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Isolation and toxicity test of *Bacillus thuringiensis* from Sekayu region soil, South Sumatra on *Spodopteralitura*

S R Afriani[1], Y Pujiastuti[1*], C Irsan[1] ,N Damiri[1] S Nugraha[2] and E R Sembiring[2]

[1)] Study Program of Crop Science, Post Graduate Program, Sriwijaya University, Palembang, South Sumatra, Indonesia
[2)] Research Center for Biotechnology, Indonesian Institute of Sciences, Jakarta, Indonesia

*Email: ypujiastuti@unsri.ac.id

Abstract. This study aimed to obtain bacterial isolates *B.thuringiensis* potential as a biological control against pests *Spodoptera litura*. The research was conducted at the Laboratory of Pest and Disease Department, Agricultural Faculty of Sriwijaya University, Campus InderalayaOgan Ilir, South Sumatera, from March to June 2017. The study was conducted with survey method and laboratory trial. The results showed that of the 50 soil samples from three villages selected through morphological observation, reaction staining, KOH test, catalase test, producing 13 bacterial isolates. Screening of the 13th toxicity of the isolates suspected *B.thuringiensis* against *S. litura* larvae was investigated. Based on the toxicity screening test the following facts were obtained: five isolates ie KJ_2M_2, KJ_3E1, KJ_3JB_1, KJ_3D_3 and KJ_3D_5 were lower toxicity than Dipel, two isolates ie KJ_3K_4 and KJ_3D_3 which had the same toxicity to Dipel. Five isolates the KJ_3E_3, KJ_3BW_5, KJ_3JB_5, KJ_3D_1 and LC_2, LC_3 known to have effectiveness until the seventh day reached 40%. There was one isolate that is KJ_3BW_5 which was more effective compared to Dipel as comparison.

Keywords : .*Bacillus thuringiensis, Toxicity, Spodoptera litura*

1. Introduction

Green mustard (*Brassica rapa*varparachinensis L.) is a vegetable that is in great demand and consumed by many people. Green mustard contains many vitamins and antioxidants [1]. The demand for green mustard is increasing, to meet the needs and demand of green mustard are needed to improve their production. Increased production of green mustard greens in Indonesia often experience some obstacles. Among these obstacles is attacked by *Spodopteralitura*larvae.

S. litura is an important pest that can cause damage to many types of vegetable plants of Bracicaceae and Solanaceaein Indonesia. Loss of results due *to Spodopteralitura* attacks in some countries such as Japan reached 80 percent, America 90 percent whereas in Indonesia the yield loss ranged from 23 to 45 percent [2]. In certain areas of vegetable plantation areas in highland of Java and Sumatra islands, the yield loss due to pests can reach 75 to 90 percent [3,4].

The use of synthetic insecticides in vegetable crops in the lowlands is quite intensive. Synthetic insecticides can rapidly decrease pest populations, thus preventing the spread of pest attacks. The use of synthetic insecticides in vegetable crops is presumably excessive in terms of type, composition, dose, time, and interval [5]. Generally farmers emphasize pest control efforts using synthetic pesticides [6,7]. Loss of yield and cost of vegetable pestscontrol globally reaches US 1 billion. It has been reported that in one season farmers use 3-4 types of insecticides with spraying frequency 11-15 times in the dry season and 6-10 times in the rainy season. These conditions resulted in the presence of high insecticide residues

in the product[8,9].Therefore, new alternatives are needed to reduce pest populations and not cause negative impacts on the environment. Control of insect pests by using natural enemies from these insect pests. The natural enemiy used in this study is *Bacillus thuringiensis*, the entomopathogenic bacteria.

B. thuringiensis is a gram-positive bacterium that is rod-shaped and forms a spore. The hallmark of *B. thuringiensis*is to have protein crystals, which are toxic to insects [10]. *B. thuringiensis* is highly toxic to host insects of Lepidoptera order such as *S. litura*, thus harmless to natural enemies, as well as non-targeted living creatures such as humans and other organisms [11]. Although there have been many widely used commercial products and have identified various types of Cry proteins, but isolation and identification of *B. thuringiensis* strains are still underway. Because a large number of insect pests can not be controlled by using existing toxins. In addition, new *B. thuringiensis* strains are also required to provide alternatives when insect resistance appears to certain Bt strains [12]. This study aims to isolate *B. thuringiensis* and perform the best toxicity test of *B. thuringiensis* isolates for *Spodopteralitura* larvae on green mustard greens (*Brassica rapa varparachinensis* L.).

2. Materials and Methods

2.1 Place and Time

The research was conducted at the Laboratory of Pest and Disease Department, Agricultural Faculty of Sriwijaya University, Campus InderalayaOganIlir, South Sumatera, from March to June 2017.

2.2 Soil Sampling

Soil samples were taken from Sekayu sub-district, MusiBanyuasin, South Sumatera with 10 sampling points. *B.thuringensis*exploration was done by sampling the soil from the green mustard plant site at a depth of 10-20 cm. Soil taken as much as 250 g at each sampling point. The soil sample is then inserted into a plastic bag, tied tightly, labeled location and date of capture. The soil sample is taken to the laboratory and stored in the refrigerator to be isolated.Prior to the selection of *B.thuringensis*, bacteria were first identified.

2.3 Isolation of Bacillus thuringiensis

The soil samples taken from the field were isolated using [13] methods in the following manner. As much as 1 g of soil sample, put into a test tube containing 9 mL of sterile water. The suspension is shaken to homogeneous and heated to a temperature of about 80 ºC for 10 minutes. Suspension made series dilution from 10^{-2}-10^{-6}. From aseptic dilution 10^{-6}were taken as much as 0.1 mL suspension using a micropipet flattened on a NA medium with a spreading cup method with 5 replications. The plate was isolated and placed in reverse position at 28-30 ºC for 48 hours.

2.4 Identification of Bacillus thuringiensis

*2.4.1 Morphological Observations.*The bacteria were grown on NA media and were observed at 48-96 hours after inoculation. The observed parameters are colony shape, colony color, colony edge, colony surface, and colony elevation. Selection early, from many colonies that grow selected characteristics of the colony with morphological features: rod-shaped cells, motile, gram positive, circular colon, color white and yellowish colonies. Colonies of bacteria showing positive features as bacteria *B. thuringensis* were made pure cultures and then stored in a room with a temperature of 4 ° C as the stock to be used during the study.

2.4.2 *Gram staining*.Gram staining is used to distinguish bacteria that are separated generally in two major groups: gram-positive and gram-negative. Stages of gram staining of the bacteria is taken by using ose needle, then leveled in the middle of the object glass to form a thin layer and fixed. After that flood the spread of bacteria with a primary dye that is crystalline violet for 1 minute, then washed with running water, and dried wind. Flush it with iodine for two minutes, washed with water and drained. Then wash the spread with a pale solution of 95% alcohol, dropwise until the purple dye no longer appears to flow

from the object glass. Then flood again with a counter-dye of safranin for 30 seconds, then wash and absorb excess water on the spread by pressingthe paper absorb carefully on it. Furthermore, microscopic observations were made.

2.4.3 *KOH Test*. KOH test is done by taking and putting 1 end of needle ose culture *B. thuringiensis* on glass preparations that had been spilled with 3% KOH solution. If bacteria are not slimy then these bacteria are gram-positive bacteria, while bacteria that produce mucus is a gram-negative bacteria. KOH test is used to distinguish gram-positive bacteria and gram-negative bacteria.

2.4.4 *Catalase Test*.The catalase test is carried out by taking and putting the end of the needle culture of*B. thuringiensis* bacteria on the preparatory glass which had previously been spilled with a solution of hydrogen peroxide (H_2O_2). Then stirred to produce bubbles, in positive catalase bacteria to produce bubbles.Whereas in the negative catalase bacteria do not produce bubbles. The catalase test was used to determine the catalase activity in the bacteria tested.

2.4.5 Breeding of Insect Bulk. Insect test used is *Spodopteralitura* obtained from farmer's land in SakoBorang, Palembang City, South Sumatera. Then the *Spodopteralitura* larvae were brought to the laboratory and maintained in a plastic container with a size of 23 cm x 50 cm covered with gauze. Then each larva is separated according to stadia. The insect test food is Chinese cabbage leaf that is replaced daily with a new one. The length of larval development is calculated until imago form. The new Imago appears separated into a plastic container that has been laid with cotton that has been previously immersed in honey to feed the imago. The test insect to be used for the efficacy test is *S.litura* instar 3 larvae from second line (F_2).

2.4.6B.thuringiensistoxicity screening test against S.litura. The treatment in the screening test was performed by administering *B.huringensis* suspension on Chinese cabbage leaf for insect larvae of *S. litura*. Prior to testing, first calculated the density of spores using haemocytometer. Each chinesecabbage leaf weighed 2.5 g. Then each chinese cabbage leaf was immersed until submerged entirely into 20 mL suspension of *B. thuringensis* and 80% tween solution for 3 minutes. Chinese cabbage leaves are dried before infestation of the larvae. Each piece of chinese cabbage leaf is inserted into a plate with a size of 12cm x12cm which has been given tissue paper. In each plate plot 10 larvae were added. As a positive control in the form of dipel and negative control of aquadest.

3 Results and Discussion

The isolation results from 10 soil samples obtained from Kayuara outlet lane 2 (3 soil samples), Kayuaralane 3 (4 soil samples), and Lumpatan (3 soil samples). The samples of soil examined were 50 isolates with traits such as the Bacillus genus. 38 isolates from the total of Bacillus colonies examined had morphological and microscopic features of *B. thuringensis*. However, only 38 isolates showed the characteristics of *B. thuringensis* such as round, white, elevated and wrinkled edges (Fig. 1). This is supported by [14] revelation that the features of *B. thuringensis* have a circular shape, rough and smooth surface of the colony, glossy and slightly glossy, yellowish white and white colonies. Microscopically, it can be seen that the form of bacterial cells suspected of *B. thuringensis* isolation has a stem cell shape and belongs to Gram positive bacteria with Gram staining (Figure 2).

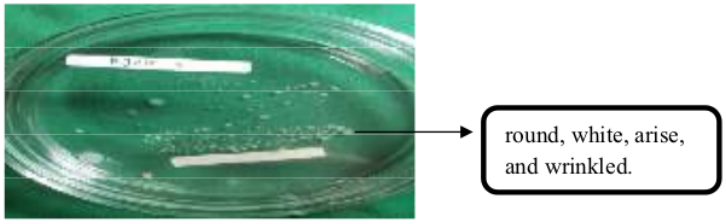

Figure 1. Morphology of bacterial colonies suspected *B thuringiensis* bacteria.

Figure 2. Bacterial vegetative cells *B. thuringiensis*have rod-shaped cells and includegram-positive bacteria, because they are purple after gram staining in 400 X magnification.

Of the 38 selected isolates were selected by KOH test and catalase test. Based on KOH test conducted, if the bacteria is lifted when lifting the needle ose then the bacteria is gram negative. In this case, the tested *B. thuringiensis* bacteria is not elevated, it can be said that this bacterium is gram positive (Figure 3). This is in line with the gram staining reaction that has been done that *B. thuringiensis* includes gram positive bacteria. While the catalase test aims to determine the reaction of catalase test in bacteria. The positive reaction of the catalase test is shown by the formation of bubbles. Conversely, if the reaction is negative, then bubbles are not formed. *B. thuringiensis*bacteria have catalase enzymes, so the catalase enzyme will work to break the H_2O_2that produces bubbles derived from the formation of oxygen gas (Figure 4). Screening of 38 selected isolates using KOH test and catalase test showed 13 isolates which will then be tested toxicity to *Spodopteralitura* larvae.

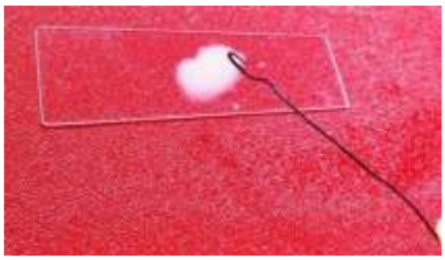

Figure 3: KOH test on *B. thuringiensis*bacteria using KOH solution that does not produce mucus (gram positive).

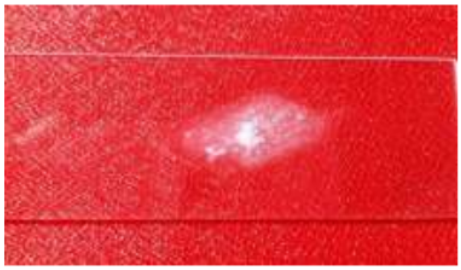

Figure 4: The catalase test on *B. thuringiensis* bacteria using H_2O_2 solution that produces bubbles.

The soil samples from Line 3 have the most isolates and line 2 has the fewest isolates. It is presumed that in the soil samples taken in line 3 have considerable sources of minerals, carbon, and nitrogen for the growth *of B. thuringiensis.*Environmental factors such as pH, oxygen solubility and temperature support bacterial growth.The low acquisition of isolated bacteria in Line 2, is most likely influenced by the content of crystal-producing bacterial spores in soil samples and viability of the spores. In relation to these habitats, multiple soil sampling is required because the discovery of entomopathogenic bacteria at any given moment is influenced by many factors including rain and other factors. There is a possibility when sampling of soil is found entomopathogenic bacteria in a particular place, but at other times can not be found again, and vice versa [14].

3.1 Screening toxicity of B.thuringiensis isolates against S.litura larvae

From selection result through morphological observation, coloring reaction, KOH test, and catalase test, 13 subsequent selection of isolates were tested for toxicity to *Spodopteralitura* instar three.

Table 1.*B.thuringiensis* toxicity screening test results against *S. litura l*arvae.

No	Isolate code	Village	Mortality (%)
1.	KJ_2M_2	KayuaraJalur 2	20
2.	KJ_3E_1	KayuaraJalur 3	13
3.	KJ_3E_3	KayuaraJalur 3	40
4.	KJ_3BW_5	KayuaraJalur 3	40
5.	KJ_3JB_1	KayuaraJalur 3	30
6.	KJ_3JB_5	KayuaraJalur 3	40
7.	KJ_3D_1	KayuaraJalur 3	40
8.	KJ_3D_3	KayuaraJalur 3	30
9.	KJ_3D_5	KayuaraJalur 3	13
10.	KJ_3K_4	KayuaraJalur 3	30
11.	LC_2	Lumpatan	40
12.	LC_3	Lumpatan	40
13.	LKB_1	Lumpatan	80
14.	Control (Aquadest)	-	7
15.	Dipel (*B.thuringiensis* commercial) as a comparison	-	30

In general, the mortality of treated *S. litura* larvae was different from that of aquades. According to the results of toxicity tests against *S.litura.B. thuringiensis* isolates obtained 5 (KJ_2M_2, KJ_3E_1, KJ_3JB_1, KJ_3D_3, KJ_3D_5) the toxicity is lower than the Dipel, a synthetic chemical insecticide, as a comparison, 2 isolates (KJ_3K_4 and KJ_3D_3) the same as the comparative toxicity. 5 isolates (KJ_3E_3, KJ_3BW_5, KJ_3JB_5, KJ_3D_1, LC_2, LC_3) that its effectiveness until the seventh day reached 40%. There is 1 isolate (KJ_3BW_5) which is more effective than comparison (Dipel). It is suspected that isolates have the same toxicity and are higher compared toDipel, since they have protein crystals that are characteristic of *B. thuringiensis*.

Factors that affect susceptibility of insect larvae are the type or type of protein crystal, alkaline pH of the alkaline insect digestive tract, and proteolytic enzymes [15]. This crystal contains a protein called δ-endotoxin, which is lethal when eaten by sensitive insects. Insect digestion can turn Bt-proteinsinto a shorter, toxic polypeptide. Toxins that have been actively interacting with epithelial cells in insect midgut. Evidence has shown that this Bt toxin causes the formation of pores (a very small hole) in the cell membrane in contribution digestion and disturbing the osmotic balance of the cells. Because the osmotic balance is disrupted, the cells become swollen and rupture and cause insect death [15].According to [16],when spores and protein crystals are eaten by sensitive insects, paralysis results in the death of the host. The bacterial crystals will dissolve in the gastrointestinal tract, in which bacteria secrete toxins that can kill insects. [17]states that insect sensitivity to destruction varies and the order of Lepidoptera has more sensitivity.Protein crystals are ingested by insects will dissolve in the alkaline environment of the insects. In target insect, the protein will be activated by an enzyme digesting protein enzyme. Protein which is activated will attach to the receptor protein located on the surface of the intestinal epithelial cells. The attachment resulted in the formation of pore or holes in the cell so that the cell undergoes lysis in the end the insect will experience indigestion and die.

Larva *S. litura*. Infected by *B. thuringiensis*shows more slow movement symptoms, decreased feeding activity, and reduced touch response. Dead larvae are blackish brown soft body. When touched the caterpillar skin will break and remove the black liquid and foul-smelling. The emergence of black color according to Steinhaus[18] caused by the body's bacteria to the haemocoel so that the blood cells become poisoned. This behavior change causes the caterpillar to become anxious and paralyzed. This paralyzed caterpillar shows that the condition of the caterpillar has been weak or the caterpillar defense system works so well that the spores.

4 Conclusion

From the results and discussions, it can be concluded that of the 50 soil samples from three villages selected through morphological observation, reaction staining, KOH test, catalase test, producing 13 bacterial isolates. Screening of the 13thtoxicity of the isolates suspected *B.thuringiensis* against *S. litura* larvae was investigated. Based on the toxicity screening test the following facts were obtained: five isolates ie KJ_2M_2, KJ_3E1, KJ_3JB_1, KJ_3D_3 and KJ_3D_5 were lower toxicity than comparison, two isolates ie KJ_3K_4 and KJ_3D_3 which had the same toxicity as Dipel. Five isolates namely KJ_3E_3, KJ_3BW_5, KJ_3JB_5, KJ_3D_1 and LC_2, LC_3 known to have effectiveness until the seventh day reached 40%. There was one isolate that is KJ_3BW_5 which was more effective than comparison.

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