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Larvicide effects of *Bacillus* sp isolated from soil against fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract

Prolonged use of pesticides was known to have a negative impact on the environment, health, and living organisms. Therefore alternative pest control techniques need to be developed continuously. One pest eradication techniques that is believed to be safe for the environment is biological control. This study intended to evaluate the toxicity properties of 7 different isolates suspension of *Bacillus* sp. isolated from soil in a botanical garden in West Lampung against *Spodoptera frugiperda* larvae. Morphological characterization of protein crystals which are toxic to insects contained in the suspension was carried out microscopically at a magnification of 1000x. Then, each isolate suspension was tested for its toxicity properties on the 3rd instar larvae of *S. frugiverda*. The protein crystal morphology of the *Bacillus* sp isolate coded TB7 was similar to that of *L. sphaericus*. While the other six isolates (TMA 26, TBA 4, TBA 7, TB 5, BP 14, and TSR 6) had morphological characteristics similar to that found in the *B. thuringiensis*. The results of larvicide test showed that *Bacillus* sp isolates coded TMA 26, TBA 7, BP 14 and TB 5 caused mortality on day 3. TBA 4 isolates killed larvae on day 4, while isolates coded TSR 6 and TB 7 killed larvae on day 5. In conclusion, *Bacillus* sp isolate isolated from soil has toxic properties to the larvae of *S frugiperda*. So it is suggested that the bacterial isolate *Bacillus* sp can be used as a biological control agent for insect pests.

Keywords: Ulat Grayak; Fall Armyworm; *Spodptera frugiperda*; Biological Control Agent; *Bacillus* Bacteria

1. Introduction

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is an invasive insect pest native to the tropical regions of the western hemisphere from the United States to Argentina. This insect pest, which in Indonesia is known as *ulat grayak*, has spread in various country in the world and damaged 80% of crop plant including maize, sugarcane, rice, sorghum, vegetables, cotton, and grasses [1, 2].

Armyworm is a polyphagous insect with the plant of family Gramineae as the most preferred host. Plant parts that are commonly infested by *S. frugiperda* are the shoot meristematic tissues and young leaves. Another trait that distinguishes this insect from other insect pests is that it is unable to go dormant in extreme environmental conditions [3, 4].

So far, plant pest control in Indonesia is still very dependent (95.29%) on chemical pesticides. This is because its use is considered more effective, easy, and economical. However, prolong use of synthetic pesticides is known to leave residues that accumulate in the soil which then have a negative impact on health, the environment and soil organisms and the surrounding plants [5, 6].

One strategy to reduce or eliminate the adverse effects of using synthetic pesticides is to apply biological control [7].There are many types of biological agents that can be used in controlling plant pest including microorganisms such as entomopathogenic bacteria. acteria of *Bacillus* spp. such as *Bacillus cereus*, *Bacillus anthracis* and *Bacillus*

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thuringiensis are among the entomopathogenic that can be used as biological control agents. These bacteria are known to produce protein crystals, the secondary metabolites, that toxic on certain insects.The bacterial *toxins* include enterotoxin (Cereulide), bipartite exotoxins: protective antigen-lethal factor (PA-LF) and PA-edema factor (PA-EF), Cry and Cyttoxin [8].

There are several researchers in Indonesia who have proven the potential of *Bacillus* sp. as a biological pest control agent on the armyworm, *S. frugiperda.* By using *Bacillus* sp. isolate of SLBE2.1BB strain, Adwiyah (2022) found that the secondary metabolite extract of the bacteria causing mortality in *S. frugiperda* larvae up to 35% [9]

Furthermore, Mahmud (2022) reported that a bioinsecticide extracted from *B. thuringiensis*was effective in killing armyworms with a mortality of up to 70-80% [10]. Another study used the same bacterial strain found that administration of *B. thuringiensis* suspension for 36 hours caused 88% mortality of the fall armyworm larvae [11].

Our research whose results are presented in this paper is intended to evaluate the toxic effects of secondary metabolites extracted from 7 different isolates of *Bacillus* sp. isolated from soil in a botanical garden in Lampung, Indonesia.

2. Material and methods

2.1. Bacterial isolates

The bacterial isolates used in this study consisted of 7 *Bacillus* sp isolates isolated from the soil in Liwa Botanical Gardens, West Lampung Regency of Lampung Province, Indonesia. The seven bacterial isolates (isolate codes: TMA 26, TSR 6, BP 14, TBA 7, TBA 4, TB 7, and TB 5) were then inoculated with nutrient agar (NA) and incubated for 24 hours at room temperature. The seven isolates were coded as follows: TMA 26, TSR 6, BP 14, TBA 7, TBA 4, TB 7, and TB 5. Furthermore, macroscopic observations were made of the colony morphology including form, margin, color, and elevation.

2.2. Characterization of protein crystal morphology

Morphological characterization of bacterial secondary metabolite protein crystals following the staining technique introduced by Sharif and Alaeddinoĝlu(1988) using coomassie brilliant blue solution [12].The composition of the coomassie brilliant blue solution is as follows: 0.1% coomassie brilliant blue dye, 30% methanol, and 5% acetic acid. Protein crystal staining was started Staining of protein crystals begins with taking one loop of 72-hour-old Bacillus sp. isolate which is inoculated on a sterile glass object.

Furthermore, the preparations were fixed by passing them over a Bunsen flame. After that, coomassie brilliant blue dye was added and left for 3 minutes. The object then is rinsed under running water and dried. Observations were made under a microscope with a magnification of 1000x. The protein crystal will stain blue because it binds the coomassie brilliant blue dye, while the spores will not because they cannot bind the dyes [13, 14]..

2.3. Bioassay test against fall armyworm larvae

Insect pest tested in this study were 3rd instar larvae of fall armyworm *S. frugiperda.* A total of 24 larvae were divided into 8 groups, consisting of 7 groups according to the isolate code (type) and 1 control, 3 worms each. The test larvae were put into a container and fed cabbage that had been smeared with isolate suspension of *Bacillus* sp. at a concentration of 3x10^{8}cells/ml. The concentration is determined by equalizing the turbidity level of the bacterial suspension in liquid medium with Mc Farland 1 solution. Then, the container is covered with clear plastic and given sufficient air holes. The time of larval death was observed for 6 days [15].

2.4. Data analysis

The results of this study are presented in the form of photos and numbers which are then analyzed descriptively.

3. Results and discussion

3.1. Morphological characteristic of protein crystal

Microscopic characteristics of protein crystal morphology of 7 bacterial isolates of *Bacillus* sp. stained with coomassie brilliant blue solution at 1000x magnification are presented in Figure 1. The blue color of protein crystals given coomassie brilliant blue dye is formed through ionic interactions between the sulfonate groups and the positive amino

groups. However, the spores cannot bind the dye so that the spores are colorless. With regard the protein crystals outside the spore, it is caused by an alkaline pH which causes autolysis of the spores [13].

Bacterial groups of *Bacillus* sp. which are known to have a distinctive character in forming protein crystals are *B.thuringiensis* and *Lysinibacillus sphaericus* [16,17].Protein crystals extracted from *B. thuringiensis* have toxicity properties against the Order Diptera, Coleoptera and Lepidoptera. Meanwhile, protein crystals produced by *L. sphaericus* showed a toxic effect on mosquito larvae, especially *Culex* sp. and *Anopheles* sp. [8, 18]

The morphology of *L. sphaericus* observed using phase-contrast microscopic techniques has the characteristics of a sphaerical (round) shape, spores located terminally, and its sporangia are swollen. In contrast, *B. thuringiensis* has the characteristics of broad bacterial cells with spore-shaped ellipses, spores situated sub-terminally, un-swollen sporangia and the protein crystals appear darker than the spores [16].

The results of the crystal characterization carried out in this study showed that the protein crystal morphology of the *Bacillus* sp isolate coded TB7 was similar to the protein crystals of *L. sphaericus*. While the other five Bacillus sp isolates (TMA 26, TBA 4, TBA 7, TB 5, BP 14, and TSR 6) had the following morphological characteristics. Protein crystals are found at the end of the bacterial cell, spaced apart from the spores, and are oval and solid round in shape. In general, the morphology of the protein crystals formed by the five isolates is similar to that of *B. thuringiensis* protein crystals.

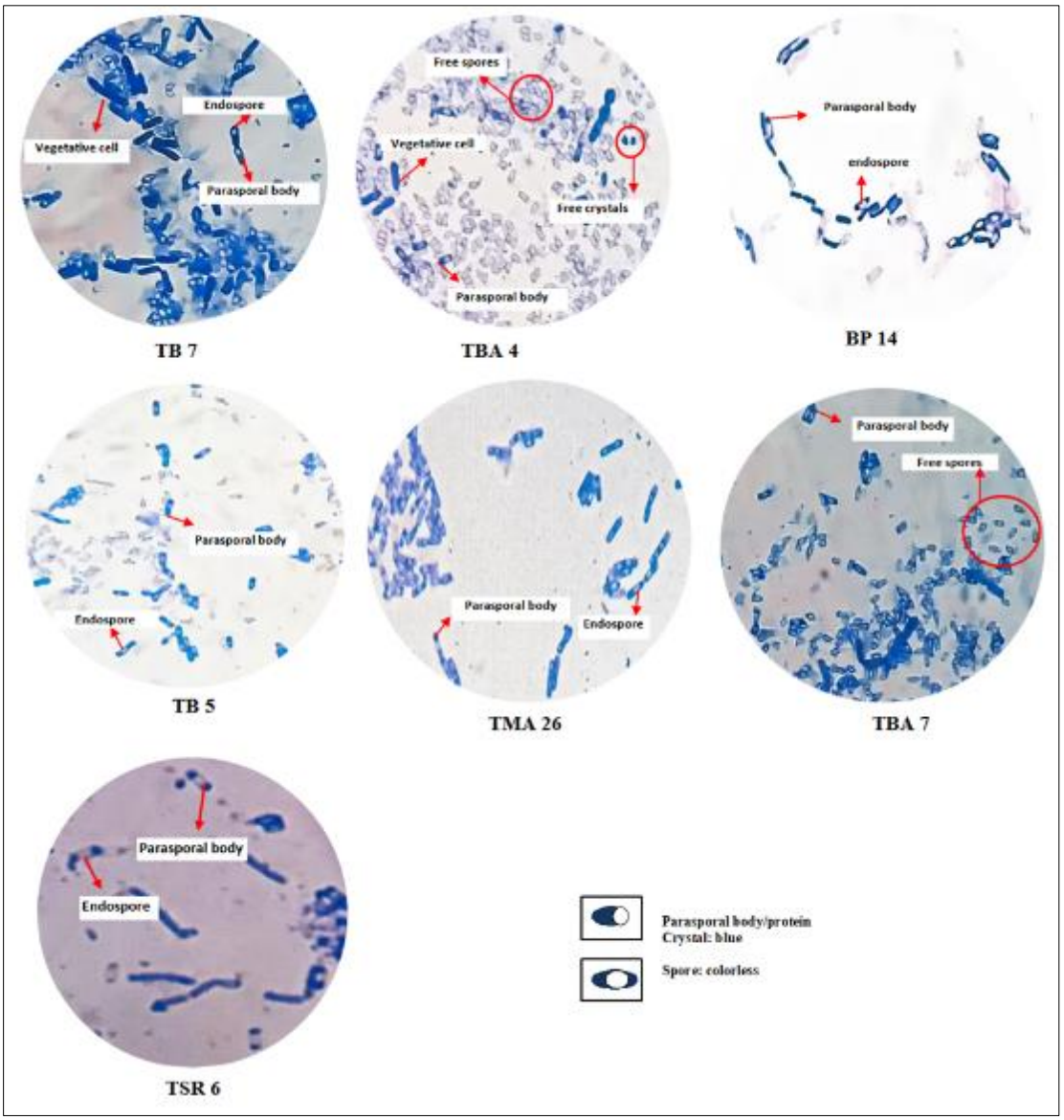

Figure 1 Morphological characteristic of protein crystals of *Bacillus* sp isolates under microscope at a magnification of 1000x.

The protein crystals can form crystal inclusions in various shapes including bipyramidal, cuboidal, round, oval and irregular. The spherical protein crystal type *Cry4* found in *B. thuringiensis* ssp. *israelensis* (Bti) known to have toxicity activity against the insect of order Diptera. Whereas the bypiramidal and cuboidal protein crystals extracted from *B. thuringiensis* var. *kurstaki* revealed to have toxic effect on Lepidopteran and Coleopteran insects [19,20].

The bacterium *B. thuringiensis* synthesizes protein crystals during sporulation, precisely in the stationary phase. This protein is known as ICP (insecticidal crystal protein) or δ-endotoxin which has toxicity to target insects, namely the Diptera, Lepidoptera and Coleoptera orders [21-23].

There are 8 classes of protein crystals known to be made by *B. thuringiensis*, namely CryI is toxic to the Order Lepidoptera, CryII is toxic to the Order Diptera and Lepidoptera, CryIII is toxic to the Order Coleoptera, CryIV is toxic to the Order Diptera, CryV is toxic to the Order Lepidoptera and Coleoptera, CryVI is toxic to Nematodes, CryIX is toxic to the Order Lepidoptera and CryX is toxic to the Order Lepidoptera [24].

The optimistic view that *B.thuringiensis* can be used as a biocontrol agent against Lepidoptera and Diptera insects because it produces δ-endotoxin is strengthened by the results of the bacterial protein crystal bioassay test for 48 hours against *Plutella xylostella* reported by Ervina 2020. Meanwhile, the toxicity activity of the *Binary toxin* (*Btx*) from *L. sphaericus*against *Culex quinque fasciatus* and *Aedes aegypti* was revealed by Santana-Martinez (2019) using formulation of vegetative cells of the bacteria [14, 25].

3.2. Larvicide effects of the isolates

Bioassay test of 7 bacterial isolates suspension of *Bacillus* sp against *S. frugiperda* insect carried out in this study proved to cause mortality on the larvae. The mortality responses of the test larvae are characterized by changes in physical appearance of the larvae such as changes in body color from greenish to black (melanization), shrinking and undergoing lysis (Figure 2).

A B C

Figure 2 Photographs showing mortality responses of 3rd instar of *S. frugiperda* larvae treated with *Bacillus* sp isolates. (A) Physical appearance of the larvae before treatment, (B) Shrunk and melanized body of dead larvae, (C) Lysed larval body

The characteristics of the death response are in accordance with the results of previous studies by other researchers which stated that the bodies of *S. frugiperda* larvae that died from the toxin from Bacillus sp isolates would be brown-black in color, wrinkled, curved, dry, and stiff [26]. Changes in the physical appearance of the larvae body are very likely related to the damage and swelling of the digestive tract. Then, the larval body will undergo melanization and lysis in a few days later [19].

There are several initial symptoms shown by larvae infected with bacterial isolates, namely: less active movements, decreased appetite, diarrhea, and discharge from several parts of the body [27] The death of caterpillars after administration of Bacillus thuringiensis isolate is most likely due to δ-endotoxin affecting the digestive system of insects (larvae) and disrupting the osmotic balance of their cells [14].

Furthermore, the number of deaths and lethal time of larvae due to administration of Bacillus bacterial isolate suspension can be seen in Figure 3.

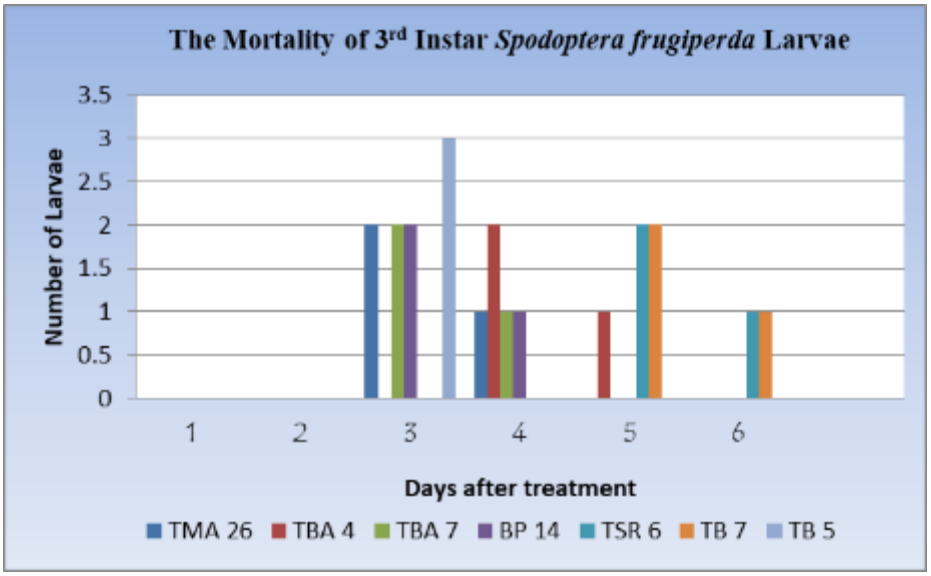

Figure 3 Mortality numbers and lethal time of *S. frugiperda* larvae by *Bacillus* sp. isolates suspension treatment

The numbers on the bar chart in Figure 3 can be described as follows. Bacillus isolates coded TMA 26, TBA 7, BP 14 and TB 5 showed the shortest lethal time where death occurred on day 3. TBA 4 isolates killed larvae on day 4, while isolates coded TSR 6 and TB 7 killed larvae on day 5.

The difference changes in larval body structure after application of Bacillus sp suspension were influenced by the duration of the toxicity mechanism needed to disrupt the digestive system, especially on the epithelial cells of the larval intestine. The duration of toxicity mechanism also depend on the amount, shape, and size of protein crystal ingested by the larvae. However, in this study, the amount, shape and size of the crystal that enters the digestive tract of the larvae unknown.

It is suspected that the mechanism of toxicity occurs on day 1 to day 4 after application. Protein crystals that enter the digestive tract with food experience toxin activation by environmental factors. Protein crystals that were originally protoxins are converted into toxins by protease enzymes. Then, the toxin attaches to receptors on intestinal epithelial cells. As a result, the digestive tract disrupted leading to mortality of the larvae [26].

The other determinant toxicity factor of *Bacillus* isolates suspension on *Spodoptera frugiperda*is the ability of the bacteria in synthesizing protease, chitinase, and lipase enzymes. These three enzymes synergize with each other in degrading body tissues of larvae that are exposed to the toxin. The protease enzyme activates the protoxin to become a toxin that damages the digestive tract of the target insect. In addition, protease and lipase also simultaneously degrade the protein and fat components in the insect's body. Protease enzymes are able to degrade proteins making up the basement membrane resulting in melanization [28, 29].

Overall, there are several factors affecting mortality of the test larvae namely the type of bacterial isolates, amount of protein crystal digested by the insect, temperature, pH, larvae condition, and technical aspect in treating the larvae. Bacterial factors that influence the toxicity mechanism include protein and spore crystal structures, molecular size, and amino acid composition in crystals that are toxic to target insects [19, 30].

4. Conclusion

Suspension of bacterial isolates of *Bacillus* sp isolated from soil in Liwa Botanical Garden containing protein crystals with morphological characteristic similar to that of found in *L. sphaericus* and *B. thuringiensis*. All of the seven isolates tested for the toxicity properties against fall armyworm larvae *Spodoptera frugiperda* showed larvicide effects with lethal time ranging from 3 to 5 days. Thus, it can be suggested that *Bacillus* bacteria can be used as biological control agents against insect pests.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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