Pak. J. Agri. Sci., Vol. 56(2), 415-420; 2019
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI:10.21162/PAKJAS/19.7122
http://www.pakjas.com.pk

VARIATION IN SUSCEPTIBILITY OF *Helicoverpa armigera* (LEPIDOPTERA: NOCTUIDAE) TO CRY1AC TOXIN

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Bt transgenic cotton, is being a vital part of a pest management program, effectively controls *Helicoverpa armigera*. However, the success of Bt technology depends on the persistent vulnerability of target pests to the Bt insecticidal proteins. The baseline susceptibility of *H. armigera* larvae was determined for first, second, and third instar larvae field collected from three locations, Faisalabad, Multan and Bahawalpur, and from a known susceptible laboratory population in 2013 and 2014.The LC_{50} ranged from 0.123 to 1.026 µg/ml, 0.148 to 1.675 µg/ml, and 0.210 to 2.761 µg/ml**,** for first, second and third instar larvae, respectively. The population of Bahawalpur was 8.34, 11.32 and 14.71-fold more resistant than a susceptible population for first, second, and third instar larvae, respectively. The population from Multan was 5.54, 7.44 and 8.99-fold more resistant that a susceptible population for first, second, and third instar larvae, respectively. The population from Faisalabad was 4.08, 4.88 and 5.23-fold more resistant than a susceptible population for first, second and third instar larvae, respectively. The MIC_{50} was 0.003 to 0.006 µg/ml**,** 0.009 to 0.088 µg/ml, and 0.014 to 0.206 µg/ml for first, second and third instar larvae, respectively. The Bahawalpur population was the most resistant followed by the population from Multan, and the population from Faisalabad had the lowest amount of Bt insecticidal resistance. The trend in lethal concentration found at the three locations in 2013 was found in the samples taken in 2014.

Keywords: Base line susceptibility, Bt cotton, *Helicoverpa armigera*, Cry1Ac toxin

INTRODUCTION

Bacillus thuringiensis (Bt) is a spore forming bacteria that produces crystal proteins called Cry toxins. Bt delivered to the plants either through sprays or incorporated genetically in the plants, exhibit insecticidal activities against various lepidopteran, dipteran and coleopteran larvae (Tabashnik *et al*., 2008). The production of cotton was revolutionized around the world in a short period of time due to the development and use of Bt cotton. However, the replacement of non-Bt cotton with Bt cotton may not be the best solution for both chewing and sucking insect pests problems due to target specificity. Without appropriate resistance management tactics, the future of this innovative control measure could be short-lived (Sayyed *et al.*, 2008).

Cotton bollworm, *Helicoverpa armigera* Hubner (Noctuidae: Lepidoptera), is one of the most harmful and cosmopolitan pest that causes major economic losses to cotton and vegetable crops. *H. armigera* is a difficult pest to manage due to its wide host range, multiple generations, high fecundity, migratory behaviour and development of insecticide resistance (McCaffery, 1998). The regular and injudicious use of insecticides can lead to resistance development in many insect pests (Sayyed and Wright, 2006), and resistance has become a major issue with *H. armigera*, world-wide (McCaffery, 1998). In Pakistan, previous studies reported moderate to high levels of resistance to pyrethroid and organo-phosphate insecticides in *H. armigera* field populations (Ahmad *et al*., 1995).

Like insecticides, the pests can also develop resistance against Bt toxins (Gujar *et al*., 2004) because the variation of expression of Bt toxins across cotton varieties allow some larvae to survive (Dong and Li, 2007). There is a serious threat of resistance development in targeted insects due to the increased planting of Bt cotton (Gould, 1998). The insects within a population that survive the actual toxins being expressed by a transgenic plant will be selected progressively as they convey the resistance to their progeny. In an artificial diet bioassay, the larvae of *H. armigera* have already developed resistance against the Cry1Ac toxin (Chandrashekar and Gujar, 2004). Moreover, the ability of *H. armigera* to develop resistance against Cry1Ac toxins under field conditions is found in the literature from India (Kranthi *et al.*, 2001), China (Liang *et al.*, 2000) and Australia (Akhurst *et al*., 2003). Low level of susceptibility to Bt insecticidal protein has also been reported in other insect species that are exposed to Bt crops under field conditions (Tabashnik *et al*., 2008). A preliminary requisite for management of resistance is the development of baseline susceptibility data. It will serve as a resistance monitoring tool for target insect. The populations of *H. armigera* from China, (Wu *et al*., 2006), India (Gujar *et al*., 2004;

Chandrashekar *et al*., 2005) and Australia (Dang and Gunning, 2002) have been studied, and baseline susceptibility data for *H. armigera* to Cry1Ac toxins has been established. However, no base line susceptibility data are available for this pest in Pakistan.

MATERIALS AND METHODS

***Insect source*:** Laboratory strains of *H. armigera* culture were developed by collecting late instar larvae from Faisalabad, Bahawalpur and Multan during August, September 2013 and 2014 (Fig. 1).

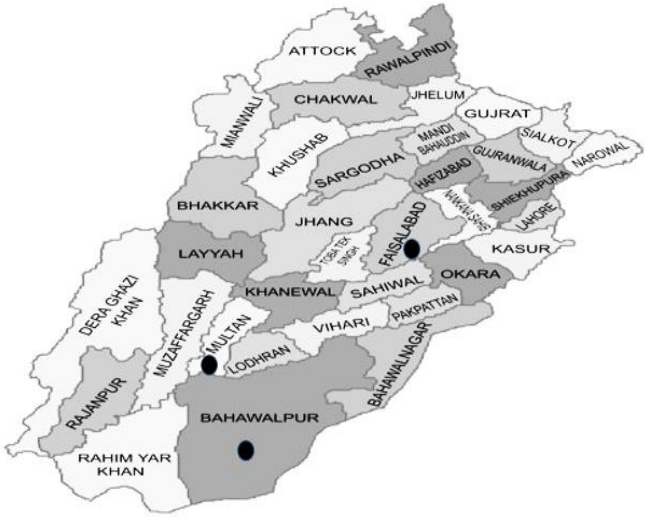

Figure 1. Map of Punjab Pakistan showing *H. armigera* populations collection localities in 2013 and 2014.

The populations were designation as FSD, BWP and MLT. Larvae were collected in the field were held individually in glass vials (Jalali *et al.*, 2004) containing diet and were transported to the Insect Biodiversity and Biosystematics Laboratory, Department of Entomology, University of Agriculture Faisalabad. The susceptible laboratory population was used as a reference population. The susceptible laboratory strain was obtained from the Nuclear Institute of Agriculture and Biotechnology (NIAB), Faisalabad.

Rearing procedure: Larvae were maintained at 26±4°C and 75±5% RH under 16h: 8h (Light: Day) cycle on artificial diet until pupation. Pupae were collected, put into petri plates, and placed in plastic containers for adult emergence. Adults were kept in open plastic containers, covered with muslin cloth for egg laying. Adults were fed with a 10 percent honey solution. The egg laden muslin cloth was cut into small pieces and surface sterilized in 0.05% sodium hypochlorite solution. The sterilized eggs were placed in a 500 ml plastic jar and held at 26±4ºC and 75±5% R.H under 16h: 8h (Light: Day) cycle to allow egg hatch to occur. The F1 generation neonate, second and third instar larvae were used for bioassays.

***Bacillus thuringiensis* toxin:** Cry1Ac toxin was stored at -80°C and the toxin was freshly prepared for each assay using the method of Sayyed *et al.* (2000).

***Bioassay method*:** Diet incorporation bioassays were conducted using seven concentrations (16 to 0.25 µg/ml) of Cry1Ac toxin (Dulmage *et al.*, 1971). Approximately 5ml of diet containing a toxin concentration was put into a small, aerated cup. Four replications were used for each bioassay. All bioassays were carried out in a controlled environment room at 26±4ºC and 75±5% RH with a 16:8 h (Day: Light cycle). After seven days, mortality and moult inhibition were recorded.

***Data analysis*:** The mortality data was corrected by Abbott formula (Abbott, 1925) and Probit analysis was done to calculate LC_{50} and MIC_{50} using Mini Tab Software 18.

RESULTS

***Toxicity of Cry1Ac toxin against H. armigera susceptible and field populations during 2013*:** Populations of *H.*

Table 1. Lethal conc. (LC_{50}) for susceptible and field populations of *H. armigera* to Cry1Ac toxin during 2013.

Instar	Pop	LC_{50}	Fiducial limit	Equation	χ^2	R. R
1st	SS	0.123±0.030	0.066- 0.182[a]	0.629X+1.318	2.416	1.000
	FSD	0.502±0.070	0.367-0.646[b]	0.487X+0.335	3.928	4.081
	MLT	0.682±0.090	0.512-0.867[bc]	0.466X+0.178	2.050	5.544
	BWP	1.026±0.126	0.790-1.294[cd]	0.443X-0.011	2.949	8.341
2nd	SS	0.148±0.034	0.083-0.217[a]	0.542X+1.037	3.439	1.000
	FSD	0.722±0.111	0.513-0.954[b]	0.388X+0.125	2.353	4.878
	MLT	1.101±0.157	0.811-1.439[bc]	0.372X-0.036	1.345	7.439
	BWP	1.675±0.246	1.238-2.334[c]	0.337X-0.174	0.311`	11.317
3rd	SS	0.210±0.051	0.116-0.316[a]	0.398X+0.621	6.511	1.000
	FSD	1.099±0.152	0.818-1.423[b]	0.387X-0.036	0.394	5.233
	MLT	1.889±0.259	1.434-2.480[c]	0.361X-0.230	0.225	8.995
	BWP	2.761±0.357	2.154-3.607[cd]	0.395X-0.401	2.425	13.147

† Pop- Population, SS- lab susceptible population, FSD- Faisalabad population, MLT- Multan population, BWP- Bahawalpur population;
*Different letters in the same column indicate significant differences due to non-overlapping basis of 95% CI.

armigera from Faisalabad, Multan, and Bahawalpur in 2013 showed variable responses to Cry1Ac as reflected in the LC_{50} values for first, second and third instar larvae (Table 1). For all instars, the LC_{50} value for the susceptible population was lower than the field collected larvae (Table 1). The LC_{50} values of the field populations were lowest for the population from Faisalabad, followed by the population from Multan and highest for the population from Bahawalpur (Table 1). The LC_{50} values ranged from 0.123 to 1.026 µg/ml for first instar larvae, 0.148 to 1.675 µg/ml for second instar larvae, and 0.210 to 2.761 µg/ml for third instar larvae. The data showed variation in susceptibility levels among all populations, up to 8.341-fold for first instar larvae, 11.32 for second instar larvae and 13.147 for third instar larvae. The moult inhibitory concentration (MIC_{50}) ranged from 0.003 to 0.006 µg/ml, 0.009 to 0.088 µg/ml and 0.014 to 0.206 µg/ml for first, second and third instar larvae, respectively. The data also depicted up to 23.33 (for first instar), 9.78 (for second instar) and 14.71(for third instar) fold variations in susceptibility level among all populations (Table 2).

***Toxicity of Cry1Ac toxin against H. armigera susceptible and field populations during 2014*:** Variability in the LC_{50} values also occurred among the larval instars and geographic populations in 2014 (Table 3). The LC_{50} values ranged from 0.112 to 1.215 µg/ml for first instar larvae, 0.142 to 2.066 µg/ml for second instar larvae, and 0.191 to 3.090 µg/ml for third instar larvae (Table 3). As the larvae aged, the susceptibility level decreased. There was a 10.84-fold decrease in susceptibility for first instar larvae, 14.549-fold decrease for second instar larvae, and 16.18-fold decrease for third instar larvae among the populations. The MIC_{50} values recorded for first, second and third instar larvae were as 0.003 to 0.076 µg/ml, 0.008 to 0.097 µg/ml, and 0.012 to 0.256 µg/ml respectively (Table 4). After baseline analysis of all populations, there was 25.33-fold variation for first instar larvae, 12.125-fold for second instar larvae, and 21.33-fold

Table 2. Moult inhibitory concentration (MIC_{50}) for susceptible and field populations of *H. armigera* to Cry1Ac toxin during 2013.

Instar	Pop	MIC_{50}	Fiducial limit	Equation	χ^2	R.R
1st	SS	0.003±0.006	0.000-0.031[a]	-0.392X-2.204	1.130	1.000
	FSD	0.045±0.271	0.007-0.110[bc]	-0.317X-1.238	1.885	15.000
	MLT	0.032±0.020	0.005-0.083[b]	-0.306X-1.044	1.044	10.667
	BWP	0.070±0.033	0.191-0.148[c]	-0.294X-0.782	1.984	23.333
2nd	SS	0.009±0.010	0.000-0.426 [a]	-0.424X-1.982	1.847	1.000
	FSD	0.022±0.175	0.002-0.070 [ab]	3.929X-3.787	0.745	2.444
	MLT	0.053±0.032	0.009-0.1312[abc]	-0.249X-0.730	1.313	5.888
	BWP	0.088±0.054	0.015-0.214 [bcd]	4.811X-2.422	0.844	9.777
3rd	SS	0.014±0.012	0.000-0.047 [a]	-0.383X-1.628	0.641	1.000
	FSD	0.038±0.025	0.005-0.105 [b]	-0.246X-0.798	0.757	2.714
	MLT	0.087±0.044	0.020-0.192 [bc]	-0.245X-0.597	0.331	6.214
	BWP	0.206±0.084	0.067-0.392[bcd]	-0.227X-0.359	0.247	14.714

† Pop- Population, SS- lab susceptible population, FSD- Faisalabad population, MLT- Multan population, BWP- Bahawalpur population;
*Different letters in the same column indicate significant differences due to non-overlapping basis of 95% CI.

Table 3. Lethal concentration (LC_{50}) for susceptible and field populations of *H. armigera* to Cry1Ac toxin during 2014.

Instar	Pop	LC_{50}	Fiducial limit	Equation	χ^2	R. R
1st	SS	0.112±0.029	0.057-0.171 [a]	0.614X+1.342	2.028	1.000
	FSD	0.577±0.078	0.428-0.739 [b]	0.477X+0.261	2.634	5.151
	MLT	0.832±0.013	0.637-1.046 [bc]	0.466X+0.085	1.858	7.428
	BWP	1.215±0.144	0.949-1.523 [cd]	0.445X-0.087	1.964	10.848
2nd	SS	0.142±0.034	0.079-0.211 [a]	0.534X+1.042	3.667	1.000
	FSD	0.871±0.127	0.632-1.137 [b]	0.386X+0.053	3.229	6.133
	MLT	1.374+0.188	1.032-1.784 [bc]	0.373X–0.119	3.173	9.676
	BWP	2.066+0.276	1.584-2.702 [cd]	0.372X–0.270	0.814	14.549
3rd	SS	0.191+0.504	0.100-0.296 [a]	0.383X–0.633	5.038	1.000
	FSD	1.335+0.177	1.011-1.720 [b]	0.387X–0.111	0.718	6.989
	MLT	1.996+0.261	1.538-2.591 [bc]	0.381X+0.434	0.435	10.450
	BWP	3.090+0.347	2.493-3.895 [cd]	0.469X-0.529	2.823	16.178

† Pop- Population, SS- lab susceptible population, FSD- Faisalabad population, MLT- Multan population, BWP- Bahawalpur population;
*Different letters in the same column indicate significant differences due to non-overlapping basis of 95% CI.

Table 4. Moult inhibitory concentration (MIC_{50}) for susceptible and field populations of *H. armigera* to Cry1Ac toxin during 2014.

Instar	Pop	MIC_{50}	Fiducial limit	Equation	χ^2	R. R
1st	SS	0.003±0.006	0.000-0.031[a]	-0.392X-0.139	1.130	1.000
	FSD	0.027±0.017	0.004-0.072[b]	-0.319X-1.148	2.022	9.000
	MLT	0.037±0.022	0.006-0.094[bc]	-0.295X-0.965	2.416	12.333
	BWP	0.076±0.036	0.020-0.159[bcd]	-0.288X-0.743	2.552	25.333
2nd	SS	0.008±0.102	0.000-0.040[a]	-0.418X-2.007	1.907	1.000
	FSD	0.023±0.018	0.002-0.075[ab]	-0.243X-0.907	0.764	2.875
	MLT	0.057±0.034	0.010-0.140[bc]	-0.242X-0.695	1.192	7.125
	BWP	0.097±0.056	0.017-0.232[bcd]	-0.203X-0.474	0.782	12.125
3rd	SS	0.012±0.011	0.000-0.045[a]	-0.380X-1.660	1.147	1.000
	FSD	0.041±0.028	0.005-0.112[ab]	-0.237X-0.755	0.358	3.417
	MLT	0.091±0.048	0.021-0.203[abc]	-0.237X-0.566	0.378	7.583
	BWP	0.256±0.095	0.093-0.462[bcd]	-0.231X-0.315	0.549	21.333

† Pop- Population, SS- lab susceptible population, FSD- Faisalabad population, MLT- Multan population, BWP- Bahawalpur population;
*Different letters in the same column indicate significant differences due to non-overlapping basis of 95% CI.

for third instar larvae among all populations. Overall, the population collected from Bahawalpur was found more resistant to the Cry 1Ac toxin in comparison to the Multan and Faisalabad populations. The results also indicated that the level of resistance not only increased with larval developmental stage but also with time as indicated in 2013 and 2014 analyses.

DISCUSSION

The insecticidal protein in Bt cotton is considered one of the best insect pest management tools due to its eco-friendly and target specific natures. The Bt cotton hybrids have been planted in commercial fields for more than two decades, and some scientists have presumed that resistance would evolve (Krieg and Langenbruch, 1981). But, resistance to Bt was documented in a field population of diamond back moths in Hawaii thirty years after commercialization (Ferre *et al*., 1991; Tabashnik, 1992). Intensive use of commercial Bt genotypes resulted in resistance development in field populations in other countries including Thailand, China, Japan and Philippines (Liu and Tabashnik, 1997). Commercial Bt cotton was released for the first time in Pakistan in1996. It quickly found favour with farmers because of the tremendous reduction in the number of insecticide applications needed against bollworm (Kranthi *et al*., 2005). The prolonged exposure of insect pests to Cry toxins in large scale plantings of Bt cotton increased selection pressure on the insects to develop resistance rapidly (Tabashnik *et al*., 1994; Gould, 1998; Shelton *et al.*, 2002; Ferre and Vanrie, 2002). The establishment of susceptibility baseline data is necessary for early detection of insecticide resistance problems. In the present study, baseline susceptibility of Cry1Ac was established for three geographic populations of *H. armigera*. Our results showed variation in LC_{50} values in 2013 and 2014 among first, second, and third instar larvae in all populations. The variation in susceptibility among the tested insects depended on the age of the insect and susceptibility decreased with the age of insect. The baseline susceptibility of *H. armigera* to Cry1Ac was studied in China (Wu *et al*., 2006; Gao *et al*., 2011), India (Gujar *et al*., 2007), and Australia (Bird and Akhurst, 2007), and most of the studies reported low susceptibility of the tested populations. Our results are in line with Gujar *et al*. (2007) who evaluated the susceptibility of *H. armigera* to CryIAc toxin using a diet contamination bioassay method and reported LC_{50} values between 0.023 to 0.372 µg/g. They also stated that these variations in susceptibility of field-collected populations may be due to genetic differences, host crops, and/or agro-climatic conditions. Our findings are in line with Fakrudin *et al*. (2003), Jalali *et al*. (2004), Avilla *et al*. (2005), Kalia *et al.* (2013), Salunke *et al.* (2014) and Rao *et al*. (2015) who worked in different parts of the world and reported significant variation in susceptibility level of *H. armigera* to Bt toxins.

***Conclusion*:** This study establishes a benchmark for the susceptibility of *H. armigera* field populations collected from the core cotton producing areas of Punjab, Pakistan. The threat of Bt resistance development in *H. armigera* is a major concern for the cotton industry. Regulatory agencies should create and implement a mandatory monitoring system for resistance in *H. armigera* populations in cotton fields. Our baseline susceptibility data provides important information regarding variation in the susceptibility of *H. armigera* to Cry1Ac toxins. This data will aid in future development and implementation of resistance monitoring and management programs for *H. armigera* in Pakistani cotton.

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