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THE EXPLORATION of *Bacillus thuringiensis* Berliner AND *Bacillus cereus* Frank AT MOUNT MASARANG FOREST

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Abstract:- -Farmers have been using insecticides heavily to control the spread of Lepidoptera pests that attack Cruciferae in North Sulawesi. One solution to the problem of over use of pesticides is to find an environmentally friendly agent to control those pests. We have investigated *Bacillus thuringiensis* (Bt) and *Bacillus cereus* (Bc) at Mount Masarang forest with a high level of virulence against *Plutella xylostella, Crocidolomia binotalis* and *Spodoptera litura* found in cabbages. The spread-plate method was applied to isolate the bacteria, followed by the method of dying leaves to examine the pathogenicity and probit analysis to determine LC_{50} and LC_{90}. The isolation of *B. thuringinsis* and *B. cereus* from 227 soil samples based on the prevailing wind direction, found 801 *B. thuringinsis* isolates and 389 B.cereus isolates. Furthermore, LC_{50} determined in 15 isolates of *B. thuringinsis* against *P. xylostella* range from $2{,}414 \times 10^7$ to $3{,}747 \times 107$, whereas in 6 isolates of *B. cereus* against *P. xylostella* range from $2{,}703 \times 10^7$ to $3{,}228 \times 10^{7.}$

Keywords:- Cruciferae; *Bacillus thuringiensis*; *Bacillus cereus*; *Plutella xylostella*; *Crocidolomia binotalis*; *Spodoptera litura*

I. INTRODUCTION

Mount Masarang is a conservation zone of 500 hectares in area, situated close to Tomohon city in North Sulawesi. Most parts of the area are managed by The Masarang Foundation since 2001. The projects of this Foundation deal with natural conservation, economic and social potential of the local population, local customs and traditions, science and technology. The central vision is concerned with environmental preservation through local empowerment.

One of the Mt. Masarang potential which was not explored and exploited is the availability of entomopathogenic bacteria species such as *Bacillus thuringiensis* and *Bacillus. cereus*. These strains of bacteria probably have high virulence level against lepidopteran pests on cabbage because they have not in contact

previously. Diamondback moth, *P. xylostella*, become resintant to a strain of *B. thuringiensis* after 30 years continuous contact [1].

B. thuringiensis can be isolated from many sources, such as soil with high population and infected insects in the field [2]. In Indonesia, research has begun on finding bacteria to be developed as microbial insecticide, and some Bacillus bacteria such as *B. spaerichus* and *B. cercus* have been found to be able to kill mosquito *Culex qunquefasciatus* [3]. *B. thuringiensis* from the land around Manado has isolated and experimented against Spodoptera larvae [4].

According to [5] that Bt strains produce two types of toxins. The first/main type is Cry (Crystal) toxins that are symbolized by different Cry genes. This toxin classifies Bt types. The second type is Cyt (Cytolytic) toxins which together with other toxins increase the effectiveness of insect control. More than 50 genes that symbolize Cry toxins have been sequenced recently from the 50 groups based on the sequence similarity.

Another *Bacillus* species used as an agent of insect biological control is *Bacillus cereus*. Certain strains of these bacteria are able to produce anti-toxin compounds including toxins on fungi that attack some plants [6]. *B. cereus* can produce proteinaceus and nonproteinaceus toxins that can control pest insects such as *Anthonomus grandis, Spodoptera littoralis* and *Aphis fabae* [7].

B. cereus can be isolated from soil samples in Minahasa and South Minahasa regency as well as Tomohon city. These bacterial isolates have the potential to be controlling agents against cabbage pests [8].

This paper discusses our research investigating the strains of *B. thuringiensis* and *B. cereus* entomopathogenic and the level of pathogenicity of these bacteria against the larvae of *Plutella xylostella*, *Crocidolomia binotalis* and *Spodoptera litura*. The significance of this research is the finding of bio-insecticidal agents against Lepidoptera pests that attack Cruciferae plants.

II. METHODS

A. Isolation of *B. thuringiensis* and *B. cereus* from soil

Taking soil samples was based on the valley (basin) and land elevation approach. Sampling spots were the valleys in all directions (North, East, South and West) at elevation intervals of 100, 200, 300, 400, 500, 600, and 700 m. From each elevation (starting from 100 m) 7, 5, 5, 3, 3, 1 bags of soil were taken, respectively. The distance between each sample spot was 2 meters. 50 grams of soil was taken by using a sterile spatula at 5-10 cm depth under the surface.

The spread-plate method was used to isolate *B. thuringiensis* and *B. cereus*. One gram of soil sample from each location was suspended into 9 ml sterile water in a test tube. This soil was diluted 10,000 times. After diluting the soil, the tube was heated into a water bath with temperature 80 °C for 10 minutes. Then, 0.5 ml suspension was taken from each dilution using a micropipette and was plated using the spread-plate method to

NA media. These plates, after being wrapped with plastic in inverted position, were incubated at room temperature for 48 hours [9]. These cultures were observed by naked eye on a compound microscope to identify the Bt and Bc.

B. Pathogenicity Test

Pathogenicity-tested isolates were multiplied into 4 plates filled with NA. The number of cell (cell concentration) was counted using a hemasitometer (Mod-Fuch' S Rosental: depth 0,2 mm, 1/16 mm2) following the procedure:

- Add 15 ml sterile water to each large petri dish (4 in total) in which B. thuringiensis and B. cereus have grown for 7-10 days and scrape the colonies using a round ose needle.
- Pour this suspension into a 500 ml glass beaker whose surface is covered with three layers of gauze so the media and other matter is filtered out.
- Pour again 10 ml aquadest into each petri plate and agitate, then re-pour into the beaker to rinse out the rest of the bacteria.
- Transport the suspension in the glass beaker to make it homogeneous using 1 ml eppendorf pipette.
- Use the 1 ml pipette to pour the bacterial suspension into the hemasitometer and observe under a compound microscope at 400×. Each box (up and down) contains 9 big square (BS) and measures 3 BS taken diagonally. Then count the average amount of conidia per small square (SS) (1 BS has 16 SS). Model of hemasitometer used is that if 1 SS contains 1 bacteriumal cell then 1 ml of suspension contains 80,000 bacterial cells.

The concentration count is based on the formula:

$$C_1 V_1 = C_2 V_2$$

wher:

V_1 = Bacterial suspension volume that will be poured into sterile water.

C_1 = Bacterial concentration per ml suspension obtained by scraping.

C_2= Expected concentration (10^5, 10^6, 10^7, and 10^8)

V2= 250 ml sterile water.

The Dying leaves method of Hamilton and Atia in [10] is used in pathogenicity test of Bt and Bc. Referring to the research of [11] and [12], five concentrations, from 1 x 10^5 to 1 x10^8 and 1 control of sterile water, are applied. Observation of symptoms and the behavior of the three pests and the death of larvae ismade at the 12^{th}, 24^{th}, 48^{th}, 72^{nd} and 96^{th}hour after treatment.

Mortality data were used to get the value of LC_{50} to LC_{90}. Probit analysis was applied to analyze the data in order to get the LC values. If the mortality of larvae during the control treatment is bigger than 0% but smaller than 20%, the larvae mortality is corrected by using the Abbot formula [12]:

$$Pp - Pk$$

$$Pt = \frac{Pp - Pk}{100 - Pk} \times 100\ \%$$

Pt = Percentage of corrected larvae death

Pp = Percentage of treated larva death

Pk = Percentage of larva death on control

III. RESULTS AND DISCUSSION

A. Isolation of B. thuringiensis and B. cereus

Isolation of *B. thuringiensis* and *B. cereus* from 227 samples of soil based on wind directions at MountMasarang forestbrings the results of 801 *B. thuringiensisisolates* andand 389 *B.cereus* isolates (see table 1). From 801 Bt isolates, 15 potential isolates are chosen for pathogenicity test against *P. xylostella, C. bonotalis and S. litura*. 5 isolates are tested against each pest.

Table 1. The amount of soil samples and *B. thuringiensis* and *B. cereus* from Mt. Masarang area

Direction	Number of Soil Samples	Number of Bt	Number of Bc
North	50	188	70
East	58	204	117
South	50	182	77
West	69	227	125
Total	227	801	389

Table 1 shows that number of isolates of *B. thuringiensis* and *B. cereus* are different for each direction. The difference is due to the soil composition. The location of some of the soil samples is close to cultivated areas, which makes it different from samples in other areas. The northern part with brown-yellowish soil is assumed to be the area of podsolic soil which is the characteristic of mountain land. The eastern part with brown-blackish soil, somewhat moist, has more colonies. Presumably this area has more organic materials than in the north and south. Thus, bacteria play a role as degrader that makes land fertile for crops. The western location has brown-blackish soil where mostly palms, bamboos and cempaka trees grow. Roots are the best habitat for microbes to grow as the interaction between roots and bacteria increases the nutrient supplies for both.

Some factors influenced the finding of entomopathogenic bacteria in certain location, such as erosion, vegetation and rain. Once these bacteria are found in a location, it does not mean that they can be found again in the same place at different times [2,10].

B. Pathogenicity Test

Pathogenicity of 15 Bt isolates against the larva of *P. xylostella, C. binotalis* and *S. litura* (5 isolates for each larva) as well as 6 Bc isolates against *P. xylostella* were analyzed using probit analysis to count LC_{50}-24 hours and LC_{90}-24 hours (see Table 2). The data shows that in general the Bt concentration that killed 50% of *P. xlostella, C. binotalis* and *S. litura* varied. 3 Bc isolates have the same concentration to kill *P. xylostella* larva, while 3 other isolates varied.

Insects that are constantly in contact with Bt and their toxins might have a high genetic cellular immune system. They undergo physiological adaptation that fortifies them with insecticidal immune action from Bt. This is known as cellular resistance mechanism. The genetic-cellular immune mechanism consists of non-specific responses against organisms both vertebrate and invertebrate triggered by the interaction with pathogens. Genetic immunity includes the changes of mucosal surface activities (membranes that synthesize liquid like mucus) that increase secretion of prothealitic enzyme and antimicrobial molecules. Most invertebrates such as insects rely on genetic cellular responses to immunize themselves against their own microbes and toxins. On the other hand, vertebrates like mammals produce their own cells and antibodies to fight against microbes. Entomopathogenic activities of Bt are produced by chain reactions of Cry toxins digested by susceptible insect larvae which include solubilization and enzymatic process, interaction with the middle epithelium intestine of the insect, and interference on structural integrity and function of the epithelium leading to the damage of the entire membranes and insect death. Genetic behaviors and adaptive responses like the changes in protein expression and the condition of the middle intestine of larvae can disturb the action of toxin and the intestinal membranes become immune /not influenced by Cry toxins [13].

Table 2. Pathogenicity of *B. thuringiensis* against larvae *P. xylostella, C. binotalis* and *S. litura* expressed with value of LC_{50}– 24 hours and the value of LC_{90}– 24 hour.

Bacteria	Isolate No	Isolate Code	LC_{50} (spora/ml)	LC_{90} (spora/ml)	Larvae
B. thuringiensis	**33.2**	**MTCt**	2.560×10^{7}	1.171×10^{9}	***P. xylostella***
	101.2	**MBNt**	2.689×10^{7}	3.388×10^{9}	
	150.2	**MSAt**	2.978×10^{7}	2.364×10^{9}	
	156.2	**MTSet**	3.125×10^{7}	1.047×10^{10}	
	104.2	**MSSpt**	3.404×10^{7}	4.551×10^{9}	
	148B.1	**MSCt**	2.414×10^{7}	2.038×10^{9}	***C. binotalis***
	120.3	**MTKT**	2.576×10^{7}	1.717×10^{10}	
	64.1	**MUAt**	2.753×10^{7}	9.171×10^{11}	
	124.1	**MTFT**	3.125×10^{7}	1.047×10^{10}	
	33.3	**MBNt**	3.747×10^{7}	9.946×10^{9}	

	115.1	**MTAt**	**2.533×10^{7}**	**3.817×10^{9}**	***S. litura***
	98.4	**MUSot**	**2.700×10^{7}**	**5.069×10^{9}**	
	146.4	**MSAt**	**2.932×10^{7}**	**1.207×10^{10}**	
	112.1	**MTPt**	**3.125×10^{7}**	**1.047×10^{10}**	
	58.2	**MBSt**	**3.404×10^{7}**	**4.551×10^{9}**	
B. cereus	**98.3**	**MUSc**	**2.703×10^{7}**	**7.922×10^{9}**	***P. xylostella***
	107.5	**MTFc**	**2.877×10^{7}**	**6.916×10^{9}**	
	129.3	**MTDc**	**2.877×10^{7}**	**6.916×10^{9}**	
	149.2	**MSSc**	**2.877×10^{7}**	**6.916×10^{9}**	
	14.1	**MBWc**	**2.885×10^{7}**	**4.888×10^{9}**	
	25.1	**MBK_1c**	**3.228×10^{7}**	**5.245×10^{9}**	

B. thuringiensis produces one family of insecticidal-crystal proteins known as delta endotoxins which have recently been developed and used as environmentally-friendly pesticides. Cry proteins are insoluble inclusions. They consist of one or more inactive pro-toxins. Larvacidal proteins, particularly in lepidopteran are classified as insecticidal proteins Cry I. These proteins are activated by proteases within middle-alcaline intestine. Toxins are tied to specific receptors on the surface of epithelium cells of the middle intestine. These receptors can catalyze toxin conformational changes to make toxins get into membranes and ion permeability changes in intestinal membranes that cause cell lysis and insect death. Proteins that tie Cry I toxins are found in brush border membrane of middle intestine cells of the reported lepidopteran insects. Aminopetidase-N (APN) has been identified as putative receptors which are assumed as Cry I Ac toxins of *Manduca sexta*, *Heliothis virescens* and *Lymantria dispar*. Moreover, different APNs are also demonstrated to interact with Cry Iab toxins and/or Cry Iaa in *Bombyx mori*. The difference is that bigger brush border glycoprotein membrane is related to Cadherins, which is also reported to bind Cry IA toxins in *M. sexta* and Cry Iaa toxins in *B. mori* [14].

Toxicity of crystal inclusions is described as follows; a complex process includes several phases after being digested by insects. It covers (1) crystal solubilization to release Cry proteins in the forms of their pro-toxins, (2) activation of pro-toxins by middle intestine proteases, (3) binding toxins in one of middle intestinal receptors, and (4) forming pores/holes. Insects become resistant against Bt by changing one or more phases of the above process. Resistance against Bt as reported occurs to *Plodia interpunctella* and to other insects that have developed their resistance against one or more Cry proteins. Thus, resistance against Bt is not only found in *P. xylostella* population in the field but also in the strains of *Heliothis virescens*, *Spodoptera exigua*, *Trichoplusia ni*, and other species [15].

Well-characterized resistance mechanism shows significant changes on the binding of Cry proteins with middle intestinal receptors.Some strains of *P. interpunctella*, *P. xylostella* and *H. virescens* show less capacity to binding Cry IA proteins. Another different mechanism covers changes on the activities of intestinal

proteinase that interact with Bt toxins as described in *P. interpunctella* and *H. virescens*.The absence of main intestine protease that associateswith Cry IAc pro-toxin activation as presented in 198 colonies of P. *interpunctella* makes them resistant against Cry IAb and Cry IAc. Genetic studies on this colony prove that there is a connection between the absence of this protease; and the resistance against Bt. Strain CP73-3 of *H. virescens* shows that compared to the sensitive control strain, the process occurs more slowly when Cry IAc turns into active toxins while these toxins degrade faster. Ultimately, a repair mechanism of damaged cells is believed to contribute more to the resistance of H. virescens CP73-3 strain [16].

In brush border epithelium membrane vesicles (BBMV) of *H. virescens*, toxins Cry IAa, Cry IAb, Cry IAc, Cry IFa and Cry IJa use one tie (receptor A) together. Cry IAb and Cry IAc have one additional tie (receptor B) and Cry IAcone further (receptor C) according to the places of ties in this model. Changing receptor A might decrease visibility and resistance possibility against all toxins Cry IA, Cry IFa and Cry IJa. This mechanism occurs to Cry IAc of *H. virescens* YHD strain [17].

Facts show that the resistance of *H. virescens* against CXC strain is not due to the changes of toxin ties on middle intestine receptors. Rather, this strain resistance is the result of change in the general mode of how Cry IAc toxin and Cry 2A toxin work. Because these toxins seem to recognize different receptors of *H. virescens*, there is only one possibility of phase changes before the tying process by this receptor strain. Such a change occurs on solubilizationor processing of Cry toxins inthe middle intestine in which CXC strain can produce resistance for Cry IA and Cry 2A. This mechanism will be consistent with the sensitivity level generated to other Bt toxins like the cases found in Cry IAa, Cry IAb, and Cry IFa [17].

Table 2 also shows that some of the LC50 and LC90 of *B. cereus* isolates against *P. xylostella* are different. In Bengal, India, have been found 1412 larvae of *Anopheles subpictus* not healthy due to the infection that occurs naturally from *B. cereus*. In laboratory conditions, this bacterial suspension increases the mortality of *A. subpictus* larvae from 43.57% to 93.78% 3 to 6 hours after treatment [18].

The absence of Cry plasmids of B. cereus is the main characteristic that makes it different from *B. thurringiensis.* No plasmids release any insecticidal exotoxins that are heat-stable [Krieg and Lysenko, 1979 in 7]. Six strains of Six strains of *B. cereus* produce some non- proteinaceous exotoxins [7].

C. Symptoms of larvae infected with *B. thuringiensis* and *B. cereus* and further research development

1. *B. thuringiensis*

Observation shows that larvae of *P. xylostella, C. binotalis* and *S. litura* infected with *B. thuriengiensis* move slowly and eat less. Such symptoms occur just after treatment. In general, this research shows that the movement of the infected larvae becomes weak against the given touch stimulus. The color of the dead larvae changes gradually from light green to black. When they are stretched out, they smell acidic and the digestive organs are damaged. The bodies become soft although the integumentsare still in good condition.

Another symptom is that 48 hours after the larvae die, an unpleasant odor appears due to the protein denaturation within the body tissues. These larvae gradually become dry and shrunken.

Observation on larvae that turn into pupae shows that the metamorphosis does not happen perfectly as they become smaller and their color changes from shiny brown to black. Other larvae turn into imago and this metamorphosisis not perfect either. Their wings shrink and there are folds in their thorax.

2. *B. cereus*

Larvae *P. xyllostella* change their behavior after eating the feed during treatment. The infected larvae move slowly away from the given feed which indicates that they have no appetite. Their faeces becomes black and body texture becomes soft. When they are touched, an unpleasant odor appears as black liquid comes out of the bodies. Their color becomes black because bacteria have penetrated through haemocol making the blood cells poisoned [19].

Larvae that become pupae do not develop perfectly at the low concentration $10^{5.}$ They become smaller. If they can turn into imago, still they are not able to lay eggs.

IV. CONCLUSION AND SUGGESTION

From this research, some major conclusions can be drawn as follows: (1) Mt. Masarang forest has *B. thuringiensis* and *B. cereus* as effective and efficient controllers against Lepidoptera pests found in cabbages, and (2) 15 strains of *B. thuringiensis* are very virulent against *P. xyllostella, C. binotalis* and *S. litura*; and 6 strains of *B. cereus* against *P. xylostella*. These bacteria strains were very virulent against lepidopteran pests probably because they have never contacted with those pests so that the pests haven not developed their resistance yet.

Further research is urgently needed in order to formulate these entomopathogenic bacteria into wet table powder with a lower price than synthetic insecticides with fewer adverse affects on the environment.

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