

Isolation and selection of maize plants rhizobacteria with the potential of entomopathogens against *Spodoptera litura* (Lepidoptera: Noctuidae)

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Abstract. Nelly N, Khairul U, Putri AY, Hamid H, Syahrawati MY. 2020. Isolation and selection of maize plants rhizobacteria with the potential of entomopathogens against *Spodoptera litura* F (Lepidoptera: Noctuidae). *Biodiversitas* 21: 753-758. *Spodoptera litura* is a polyphagous pest that can be controlled with entomopathogen. Furthermore, Rhizobacteria derived from the rhizosphere of corn plants are expected to control this pest. This research, therefore, was conducted to obtain the entomopathogenic rhizobacteria to control *S. litura* derived from the rhizosphere of corn plants. Data sampling was carried out at corn plantations in West Pasaman Regency, while Isolation, selection, and testing of *S. litura* were conducted at the Biological Control Laboratory of the Plant Protection Department, Faculty of Agriculture, Andalas University. The method uses serial glowing techniques cultured on Nutrient Agar (NA) media, with the selected isolates tested for their ability as an entomopathogen against *S. litura*. The feed dip method given to *S. litura* larvae was used to test for rhizobacteria virulence determination with entomopathogen, used to observe the larvae mortality rate, percentage of pupae, and adult formed. Furthermore, observation of morphological and physiological characters was conducted on entomopathogenic rhizobacteria, with hypersensitive testing conducted on *Mirabilis jalapa* plants. The results of rhizobacterial isolation from corn rhizosphere obtained 12 entomopathogenic isolates. Deadly larvae of *S. litura* 51-91.11%, 0.00-8.89% pupae, and 0.00- 6.67% adult were formed. The gram test results obtained approximately 8;4 positive (+) and four negative isolates (-), respectively. Hypersensitivity test results found that one of 12 isolates caused necrotic reactions in *Mirabilis jalapa*. Therefore, a total of 11 rhizobacteria isolates are entomopathogenic and safe to be used as *S. litura* biological control agents.

Keywords: Control, corn rhizosphere, entomopathogen, Rhizobacteria, *Spodoptera litura*

INTRODUCTION

Armyworm (*Spodoptera litura* L.) is a polyphagous pest with a broad range of hosts such as corn, soybeans, peanuts, cabbage, sweet potatoes, tobacco, spinach, ornamental plants, and others. In the vegetative and generative phase of soybean plants, these pests eat young leaves (leaving leaf bones) and pods (Laoh et al. 2003). According to Marwoto and Suharsono (2008), the damage and yield loss due to this pest is approximately 80%, with *S. litura* capable of attacking maize from vegetative to generative growth stages.

Crop pest control is often conducted using synthetic insecticides. However, its continuous and unwise usage causes negative impacts. Some of these include the occurrence of resistance, resurgence, environmental pollution and accumulation of residues in plants, thereby, making it harmful to humans and the various animal species that eat them (Untung 2006).

One effort to reduce the negative impact is to use microorganisms found in the soil around the roots of plants such as bacteria. Groups of bacteria that live in the rhizosphere colonize roots, live in symbiosis by utilizing exudate roots of plants called rhizobacteria (Akhtar et al., 2012). Rhizobacteria from the corn rhizosphere were also used to control diseases in maize (Rahma et al. 2018). Prischmann et al. (2008) identified specific *Serratia* strains associated with rootworms that may have potential as

biological control agents and additional *Serratia* biotypes associated with corn rhizosphere that can function as plant growth-promoting agents. Dar et al. (2018) further argue that rhizobacteria characterization taken from the walnut rhizosphere in the western part of the Himalayas obtained as many as 90 isolates that function as plant growth-promoting activities.

Another role of microorganisms found in the rhizosphere, such as rhizobacteria, was as a biological control agent for insect pests or entomopathogens (Simatupang, 2008). The utilization of bacteria as biological control agents has been widely reported. Salaki (2011) obtained 202 isolates of *Bacillus cereus* from the soil. The isolates were tested on 15 isolates, and only ten isolates caused the mortality rate of test larvae to be more than 50%. Khaeruni et al. (2012) obtained *B. thuringiensis* isolates from soil could cause the death of larvae *Crocidolomia pavonana* up to 100%. Sari (2016), reported that *Serratia* sp. was able to cause mortality in *S. litura* by 88%. However, few studies provided information related to the potential of entomopathogenic bacteria originating from the rhizosphere of corn plants is used as biological agents for *S. litura* control. Several types of research have been conducted to obtain entomopathogenic rhizobacteria, such as *S. litura* controlling agents.

MATERIALS AND METHODS

Study site

The research was performed from October 2017 to January 2018, with bacterial isolation and entomopathogenic tests conducted at the Microbiology Laboratory, Department of Plant Protection, Faculty of Agriculture, Universitas Andalas, Padang, Indonesia. Data sampling was conducted at corn plantations in West Pasaman Regency, Indonesia.

Isolation and characterization of morphological Rhizobacteria

The rhizobacterial separation was conducted using serial dilution techniques. A total of 1 gram of soil sample was put into each test tube containing 9 ml of sterile aqua dest, homogenized with vortex, and diluted to 10^{-7} ml. The suspension stocked on the media is the result of diluting 10^{-6} and 10^{-7} ml, with the suspension taken at 0.1 ml, and put in a test tube containing 9 ml Nutrient Agar (NA) liquid media, as well as a homogeneous vortex substance. A suspension in NA was put into a petri dish and incubated at room temperature for 48 hours. The rhizobacterial isolates were selected with dominant colony features, in different shapes and characteristics by observing the color, shape, elevation, edges, and size of the colony. Selected colonies were purified with the same media by the scratch method and incubated for 48 hours. A single bacterial colony was transferred aseptically into a microtube containing 1 ml of sterile distilled water and stored in a refrigerator (Yanti et al. 2013).

Rearing of *Spodoptera litura*

Spodoptera litura larvae were obtained from vegetable growing fields, where larvae are kept in plastic boxes covered with gauze and fed with long bean leaves, which were washed clean. When the larvae enter the prepupa stage, it was transferred into plastic boxes using sawdust, after which it was closed. The adult emerged from the pupae was fed with 10% honey. The honey was dipped with cotton and hung over a cage. Inside the confinement, the white paper is given as a place for laying eggs, and those laid by an adult is taken by cutting the paper which is transferred to a box ($7 \times 10 \times 5 \text{ cm}^3$). The egg-raising box was lined with filter paper, and the eggs are hatched into larvae and kept till they were inserted into a test insect. A total of 15 larvae were put into plastic boxes, with each fed with long bean leaves, which were replaced every day.

Rhizobacteria isolate testing as entomopathogen

The leaf dipped method was utilized in this research, with each rhizobacterium isolate suspended using a population density of 10^8 cells/ml, by comparing it with a scale 8 of *McFarland* solution. Long bean leaves measuring 4 cm x 4 cm that has been washed thoroughly, then dipped in a bacterial suspension for approximately 5 minutes and allowed to dry. Next, 15 individuals of *S. litura* larvae were put into each insect box. For the control treatment, long bean leaves were soaked with sterile distilled water. The bacterial suspension was given for two

days. On the next day, the larvae were given to eat long bean leaves without treatment. Observations were made as follows:

Larvae mortality (%)

Observations were made by counting the number of dead larvae starting from the first day after the application of Rhizobacteria before the formation of pupae. The mortality rate was calculated using the following formula:

$$M = \frac{n}{N} \times 100\%$$

M: larvae mortality (%)
n: number of dead larvae
N: number of larvae used

The treatment effectiveness was calculated formulas follows:

$$E = \frac{P - K}{P} \times 100\%$$

E: Effectiveness
P: Treatment
K: Control

Virulence of rhizobacteria was classified following level (Rusmana and Hadioetomo, 1994): (i) High virulence, assuming the mortality value is above 50%. (ii) Moderate virulence, assuming the mortality value is 30% - <50%. (iii) Low virulence, assuming the mortality value is <30%. (iv) Not virulent, assuming the mortality value is 0%.

The time required by bacteria to kill 50% of test insects was determined by calculating the lethal time 50 (LT50) value using probit analysis.

Percentage of pupae formed

Observations were made by counting the number of pupae from each treatment, with the percentage calculated as follows:

$$P = \frac{b}{N} \times 100\%$$

P: Percentage of pupae formed
b: Number of pupae formed
N: Number of larvae used

Percentage of adult formed

Observations were made by counting the number of an adult from each treatment. The percentage formed is calculated using the formula below:

$$I = \frac{d}{N} \times 100\%$$

I: percentage of adult formed
d: number of imago formed
N: number of larvae used

The effectiveness of the treatment was calculated using the formula:

$$E = \frac{K - P}{K} \times 100\%$$

E: Effectiveness

K: Control

P: Treatment

Data analysis

All data were evaluated using Analysis of Variance (ANOVA) and further tested with LSD at 5% level using STATISTIX ver. 8.0 (Analytical Software for Windows, 2003).

Physiological characterization of entomopathogenic rhizobacteria

Gram test

Gram tests were performed on entomopathogenic isolates to determine whether the bacterial properties are positive or negative. One drop of 3% KOH solution was placed on the slide with a dropper. Two days old pure rhizobacteria culture was taken as much as one ose and mixed with the solution. If clots occur, the bacteria were gram-negative, and vice versa, if they do not clot, the bacteria were gram-positive (Klement et al., 1990).

Hypersensitivity reaction test

Hypersensitivity reaction test was performed using an indicator plant (*Mirabilis jalapa*). Rhizobacterial suspensions with a population density of 10^8 cells/ml were infiltrated intracellularly using 1 ml injection in the leaf underwater tissue, which was conducted before its saturation, and incubated for 2 x 24 hours. The reaction was characterized by the appearance of necrotic symptoms in the infiltrated part of the leaf (Klement et al. 1990). The presence of necrotic symptoms shows that the bacterium is pathogenic and cannot be used further as an entomopathogen.

RESULTS AND DISCUSSION

Isolation and morphological characterization of rhizobacteria

Isolation results from corn soil samples of corn plants obtained 74 bacterial isolates, with diverse morphological characteristics of the entomopathogen. Colony shape (round, irregular, rhizoid, thread), elevation (convex, flat, semicircular surface), colony surface shape (slightly slimy, slimy, and rough), colony color (white) (Fig 1). Rhizobacterial colonies formed in the Petri dish on NA culture media are seen in Figure 1a. These isolates are used for the treatment of *S. litura* larvae, with its pure culture stored in a refrigerator.

Rhizobacteria as a biocontrol of *S. litura*

Larvae mortality

Spodoptera litura larvae mortality treated with rhizobacterial isolates was 0.00-91.11% with significantly

different results on control without rhizobacteria treatment ($P = 0.00$). Rhizobacterial isolates with high virulence rates were then tested for their ability to *S. litura* larvae, which looked significantly different from controls. The LT_{50} values for each isolate tested varied and were on the average, which indicates that they had high virulence ability to kill *S. litura* larvae in a short time. Table 1 shows that Rz.M2.3 isolate had the shortest LT_{50} value of 1.09 days, while the most extended LT_{50} value of Rz.R3.3 isolate was 4.52 days. The name or isolate code; Rz (Rhizobacteria). M (isolate from the land of Monoculture) R (isolate from Replanting soil), and number (sample number).

The cumulative mortality rate developed by *S. litura* larvae after inoculation with rhizobacterial isolates is seen in Figure 2. In the picture, the level of *S. litura* mortality in control was always below the rhizobacterial isolates.

Symptoms of larvae infected with rhizobacteria showed reduced feeding activity, movements become sluggish and less sensitive to the touch, larvae feces become runny (diarrhea), and green liquid comes out of the mouth. *S. litura* larvae that die from rhizobacteria infection show a blackish body, curved, soft, and emits a foul odor. Furthermore, the larvae dried and shrink with the integument still intact (Figure 3).



Figure 1. Rhizobacteria from corn roots. A. Bacteria culture isolated 48 hours in NA media, B. Pure culture isolates in NA media 48 hours.

Table 1. Mortality of *Spodoptera litura* larvae treated with rhizobacterial isolates with high virulence and LT_{50} values

Treatment	Mortality of larvae (%) \pm SD	LT_{50} (days)	Effectivity (%)
Rz. M3.1	91.11 \pm 15.40 a	1.13	97.56
Rz. M2.3	86.67 \pm 6.67 ab	1.09	97.44
Rz.R5.2	86.67 \pm 6.67 ab	2.23	97.44
Rz.M1.5	86.66 \pm 6.67 ab	3.15	97.44
Rz.M5.2	84.44 \pm 21.43 abc	1.68	97.37
Rz.M2.2	75.56 \pm 7.70 abc	2.22	97.06
Rz.M2.1	75.55 \pm 3.85 abc	3.90	97.06
Rz.M3.3	73.33 \pm 6.67 abc	3.89	96.97
Rz.M4.4	68.89 \pm 13.88 abc	4.27	96.78
Rz.M3.2	66.66 \pm 30.55 bc	3.34	96.67
Rz.M1.4	64.44 \pm 10.18 bc	3.97	96.55
Rz.R3.3	51.11 \pm 13.88 c	4.52	95.66
Control	2.22 \pm 3.85 d		

Note: The numbers followed by the same lowercase letters in the same column are not significantly different according to LSD at the 5% level

The number of pupae formed

Pupae formed by larvae survive after being treated with rhizobacterial isolates, which showed significant differences in the percentage of pupae formed ($P < 0.005$) with the highest from 12 isolates tested to produce 8.89%. While isolates of Rhizobacteria (Rz) from the land of Monoculture 1st (M1.3) and Replanting land 5th (R5.2), the sample number (3 and 2). Isolates code is Rz.M1.4, Rz.M3.1, Rz.M3.2, Rz.M5.2, and Rz.R5 had the lowest percentage of pupae formed at 0%, with pupa control, of 80.00% formed (Table 2).

The number of adults emerged

The adult was due to the treatment of larvae with rhizobacteria (Rz) from corn rhizosphere, shown significant differences with control. All of the adults were normally formed (Table 3).

Physiological characterization of Rhizobacteria

Entomopathogenic rhizobacterial isolates are characterized by their physiological properties, namely gram reactions, hypersensitivity, and endospores formation, with the characterization results shown in Table 4. In the gram test results, four gram-negative isolates characterized by clot formation in the rhizobacterial (Rz) colony, and eight gram-positive structures in the rhizobacterial colony were utilized.

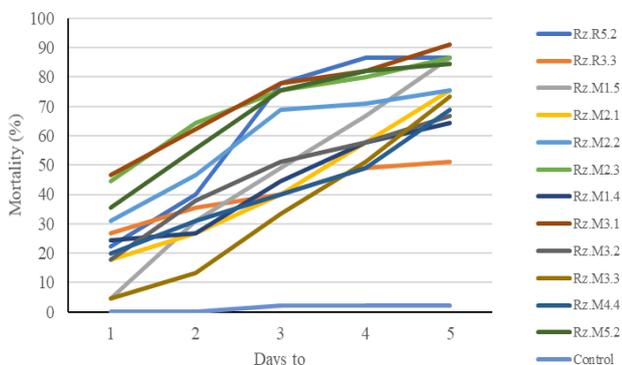


Figure 2. The cumulative mortality rate of *Spodoptera litura* larvae was treated by rhizobacteria (RZ) isolates and control.

Table 2. Percentage of pupae formed and their effectiveness after being treated with and without rhizobacteria isolates

Treatments	Formated pupae (%) ± SD		Effectivity (%)
	Normal	Abnormal	
Control	80.00 ± 3.85 a	4.45 ± 3.85	
Rz.M2.2	8.89 ± 3.85 b	2.22 ± 3.85	88.89
Rz.M4.4	8.89 ± 3.85 b	2.22 ± 3.85	88.89
Rz.M2.1	4.45 ± 3.85 bc	4.45 ± 3.85	94.44
Rz.M3.3	4.45 ± 3.85 bc	6.67 ± 6.67	94.44
Rz.M1.5	4.44 ± 7.70 c	4.46 ± 3.85	97.23
Rz.M2.3	2.22 ± 3.85 c	0.00 ± 0.00	100.00
Rz.R3.3	2.22 ± 3.85 c	4.45 ± 3.85	100.00
Rz.M1.4	0.00 ± 0.00 c	0.00 ± 0.00	100.00
Rz.M3.1	0.00 ± 0.00 c	0.00 ± 0.00	100.00
Rz.M3.2	0.00 ± 0.00 c	0.00 ± 0.00	100.00
Rz.M5.2	0.00 ± 0.00 c	0.00 ± 0.00	100.00
Rz.R5.2	0.00 ± 0.00 c	0.00 ± 0.00	100.00

Note: The numbers followed by the same lowercase letters in the same column are not significant with a difference according to LSD at the 5% level

Table 3. Percentage of *Spodoptera litura* adult emerged due to treatment of larvae with rhizobacteria isolate

Treatments	Adult emerged (%) ± SD		Effectivity (%)
	Normal	Abnormal	
Control	80.00 ± 0.00 a	0.00 ± 0.00	0.00
Rz.M2.2	6.67 ± 6.67 b	0.00 ± 0.00	91.66
Rz.M3.3	4.44 ± 3.85 b	0.00 ± 0.00	94.45
Rz.M1.5	4.44 ± 7.70 b	0.00 ± 0.00	94.45
Rz.M4.4	4.44 ± 7.70 b	0.00 ± 0.00	94.45
Rz.R3.3	4.44 ± 7.70 b	0.00 ± 0.00	94.45
Rz.M2.1	2.22 ± 3.85 b	0.00 ± 0.00	97.23
Rz.M1.4	0.00 ± 0.00 b	0.00 ± 0.00	100.00
Rz.M2.3	0.00 ± 0.00 b	0.00 ± 0.00	100.00
Rz.M3.1	0.00 ± 0.00 b	0.00 ± 0.00	100.00
Rz.M3.2	0.00 ± 0.00 b	0.00 ± 0.00	100.00
Rz.M5.2	0.00 ± 0.00 b	0.00 ± 0.00	100.00
Rz.R5.2	0.00 ± 0.00 b	0.00 ± 0.00	100.00

Note: The numbers followed by the same lowercase letters in the column are not significantly different according to LSD at the 5% level

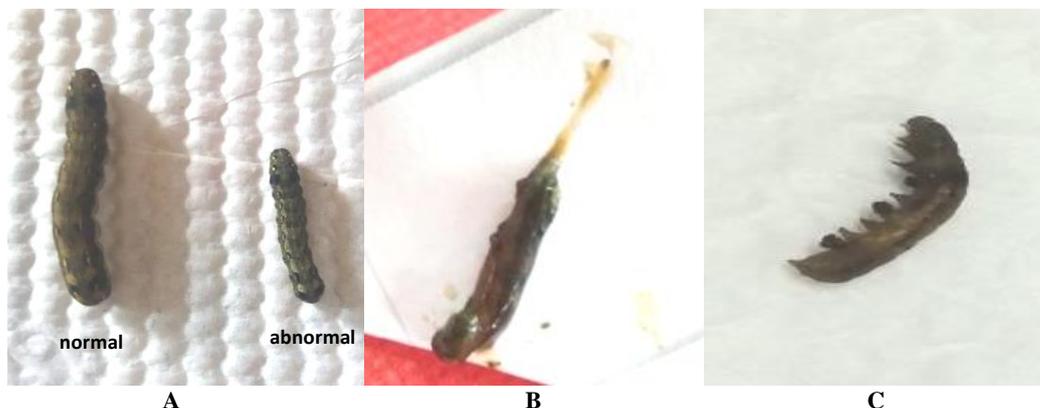


Figure 3. Symptoms of *Spodoptera litura* larvae infected by rhizobacteria. A. Reduced growth, B. Larvae feces, C. Larvae die

Table 4. Physiological characteristics of rhizobacteria that are entomopathogenic to *Spodoptera litura*

Isolate code	Reaction gram	Reaction hypersensitive	Endospore
Rz.M1.4	+	-	-
Rz.M1.5	+	-	-
Rz.M2.1	+	-	-
Rz.M2.2	-	-	-
Rz.M2.3	+	-	+
Rz.M3.1	+	-	-
Rz.M3.2	-	-	-
Rz.M3.3	-	+	-
Rz.M4.4	+	-	-
Rz.M5.2	-	-	-
Rz.R3.3	+	-	-
Rz.R5.2	+	-	-

Note: +: produce physiological characters; -: does not produce physiological character

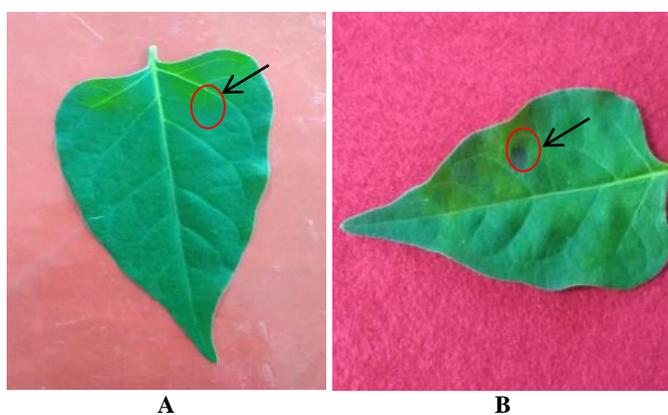


Figure 4. Hypersensitive reactions in *Mirabilis jalapa* plants at two days after inoculation. A. Hypersensitive adverse reactions (-), B. positive reactions (+)

The hypersensitive reaction test results showed an isolate with positive Symptoms characterized by the appearance of necrotic on the leaves of *Mirabilis jalapa* plant due to Rz.M3.3. The necrotic symptoms that appear on the leaves indicate that rhizobacteria are pathogenic to the plant (Figure 4).

Discussion

Rhizobacteria isolated from different locations showed different morphological characters. Rhizobacteria from the rhizosphere of corn plants are thought to be influenced by chemical and biological activity in the roots. It is in line with Widyati's (2013) statement in which chemical and biological activities were more intensively influenced by chemical compounds produced by roots and microorganisms which exist in the rhizosphere. Simatupang (2008) stated that there are more microorganisms populations in the rhizosphere, and their presence increases species diversity within the natural plant community.

The results showed that the treatment of 12 rhizobacterial isolates had a high virulence rate with larval mortality values between 51.11-91.11% (Table 2). Deaths

on larvae occurred after one day of administration of rhizobacterial isolates and continued to increase as it entered the pre-pupae period. Factors that influence the different virulence levels of each rhizobacterial isolates were caused by differences in the ability to produce enzymes and toxins during the infection process in insects upon contact with the cuticle in the hemocoel (Tanada and Kaya 1993; Dubovskiya et al. 2008).

The death of larvae *S. litura* is assumed due to the toxins found in plants and areas of the rhizosphere, thereby causing interference in the hemolymph. In some insects, the toxin work mechanism begins to activate after the bacteria entered the larvae's stomach. Aguskrismo (2011) cit Tampubolon et al. (2013) stated that protein crystals dissolve in an alkaline environment in the insect gut, and activated by its digestive enzymes. The active protein is attached to the receptor on the surface of intestinal epithelial cells. The attachment results in the formation of pores or holes in the cell; therefore, it undergoes lysis. The crystal structure of proteins influences the pathogenicity of bacteria to insects. Protein crystals play an important role because of their toxin activity, in which one strain may have bonds that are more easily broken down by enzymes produced by insects and the size of protein molecules that follow the crystals, and the composition of amino acid molecules and carbohydrate content in crystals (Castagnola and Stock 2014; Khaeruni et al. 2012)

The bacteria mechanism which causes death in larvae is yet to be specified. It is suspected that bacteria attack the hemocoel by multiplying itself in the insect's hemolymph by producing toxins, thereby, killing insects.

Bacterial-infected larvae showed symptoms of larvae losing their appetite. The larvae move away from their food, the movements become slow, and the body turns black. Besides, the larval stage also lasts longer when compared to the control (Tanada and Kaya, 1993; Dubovskiya et al. 2008). While the symptoms caused by it include a curved body, which becomes soft and experiences foul odor, with dead larvae, which dries out and shrinks with an intact integument, this is in line with Salaki (2013) opinion, where *S. litura* larvae infected by *Bacillus thuringiensis* show symptoms of larvae moving slowly, releasing fluid from the mouth and anus (diarrhea). These symptoms are typical of bacteria-infected larvae.

LT₅₀ values indicate differences in the time of death in *S. litura* larvae treated with rhizobacteria (Table 3). Isolate Rz.M2.3 was the shortest LT₅₀ for 1.09 days, while Rz.M4.4 isolate was the most extended LT₅₀ value of 4.28 days. The difference from each isolate is a result of several factors, including how bacteria enter the target insect's body, the number, and resistance (Rini et al. 2016). According to Yudiawati (2016), a biological agent is marked good when it can cause death in the target pest within a short period.

In the prepupae stage, larvae failed to form due to a bacterial infection leading to the disruption of the body's metabolism with the energy to enter the pupae stage. The larvae fail to form pupae generally dig up sand, and during formation, the process becomes abnormal and eventually dies in the soil. However, the surviving larvae result in the

formation of deformed or abnormal pupae, with the characteristics of shrinking body shape, dry surface, blackened color, abdominal segments are not attached, the structure of prepupa to pupae is not perfect, does not move when touched (Sari 2016). Normal pupae that were formed can affect the percentage of imago formation. From 12 isolates used, no created imago was deformed or abnormal. An abnormal pupa causes it will fail to become an imago and will die. Escape from the puparium was facilitated by eversion of a membranous sac on the head of the emerging adult, the ptilinum (Gullan and Cranston 2014).

Based on the hypersensitive reaction test conducted on 12 selected isolates in plants (*Mirabilis jalapa*), a bacterial isolate showed a positive reaction, namely Rz.M3.3. It was demonstrated by the appearance of necrotic symptoms around the infiltration mark of bacterial suspension after an incubation period of 24 hours. Hypersensitivity reactions are fast and allocated processes of cell death. This reaction appears to protect the tissue against pathogenic infections (Zhu et al. 2000). Bacterial isolates that exhibit necrotic symptoms were not selected as isolates that could potentially be entomopathogenic, because they considered the safety factor of these isolates. One factor that must be considered in the use of biological control agents is the safety of these agents for plants.

In conclusion, the results of isolation and selection of rhizobacteria from maize plants found entomopathogenic bacteria. Rhizobacteria have the potential to be *S. litura* biological control agents. Rhizobacterial isolates cause mortality of *S. litura* larvae 51-99.11%, and adults formed a maximum of 6.67%. Gram test results, it can be stated that of 12 isolates, 11 isolates are safe to be used as biological control agents.

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