Effectivity of Bacillus thuringiensis from Soil in Freshwater Swamps against Epilachna sp. Larvae

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Effectivity of *Bacillus thuringiensis* from Soil in Freshwater Swamps against *Epilachna* sp. Larvae

Efektivitas Bacillus thuringiensis Asal Tanah Rawa terhadap Larva Epilachna sp.

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ABSTRAK

Bacillus thuringiensis adalah bakteri entomopatogen yang berasal dari tanah dan telah banyak digunakan sebagai bahan aktif dalam pembuatan bioinsektisida. Serangga target sangat khusus dan sangat tergantung dari jenis kandungan proteinnya. *Epilachna* sp. merupakan serangga hama penting karena baik larva maupun imagonya berperan sebagai hama pemakan tanaman. Penelitian bertujuan untuk mengamati efektivitas *B. thuringiensis* terhadap larva *Epilachna* sp. Bio-insektisida *B. thuringiensis* dibuat dari isolat asal tanah rawa Sumatera Selatan (SMR04). Larva *Epilachna* sp dipelihara dengan menggunakan pakan daun takokak *Solanum torvum* di laboratorium. Rancangan yang digunakan adalah Rancangan Acak Lengkap, dengan 6 perlakuan dan 5 ulangan. Perlakuan berupa konsentrasi spora yang terkandung dalam larutan bioinsektisida meliputi: 1 x 10^8, 1 x 10^7, 1 x 10^6, 1 x 10^5 spora/mL, bioinsektisida komersil dan tanpa perlakuan sebagai kontrol. Setiap ulangan menggunakan 10 ekor larva instar kedua. Hasil penelitian dan hasil uji statistik menunjukkan mortalitas larva pada perlakuan *B. thuringiensis* berbeda nyata dengan perlakuan bio-insektisida komersial. Mortalitas tertinggi perlakuan bio-insektisida terjadi pada konsentrasi 10^8 spora/mL, yaitu sebesar 40,00% dan terendah pada konsentrasi 10^5 spora/ml yaitu sebesar 18,01%. Pada perlakuan larva, nilai LT_{50} terendah perlakuan bioinsektisida pada konsentrasi 10^8 spora/ml yaitu 79,37 jam. Pengendalian larva yang termasuk dalam ordo Coleoptera masih belum memuaskan, mengingat adanya kandungan protein pada *B. thuringiensis* strain SMR04 yang tidak sesuai dengan jenis protein yang dibutuhkan.

Kata kunci: *Bacillus thuringiensis*, bioinsektisida, *Epilachna* sp., mortalitas

ABSTRACT

Bacillus thuringiensis is an entomopathogenic bacterium isolated from the soil and has been widely used as an active ingredient in the manufacture of bioinsecticides. The target insects are very specific and depend on the type of protein content. *Epilachna* sp. are important insect pests because both larvae and adults as plant-eating pests. The research aimed was to investigate the effectivity of *B. thuringiensis* against the larvae of *Epilachna* sp.. *B. thuringiensis*- bio-insecticide was prepared using isolates originally from freshwater

swamp soil of South Sumatra (SMR04). *Epilachna* sp larvae were mass-reared with *Solanum torvum* leaf feed in the laboratory. The design used was a completely randomized design, with 6 treatments and 5 replications. Treatments were spore concentration contained in the bioinsecticide solution included: 1 x 10^8, 1 x 10^7, 1 x 10^6, 1 x 10^5 spores/mL, commercial bio-insecticide and without treatment as a control. Each replication used 10 individual of the 2nd larvae. Statistical test results showed larval mortality in *B. thuringiensis* treatment was significantly different from commercial bio-insecticide treatment. The highest mortality of bio-insecticide treatment occurred at a concentration of 10^8 spores/ml (40.00%) and the lowest was at a concentration of 10^5 spores/mL (18.01%). The lowest LT_{50} value of bio-insecticide treatment was at a concentration of 10^8 spores/mL, namely 79.37 hours. The control of larvae included in the Coleoptera order was still not satisfactory yet, considering the presence of protein content in *B. thuringiensis* strain SMR04 which did not match the type of protein required.

Keywords: *Bacillus thuringiensis*, bioinsectide, *Epilachna* sp., mortality

INTRODUCTION

Epilachna sp., a polyphagous insect pest, spread from Southeast Asia, South Asia to Australia (Kroschel et al., 2020) with the main host plant is family Solanaceae such as tomatoes, potatoes, chilies and eggplants. In India, the leaf-eating javelin beetle (*Epilachna* sp.) is reported to cause serious damage to the Solanaceae family (Ali et al., 2017; Patil and Gaikwad, 2019). In Indonesia, this pest is the main pest of *Solanum torvum* with its common name eggplant pipit, eggplant rimbang (Malay), takokak (West Java) and eggplant cepoka (Central Java). These plants grow wild whose fruit is used as a vegetable or spice and parts of the fruit, leaves and stems can be used as traditional medicine. This plant is one of the host plants for *Epilachna* sp. (Apriliyanto and Setiawan, 2019).

Farmers use insecticides mostly to control *Epilachna* sp. The use of insecticides is estimated to be a trend for the next few decades because of its broad spectrum. Moreover, they are very fast in killing insect pests (Supriadi, 2013). Improper use of pesticides can endanger the health of farmers and consumers, non-target insects (including predators, parasitoids, and pollinators) and have an impact on environmental pollution (Hanifa, 2013; Yuantari et al., 2013).

For this reason, it is necessary to try alternatives to reduce the use of pesticides through control using non-chemical substances. According to Brunner-Mendoza (2018), this method will not damage the environment and will not kill non-target organisms. In this case, a bio-insecticide with entomopathogenic bacteria *B. thuringiensis* used as an active ingredient.

B. thuringiensis isolate code SMR 04 was isolated from soil of freshwater swamps in Ogan Ilir district, South Sumatera Province. Soil sample was taken when swamp land was not cultivated by rice. This isolate was bioassayed against *Spodoptera litura* (Lepidoptera: Noctuidae) (Pujiastuti et al., 2018a), *Coptotermes curvignathus* (Isoptera: Termitidae) (Pujiastuti et al., 2018b) and other insects (Pujiastuti et al., 2020a).

These bacteria belong to the Bacillaceae family, which produces protein crystals during the sporulation phase (Valicente et al., 2010). These bacteria are entomopathogenic bacteria, with a specific target host so that they are safe against non-target insects. In general, these bacteria infect target insects using their spores and proteins. The specificity of the host is obtained because these bacteria produce proteins during sporulation. When the protein is ingested in the larval midgut, with the help of the protease enzyme, the protein will break down from large molecules into smaller proteins namely protoxins. This process occurs under conditions of high pH. If the pH conditions

are not suitable with required conditions, there will be no change from protoxin to toxin (Valicente et al., 2010). Based on the description above, a study was conducted to to investigate the effectivity of *B. thuringiensis* against the larvae of *Epilachna* sp.

MATERIALS AND METHODS

The research was conducted at Entomological Laboratory, Plant Protection Department, Faculty of Agriculture, Sriwijaya University. *B. thuringiensis* isolate used was an isolate from laboratory collections (*B. thuringiensis* SMR-04). The isolate was isolated from the soil of freshwater swamps. The test insects used were *Epilachna* sp. larvae from laboratory mass rearing.

The study was designed using a completely randomized design (CRD) with 6 treatments and each treatment was consisting of 5 replications. Each replication consisted of 1 petri dish containing 10 instar larvae. The *B. thuringiensis* treatments were :

L0 = Control (Aquades); L1 = Insecticide commercial (profenofos 1 ml/L); L2 = *B. thuringiensis* x 10^5 spores/mL; L3 = *B. thuringiensis* 1 x 10^6 spores/mL; L4 = *B. thuringiensis* 1 x 10^7 spores/mL; L5 = *B. thuringiensis* 1 x 10^8 spores/mL.

Preparation of Test Insects

Epilachna sp. was obtained in the eggplant garden in a vegetable center in Tanjung Pering Village, Inderalaya Utara District, Ogan Ilir Regency, South Sumatra. Adults were maintained in the laboratory, using a plastic container (d=10 cm, h=20 cm). The top of the plastic container was covered by gauze. Fresh takokak leaves were given as their feed every day. In order to get hygienic condition, everyday plastic container was cleaned from beetle dung. The test insects used were *Epilachna* sp. at second instar larval stage.

Preparation of Test Plants

The test plant used was the leaves of the *takokak* plant. The leaves were obtained from the wild-growing takokak plant. The leaves used were young leaves. The leaves were cut with the size of 8 x 8 cm. All leaves were changed every 24 hours during observation in order to investigate insect's ability to eat leaves after the application of bioinsecticide.

Preparation of *Bacillus thuringiensis*

The formulation of bio-insecticide was started with the preparation of seed culture [9] by placing one lope of *B. thuringiensis* Isolate SMR-04 in 10 mL of Nutrient Broth (NB) media, and was shaken for 12 hours at 200 rpm. Then, 5 mL of this culture was taken and transferred to 10 mL of NB media and was shaken for 12 hours at 200 rpm. Seed culture was were poured into Erlenmeyer flask contained with 100 ml of NB as growth media of B. thuringiensis. The growth media were then agitated in the shaker at 200 rpm for 72 hours. Spores counting was conducted by using Haemocytometer at 400 x magnification before application. The treatment used was a bioinsecticide solution with spore density of: 1 x 10^5 spores/mL, 1 x 10^6 spores/ mL, 1 x 10^7 spores/mL, and 1 x 10^8 spores/ml.

Application of Bio-insecticide to Test Insects

Application of bioinsecticide was carried out by spraying a *B. thuringiensis*-based bioinsecticide solution according to the dosage on the prepared takokak leaves. One leaf was sprayed twice, namely on the front and back of the leaves with a spray volume of 1 mL each. The leaves that had been sprayed were dried and put into a petri dish. After that, 10 larvae were infested per replication. Furthermore, observations were made every 8 hours for 7 days for larvae. The non-dead larva stage test insects were counted and their development was

observed until they formed pupae and/or adult. To calculate mortality, the following formula was used: P= a/b x 100%, in which P= mortality, a= number of larvae died and b= number of all larvae observed. In addition, from the mortality results, LT_{50} value and the percentage of larvae became pupa and adult were calculated.

Infection Symptoms of *Epilachna* sp. Larva

Observations were made by observing changes in behavior, symptoms of illness (indicated by reduced appetite) and mortality of test larvae due to application of bioinsecticides. These were done every 8 hours for 7 days.

Leaf Damage Intensity

Observation of the intensity of leaf damage was carried out 7 times. There was a method of observation by counting damaged leaves and categorizing them into each damage scale and counting the number of leaves for each observed leaf. To calculate the intensity of damage, the formula is used:

$$I = \frac{\Sigma (ni \times vi)}{Z \times N} \times 100\%$$

I = intensity of leaf damage (%),
vi = Value (score) of leaf damage based on the area of the affected leaf, namely:
n = The number of leaves that have the same damage value (score)
Z = the highest score
N = Number of leaves observed
0 = No damage at all
1 = Area of leaf damage> 0 - ≤ 25%;
2 = Area of damage to leaves> 25 - ≤ 50%;
3 = Area of damage to leaves> 50 - ≤ 75%;
4 = Area of leaf damage> 75 - ≤ 100%

Data Analysis

Larval mortality data were analyzed using Analysis of Variance (ANOVA) with Ms. Excel 2010, while LT_{50} was calculated using probit analysis with SPSS 16.00.

RESULTS

Mortality of *Epilachna* sp. Larva

Mortality test of Epilachna larvae used second instar in which the length of larvae was were 1.0-1.2 cm in size. Bioinsecticide application was carried out by spraying evenly on the leaf surfaces. Duration of observation was 7 days. The highest mortality was in the treatment of 10^8 spores/ml (40%) (Table 1).

Table 1. Mortality of *Epilachna* sp. larvae due to application of *Bacillus thuringiensis*

Treatment	Mortality (%)
L0 (Control/aquadest)	2.02^{a}
L1 (*Bt* Commercial)	99.98^{e}
L2 (Bt 1 x 10^5 spores/mL)	18.01^{b}
L3 (Bt 1 x 10^6 spores/mL)	22.00^{bc}
L4 (Bt 1 x 10^7 spores/mL)	32.00^{cd}
L5 (Bt 1 x 10^8 spores/mL)	40.00^{d}

Note: The numbers followed by different letters in the same column were significantly different at DMRT 5%

Symptoms of Attack and Death of Test Larvae

Observations of tested insects indicated within a few hours of application, larvae began to eat the leaves treated with *B. thuringiensis*. When eating leaves exposed to *B. thuringiensis*, there was no painful reaction showed by larvae. After 24 hours of feeding, larvae began to show symptoms of weakness, consumed fewer leaves, and seemed to become inactive. The last symptom was the number of spines of larval body were damaged and gradually they stopped moving (Figure 1).

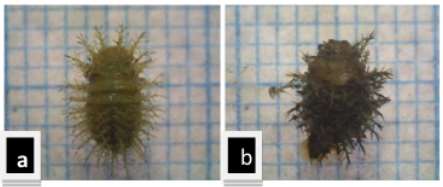

Figure 1. *Epilachna* sp.

Note: (a) Healthy larvae, (b) *Bacillus thuringiensis*-infected larvae

Leaf Damage Intensity

Observation of intensity of leaf damage was carried out 24 hours for 7 days. There was a difference in the intensity of leaf damage. The highest damage was on L5 treatment on the second day (85%) while the lowest one was on 7th day (0%) (Figure 2).

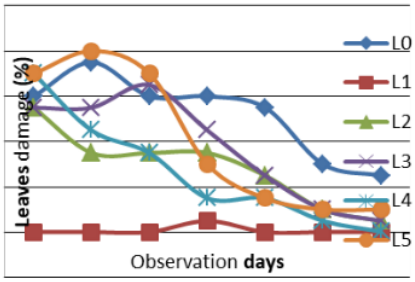

Figure 2. Leaves damage (%) consumed by *Epilachna* sp. larvae during 7 days observation

In the 3-first days, the highest damage intensity occurred in L5 (10^8 spores/ml). It can be assumed infested larvae in each treatment entered instar 4 earlier than other treatments. Kaleka et al. (2019) reported 4th instar was more resistant to possible environmental stresses and possessed a very high feeding ability. However, the higher the number of leaves eaten, the more the amount of B. thuringiensis entered the larva's body. Therefore, leaf damage of L5 was highest compare to other treatments (Table 2).

Table 2. Average of leaves damage (%) consumed by *Epilachna* sp.

Treatments	Average of Leaves Damage (%)
L1 (Bt Commercial)	52.14 [d]
L2 (Bt 1 x 10^5 spores/mL)	0.89 [a]
L3 (Bt 1 x 10^6 spores/mL)	28.57 [bc]
L4 (Bt 1 x 10^7 spores/mL)	37.14 [c]
L5 (Bt 1 x 10^8 spores/mL)	26.4 [b]
L1 (Bt Commercial)	40.7 [c]

Note: The numbers followed by different letters in the same column were significantly different at DMRT 5%

Lethal Time Value (LT_{50})

The LT_{50} value was a numerical value (in the form of time) which indicated 50 percent mortality of tested insects. The lowest LT_{50} value was achieved in the positive control treatment (commercial insecticide) at 88.69 hours. In treatment application, the lowest LT_{50} was L5 treatment (79.37%) (Table 3).

Table 3. Lethal Time (LT_{50}) value of *Bacillus thuringiensis* treated to larva Epilachna sp. larvae

Treatments	LT_{50} (h)	Fiducial Limits (h)	
		Lower Limits	Upper Limits
L1 (Bt Commercial)	88.69	61.18	118.33
L2 (Bt 1 x 10^5 spores/mL)	173.68	160.12	189.02
L3 (Bt 1 x 10^6 spores/mL)	149.78	138.00	162.84
L4 (Bt 1 x 10^7 spores/mL)	110.31	100.11	120.97
L5 (Bt 1 x 10^8 spores/mL)	79.37	69.06	89.52

Percentage of Pupa and Adult Formation

Most of the test larvae used did not experience death. They were still alive and able to continue to the next stadium, namely pupae. This was followed by the number of pupae which turned into an adult. The changes of larvae into pupa and adult were presented in Table 4.

Table 4. Number of pupae and adult of Epilachna from alive treated larvae

Treatments	Larvae to Pupae (%)	Pupae to Adult (%)
L1 (Bt Commercial)	97.98[e]	97.9[8e]
L2 (Bt 1 x 10^5 spores/mL)	0.03[a]	0.03[a]
L3 (Bt 1 x 10^6 spores/mL)	82.00[d]	62.00[d]
L4 (Bt 1 x 10^7 spores/mL)	78.00[d]	58.00[d]
L5 (Bt 1 x 10^8 spores/mL)	68.00[c]	38.00[c]
L1 (Bt Commercial)	60.00[b]	28.00[b]

Note: The numbers followed by different letters in the same column were significantly different at DMRT 5%

DISCUSSION

Mortality of larvae was significantly different among treatments. These results may occured because the death larvae was caused by the number of spores consumed by Epilachna larvae. In the treatment with the highest density of 10^8 spores/mL (L5), the highest mortality was 40%. This was

supported by Pujiastuti et al. (2020b) which stated a large number of spores ingested in the larvae's midgut will cause many deaths. In the midgut, proteins present in Bt were broken down into smaller molecules, which were called toxins (Sansinenea, 2012.). At that time, the toxin will be absorbed into midgut membrane layer and cause membrane proliferation. With increasing time, the larvae will experience to die. Death or mortality of test larvae due to consuming spores and proteins derived from *B. thuringiensis* usually occurred within 24 hours of consuming these substances. Pujiastuti et al. (2018a) reported tested larvae can show symptoms of death within 24 hours of application. This showed that during that time, the proteins contained in *B. thuringiensis* cells began to react and cause poisoning in the midgut. In addition, *B. thuringiensis* spores also began to grow in the test larvae, leading to the death process.

In Figure 2, it can be seen control treatment (L0) experienced the highest damage on the second day. This showed that without treatment of *B. thuringiensis*, the test larvae consumed large amounts up to 70%. In this application treatment, damage of leaves has decreased. It was suspected larvae was entering the feeding rest phase to molting. In the treatment with chemical insecticides, it appeared damage of leaves almost reached 0 %, which was meant tested larvae did not want to consumed leaves exposed with chemical insecticides. In this case, several studies have shown similar results, such as chemical insecticide treatment can kill the test insects quickly (Supriadi, 2013; Smith et al., 2018). In addition to quickly killing the tested insects, the use of chemical insecticides also showed target insect mortality will be more varied considering of broad-spectrum (Chowański, 2014). Leaf damage decreased on the fifth, sixth, and seventh days. In addition to mortality, the larvae have undergone a resting phase of eating at the time of molting and entering the pupa phase (Khaliq, 2014). In the insecticide treatment, the intensity of damage was low, but the mortality of larvae was the highest compared to other treatments (Table 1). This could be suspected insecticide applied to the leave was evaporated. According to Damalas et al. (2011), this occured because of low humidity and high temperature. This vaporized insecticide can be inhaled by the test insects leading to death.

In Table 4, there was a significant differenrence among treatments in treating larvae into pupa and pupa into adult. Compared with control (no treatment) and positive control (commercial bioinsecticide), all treatments showed significantly different. Likewise, in the change from pupa to adult phase, there was a similar tendency of larvae to become pupa. However, it appeared treatment of 10^5 spores ml showed the highest rate of change in larvae became pupa (82%) and pupae became adult (62%). This showed that a small number of sporeswill cause low mortality (Pujiastuti et al., 2020b). Low mortality in larval treatment caused a chance of larvae to live and change to pupal stage. The change was strongly influenced by metabolic process in the larva's body (Kaleka et al., 2019). If there was a disruption in metabolic process, larvae will face difficulty turning into a pupa. This was in line with the opinion of Jouzani et al. (2017) which reported *B. thuringiensis* also inhibited the growth of insect pests, especially when pupae will hatch into adult. It approved that some larvae which enter pupal phase will turn into an adult (Kaleka et al., 2019).

CONCLUSION

The highest mortality of bio-insecticide treatment occurred at a concentration of 10^8 spores/ml (40%) and the lowest was at a concentration of 1 x 10^5 spores/mL (18.01%). In larvae treatment, the lowest LT_{50} value was at a concentration of 1 x 10^8 spores/mL, namely 79.37 hours. Controlling larvae of Coleoptera order was

not satisfactory, considering the presence of protein content in *B. thuringiensis* strain SMR04 which does not match the type of protein required.

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