates showed mortality data below 100% (88-98%). Body weight and length showed significant differences on day 0, 6 and 12 after application. Symptoms of infection begin with a change in skin color from white to brown, dark brown and black. Death was characterized by a soft body texture and wet rot.

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