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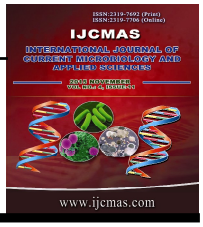

Original Research Article

Baseline Susceptibility of *Helicoverpa armigera* (Hubner) to cry Toxins of Codon Optimized *cry 1Ac* Gene and its Mutant Gene

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A B S T R A C T

The susceptibility of, *Helicoverpa armigera* (Lepidoptera: Noctuidae), to three *Bacillus thuringiensis* Cry proteins namely native *Cry1Ac*, a codon optimised *Cry1Ac* and a mutant of codon optimised *Cry1Ac* was evaluated by bioassay. The toxicity of each Cry protein to *H. armigera* larvae was determined by the diet contamination method for second instar larvae. The concentration of cry proteins to be used for bioassay was determined by quantitative ELISA. For each of the proteins used, dose–mortality and the median lethal concentration (LC_{50}) were determined. On the basis of the LC_{50} values obtained among the evaluated Cry proteins, the codon optimised *cry1Ac* was found to be the most toxic to *H. armigera*. The study provides an initial benchmark to assess the susceptibility of *Helicoverpa armigera* to codon optimised and its mutant Cry gene proteins, thus providing the data for the use of these genes in development of transgenic plants for Helicoverpa resistance.

Introduction

Bacillus thuringiensis is a gram-positive bacteria belonging to phylum Firmicutes. It is ubiquitous, spore forming bacterium producing proteinaceous inclusion bodies called δ-endotoxin during sporulation which have insecticidal action (Gajendra Babu *et al.,* 2002). This soil dwelling bacterium is commonly used as a biological pesticide and is also alternatively used for extracting cry proteins that are used as insecticide. It is an effective insecticide which is relatively harmless to the natural enemies and is safe to higher animals and humans.

These cry proteins are produced during the growth cycle of the bacteria (Hofte and Whiteley, 1989). Cry protoxins are typically 130kDa or 70kDa proteins. When ingested by susceptible insects/larvae, these crystal proteins are exposed to the alkaline environment of the gut and they are solubilized and proteolytically processed at the N-terminus and/or the C-terminus by midgut proteases to yield the active protease-resistant toxin (Deist *et al.,* 2014). These activated toxins then bind to the receptors present on the brush border membrane vesicles of the midgut epithelium

and perforate cell membrane, which leads to ionic imbalance and eventually insect death (Gill *et al.,* 1992).

Since the identification and cloning of the first Bt insecticidal crystal protein gene (Cry) in 1981, the number of genes coding for this novel insecticidal proteins has continuously increased. In the past decades, more than 700 cry gene sequences that code for crystal proteins have been identified and large plasmids appear to be the usual location for these genes (Palma *et al.,* 2014).

Cry toxins have significant but specific toxicity against many typical insect orders, such as lepidoptera (butterflies and moths), diptera (flies and mosquitoes) and coleoptera (beetles and weevils). Newly emerging toxins have presented their toxicities towards other insect orders such as Hymenoptera, Orthoptera, Hemiptera, Isoptera, Mallophaga, Thisanoptera, and some pests, such as nematodes and mites (Xu *et al.,* 2014).

Recently the cry proteins have been expressed in transgenic plants to confer inherent pest resistance. Bt transgenic crops have been overwhelmingly successful and beneficial, leading to higher yields and reducing the use of chemical pesticides. However, their deployment has attracted some criticism particularly with regard to the potential evolution of pest-resistant insect strains (Sanahuja *et al.,* 2011).

Many strategies that have been employed to enhance toxicity against specific target species including those that have evolved field resistance to Bt crystal protein. These strategies include toxin truncation, modification of protease cleavage sites, domain swapping, site-directed mutagenesis, and peptide addition (Deist *et al.,* 2014).

Helicoverpa armigera is a polyphagus insect pest of worldwide occurrence. It is reported to cause major crop damage to cotton, pulses and some vegetables (Krishnappa Chandrashekar *et al.,* 2005). *H. armigera* has a history of developing resistance to most of the conventional insecticides. Many experiments in laboratory have shown that *H. Armigera* is capable of developing resistance to *Cry1Ac* that is mostly expressed in the transgenic crops.

The toxicity of *cry* proteins has to be studied to determine the baseline that can be used in monitoring resistance to *Cry1Ac* which is a primary regulatory requirement for transgenic crop technology (Witkowski *et al.,* 1999).

The present study compares the toxicity of *Cry1Ac* with its codon optimized gene (*Cry1Ac-M)* and its mutant (*Cry1Ac-MM*) to *H. armigera*. To our knowledge, no published studies have evaluated the larvicidal activity of mutants of *Cry1Ac* on *H. armigera*. Hence, this study would provide important guidelines to determine the potential usage of *Cry1Ac* mutant genes in *H. armigera* management.

Materials and Methods

Cry genes and Bacterial strains

The purpose of mutagenesis of *Cry1Ac* was to look for possible mutants with more toxicity to the target insects.

The modifications were done as mentioned below:

Cry1Ac-M **–**The modified gene was custom synthesized at Gene Art, Germany. The native *Cry1Ac* (3.5kb) out of which 1857bp of toxic domain was considered. During the process of optimization out of 620 codons

259 were altered, without changing the amino acid sequence and the GC content of Native *Cry1Ac* was raised to 42.60% from 37.80%. The fragment was cloned into pGA4 (Amp^R) using forward Kpn1 and reverse Sac1 restriction sites and the construct was named as pMKK1008 (Mohan, 2008).

Cry1Ac-MM – The codon optimized *Cry1Ac-M* cloned from truncated version (1.85kb) of Native *Cry1Ac* was considered for mutagenesis. Here the *Cry1Ac-M* gene was released from pMKK18122 construct, containing *Cry1Ac-M* in pYES2C/T vector by digestion with Kpn1 and BamH1.The 1.85kb modified fragment was inserted into Kpn1 and BamH1 sites of pUC18 vector and random mutations were created (Ashwini, 2011).

Preparation of cry toxin /cry toxin samples

Toxins of *Cry1Ac, Cry1Ac-M and Cry1Ac-MM* were extracted by following the protocol described. *E. coli* cells were grown in LB with 100mg/ml ampicillin at 37 for 6–8 hours. 1:100 dilutions of these cultures were grown till OD = 600 (approximately 8 hours).

The cells were pelleted at 5000 rpm for 15 minutes at 4°C and suspended in 1X PBS, further the suspension was sonicated till it was clear and non-viscous. Triton X-100 (1%) was used to solubilise the proteins. The suspension was then incubated on a rocker for 10minutes at 4^0C. The supernatant containing the toxin was collected by centrifugation at 5000rpm for 30 minutes at 4^0c and quantified using Nanodrop. Protein concentration was made uniform using buffer and stored at -20C till further use.

Collection and maintenance of *H.armigera*

About 350 eggs were collected from the National Bureau of Agriculturally Important Insects (NBAII), Bangalore to obtain a stock culture of *H. armigera*. The larvae obtained were cultured on the semi synthetic diet containing chickpea powder (30g), yeast (15g), agar (4g), Wessons salt (2g), casein (1.5g), ascorbic acid (1g), multivitamin tablet, sorbic acid (0.3g), methyl parahydroxy benzoate (0.6g). The larvae were inspected regularly to ensure that they remained pathogen free and were maintained in the culture room with temperature 27–29°C. Larvae were reared on a bioassay tray till pupation. Moths were kept in a plastic jar and fed on 10% honey solution. A layer of black cloth was placed on the inner surface for oviposition.

ELISA

The primary analysis was done by Qualitative ELISA using Envirologix-Quickstix strips for *Cry1Ab/Cry1Ac* by Immunochromatography (lateral flow).The Quantitative ELISA was done by Envirologix- Quantiplate kit for Cry1Ab/Cry1Ac, APOO3QV- V50, 1 plate and kit lot 273522A.

Bioassay

Bioassay was carried out by a diet contamination method for the second instar larval stage of *H. armigera*. The toxic solutions of *Cry1Ac, Cry1Ac-M, and*

Cry1Ac-MM were thoroughly mixed with a known weight of semi-synthetic diet at room temperature. Different concentrations of the toxins in water were added to the diet after cooling to 40^0C. The diets were mixed thoroughly and poured in a tray (128 cells, 17 1/2 L, 8 1/4 W and 1 H) with each cell serving as one treatment. Five concentrations were used for each bioassay with ten neonates for each concentration and 3 replicates per concentration. Negative control as BL21 (untransformed) cells and media without any *Cry* protein were also included in the assays. A minimum of 250 larvae's (3x50-3 cry toxins, 1x50-BL21 and 1x50-without any toxin) were used for each bioassay and mortality was pooled for all concentrations. The trays were packed with pressure sensitive adhesive perforated tape. Concentrations which gave corrected mortality between 20% and 80% at 72 hours were used for the calculations of median lethal concentration (LC_{50}) and 95% Fiducity limits (FL). The mortality was checked on 24, 48 and 72 hours from the date of experiment. The larvae were marked dead if they did not move when prodded.

Statistical analysis

The statistical analysis was done by 'Statistical packages for Social Sciences' by probit analysis.

Results and Discussion

Quantification of cry proteins

The Quickstix test was done with *Cry1Ac, Cry1Ac-M and Cry1Ac-MM*. In the strip containing BL21 sample, only the assay band was observed. This indicated the absence of cry proteins and presence of analyte. It also indicates that 2 spurious bands are not formed due to analyte. In samples containing the *Cry1Ac, Cry1Ac-M* and *Cry1Ac-MM*, both assay band and expression band were detected as shown in figure 1. This indicated that *Cry1Ac-M* and *Cry1Ac-MM* were similar to *Cry1Ac* in the antibody based detection and the concentration of these cry proteins could be detected using quantitative ELISA.

The concentration of *Cry1Ac, Cry1Ac-M* and *Cry1Ac-MM* was analysed by Quantitative ELISA as shown in figure 2 and found to be 3.54ppb, 2.24ppb and 3.80ppb respectively. The cry proteins were diluted to 2.24ppb, the lowest obtained concentrations among the 3 *cry* proteins analysed. These cry proteins of equal concentration were used in bioassay to check the toxicity of cry toxin against *Helicoverpa armigera*.

Susceptibility of *H. armigera* to Cry proteins

Results of the probit analysis of the dose mortality bioassays with 2nd instars larvae of *H. armigera* are shown in the table 1. Based on the LC_{50} values obtained among the Cry proteins evaluated, the larvicidal activity of *Cry1Ac-M* isolate was found to be highly toxic to the *H. armigera* with the LC_{50} value of 0.32ppb followed by *Cry1Ac* isolate (LC_{50}=0.36ppb) and *Cry1Ac-MM* isolate (LC_{50}=0.40ppb). The order of toxicity of Cry protein was as follows: *Cry1Ac-M* isolate >*Cry1Ac* isolate >*Cry1Ac-MM* isolate. In control the mortality ranged from 0- 10%.

The use of insecticidal cry proteins of *Bacillus thuringiensis* in insect pest control has emerged as a useful alternative to chemical insecticides, especially with the development of transgenic plants expressing Cry toxins. But the development of

resistance to cry proteins has also become a major issue (Gassmann *et al.*, 2014; Kranthi *et al.*, 2006; Tabashnik *et al.*, 2012).

A mandatory resistance management tactic known as high dose plus refuge strategy is in place in many countries. But in countries with less regulated cropping systems where refuges are not required, insect resistance will be a major problem.

Table.1 Toxicity of *Cry1Ac, Cry1Ac-M and Cry1AC-MM a*gainst *Helicoverpa armigera*

Isolates	LC_{50}(ppb)	Fiducial limits		LC_{95}(ppb)	Regression equation (Y=a + bx)	X^2
		Lower limit (ppb)	Upper limit(ppb)			
Cry1Ac	0.36	0.22	0.56	9.03	Y= 0.516+0.14x	3.16
Cry1Ac-M	0.32	0.20	0.52	8.90	Y=0.550+0.16x	3.90
Cry1Ac-MM	0.40	0.28	0.65	6.19	Y=0.540+0.18x	1.00

Figure.1 Quickstix strips based detection of cry proteins

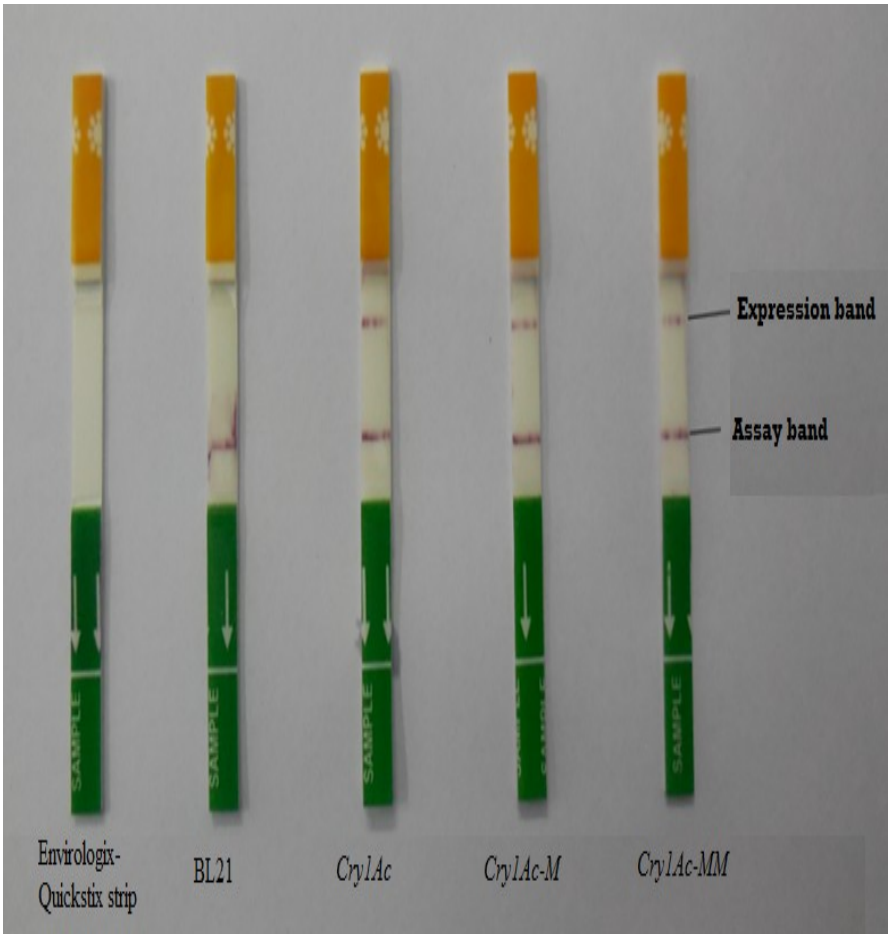
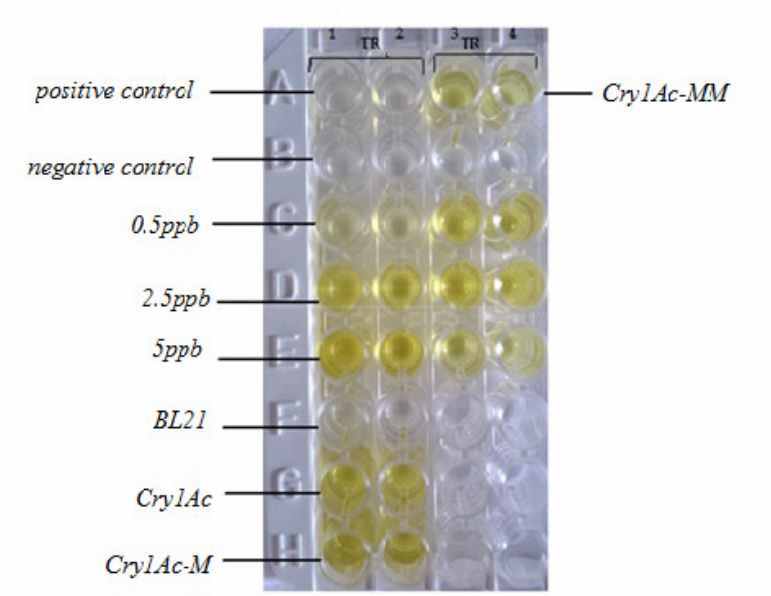


Figure.2 Estimation of *Cry1Ac*, *Cry1Ac-M* and *Cry1Ac-MM*, using ELISA assay. TR: technical replications; A1 &A2: Blank; B1& B2: Negative control; C1,C2,D1,D2,E1,E2: Positive control; F1&F2:BL21; G1 &G2: *Cry1Ac*;H1&H2: *Cry1Ac-M*; A3&A4 *Cry1Ac-MM;* B3,B4,C3,C4,D3,D4,E3,E4:Replication 2



Many ways to counter insect resistance need have been developed. One of the different strategies that could be used to cope with insect resistance is the use of modified Cry toxins (Bravo *et al.,* 2011). Generally susceptibility of *Helicoverpa armigera* to Cry toxins are measured via a diet-incorporated assay by estimating Lethal concentration of mortality (LC50) (Ali *et al.,* 2006). Since naturally many cry proteins are found in *Bacillus thuringiensis* strains it becomes difficult to determine the toxicity of single cry protein. This can be avoided by the use of *E. coli* as a host to express cry proteins. This strategy will also lead to elimination of the interaction between spores and crystals. Hence all the cry genes to be studied were cloned into pET vector and expressed in *E. coli.* The isolated protein was used to determine larvicidal activity. The observed larval mortality during bioassays was statistically identical to that observed for water control, suggest that the BL21 can be used as an expression host for bioassays of the cloned cry genes (Hua Li and Bouwer, 2012a,b).

The results of the present investigation gave the detailed information about the susceptibility of *H. armigera* larvae to cry toxins from codon optimized Cry1Ac toxin and its mutant *(Cry1Ac-M* and *Cry1Ac-MM* respectively) in comparison to cry toxin of the native *Cry1Ac*. The median lethal concentration (LC_{50}) of *Cry1Ac* toxins and larval mortality due to its treatments were studied. Among the tested samples the codon optimized *Cry1Ac* gene *Cry1Ac-M* toxin was found to be the most efficient when compared with native *Cry1Ac* and mutated *Cry1Ac* gene (*Cry1Ac-MM*). Many workers have reported toxicity of cry proteins against the larvae *H. armigera* for Cry1Ac (Goud *et al.,* 1995; Chakrabarti *et al.,* 1998; Gujar *et al.,* 2000; Hua Li and Bouwer, 2012a,b) but very few reports on the toxicity of cry proteins of codon optimized and mutated Cry genes has been reported (Kim *et al.,* 2008; Xu *et al.,* 2009; Xue *et al.,* 2005). But till now no reports on the toxicity of cry toxins from codon optimized and mutated cry genes on *Helicoverpa armigera* has been reported hence our work acts as a baseline for the study of susceptibility of *Helicoverpa armigera* to the codon optimized and mutant genes of *Cry1Ac* expressed in *E. coli* DH5-α.The perusal data showed that *Cry1Ac* as

the most toxic but with our results of LC_{50} indicating *Cry1Ac-M* to be the more toxic, there is a scope for this gene to be used as an alternative to *Cry1Ac* or to be used in the pyramiding of different cry genes for controlling *Helicoverpa armigera*.

In conclusion, the present study showed a brilliant future in the use of the codon optimized cry genes for controlling insect pests, especially with developing transgenic plants for *Helicoverpa armigera* resistance.

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