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Toxicity of *Bacillus thuringiensis* crystal proteins against eri silkworm, *Samia cynthia ricini* (Lepidoptera: Saturniidae)

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Abstract

Ten purified crystal proteins of *Bacillus thuringiensis* (Bt) were tested at concentrations ranging from 2.93 to 3000 ng/cm^2 for their toxicity to eri silkworm through protein paint bioassays using castor leaves. Based on LC_{50} values, Cry1Aa (2.6 ng/cm^2) was highly toxic followed by Cry1Ac (29.3 ng/cm^2) and Cry1Ab (68.7 ng/cm^2). The Cry1Ca and Cry1Ea proteins were moderately toxic to eri silkworm larvae and resulted in 23% and 28% mortality, respectively at the highest concentration tested (3000 ng/cm^2). Only reduction in larval weight was observed with Cry2Aa, Cry1Da and Cry9Aa proteins while Cry3Aa and Cry1Ba proteins were found to be nontoxic.

Key words: Bacillus thuringiensis, Crystal proteins, eri silkworm, insect bioassays, *Samia ricini*

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1. Introduction

Castor (*Ricinus communis* L.) belonging to euphorbiaceae family has gained importance as its seed produces approximately 40 to 60% of oil which is rich in the unique hydroxy fatty acid, ricinoleic acid. Derivatives of castor oil are used in the manufacturing of soaps, lubricants, paints, dyes, ink coatings, cold resistant plastics, waxes and polishes, perfumes and also in pharmaceuticals. India produces 1.69 m tons with a cultivable area of 1.04 m ha (FAOSTAT, 2014) and is one of the world's largest producer of castor seed meeting 80% the global demand for castor oil. The average productivity of castor is low in India (973 kg/ha) due to a number of biotic stresses including insect pests. Under protected conditions, castor yields are at least 25% higher (1,200–1,400 kg/ha). The defoliators (castor semilooper and *Spodoptera litura*), sucking pests (leaf hopper and thrips) and capsule borer cause economic losses to rainfed castor. Castor semilooper, *Achoea janata* L. (Noctuidae: Lepidoptera) is a regular and serious pest on castor and the older larvae are voracious feeders which often defoliate the plants completely compelling the farmers to abandon the fields. The tobacco caterpillar, *Spodoptera litura* (Fabr.) (Noctuidae: Lepidoptera), a major defoliator of castor is highly polyphagous and widely distributed in India. It causes huge defoliation and attacks capsules and bore into the stems resulting in breakage and falling of castor plants during heavy infestation or epidemics (Lakshminarayana and Raoof, 2005). Mechanical control of *Spodoptera* in its gregarious stages and hand picking of older larvae of semilooper are the only effective management strategies suggested in castor (Lakshminarayana and Raoof, 2005).

Castor belongs to a monotypic genus and the genetic variability available in the germplasm for resistance to foliage feeders is rather limited. Hence, attempts were made to broaden the genetic diversity via transfer of genes from other biological sources through genetic

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transformation for development of transgenics. Both *Agrobacterium*-mediated and direct gene transfer methods (Sujatha and Sailaja, 2005; Sailaja et al., 2008) were used for development of transgenic events conferring resistance to foliage feeders through deployment of *cry1Ab* (Malathi et al., 2006), *Cry1EC* (Sujatha et al., 2009), *cry1AcF* (Kumar et al., 2011) and *Cry1Aa* (unpublished) genes.

Traditionally, castor leaves are fed to larvae of eri silkworm. Eri silkworm (*Samia cynthia ricini*) is an important economic insect used in the production of valuable silk popularly known as `vanya silk` and the process is called eri culture. In India, it is widely distributed in North-Eastern India and is more prevalent in Assam and some parts of Bihar, West Bengal and Orissa followed by Andhra Pradesh, Meghalaya, Nagaland, Manipur, Arunachal Pradesh, Jharkhand, Chhattisgarh and Karnataka. Among the non-mulberry silks, only eri silk production is on an increasing trend. India stands second in the world in production of vanya silk and the production of 1,485 metric tonnes in 2006-07, has significantly increased to 2,460 metric tonnes in 2009-10. Recently, the importance of eri silkworm larvae in terms of economic and nutritional value as a source of edible oil with α- linoleic acid has been realized (Longvah et al., 2012).

Although eri silkworm is polyphagous and multivoltine in nature, the larvae mainly feed on castor leaves when compared to other host plants (Dayashankar, 1982; Devaiah et al., 1985). Keeping in view the development of transgenic events in castor through incorporation of different *Bacillus thuringiensis* (Bt) genes, it is felt essential to determine the safety of the Bt proteins against eri silkworm which is a foliage feeder. Frankenhuyzen (2009) reviewed and presented a detailed account of the insecticidal activity of 125 Bt toxins tested in about 1700 bioassays against 163 test species but information with regard to the reaction of the genus *Samia* to Bt proteins is not documented. The present study has been undertaken to assess the toxicity of

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purified Bt crystal proteins to eri silkworm so that the information generated through these studies will enable the researchers to develop transgenic castor with ecologically safer crystal proteins.

Ten *E. coli* strains harboring Bt δ-endotoxin genes (ECE52- *Cry1Aa*, ECE53- *Cry1Ac*, ECE54- *Cry1Ab*, ECE125- *Cry1Ca*, ECE126- *Cry2Aa*, ECE127- *Cry1Ea*, ECE128- *Cry1Ba*, ECE129- *Cry1Da*, ECE130- *Cry9Aa*, ECE131- *Cry3Aa*) cloned in hyper expressing vectors (pKK223-3, pTZ19R, pSB1402) were obtained from Dr. Zeigler, *Bacillus* Genetic Stock Centre (BGSC), Ohio State University, Ohio. The strains were maintained on MacConkey agar with 50 µg/ml ampicillin and used for toxicity studies. Purification of insecticidal crystal proteins (ICPs) was done according to Lee et al. (1992) from *E. coli* clones grown on LB medium with 100 µg/ml ampicillin for 72 h with shaking at 200 rpm at 37 ^{0}C. The purified crystal proteins were solubilised in buffer with 50 mM sodium carbonate, pH 9.5; 10 mM DTT (dithiothreitol) and incubated at 37 ^{0}C for 2 h with slow shaking (150 rpm). The supernatant collected after centrifugation referred to as protoxin was activated into toxin by digestion with 5% (by mass) trypsin and incubated at 37 ^{0}C for 3 h followed by centrifugation at 12,000 rpm for 15 min and the supernatant with the toxin was collected. The protoxin and toxins were separated on 8% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE).

The eri culture was established in the laboratory of IIOR, Hyderabad by rearing egg masses collected from Regional Eri Research Station, Central Silk Board, Mahaboobnagar, Telangana, India on castor leaves. Bioassays against neonate larvae of eri silkworm were done using leaf paint assay. Eleven concentrations (0-3000 ng/cm^2) of each crystal protein were applied on both sides of the castor leaf (40-50 days old plants) of 8 cm diameter using soft paint brush and air dried for 15 min. Ten neonate larvae were released for each treatment and each

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treatment was replicated three times and repeated twice. Assays were carried out at ambient temperature. Leaf material in the petri dishes was changed every 2 days up to 8 days. Observations on larval mortality were recorded at 2 days interval till 8 days after treatment (DAT) and larval weight gain of surviving larvae was recorded at 8 DAT. The results of the larval bioassays were analysed using probit analysis according to Finney (1971) using the Statistical Analysis System (SAS) version 9.1. The LC_{20}/LC_{50} and EC_{20}/EC_{50} values were defined as the concentration of crystal proteins at which the larvae showed 20/50% mortality and 20/50% larval weight reduction, respectively. The ratio between weights of larvae on treated and untreated leaves at day 8 was used to calculate the reduction in larval weight of surviving larvae.

Significant differences in mortality of eri silkworm larvae were observed when tested with varied concentrations of Bt crystal proteins. Among the ten crystal proteins tested, 100% mortality was observed with 11.7-3000 ng/cm^2, 187.5-3000 ng/cm^2, 375-3000 ng/cm^2 concentrations of Cry1Aa, Cry1Ac and Cry1Ab proteins, respectively (Fig. 1). Larval mortality evaluated by probit analysis and data presented in table1 indicated high larvicidal activity of Cry1Aa with LC_{50} value of 2.6 ng/cm^2 followed by Cry1Ac (29.3 ng/cm^2) and Cry1Ab (68.7 ng/cm^2) proteins. Cry1Aa was the most potent toxin and resulted in 100% mortality even at low concentrations (5.86 ng/cm^2) at 8 DAT whereas Cry1Ab and Cry1Ac proteins showed only significant reduction in weight gain of surviving larvae. On leaves coated with Cry1Ca and Cry1Ea proteins, 23% and 28% mortality, respectively at 3000 ng/cm^2 was recorded while the other proteins were found to be mildly toxic or non-toxic.

Differences in growth of the surviving larvae due to various treatments were highly significant. On leaves treated with Cry1Aa protein, larvae survived on the lowest concentration tested (2.93 ng/cm^2) and weight of surviving larva was 25-fold less (6 mg) as compared to

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control (150 mg). Larval growth was significantly reduced on leaves treated with Cry1Ac and Cry1Ab proteins with EC_{50} values of 6.8 and 66.3, respectively (Table 1). The proteins Cry1Ca, Cry2Aa, Cry1Da and Cry9Aa with no significant effect on larval mortality showed clear effect on larval growth with EC_{50} values ranging from 193.7 to 996.3 ng/cm^2. The toxins Cry1Ba and Cry3Aa had no effect either on larval mortality or growth.

The main aim of this study was to assess the efficacy of *B. thuringiensis* crystal proteins against neonate larvae of eri silkworm and generate information on the lethal concentration of the ten different crystal proteins by using leaf paint bioassays. In the present study, Cry1Aa showed most toxicity to eri silkworm neonates with LC_{50} value of 2.6 ng/cm^2. We had previously found that Cry1Aa protein was toxic to *Spodoptera litura* (Lakshminarayana and Sujatha, 2005) and resulted in 100% mortality of castor semilooper in 3 DAT (Sujatha and Lakshminarayana, 2005). The Cry1Ac and Cry1Ab proteins were less toxic when compared with Cry1Aa against neonate larvae of eri silkworm. Likewise in our earlier studies, these proteins were found to be moderately toxic to the two major foliage feeders (Lakshminarayana and Sujatha, 2005; Sujatha and Lakshminarayana, 2005). However, the EC_{50} and LC_{50} values recorded for the three toxic proteins for eri silkworm larvae were comparable with those for castor semilooper (Sujatha and Lakshminarayana, 2005) while were far less than those for *Spodoptera litura* (Lakshminarayana and Sujatha, 2005). Since castor transgenic events conferring resistance to foliage feeders are being developed through deployment of fusion genes like *cry1EC* and *cry1AcF* (Sujatha et al., 2009; Kumar et al., 2011) it is essential to determine the effects of the individual toxins used in these chimeric genes.

Thus, among the 10 insecticidal crystal proteins tested, Cry1Aa was highly toxic to the larvae of eri silkworm followed by Cry1Ac and Cry1Ab proteins. The crystal proteins Cry1Ca

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and Cry1Ea were moderately toxic while the other proteins tested were non-toxic to eri silkworm larvae. This is the first report to evaluate the toxicity of crystal proteins against eri silkworm and the information serves as a benchmark for deployment of Bt δ-endotoxin genes in castor which serves as a source of leaves for rearing beneficial insects like eri silkworms. Owing to the sensitivity of eri silkworm larvae to Cry1Aa, Cry1Ab and Cry1Ac proteins, castor transgenic events harbouring these genes should be assayed against eri silkworm in addition to the lepidopteran pests for which the respective genes are deployed. Further, studies need to be taken up for identification of proteins that are toxic to the target pests of castor but not attacking eri silkworm for development of genetically modified castor.

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Table 1

Dose-mortality responses and weight reduction of eri silkworm neonate larvae on castor leaves treated with Bt Cry toxins at 8 days after treatment.

Toxin	LC_{20}[a] (ng/cm^2)	95% Fiducial limits		LC_{50}[a] (ng/cm^2)	95% Fiducial limits		Slope ± SE	EC_{20}[b] (ng/cm^2)	95% Fiducial limits		EC_{50}[b] (ng/cm^2)	95% Fiducial limits		Slope ±SE
		Lower	Upper		Lower	Upper			Lower	Upper		lower	upper	
Cry1Aa	1.4	1.0	1.8	2.6	2.1	3.0	3.2±0.3	ND	-	-	ND	-	-	-
Cry1Ab	29.3	15.6	43.6	68.7	46.5	101.0	2.3±0.1	26.6	0.9	67.8	66.3	8.6	132.8	2.1±0.5
Cry1Ac	11.3	6.0	17.0	29.3	19.7	43.5	2.0±0.1	2.0	0.3	5.3	6.8	1.8	13.6	1.6±0.3
Cry1Ca	1332.6	445.0	2525.2	3104.3	1804.0	$31.5x10^3$	2.3±0.5	5.3	$5.3x10^{-6}$	84.5	193.7	0.6	1523.0	0.5±0.2
Cry2Aa	6206.4	-	-	$17.4 x10^3$	-	-	1.9±1.2	31.9	16.6	51.9	996.3	644.9	1747	0.6±0.1
Cry1Ea	3422.1	2161.4	$29.1 x10^3$	8934.0	4246.1	$202.1x10^4$	2.0±0.8	51.5	25.3	90.4	9837.0	3792.0	$43.6x10^3$	0.4±0.1
Cry1Ba	8805.6	-	-	$27.1x10^3$	-	-	1.7±1.3	2106.0	-	-	$89.6x10^5$	-	-	0.2±0.3
Cry1Da	5103.0	-	-	$14.0 x10^3$	-	-	1.9±1.0	19.0	-	-	312.7	-	-	0.7±0.4
Cry9Aa	-	-	-	-	-	-	-	18.0	11.4	26.4	439.7	314.4	650.1	0.6±0.1
Cry3Aa	-	-	-	-	-	-	-	2.2	-	-	$2.6x\ 10^{20}$	-	-	0.04±0.1

[a] LC_{20} and LC_{50} denote the concentration of Cry protein causing 20 and 50% mortality, respectively.

[b] EC_{20} and EC_{50} denote the concentration of Cry protein causing 20 and 50% larval weight reduction, respectively.

- is used in cases where values could not be estimated as the $EC_{20/50}$ and $LC_{20/50}$ values were substantially higher than the highest concentration tested.

ND - indicates not determined as there were no surviving larvae.

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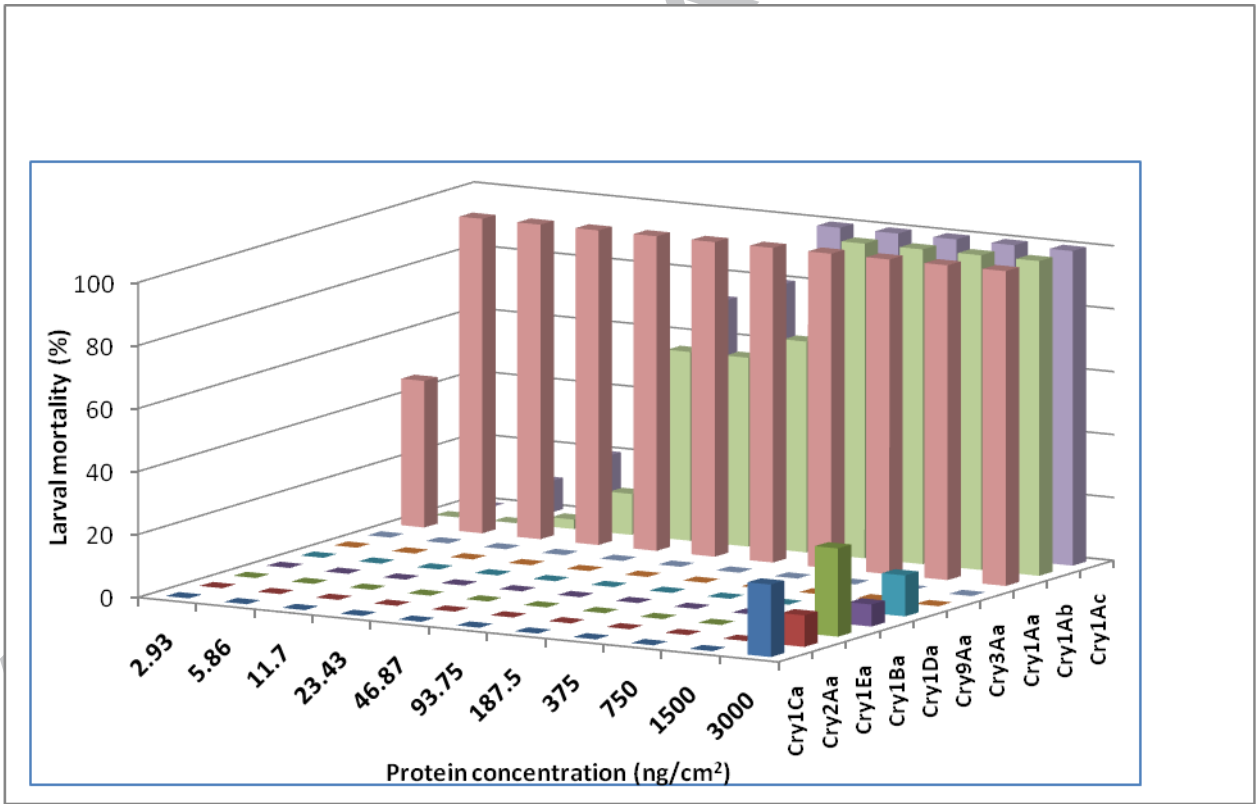
Figure legends

Fig. 1. Mortality (%) of eri silkworm larvae due to Bt δ-endotoxin proteins at different concentrations (2.93-3000 ng/cm^2) recorded at 8 DAT.

Figure 1



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Graphical abstract

Highlights

- Efficacy of *Bacillus thuringiensis* (Bt) proteins against *Samia cynthia* determined.
- Cry1Aa, Cry1Ab, Cry1Ac proteins highly toxic to eri silkworm.
- Castor transgenics harbouring Bt genes should be bioassayed for eri silkworm.

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