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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (9): 1639-1649, 2006
ISSN 1028-8880

Cross Resistance of Cry1Ac Resistant Cotton Bollworm, *Helicoverpa armigera* to Spore-δ-endotoxin of Various *Bacillus thuringiensis* (Berliner)

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Abstract: The present study elaborates on the results of cross-resistance of Cry1Ac resistant *Helicoverpa armigera* (Hübner) to spore-δ-endotoxin complex of Bt strains (HD-1, HD-4, HD-8, HD-57, HD-133, HD-234, HD-263, HD-282, HD-551, HD-744 and Bt-based biopesticide. In this study, all the bioassays were done on larvae of 55 th, 60 th and 61st Cry1Ac selected generations of *H. armigera*. The resistance ratios (RR) among Cry1Ac selected generations varied from 172 to 417 fold in different generations. Cry1Ac selection exhibited low resistance ratios (2.722, 2.756) against HD-1 and HD-8 among all the HD strains tested and the slopes for HD-1 and HD-8 were highly significant (1.49 and 1.31, respectively). The test insect also showed low resistance ratios 3.959 and 4.385 for HD-551 and HD-57 respectively with significant slopes 1.18 and 2.36 accordingly. The spore-crystal toxins of strains of HD-1, HD-8, HD-263 and Bt-based biopesticide caused significant reduction in larval weight (%) with regressions $y = 0.011x+93.912$, $R^2 = 0.7269$, df = 4, $p<0.05$ and $y = 0.03x+85.332$, $R^2 = 0.6982$, df = 4, $p<0.05$, $y = 0.0458x+79.58$, $R^2 = 0.7271$, df = 4, $p<0.05$ and $y = 0.0776x+59.169$, $R^2 = 0.8273$, df = 4, $p<0.05$, respectively against Cry1Ac selection. Significant slopes 1.49, 1.31, 2.36, 1.67, 1.18, 1.12 and 2.09 for HD-1, HD-8, HD-57, HD-263, HD-551, HD-744 and Bt-based biopesticide, respectively indicated fast interaction between spore-crystal toxins of Bt strains and the Cry1Ac resistant insect. Similarly for susceptible *H. armigera*, significant regression trends in term of percent larval weight reduction were observed for HD-263 (equation $y = 0.0374x+82.173$, df = 4, $p<0.05$, $R^2 = 0.7098$), HD-1 ($y = 0.0501x+76.35$, df = 4 $p<0.05$), HD-8 ($y = 0.0584x+68.508$, df = 4, $p<0.05$) and Bt-based biopesticide ($y = 0.0968x+57.063$, df = 4, $p<0.05$). Previous studies conducted by other workers indicated synergistic impact between spores and crystals of Bt strains against resistant insects. Present results suggest that the spore-crystal complex (perhaps due to synergistic interaction between or among the spore and crystal components) may have caused synergism due to which a Cry1Ac selection was knocked down. Thus, spore-crystal complex-based formulations of Bts can be developed for use as an effective tool to manage insect populations that have development resistance to single toxin (Cry1Ac) produced in transgenic crops (e.g., Bt-Cotton).

Key words: Spore-δ-endotoxins, *Bacillus thuringiensis*, *Helicoverpa armigera*, Cry1Ac resistant cotton bollworm, resistance management

INTRODUCTION

Helicoverpa armigera, a major pest of many crops throughout parts of Europe, Asia, Africa and Australia and most of the research on the control of *H. armigera* has been done in cotton, because this crop has major economic importance and exceptionally high rate of pesticide application (Glenn *et al*., 1994). In China since 1990, the control of *H. armigera* has become difficult due to development of high level of resistance to chemical pesticides (Wu and Guo, 1997; Wu *et al*., 1997). Transgenic cotton expressing δ-endotoxin from *Bacillus thuringiensis* (Bt) did not only contribute in managing *H. armigera* populations but also resulted in an increase in cultivation of Bt-cotton in northern China covering an area of 1.067 million ha (Wu *et al*., 2000, 2002; Qu *et al*., 2001).

The Cry1Ac protein has been reported to be the most toxic of the *B. thuringiensis* insecticidal proteins to *H. armigera* (Padidam, 1992; Liao *et al*., 1996) and this protein is 30 times less toxic to *H. virescens* than to *H. armigera* being the prime target of transgenic cotton in United States (Luttrell *et al*., 1999; Liao *et al*., 2002). As the cotton plants mature, their insecticidal activity

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decreases and in population, some *H. armigera* larvae are capable to survive and complete development on the transgenic cotton, thus reduced toxicity of mature transgenic cotton provides an opportunity for the insect to develop resistance to Cry1Ac (Akhurst *et al*., 2003).

Transgenic crops producing toxins encoded by Bt genes are important for pest management (Liu *et al*., 2001) but widespread use of Bt crops favors evolution of resistance by pests (Gould and Tabashnik, 1998). Documented mode of resistance to Bt is characterized by >500-fold resistance to at least one Cry1A toxin, recessive inheritance, little or no cross-resistance to Cry1C and reduced binding of at least one Cry1A toxin (Tabashnik *et al*., 1998) and high level of cross-resistance to Cry1Aa, Cry1Ab, Cry1Ac, Cry1F and Cry1J was observed in experiments using Cry1C resistant diamondback moth.

Liang *et al.* (2000) conducted studies on the cross resistance of cotton bollworm, *Helicoverpa armigera* to *Bacillus thuringiensis* and observed significant disorientation in weight of 7 days old larvae and adult emergence in resistant *H. armigera* populations due to feeding of Bt pesticide, Bt protoxin and Bt transgenic cotton. Muller-Chon *et al.* (1996) also conducted studies on *Spodoptera litoralis* resistance to Cry1C and cross-resistance to other *Bacillus thuringiensis* crystal toxins and reported partial cross-resistance to Cry1D, Cry1E and Cry1Ab.

In addition to studies conducted on evaluation of cross-resistance, Liu *et al.* (1998) reported synergism between *Bacillus thuringiensis* spores and toxins against resistant and susceptible diamondback moth (*Plutella xylostella*) and observed that spore of *B. thuringiensis* subsp. *kurstaki* increased the toxicity of crystals of *B. thuringiensis* subsp. *Kurstaki* to both resistant and susceptible larvae (*B. thuringiensis* subsp. *kurstaki*, resistance ratios were 1,200 for a spore-crystal mixture and 56,000 for crystals without spores).

Miyasono *et al.* (1994) found that toxin-free spores did not kill larvae, but spores increased the toxicity of *B. thuringiensis* subsp. *kurstaki* crystals to larvae from a susceptible strain of diamond back moth. The interaction observed between these spores and toxins exemplifies synergism, in which the toxicity of a mixture is greater than expected on the basis of the independent toxicity of components (Tabashnik, 1992). Tang *et al.* (1996) found synergism between *B. thuringiensis* subsp. *kurstaki* spores and each of the three individual Cry1 toxins from *B. thuringiensis* subsp. *kurstaki* against a susceptible strain of diamond moth but not against a resistant strain.

In the present study, we describe the degree of cross-resistance and larval developmental retardation (larval weight decrease) in Cry1Ac selections of cotton bollworm *H. armigera* to spore-δ-endotoxin (multiple toxins) of various strains of *B. thuringiensis* (HD-series) with a view to manage resistant insect. On the basis of interaction between spores and toxins of *B. thuringiensis* in some case (not in all), it is also possible to develop suitable Bt-based spore-crystal formulations which may prove as an effective tool to manage cotton bollworm populations that have developed resistance to single toxin Cry1Ac produced in Bt cotton.

MATERIALS AND METHODS

This study was conducted in 2004 in the Department of cotton Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, People's Republic of China.

Cry1Ac *H. armigera* selection: The resistant selection were developed as per procedure by Liang *et al.* (2000) which stated that the larvae collected from the cotton fields in Henan Province, China in 1996 and maintained in the laboratory on artificial diet developed by Liang *et al.* (1999). The adults raised from the field larvae were maintained as susceptible *H. armigera* stock culture for several generations on artificial diet. In 1997, neonates from adults of susceptible *H. armigera* were divided into two groups. The first group was reared on a normal artificial diet for bioassays for establishing baselines. The second group (about 1000 larvae) was reared on an artificial diet which contained 2.32 μg mL^{-1} Cry1Ac protein in F_1 (the pure Cry1Ac protein was procured through the courtesy of Dr. Zhang Jie of biotechnology group of this Institute). After 7 days of feeding, about 250 larvae, which developed rapidly, were transferred to normal artificial diet until moth emergence (this procedure was adopted for all subsequent generations from F_1 to F_{61}). The moths emerged from F_1 were transferred to the mating cage for production of the next generation ($F_{2)}$ and about 1000 of the resulting larvae were used for further selection in each generation. A concentration of 2.32 μg mL^{-1} Cry1Ac protein was used for resistance screening until 5th generation. Sixth to F_{10} generation larvae were allowed to feed on 2.63 μg mL^{-1} Cry1Ac protein. The concentration of Cry1Ac toxin was increased to 2.78 μg mL^{-1} for the F_{11} to F_{15} generations, 5.56 μg mL^{-1} Cry1Ac toxin from F_{16} to F_{20}, 6.67 μg mL^{-1} Cry1Ac from F_{21} to F_{25}, 8.33 μg mL^{-1} Cry1Ac toxin from F_{26} to F_{35}, 9.09 μg mL^{-1} Cry1Ac from F_{36} to F_{45}, 14.30 μg mL^{-1} Cry1Ac toxin from F_{46} to F_{61}. In this study, all the

bioassays were done on larvae of 55 th, 60 th and 61st generations. The development of resistance in *H. armigera* strain in terms of resistance ratio has been shown in Table 1.

***Bacillus thuringiensis* strains used against Cry1Ac selected *H. armigera*:** Different strains of *B. thuringiensis* procured through courtesy of 1) Dr. Alejandro (Alex) J. Rooney, United States Department of Agriculture-Agricultural Research Service (USDA-ARS), 1815 North University Street, Peoria, Illinois 61604, USA and 2) Dr. Daniel R. Zeigler, *Bacillus* Genetic Stock Center (BGSC), Department of Biochemistry, Ohio State University, 484 West Twelfth Avenue, Columbus, OH, USA were used in the present study. *B. thuringiensis* strains selected for the experimentation were HD-1, HD-4, HD-8, HD-57, HD-133, HD-234, HD-263, HD-282, HD-551, HD-744 and Bt-based biopesticide (Bt-*kurstaki* based, 16000 IU mg^{-1} potency).

The test insect: Neonate larvae of susceptible and resistant selection of *H. armigera* were obtained from the colonies maintained on corn-soybean diet (Liang *et al*., 1999) in the rearing facility of this Institute.

***Bacillus thuringiensis* spore-δ-endotoxins production:** Spore-δ-endotoxins of various *B. thuringiensis* were produced as per procedure of Ahmed (1995). Basic growth medium (BGM) 4.5 liter batch liquid medium for production of spore-δ-endotoxin: Nutrient Broth (NB) 58.50 g, Calcium chloride ($CaCl_2$ $2H_2O$) 0.36 g (Dihydrate MW 147.02), Magnesium sulphate ($MgSO_4$ $7H_2O$, MW 246.47 pH5.0) 0.90 g, Magnese sulphate ($MnSO_4$ $4H_2O$, FW 190.0) 0.225 g (Tariq, 2001; Abida, 2002; Ahmed, 1995). Components of the Basic Growth Medium (BGM) were dissolved in 4.5 L distilled water and distributed into one liter capacity conical flasks so that each flask contained 300 mL BGM. The flasks were autoclaved at 121°C, 15 lb. pressure for 15 min. Salt solution containing ($ZnCl_2$ 0.05 M, $MgCl_2$ 0.5, $MnCl_2$ 0.01 M, $CaCl_2$ 0.2 M, $FeCl_3$ 0.05 M plus 5 drops of concentrated HCl (salt solution was sterilized through 0.20 μm millipore filter) was added to the BGM at the rate of 1.00 mL salt solution/liter BGM at about 50°C for enhancing sporulation. One loop of pure Bt culture was added to each flask containing 300 mL of respective BGM batch. Flasks were incubated in rotary shaking incubator at 30±1°C with 250 rpm for 3 to 5 days (as per slow/fast growth rate of the respective Bt till more than 95% sporulation was achieved). Sporulated culture was centrifuged at 8000 rpm, 4°C for 15 min. The thick layer of sediment (pellet) of spore-δ-endotoxin complex at the bottom of centrifuge tube was carefully transferred to a sterilized beaker and covered with paraffin paper and freezed for 24 h (Tariq, 2001; Ahmed, 1995). The frozen pellet of spore-δ-endotoxin was freeze dried at -95°C and vacuum 147 mT for 36 h. The weight of the dried Bt powders was recorded and the freeze dried powders of spore-δ-endotoxins of various HD-strains were used in all the bioassays as and when needed.

Determination of colony forming units (CFU or spore) per mg of freeze dried spore-δ-endotoxin powder of various HD-strains

Preparation of dilutions of Bt powder: Ten mg of Bt powder was added to 10.0 mL sterilized distilled H_2O, mixed well so as to obtain a uniform mixture, this mixture was marked 1 (Ahmed, 1995).

1. 10 mg Bt powder + 10.0 mL sterile distilled (s.d.) H_2O
2. 1 mL of (100 μg) from mixture 1 + 9.0 mL sterile distilled (s.d.) $H_2O = 10^{-1}$
3. Transferred 10 μL from 2 mixed with 10.0 mL s.d. $H_2O = 10^{-4}$
4. Transferred 100 μL from 3 mixed with 9.9 mL s.d. $H_2O = 10^{-6}$
5. Transferred 1.0 mL from 4 mixed with 9.0 mL s.d. $H_2O = 10^{-7}$
6. Transferred 1.0 mL from 5 mixed with 9.0 mL s.d. $H_2O = 10^{-8}$

Fifty microliter was pipetted out from each of the 10^{-8} dilutions and poured on the surface of agar-basic growth-medium in the petri-plate and spread uniformly on the medium surface with the help of sterilized L-shaped glass rod. Four replicates of each dilution were set up. The inoculated plates were placed in the incubator at 28°C for 24-28 h (as per fast/slow growing colony). The colonies in each plate of respective Bt powder was counted and calculated in term of No. of CFU/mg of the Bt powder. Following formula was used for calculating the colony forming unit cfu mg^{-1}.

For example: If 15 colonies are found on a plate inoculated with 10^{-8} dilution of a Bt powder.

15 cfu×20 (50 μL spread on the plate surface x 20 = 1.0 mL) =300 cfu per mL per mg in dilution $10^{-8} = 3\times10^{10}$ cfu mg^{-1} of the original sample.

spore-δ-endotoxin preparation were determined to be 99% pure by phase contrast microscope. The plating showed that 1000 μg (1 mg) of spore-crystal powder of

HD-1, HD-4, HD-8, HD-57, HD-133, HD-234, HD-263, HD-282, HD-551, HD-744 and Bt-based biopesticide were equivalent to 7.1×10^9, 2.4×10^9, 8.3×10^9, 4.6×10^8, 4.7×10^9, 6.1×10^8, 6.6×10^9, 3.1×10^9, 4.9×10^9, 3.1×10^9 and 9.8×10^7, respectively.

Bioassay: Three hundred milligram (300 mg) powder of spore-δ-endotoxin of respective HD-strain was mixed in 50 mL sterilized distilled H_2O to get 6,000 μg concentration/mL of H_2O. From this spore-δ-endotoxin mixture, 25 mL of toxin mixture (which contained 1,500,00 μg toxin) was mixed into 300 mL insect diet in a 800 mL capacity glass jar at approximately 60.0°C so as to obtain 500 μg concentration of spore-δ-endotoxin mL^{-1} diet. Rest of the quantity (25 mL) of toxin mixture was added to 25 mL of sterilized distilled H_2O in order to further dilute to have 250 μg of spore-crystal toxin mL^{-1} diet. In this way six serial dilutions (500, 250, 125, 62.5, 31.25 and 15.625 μg mL^{-1} diet) were made for each bioassay of the *B. thuringiensis* strains (Ahmed *et al*., 1994, 1996, 1997, 1998; Ahmed, 1995). A parallel control was run for each bioassay. In some experiments, a common control was used for bioassay of more than one Bt strains under the same set of experimental conditions.

Four replications of each toxin concentration were used. Each replication consisted of 20 neonate larvae of susceptible or Cry1Ac selected population individually kept in sterilized glass tube (7.5 cm height×2.3 cm diameter) containing a diet cube and plugged with sterilized cotton wool (Liang *et al*., 1999). All the insect in the glass tubes were placed at 25±2°C for seven days at photo-period of 14:10 (L: D) and relative humidity of 90±5%. After 7 days, the individual larva in each tube was checked in term of live and dead and the weight of all the surviving larvae in respective treatments of spore-δ-endotoxins was recorded.

Cross Resistance: Cross-resistance of Cry1Ac-selected neonate larvae of cotton bollworm was evaluated by subjecting the required number of larvae to bioassays of various HD-strains and the data were recorded in term of larval weight, LC_{50}, x^2 value, slope and Resistant Ratio (RR). Neonate larvae of susceptible cotton bollworm were also subjected to bioassays against the same HD strains as that used against Cry1Ac selection for comparison and calculation of Resistance Ratio (RR).

Data Analysis: Data were analyzed using software packages POLO-PC (Anonymous, 1987) for Probit analysis. Resistance ratios were calculated by dividing the LC_{50} of the Cry1Ac resistant selection divided by the LC_{50} of susceptible insect (Muller-Chon *et al*., 1996). MSTAT-C computer program was used for computing ANOVA and DMRT.

RESULTS AND DISCUSSION

Variation in resistance ratios of the selection: Resistance ratios of the selected *H. armigera* was conducted by using two different methods A) larval growth inhibition method (Liang and Guo, 2002) from generation 46 to 55 and B) Diet Bt-bioassay method (Ahmed, 1999) for 62nd generation of Cry1Ac selection (Table 1). Despite continuous selection, there has been observed changes in resistance ratios of the insect from generation to generation (e.g., resistance ratios from 46 th, 55th and 62nd generations indicated 417.000, 172.686 and 278.102) (Table 1). Generation 55th produced abnormally low slope (Table 1).

Cross-Resistance of Cry1Ac selected *H. armigera* to various HD-strains of *Bacillus thuringiensis*: Low resistance was exhibited against spore-δ-endotoxin of HD-744. The LC_{50} for the resistant selection was 25.177 μg toxin concentration mL^{-1} diet as compared to the LC_{50} of susceptible (1.945 μg toxin concentration mL^{-1}) with 12.944 resistance ratio. High resistance (>500 RR) was observed against the toxin of HD-4 with very high LC_{50} (1111.9^3 μg toxin concentration mL^{-1} diet) with the lowest slopes 0.28 and 0.43 for Cry1Ac and susceptible *H. armigera*, respectively. Cry1Ac selection showed 2.722 resistance ratio for HD-1 which was the minimum among the toxins tested. The slopes of regression for HD-1 against Cry1Ac selection and susceptible insect were 1.49 and 1.88, respectively indicating significantly high response of the insects (Table 2).

Low resistance (14.075 resistance ratio) was observed for HD-263 with significantly high slope 1.67 and LC_{50} 116.301 μg toxin concentration mL^{-1} diet. The x^2 values 10.611 and 10.808 were highly significant for Cry1Ac selection and susceptible for HD-263 strain. Moderately high level of cross-resistance (120.11 times higher than susceptible) was demonstrated by Cry1Ac resistant *H. armigera* against HD-234. Slope for regression (0.32) of HD-234 was low with the lowest but significant x^2 value (1.639). Low resistance (2.756 RR) was found in Cry1Ac selection having significantly high slope (1.31) as well highly significant x^2 value (7.460) against the toxin of HD-8. *Bacillus thuringiensis* strain (HD-551) also exhibited low cross-resistance (3.959 RR) with significantly high slope (1.18) and x^2 value (8.038) (Table 2).

Table 1: Development of resistance in *H. armigera* strain. The selection dosage was started from 2.32 μg Cry1Ac mL^{-1} at 1st generation subsequently dosages were increased from 2.63 to 14.30 μg Cry1Ac mL^{-1} from 6 th to 45 th generations. Bioassays for estimation of LC_{50s} were conducted with: A. larval growth-inhibition method (Liang, 2002), B. diet incorporation method (Ahmed, 1999)

	Insect strain	LC_{50} (μg mL^{-1} diet, 95% CL)	Slope	Resistance ratio (RR)
A. Generation				
46	Cry1Ac resistance strain	12.51(23.02- 42.41)	1.29±0.12	417.000
55	Cry1Ac resistance strain	8.807(5.57- 13.91)	0.276±0.11	172.686
B. Generation				
62	Cry1Ac resistance strain	376.829(206.39-1154.92)	0.822±0.18	278.102

Table 2: Probit analysis results of bioassays to indicate cross-resistance of Cry1Ac-selected and susceptible *H. armigera*

Bt Toxin	Insect strain	LC_{50} (μg mL^{-1} diet) (95% CL)	χ^2	Slope	RR
HD-744	Cry1Ac selection	25.177(10.253-41.771)	4.772	1.12±0.145	12.944
HD-744	Susceptible	1.945(0.228-5.711)	3.758	1.03±0.253	
HD-4	Cry1Ac selection	1111.9[3](----ns----)	4.293	0.28±0.148	>500
HD-4	Susceptible	27.057(2.634-66.733)	3.566	0.43±0.131	
HD-1	Cry1Ac selection	41.766(9.149-86.657)	35.503	1.49±0.150	2.722
HD-1	Susceptible	15.341(5.340-25.087)	6.460	1.88±0.253	
HD-263	Cry1Ac selection	116.301(84.986-187.764)	10.611	1.67±0.142	140.075
HD-263	Susceptible	8.263 (0.103- 21.390)	10.808	1.28±0.198	
HD-234	Cry1Ac selection	221.4[3](----ns-----)	1.639	0.32±0.182	120.11
HD-234	Susceptible	1843.297(557.4-193.268[3])	0.532	0.61±0.211	
HD-8	Cry1Ac selection	62.241(28.076-105.713)	7.460	1.31±0.153	2.756
HD-8	Susceptible	22.587 (3.646- 47.519)	7.957	1.13±0.159	
HD-551	Cry1Ac selection	71.821 (28.922- 133.768)	8.038	1.18±0.146	3.959
HD-551	Susceptible	18.141 (2.607- 38.882)	6.801	1.07±0.160	
HD-282	Cry1Ac selection	1175.801 (570.610-64118.063)	3.629	0.53±0.158	259.845
HD-282	Susceptible	4.525 (0.149- 13.342)	4.237	0.87±0.169	
HD-133	Cry1Ac selection	73.597 (-----ns----)	6.539	0.44±0.120	13.415
HD-133	Susceptible	5.486(0.035- 18.376)	5.686	0.77±0.153	
HD-57	Cry1Ac selection	582.510 (434.612- 1172.775)	4.640	2.36±0.576	4.385
HD-57	Susceptible	132.819 (23.676- 362.185)	8.599	1.08±0.185	
Bt-based biopesticide	Cry1Ac selection	473.347(375.701- 745.042)	3.565	2.09±0.727	14.374
Bt-based biopesticide	Susceptible	32.929 (7.374- 67.092)	9.200	1.17±0.149	

ns= non-significant

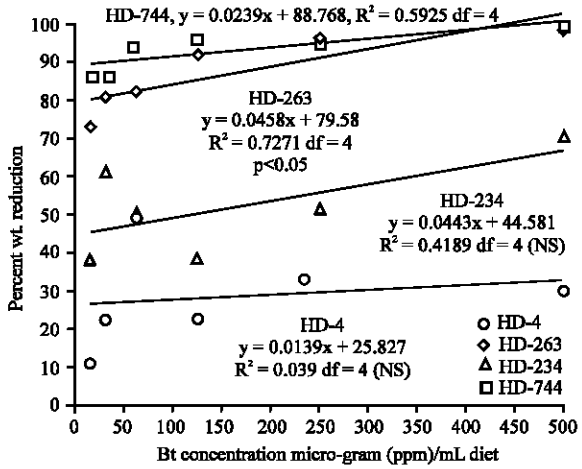

Fig. 1: Trend in larval weight reduction (%) in treatments of HD-744, HD-263, HD-234 and HD-4 against Cry1Ac selection of *H. armigera*

Cry1Ac selected population showed high resistance to the toxin of HD-282 (259.845 RR) and LC_{50} (1175.801 μg toxin concentration mL^{-1} diet). Resistance ratio for HD-133 was 13.415 with low slope value (0.44). Low resistance (4.385 RR) was exhibited by Cry1Ac selection against the toxins of HD-57 with LC_{50} (582.510 μg toxin concentration mL^{-1} diet). Slope of regression for HD-57 was the highest (2.36) among all the HD strains tested. Cry1Ac selection also exhibited resistance ratio of 14.374 against the toxins of Bt-based bio-pesticide with highly significant slope (2.09) and x^2 value (3.565) (Table 2).

Percent larval weight reduction in Cry1AC resistant insect: The linear regression (trend) of percent weight reduction in case of HD-263 was significant with equations y = 0.0458x+79.58, R^2 = 0.7271, df = 4, $p<0.05$, which also indicated comparatively higher activity of HD-263 in causing a regular and successive decrease in larval weight (Fig. 1). Percent larval weight reduction due to toxins of HD-1 and HD-8 was also significant at y = 0.011x+93.912, R^2 = 0.7269, df = 4, $p<0.05$ and y = 0.03x+85.332, R^2 = 0.6982, df = 4, $p<0.05$, respectively (Fig. 2). The spore-δ-endotoxin of Bt-based biopesticide also exhibited significant interaction (y = 0.0776x+59.169, R^2 = 0.8273, df = 4, $p<0.05$) (Fig. 3). Overall results with respect to larval weight reduction showed that only 4 strains (HD-1, HD-8, HD-263 and Bt-based biopesticide) showed significance out of 11 strains tested.

Percent larval weight reduction in susceptible insect: Regression trends in term of percent larval weight reduction were significant for HD-263 with regression

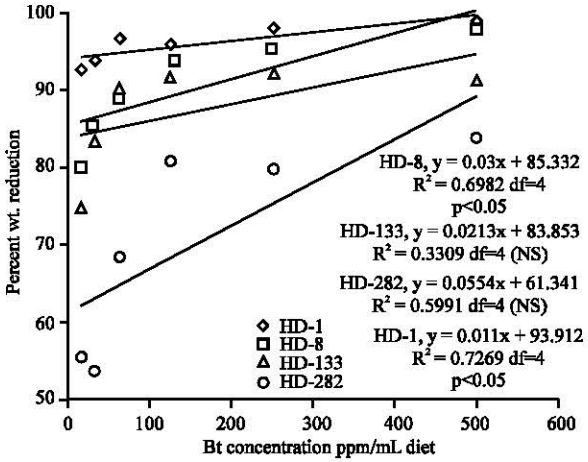

Fig. 2: Trend in larval weight reduction (%) in treatments of HD-1, HD-8, HD-133 and HD-282 against Cry1Ac selection of *H. armigera*

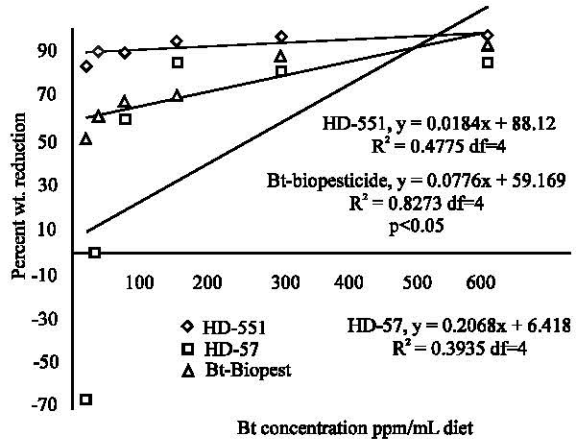

Fig. 3: Trend in larval weight reduction (%) in treatments of HD-551, HD-57 and Bt-biopesticide against Cry1Ac selection of *H. armigera*

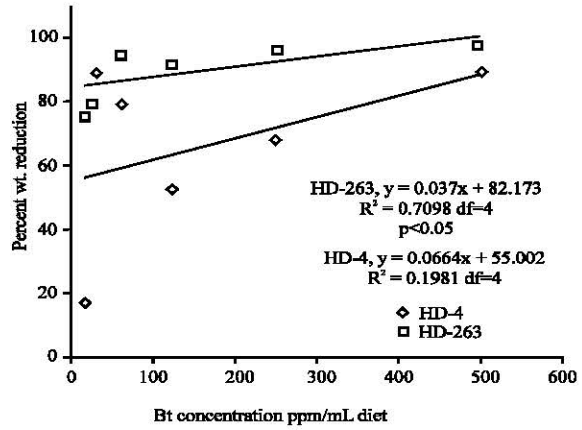

Fig. 4: Trend in larval weight reduction (%) in treatments of HD-263 and HD-4 against susceptible *H. armigera*

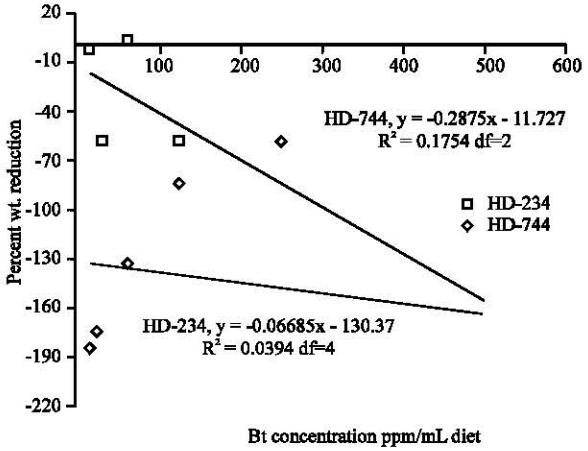

Fig. 5: Trend in larval weight reduction (%) in treatments of HD-744 and HD-234 against susceptible *H. armigera*

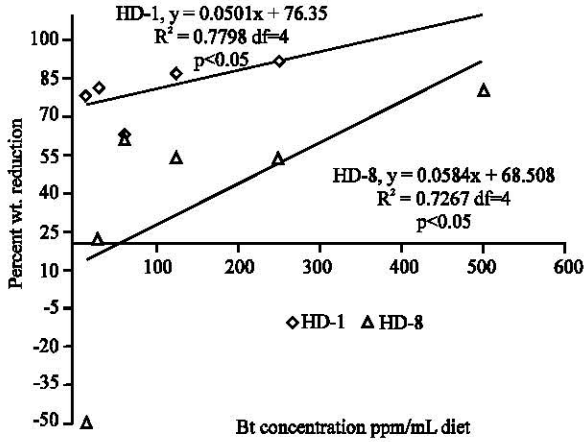

Fig. 6: Trend in larval weight reduction (%) in treatments of HD-1 and HD-8, against susceptible *H. armigera*

equation y = 0.0374x+82.173, df = 4, p<0.05, Coefficient of correlation (R^2 = 0.7098) indicated a regular and fast response of the insect. (Fig. 4). Susceptible insect indicated a successive increase in larval weight from 15.62 to 500.00 μg mL^{-1} concentration of Bt toxin of HD-234, the larval weight increase was 184.571, 172.571, 132.000, 82.857, 57.147 and 218.857% in 15.62, 31.25, 62.50, 125.00, 250.00 and 500.00 μg toxin mL^{-1}. A general trend of weight increase was also observed in case of HD-744 (Fig. 5). Susceptible insect responses in terms of weight reduction for the spore-toxins of HD-1 and HD-8 were significant at p<0.05 level with equation y = 0.0501x+76.35, df = 4, Coefficient of correlation R^2 = 0.7798, p<0.05 and y = 0.0584x+68.508, df = 4, Coefficient of correlation R^2 = 0.7262, p<0.05 (Fig. 6). The spore-δ-endotoxin of HD-282 and HD-133 did caused an irregular decrease in larval weight but their regressions were non significant

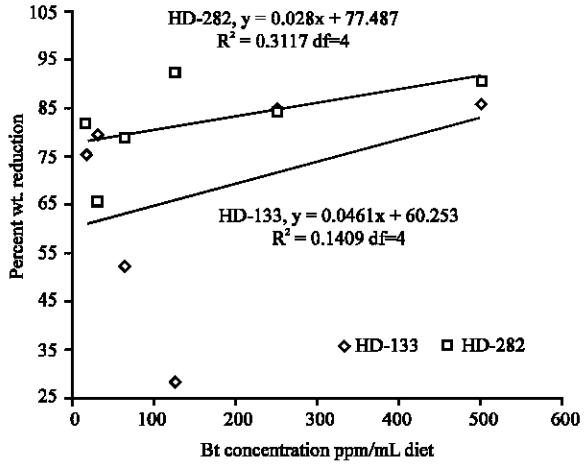

Fig. 7: Trend in larval weight reduction (%) in treatments of HD-133 and HD-282, against susceptible *H. armigera*

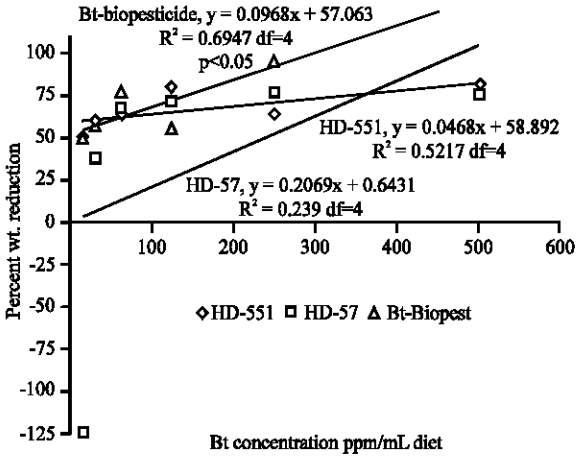

Fig. 8: Trend in larval weight reduction (%) in treatments of HD-551, HD-57 and Bt-biopesticide against susceptible *H. armigera*

(Fig. 7). The response of insect to Bt-based biopesticide was observed to be significant with regression equation y = 0.0968x+57.063, df = 4, $p<0.05$ (Fig. 8).

It is obvious that the development of insecticide resistance in *H. armigera* in many countries of the world can be dealt with by using transgenic crop plants expressing Cry1Ac insecticidal protein for managing insect pest problems. The data reported in this paper indicated that Cry1Ac resistant cotton bollworm *H. armigera* may exhibit resistance to spore-δ-endotoxin of various strains of *B. thuringiensis* to varying degrees, but there is still strong likelihood that some spore-crystal formulation of Bt may be developed and they can prove ineffective if applied against insects which has developed resistance to transgenic crop.

More importantly, there are reports which stated synergism between spores and crystal toxins of *B. thuringiensis*, therefore, the present study carries importance from the view point of synergism that exists between spores and toxins. Miyasono *et al.* (1994) observed that toxin-free spores did not kill larvae, but spores increased the toxicity of *B. thuringiensis* subsp. *kurstaki* crystals to larvae from a susceptible strain of diamond back moth. The interaction observed between these spores and toxins exemplifies synergism, in which the toxicity of a mixture is greater than expected on the basis of the independent toxicity of components (Tabashnik, 1992). Tang *et al.* (1996) found synergism between *B. thuringiensis* subsp. *kurstaki* spores and each of the three individual Cryl toxins from *B. thuringiensis* subsp. kurstaki against a susceptible strain of diamondback moth but not against a resistant strain.

Liu *et al.* (1998) observed synergism between *B. thuringiensis* subsp. *Kurstak*i spores and Cry2A, they stated that Cry2A alone did not cause significant mortality of susceptible diamondback moth but in combination of spore, the mortality of the insect was significant. They also reported significant synergism between *B. thuringiensis* subsp. *aizawai* spores and Cry1C toxin against a Bt *kurstaki* resistant diamondback moth whereas Bt subsp. spores alone did not cause significant mortality either of susceptible or resistant diamondback moth. They further reported that the extent of synergism between spores and toxins of *B. thuringiensis* depends on the type of insect, the type of spore, the set of toxins, the presence other materials such as formulation ingredients and the concentrations of spore and toxins.

Although, in the present study, we have not carried out separate tests to determine the synergistic interactions between the spores and crystal toxins of HD strains but some HD strains e.g., HD-1 and HD-263 (*B. thuringiensis* subsp. *kurstaki*) contains Cry1Ac protein. Therefore, we emphasize that according to previous reports (Liu *et al*., 1998; Tang *et al*., 1996; Tabashnik, 1992), it is very likely that some synergistic interaction may have taken place to enhance Bt effect and thus, our results indicate low resistance to HD-1, HD-263, HD-744, HD-8, HD-551, HD-133, HD-57 and Bt-based biopesticide (Table 2) to Cry1Ac resistant *H. armigera*. On the basis of interaction between spores and toxins of *B. thuringiensis* in some case (not in all), it is also possible to develop suitable Bt-based spore-crystal formulations which may prove as an effective tool to manage cotton bollworm populations that have developed resistance to single toxin Cry1Ac produced in Bt cotton.

Resistance between *B. thuringiensis* strains and toxins has been reported in a number of insect

species at specific toxin levels by many researchers (Akhurst *et al*., 2003, Mrakwick *et al*., 2002; Tabashnik, 1994). Resistance Ratios (RR) in Cry1Ac selection to commercial Bt preparations (DiPel 2X, Xen Tari WDG which contains multiple insecticidal crystal proteins), MVP (contains Cry1Ac proteins) and HD-73 spore/crystal) were 2, 7, 69 and 188 with LC_{50}s 129, 90, 97 and 1876 μg toxin mL^{-1} diet, respectively (Akhurst *et al*., 2003).

Our results also indicated that Cry1Ac selection exhibited a low resistance (14-fold) to Bt-based bio-pesticide formulation with a significant slope (2.09) indicating fast interaction between the formulation and the insect in terms of mortality as compared with the slope of susceptible insect (Table 2).

Cross-resistance studies on laboratory selection of other insect (*Spodoptera littoralis*) with spore-crystal preparations of *B. thuringiensis* Cry1C toxin for 14 generations exhibited resistance ratio from 10 to >500-folds and partial cross-resistance to Cry1D, Cry1E, Cry1Ab toxins and strain *B. thuringiensis aizawai* 7.29 (Muller-Cohn *et al*., 1996). Cross-resistance to Cry1 toxins was observed in *P. interpunctella* strains showing high cross-resistance between *B. thuringiensis* strains for insects selected with HD-1 (DiPel, Abbott. North Chicago, IL) containing Cry1Aa, Cry1Ab, Cry1Ac and Cry11A (McGaughey and Johnson, 1987). Mrakwick *et al.* (2002) reported that after 25 selection of lines of light brown apple moth, *Epiphyas postvittana* reared on diet containing Cry proteins (Cry1Ac, Cry1Ba, Cry2Aa and mixture of all Cry proteins developed resistance to proteins they were selected with: Cry1Ac (23x), Cry1Ab (10x) and Cry2Aa (4x) and the lines selected with mixtures of the proteins also developed low resistance to Cry1Ac (14x) and Cry1Ba (6x). They further reported that larvae from lines selected >30 times were resistance to the formulated Bt insecticide DiPel 2X® (contains both spores and crystals Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cry1Ab).

In our study, a similar resistance pattern shown by the Cry1Ac selected *H. armigera* to the spore-δ-endotoxins of HD-1 and HD-8 exhibited very low resistance ratio (2.722 and 2.756, respectively) indicating that toxic proteins present in both the strains caused high insect mortality at minimum dose (LC_{50}s 41.766 and 62.24 1μg toxin mL^{-1} diet, respectively) (Table 2). The resistance ratios for HD-744, HD-263 and HD-133 were also similar (12.944, 14.075 and 13.415 RR, respectively) which indicated a similarity in response to resistant *H. armigera* to spore-δ-endotoxin complexes of these strains at LC_{50}s 25.177, 116.301 and 73.597 μg toxin mL^{-1} diet respectively). The insect exhibited the highest resistance (>500 RR) against HD-4 with the highest LC_{50} (1111.9^3 μg toxin mL^{-1} diet) as compared with susceptible insect. The spore-δ-endotoxin complex of HD-4 strain were not effective and the slope of the strains was also the least one (0.28) among all the strains tested (Table 2). The insect selection also showed high RR (259.847) against HD-282. The resistant selection has demonstrated increased ability to survive and develop on spore-δ-endotoxins of HD-4 and HD-282 and similar findings were observed by Liu *et al.* (2001) in case of *Pectinophora gossypiella*: they reported highly Cry1Ac resistant selections (APHIS98-R and AZP-R) of insect strains overcame the negative effect of high concentrations of Bt toxin Cry1Ac. Gould *et al.* (1995) conducted cross-resistance studies on Cry1A(c) selected *H. virescens* and reported that after 19 episodes of selection [19 times treatment with Cry1A(c)] over 30 generations, selected strain developed >500 folds resistance to this toxin and further selection resulted into resistance ratio (RR) of 10,000 folds, they further stated that selection *H. virescens* developed very high level of cross-resistance to Cry1A(a) (2,157 RR), Cry1A(b) (2,364 RR) and Cry1F (3,678 RR) as well as low resistance to Cry1C (2.50 RR) and Cry11A (2.29 to 24.91 RR).

Our results also showed that response of Cry1Ac resistant selection was high against the toxins of HD-551 and HD-57 with significant slopes (1.18 and 2.36, respectively) exhibiting high interaction in terms of mortality of the resistant insect with RR (3.959 and 4.385, respectively). The overall results in Table 2 showed that the activity of various HD strains were in the order of (from the highest to the lowest) HD-1>HD-8>HD-551>HD-57>HD-744>HD-133>HD-263>Bio-pesticide> HD 234>HD-282>HD-4.

The trend in mean percent larval weight of the Cry1Ac resistant *H. armigera* due to feeding of different spore-δ-endotoxin of various HD-strains indicated a regular decrease in larval weight with the increase in toxin concentration in almost all the HD-strains other than HD-4 and HD-57 (Fig. 1-3). Linear regressions for percent weight reduction in Cry1Ac resistant *H. armigera* due to feeding of spore-crystal toxins of HD-263, HD-1, HD-8 and Bt-based biopesticide were significant at $p<0.05$ with co-efficient of co-relation ($R^2 = 0.7271$, $R^2 = 0.7269$, $R^2 = 0.6982$ and $R^2 = 0.8373$, respectively) (Fig. 1-3).

An abrupt but significant increase by 1.65 folds in percent larval weight was observed in 15.62 μg toxin concentration of HD-57 as compared to the larval weight of the control (Fig. 3). Present observations on larval weight loss/gain corresponds with the observation of Gould *et al.* (1995) who conducted studies on Cry1Ac selection and genetic analysis of *Heliothis virescens* and

reported that resistant larvae (after 20 episodes of selection with low dose of 0.032 μg Cry1Ac toxin mL^{-1} artificial diet) showed greater mean weight (40.11±4.06 mg) than that of the susceptible (1.47±0.16 mg), they further noted similar findings of increase in larval weight (109.77±11 mg) in Cry1Ab toxin selected *H. virescens* after 20 episodes of selection with 0.16 μg Cry1Ab toxin mL^{-1} artificial diet) in comparison with the larval weight of the susceptible insect (2.42±0.29 mg). The linear regression results showed that Cry1Ac resistant cotton boll worm indicated a general and successive reduction in percent larval weight with the increase in toxin concentration of HD-1, HD-8, HD-133, HD-234, HD-263, HD-282, HD-551, HD-744, Bt-based Biopesticide, thereby exhibiting an adverse effect on the development of Cry1Ac resistant insect.

Percent mean larval weight of susceptible cotton bollworm showed that the trend of larval weight loss in HD-4 treatments is quite different from that of the trend observed in Cry1Ac selection (Fig. 4), here, in this case, we observed a significant successive loss in larval weight from treatment 31.25 to 500.00 μg toxin concentration mL^{-1} diet as compared with the control with the exception that the difference in larval weight between the control and 15.62 μg toxin concentration of HD-4 was non-significant. Linear regression for percent larval weight reduction caused by HD-263 was significant at $p<0.05$ with $R^2=0.7098$ (Fig. 4). A significant increase in percent larval weight in HD-234 treatments (2.84, 2.72, 2.32 and 3.19 folds increase in weight in treatments 15.62, 31.25, 62.50 and 500.00 μg toxin concentration mL^{-1} diet respectively as compared with the control) was noted (Fig. 5). Significant regressions $y = 0.050x + 76.35$, $R^2 = 0.7798$, at $p<0.05$, $y = 0.05584x + 68.508$, $R^2 = 0.7267$, at $p<0.05$ and $y = 0.0968x + 57.063$, $R^2 = 0.6947$, at $p<0.05$ for HD-1, HD-8 and Bt-based biopesticide respectively. Increase in larval weight at lower concentrations (15.62 μg toxin) of HD-8 and HD-57 of was observed as compared to the larval weight of the control (Fig. 6-8). In general, the spore-δ-endotoxins of HD-1, HD-8, HD-133, HD-263, HD-282, HD-551, HD-744, Bt-based bio-pesticide at higher concentrations caused reduction in larval weight. Studies conducted by Bolin *et al.* (1999) on long-term selection for resistance to *B. thuringiensis* Cry1Ac and Cry1Ab toxins in European corn borer, *Ostrinia nubilalis* and reported greater larval weight of *O. nubilalis* selected on lyophilized *B. thuringiensis* field corn (NK4334, Cry1Ab, 35S promoter from Novartis seed) leaf tissue into the diet, they also noted a significantly greater larval weight in lyophilized Bt-corn selection at lower concentration of Bt-corn tissue (0.55 μg concentration mL^{-1} diet), they further observed a successive decrease in larval weight with the increase in amount of Bt-corn tissue in selected and non-selected *O. nubilalis*. Present finding on larval weight corresponds with the finding of Bolin *et al.* (1999) that the response of selected insect may be different at certain concentration of Bt-toxin.

CONCLUSIONS

The results of our studies on Cry1Ac resistant *H. armigera* indicated that the insect did not only show low resistant to spore-δ-endotoxins of *Bacillus thuringiensis* (HD-1, HD-8, HD-57, HD-263, HD-551 and Bt-based biopesticide) strains but the toxin of these Bt strains/biopesticide preparation exhibited a high degree of larval developmental retardation. The previous studies conducted by Liu *et al.* (1998) Miyasono *et al.* (1994) Tabashnik (1992) and Tang *et al.* (1996) reported that synergistic interactions between the spores and protein crystals of *Bacillus thuringiensis*, therefore, some interaction may have taken place to enhance Bt effect and thus, our results indicated low resistance to HD-1, HD-263, HD-744, HD-8, HD-551, HD-133, HD-57 and Bt-based biopesticide to a Cry1Ac resistant *H. armigera*. On the basis of interaction between spores and toxins of *B. thuringiensis* in some case, it is possible to make efforts on the development of a suitable Bt-based spore-crystal formulations which can be used to manage cotton bollworm populations under circumstances where cotton boll worm has developed resistance to transgenic cotton.

ACKNOWLEDGMENTS

The authors extend appreciation and thanks to Dr. Alejandro (Alex) J. Rooney, United States Department of Agriculture-Agricultural Research Service (USDA-ARS), 1815 North University Street, Peoria, Illinois 61604, USA and Dr. Daniel R. Zeigler, *Bacillus* Genetic Stock Center (BGSC), Department of Biochemistry, Ohio State University, 484 West Twelfth Avenue, Columbus, OH, USA for providing various Bt strains which were used in the present study. Dr. Feeroza Khalique, Pakistan Agricultural Research Council, Islamabad 45500, Pakistan, is gratefully acknowledged for providing assistance in statistical analysis of the data.

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