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BIOACTIVITY OF A *BACILLUS THURINGIENSIS* CRY1AC TOXIN TO *SPODOPTERA LITURA*

BIOAKTIVITAS TOKSIN BACILLUS THURINGIENSIS *CRY 1 AC TERHADAP* SPODOPTERA LITURA

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ABSTRACT

The transgenic cotton expressing *Bacillus thuringiensis* toxin Cry1Ac in Indonesia has been planted since 2000 for controlling *Helicoverpa armigera*. *Spodoptera litura* is another lepidopteran insect that also attacks cotton. The objective of this research was to determine the toxicity of Cry1Ac to *S. litura*. The acute toxicity was determined using neonates of *S. litura* exposed to artificial diets treated with series concentrations of Cry1Ac ranging from 0.14 to 625 mg/ml, and larval mortality was recorded at the seventh day after treatment. The chronic toxicity was determined by exposing neonate to artificial diets treated with the sublethal concentrations (LC_5 and LC_{40}). The growth and development of treated larvae were compared with those of the control larvae. Cry1Ac was toxic to neonate with LC_{50} values of 71.9, 18.1, 24.7, and 16.2 mg/ml for *S. litura* collected from Wonosari, Bantul-1, Bantul-2, and Kopeng, respectively. Cry1Ac was more toxic than formulated *B. thuringiensis* (LC_{50} = 724.8 mg/ml). Larvae exposed continuously to artificial diets treated with sublethal concentrations of Cry1Ac (0.61 and 9.77 mg/ml) showed no significant difference on weight and length of each life stages than those of the control larvae. These indicate that the application of the tested sublethal concentrations of Cry1Ac did not affect the growth and development of *S. litura*. However, increasing concentrations of Cry1Ac (156.25 and 625 mg/ml) significantly reduced the weight of surviving larvae. These findings suggest that application of Cry1Ac to *S. litura* shows some toxicological effects. The effectiveness of the transgenic cotton in controlling *S. litura* in field situation will be discussed.

Key words: *Bacillus thuringiensis*, Cry1Ac, *Spodoptera litura*

INTISARI

Kapas transgenik yang mengekspresikan toksin Cry1Ac Bacillus thuirngiensis *telah ditanaman di Indonesia untuk mengendalikan* Helicoverpa armigera. Spodoptera litura *adalah Lepidoptera lain yang juga menyerang pada tanaman kapas. Penelitian in bertujuan mendeterminasi toksisitas toksin Cry1Ac terhadap larva* S. litura. *Pengujian toksisitas akut dilakukan dengan memberi makan larva yang baru menetas dengan pakan buatan yang telah diperlakukan dengan toksin konsentrasi 0,14-625 mg/ml, dan mortalitas larva dihitung satu minggu setelah aplikasi. Toksistas kronik dideterminasi dengan memberi makan larva dengan pakan yang telah diperlakukan dengan konsentrasi sublethal (LC_5 dan LC_{40}). Pertumbuhan dan perkembangan larva yang diperlakukan dibandingkan dengan larva kontrol. Nilai LC_{50} Cry1Ac adalah 71,9; 18,1; 24,7; dan 16,2 mg/ml untuk populasi* S. litura *berturut-turut dari Wonosari, Bantul-1, Bantul-2, dan Kopeng. Cry1Ac lebih toksik dibandingkan dengan formulasi* B. thuringiensis *(LC_{50} = 724,8 mg/ml). Larva yang diperlakukan dengan Cry1Ac konsentrasi sublethal (0,61 dan 9,77 mg/ml) mempunyai berat yang sama dengan larva kontrol, dan larva tersebut berhasil menjadi pupa dan imago*

dalam waktu yang sama. Hal ini menunjukkan bahwa pada konsentrasi tersebut pertumbuhan dan perkembangan larva tidak terhambat. Namun, apabila konsentrasi toksin dinaikkan menjadi 156,25 dan 625 mg/ml penghambatan pertumbuhan menjadi nyata. Hasil penelitian ini menunjukkan bahwa Cry1Ac mempunyai efek toksikologis terhadap S. litura. *Efektifitas Cry1Ac pada kapas transgenik untuk hama tersebut pada kondisi lapangan akan didiskusikan lebih lanjut.*

Kata kunci: Bacillus thuringiensis, *Cry1Ac,* Spodoptera litura

INTRODUCTION

Several lepidopteran pests present a major threat to the production of cotton due to a significant yield loss as a result of crop damage. The armyworm, *Spodoptera litura* F. (Lepidoptera: Noctuidae), is an important pest in cotton, and some other economical crops such as groundnuts, potatoes, chili, onions, cabbage, tobacco, and tomato. The control of *S. litura* has relied on the use of chemical insecticides. However, the effectiveness of this method is often low because the application does not fulfill the standard recommendations and unjudicious use of chemical insecticides could negatively impacts to non-target and beneficial organisms (Croft, 1990). Because of some risks posted by conventional insecticides, development of new insecticides has been directed to seek more environmentally friendly toxicant.

Bacillus thuringiensis Berliner is one of entomopathogenic bacteria that is widely used to control insect pests. *B. thuringiensis* produces many toxins with different spectrum of activities even within the same order of insect (Gould, 1999). Some of toxins produced are d-endotoxins that are effective against Lepidopteran larvae (moths and butterflies) (Magallona *et al.*, 1990). *B. thuringiensis* is considered safe to other insects as well as to animals and humans (Delannay, 1996; Fischoff, 1996; Sims, 1995).

B. thuringiensis has been evaluated for its effectiveness to control *S. litura*, and it showed that the isolates of *B. thuringiensis* were effective for *S. litura* and other species of armyworm (Luttrell *et al.*, 1998; MacIntosh *et al.*, 1990; Situmorang *et al.*, 1997). However, the first instar of *S. litura* was less susceptible to *B. thuringiensis* than those of *Crocidolomia binotalis* Zeller (Endah *et al.*, 1997).

Efficacy of *B. thuringiensis* is limited by the nature of its mode of entry. The Cry protein must be ingested in order to cause mortality. The longer the Cry protein is presented to the susceptible larvae, the greater the chances for insect control. Thus, the effectiveness of *B. thuringiensis* applied conventionally is affected by the timing and coverage of spray, feeding behavior, and inactivation of both the spores and crystals by sunlight (Baum *et al.*, 1999).

B. thuringiensis transgenic crops aim to overcome some of the delivery barriers by engineering crop to express high-level of *B. thuringiensis* toxin(s) within crops tissues continuously throughout the growing season (Whalon and Morris, 1999). *B. thuringiensis* transgenic cotton was introduced in Indonesia since 2000 for the control of *Helicoverpa armigera* Hubner. Considering the economic importance of *S. litura* and the introduction of the transgenic cotton expressing Cry1Ac (the transgenic cotton) in Indonesia, assessment on the bioactivity of Cry1Ac toxin to *S. litura* is essential from the aspects of efficacy and potency for the development of resistance, and its impact on natural enemies as a result of intoxification of *S. litura*. This study was

specifically designed to determine the acute and chronic toxicity of the Cry1Ac toxin on larvae of *S. litura* and their subsequent development.

MATERIALS AND METHODS

Insect collections. Three founding populations of *S. litura* used in this research were collected from the district of Wonosari (31 larvae), and Bantul (42 larvae), the province of Yogyakarta Special Territory, and Kopeng (15 Larvae), the province of Central Java. Larval collections were carried out in March 2002. The first generation (F1) from these populations were used for the experiments, except from Wonosari in which the larvae had been reared in an artificial diet (Table 1) for three generations before use.

Insect rearing. All collected larvae of *S. litura* were reared using an artificial diet (Table 1). This diet was a modification from that used in the laboratory of Balitbio (Budiharjo, per. com.). Newly hatched larvae were placed in plastic cups containing a cube of diet (one larva/ cup). The artificial diet was changed as necessary. Larval feces were left in the cups for pupation. Pupae were collected daily and placed in plastic jars. Newly emerged moths were fed with 10% honey solution. All life stages were maintained in an insect rearing room at 26-30^{0}C (min-max) and 55-57% r. h. (min-max) and L10:D14 photoperiod.

Preparation of the toxin solutions. Cry1Ac encapsulated in killed *Pseudomonas fluorescens* (21% AI [MVP-II] San Diego, California, USA) was used. The protein was dissolved in 10 ml distilled water to obtain a stock solution. A serial dilution was employed to prepare the tested concentrations.

Preliminary bioassay. Preliminary bioassays were carried out to determine the working concentrations that caused mortality ranging from >0% to <100%. Larval feeding bioassays were used to determine the acute and chronic toxicity of Cry1Ac. Eight concentrations of Cry1Ac ranging from 0 to 10 mg/ml were tested against newly hatched larva (<1 day old). The diet was dipped in a Cry1Ac solution or distilled water for 10 seconds and air dried for 20 minutes. Ten newly hatched larvae were transferred into plastic cups (five larvae/cup) containing a cube (1 cm^3) of control or treated diet. Larval mortality was observed every 24 hours for seven days. Larvae were considered dead if they did not move when they were probed using a camel hairbrush.

Acute toxicity of Cry1Ac. Based on the results from preliminary bioassays, eight concentrations ranging from 0 to 625 mg/ml Cry1Ac were tested against newly hatched larva. The bioassays were carried out using procedures similar with that in the preliminary bioassay. Surviving larvae were weighted at the ninth day after treatment. Formulated *B. thuringiensis* (Thuricide$^{\hat{a}}$ HP, Basle, Switzerland, 16,000 IU/mg) was purchased from the local store and used as a positive control. A similar procedure was used to prepare *B. thuringiensis* solutions. The concentration of *B. thuringiensis* used in the bioassays ranged from 0 to 2 mg formulation/ ml.

Chronic effects of Cry1Ac. The effects of Cry1Ac on growth and development of *S. litura* were tested using newly hatched larvae. Three concentrations of Cry1Ac [0 (control), 0.61 mg/ ml (the expected LC_5), and 9.77 mg/ml (the expected LC_{40})] were used in bioassays because these concentrations caused larval mortality lower than 50% leaving enough number of surviving larvae to be observed. Larvae were transferred individually into plastic cups containing a cube of control or treated diet and exposed continuously on the same diet for ten days. After ten days, diets were substituted

Table 1. The components of artificial diet for *Spodoptera litura* larvae

Components	Total amount
Agar	12.0 g
Kidney bean	62.5 g
Wheat germ	50.0 g
Casein	2.5 g
Yeast	31.25 g
Ascorbic acid	3.0 g
Sorbic acid	1.5 g
Methyl parabenzoat	2.5 g
Tetracycline	0.00625 mg
Vitamin mix Vanderzant-Adkisson[1]	5.0 g
Aquadest	550.0 ml

[1]Purchased from Bio-Serv™ (Frenchtown, New Jersey, USA)

with the new treated diet. Substitutions were repeated as necessary until all larvae became pupae. The mean weight of newly hatched larvae (initial weight) was determined by weighing a group of 20 larvae with three replications. Larvae fed with *Ricinus* leaves (natural food) were used as a positive control. Surviving larvae were weighted every five days. Pupae were collected, sexed, and weighted daily. The duration of larval, pupal, and adult stages was recorded.

Data analysis. Data of larval mortality was analyzed using probit analysis to determine LC_{50} and LC_{95} values. Probit analysis was conducted only for the data of seven days after treatment. Larval mortality was corrected using Abbots' formula (1925). Analysis of variance (ANOVA) using a completely randomized design (CRD) was used to determine the effects of different concentrations of Cry1Ac protein on growth and development of *S. litura*. The least significant different (LSD) with a= 0.05 was applied for means comparisons only when the *F*-test in the ANOVA was significant (Fisher's protected LSD).

RESULTS

Acute toxicity. Based on the LC_{50} and LC_{95} values, S. litura collected from Bantul was found to be more susceptible to Cry1Ac than those collected from Wonosari and Kopeng (Table 2). S. litura collected from Kopeng was found to be the least susceptible to Cry1Ac. To determine the relative toxicity of Cry1Ac to S. litura, bioassays using formulated B. thuringiensis was carried out using the most susceptible population (Bantul). The data showed that Cry1Ac was more toxic to S. litura than formulated B. thuringiensis (Table 2).

Larvae of *S. litura* that survived from Cry1Ac treatment gained less weight than the control larvae (Table 3). Increasing concentration of Cry1Ac resulted in more prominent growth inhibition. The control and surviving larvae obtained from Kopeng gained more weight than those collected from Bantul. This trend supports the data on the LC_{95} values, which indicate that the population of *S. litura* from Kopeng was less susceptible to Cry1Ac than that from Bantul.

Table 2. Susceptibility of *Spodoptera litura* populations to *Bacillus thuringiensis* Cry1Ac toxin and formulated *B. thuringiensis*

Toxin	Population	n	Slope	LC_{50} (95% CL)*	LC_{95} (95% CL)*	χ^2	$\chi^2(0.05)$
Cry1Ac	Wonosari	93	1.72	71.9(34.6-127.7)	651.2	3.94	7.81
	Bantul-1	280	1.03	18.1(10.6-28.8)	709.8	2.67	9.49
	Bantul-2	100	4.06	24.7(14.5-34.3)	62.6	5.27	7.81
	Kopeng	119	0.63	16.2(5.4-43.5)	6374.1	5.39	9.49
*B. thuringiensis***	**Bantul**	203	1.70	724.8(510.0-1050.0)	6717.4	9.96	5.99

* mg AI/ml for Cry1Ac and mg formulation/ml for *B. thuringiensis*.

** Formulated *B. thuringiensis* (Thuricide® HP)

Chronic toxicity. Sublethal concentrations of Cry1Ac (0.61 and 9.77 mg/ml) did not affect the growth of surviving larvae as indicated by no significant differences in control and treated larvae after they were exposed for 15 days on control and treated diet, respectively (Table 4).

Pupae formed from larvae fed with *Ricinus* leaves had lower weight than those fed with control diet or artificial diet treated with Cry1Ac, which has the same weight (Table 5). This data indicates that surviving larvae grew at the same rate as those of the control larvae. In other words, application of small amount of Cry1Ac did not affect the larvae of *S. litura* to feed on the artificial diet.

Pupae produced from larvae treated with Cry1Ac or control diet took the same length of time to develop into adults. Furthermore, these adults also lived for the same length (Table 6). Unlike the pupae and adults obtained from larvae fed with the artificial diet, the pupae produced from larvae fed with *Ricinus* leaves needed shorter time to become adult (Table 6). However, the adult longevity was similar to those from the artificial diet.

DISCUSSION

Cry1Ac was toxic to *S. litura* with its LC_{50} ranged from 18.1 to 71.9 mg/ml. However, this species is significantly less susceptible to Cry1Ac than is *H. armigera*. The LC_{50} values of Cry1Ac to *H. armigera* ranged from 0.8 to 4.6 mg/ml (Trisyono *et al.*, 2003). These differences may be due to differences in the physiological conditions of theirs alimentary canal. The midgut of *S. litura* contains low ascorbic acid, high phenol, low activity of protease enzyme, with pH ranges from 8.2 to 8.5 (Narayanan *et al.*, 1976 *cit* Endah *et al.*, 1997). In addition, Cooksey (1971) reported that variations in gut pH and enzyme content affected the toxicity of the same toxin. Proteases are needed to solubilize protoxin or crystal protein and convert it to a toxic protein in the larval gut (Aronson *et al.*, 1986). Therefore, if the activity of protease enzyme in the larval gut is low, the process of solubilization and conversion will be inhibited and it results in a decrease in the toxicity of the toxin.

Table 3. Weight of surviving larvae of *Spodoptera litura* after nine days of feeding on diets treated with Cry1Ac toxin

Cry1Ac (µg/ml)	Mean larval weight (mg/larva)		
	Bantul 1	Bantul 2	Kopeng
Control	39.7a (34)	24.1a (19)	77.5a (17)
0.15	40.9a (39)	28.5ab (15)	45.6abc (13)
0.61	37.0a (31)	46.2b (18)	96.6abcd (6)
2.44	28.2ab (27)	20.2ac (17)	33.4abc (8)
9.77	15.0b (27)	22.9a (18)	17.7bcd (6)
36.06	23.1ab (10)	2.4c (4)	44.4abc (12)
56.25	6.5cd (7)	-	7.4bcd (13)
625.00	2.7c (2)	-	4.2d (1)

Means within the same column followed by the same letters are not significantly different at α= 5% (Fisher's protected LSD). Numbers in the brackets showed the number of surviving larvae for each concentration. (-) : all larvae died

Table 4. Effects of sublethal concentrations of Cry1Ac on weight of *Spodoptera litura* larvae

Treatment	Larval weight (mean ± SEM) (mg/larva) at		
	5 days	10 days	15 days
Control: *Ricinus* leaves	40.2 ± 13.8a (44)	710.4 ± 71.5a (29)	NA
Control: artificial diet	10.0 ± 10.1b (61)	281.1 ± 41.9b (60)	772.0 ± 163.3a (14)
0.61 µg/ml Cry1Ac	8.7 ± 1.2b (61)	182.9 ± 28.0c (56)	807.1 ± 172.0a (19)
9.77 µg/ml Cry1Ac	5.7 ± 1.5b (55)	127.0 ± 17.3c (49)	1079.6 ± 98.6a (22)

Means in the same column followed by the same letters are not different at 5% significant level based on Fisher's protected LSD. Data on the fifth and tenth day was transformed using $\sqrt{x}$ while on the fifteenth day using $\sqrt{x+0.5}$ before ANOVA. NA: all larvae had pupated. Numbers in the brackets showed the number of surviving larvae for each concentration

Table 5. Pupal weight of *Spodoptera litura* formed from the larvae treated with sublethal concentrations of Cry1Ac

Treatment	Pupal weight (mean ± SEM) (mg/pupa)	
	Male	Female
Control: *Ricinus* leaves	327.3 ± 12.9a (25)	339.1 ± 12.1a (17)
Control: artificial diet	422.0 ± 19.6b (24)	473.7 ± 9.6b (29)
0.61 µg/ml Cry1Ac	429.5 ± 5.9b (18)	471.0 ± 10.0b (35)
9.77 µg/ml Cry1Ac	418.8 ± 15.5b (25)	452.1 ± 21.2b (19)

Means within the same column followed by the same letters are not significantly different at 5% significant level based on Fisher's protected LSD. Data was transformed using $\sqrt{x}$ before ANOVA. Numbers in the brackets showed the number of surviving larvae for each concentration.

Table 6. Effects of exposure to sublethal concentrations of Cry1Ac on the developmental time of *Spodoptera litura*

Treatment	Developmental time (mean ± SEM) (days)		
	Larvae	Pupae	Moth
Control: *Ricinus* leaves	12.87 ± 0.21a	6.48 ± 0.19a	5.37 ± 0.25a
Control: artificial diet	16.07 ± 0.34b	7.56 ± 0.09b	6.25 ± 0.23a
0.61 µg/ml Cry1Ac	16.91 ± 0.36b	7.72 ± 0.17b	5.53 ± 0.31a
9.77 µg/ml Cry1Ac	16.97 ± 0.36b	7.78 ± 0.05b	5.24 ± 0.48a

Means followed by the same letters within the same column show no significant differences at 5% significant level based on the Fisher's protected LSD. Exposure was employed for the newly hatched larvae.

The activity of toxin is also influenced by the midgut pH of the larvae (Burgerjon and Martouret, 1971). The midgut pH of *S. litura* varies 8.2-8.5 that is lower than the required environment needed to activate protoxin in its maximum capacity.

Cry1Ac was more toxic to *S. litura* than the formulated *B. thuringiensis* recommended for controlling lepidopteran insects (Table 4). This may be due to differences in the content. Formulated *B. thuringiensis* used in this experiment may have more than one toxin. Because each toxin has its own range of activity, differences in toxin contents will contribute to the differences in their toxicity on the same species of insect.

The application of sublethal concentrations (0.61 and 9.77 mg/ml) of Cry1Ac to *S. litura* larvae did not affect the growth and development of surviving larvae. Larvae of *S. litura* survived from the treatment of 0.61 and 9.77 mg Cry1Ac/ml were able to pupate and become adults. In addition, these larvae took the same length of time as the control larvae to complete their life cycle. These findings may indicate that larvae of *S. litura* may be able to degrade toxin or maximum conversion of the protoxin into toxin did not occur. As results, the larvae grew and developed at the same rate as the control larvae.

These finding clearly show that Cry1Ac applied at high concentration resulted some mortality on larvae of *S. litura* but at low concentrations did not impact the growth and development of larvae. The use of these findings for practical purposes, particularly in relation with the use of the transgenic cotton, demands other additional information. However, together with available information from previous research a few plausible scenarios are discussed.

The expression of the Cry1Ac protein from the transgenic cotton varied depending on geographical environment where the transgenic cotton was planted, the tissue type, and the plant age (Greenplate, 1999). However, the environmental factors had less contribution in determining the expression level of the gene than that of parental background (Adamczyk *et al.*, 2001). Similar data for the condition of Indonesia is not currently available. Therefore, the toxicological data obtained from this research could not be interpreted directly for field conditions. However, our field observations in South Sulawesi showed that the leaves of the transgenic cotton received low level of infestation of *S. litura* (unpublished data). In addition, the bolls have been reported to be susceptible to other two species of armyworms, *S. frugiperda* and *S. exigua* (Adamczyk *et al.*, 1998), indicating that the currently transgenic cotton does not provide satisfactory control for the armyworm.

Even though the currently transgenic cotton is not intended for *S. litura*, some degree of control may benefit the growers Furthermore, the ineffectiveness could be beneficial for natural enemies that use *S. litura* as their host. In other words, *S. litura* function as refugee for natural enemies, particularly fo nonselective natural enemies. Increasing the population of natural enemies in the transgenic crop will increase the probability of surviving *H. armigera* to receive attack. These surviving *H. armigera* may be the resistant individuals If this assumption is true and the natura enemies have similar preferences to the susceptible and resistant individuals of *H armigera*, the development of resistance in *H armigera* to the transgenic cotton is delayed because of an increase in mortality of resistan *H. armigera*.

Sublethal effects of toxicant may b expressed as a decrease in the insect's fitness The development of resistant to toxins of *B thuringiensis* have also been reported to b associated with fitness costs, such as growt inhibition, prolonged life cycle, and reduce fecundity (e.g., Harnoto *et al.*, 1987; Trisyon and Whalon, 1997). Our findings showed tha

sublethal application of Cry1Ac to the larvae did not cause inhibition of their growth and development. However, its effect on the fecundity of the adults produced from the treated larvae remains unknown. If a significant reduction in female fecundity occurs in *S. litura* after being exposed to Cry1Ac, one could expect that a significant reduction in the population of the next generation is possible.

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