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Full Length Article

Varietal Expression of Cry1Ac in Cotton and its Concentration Effect on *Helicoverpa armigera* under Laboratory Condition

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Abstract

Comparative response of five Bt-cotton cultivars (FH-142, FH Lalazar, VH-259, CIM-602, BH-178) and one non-Bt (CIM-608) cultivar on mortality of the 1st, 2nd and 3rd instars *Helicoverpa armigera* (Hubner) was evaluated on different plant parts of Bt cultivars and synthetic diet incorporation bioassays. The 1st instar *H. armigera* showed complete (100%) mortality after feeding on leaves, squares, flowers and bolls of Bt cotton cultivars; the 2nd instar showed 100% mortality after feeding on only leaves of Bt cultivars (FH-142 and CIM-602), whereas the 3rd instar indicated comparatively less mortality (80-95%) on Bt cultivars during the two years. Overall, the highest mortality was observed on leaves compared to squares, flowers and bolls, and relatively less mortality was observed in 3rd instar compared to the 1st and 2nd instars after feeding on plant parts. The toxicity of Cry1Ac was evaluated using diet incorporation method. The lethal concentrations of Cry1Ac against 1st, 2nd and 3rd instars of *H. armigera* were 0.34, 0.77 and 1.37 μg mL^{-1} in diet incorporation method. © 2019 Friends Science Publishers

Keywords: Bioassay; Bt cotton; Cry1Ac; *Helicoverpa armigera*

Introduction

Cotton containing *Bacillus thuringiensis* (Bt) genes is an incredible tool with inherent resistance against bollworms such as *Helicoverpa armigera* and *Pectinophora gossypiella*. After the adoption of Bt cotton in 2010, the area under cultivation of this crop increased every year in Pakistan. Due to the economic advantage of Bt cotton like reducing usage of insecticides and increasing natural enemies (Drennan and Rehman, 2013), this unique transgenic technology was found to be highly beneficial in terms of its competencies to keep the target insects under check. The cotton bollworm, *H. armigera*, is a most damaging pest of many field crops. In Pakistan and some other countries of the world, it has developed resistance to a wide range of insecticides including pyrethroid and organophosphate insecticides (Sayyed and Wright, 2006). So, there is a possibility that it might also develop resistance to Cry1Ac in Bt cotton (Gujar, 2005).

The performance of Bt cotton against bollworms is linked with the expression of Cry1Ac gene (toxin protein) in plant parts leaves, squares, flowers and bolls (Greenplate *et al*., 2000; Holt *et al*., 2002; Gutierrez *et al*., 2006; Adamczyk *et al*., 2009; Bakhsh *et al*., 2010). Variable Cry1Ac expression in different plant parts of Bt cotton cultivars favors bollworms to develop resistance against this toxin (Greenplate *et al*., 2001; Mahon *et al*., 2002). In Pakistan, single gene Bt cotton is very successful in the previous years, however, multiple reports have shown the outbreak of *P. gossypiella* and *H. armigera* which can be related to variability level of Cry1Ac expression in different plant parts (Alvi *et al*., 2012; Khan *et al*., 2018). Major alarm for large-scale use of these transgenic cotton may lead to evolution of insect pest resistance to toxins (Perlak *et al*., 2001) and there is a confirmation of such events of pest attack (Wu and Guo, 2005; Liu *et al*., 2010). Variability in toxin expression level has been observed in several studies that its toxin level is variable among Bt-cotton plant parts. It was observed that Cry1Ac toxin expression and mortality of bollworm decreased constantly as the age of plant increases (Adamczyk and sumerford, 2001; Gore *et al*., 2001; Fitt, 2003). Kranthi *et al*., (2005) observed that *H. armigera* was capable of more surviving on some particular fruiting parts, and the pest infestation exceeded economic threshold levels more readily on some specific commercial hybrids compared to others. Similarly, Khan *et al*., (2018) studied that variable expression of the toxin across some genotypes cultivated in Pakistan, and also spatial and temporal dependency in plant parts that allow larvae to survive and found that all varieties lost their expression durability in late season and their expression decreased below the critical level.

So, for the development and selection of better performing Bt cotton genotypes with suitable toxin expression, it is necessary to evaluate the plant parts performance of cultivated Bt cotton for their susceptibility against *H. armigera* in Pakistan. In addition to this, little is known whether bollworms in general avoid Bt toxin at

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concentrations that produce low level of mortality. Our study would have significance for predicting what can occur if the plant parts express variable concentrations in the lesser range and might have implications for pest resistance management.

Keeping this in view, the current study was designed to evaluate the performance of different Bt cultivars against *H. armigera* to determine the expression variability in plant parts of different cultivars. Moreover, determination of concentration effect of Bt toxin on different instars of *H. armigera* was also evaluated in the current study. This will be helpful to understand role of concentrations of Bt toxin when applied as foliar spray during the direct spray application to overcome the problem of resistance against this toxin and insecticides (Tabashnik *et al*., 2003).

Materials and Methods

Seeds of five Bt and one non-Bt varieties were sourced from different research stations located in province of Punjab, Pakistan (Table 1). Six varieties were sown at entomological research area, University of Agriculture Faisalabad, Pakistan in the 2nd week of May 2014 and 2015 in a plot size of 9.14 m x 4.57 m; each separated by 1 m apart having R x R and P x P distances of 75 cm and 30 cm, respectively. General agronomic practices recommended by Department of Agriculture, Govt. of Punjab were followed.

Bollworm Survival on Bt and non Bt cotton Components

Different instars of *H. armigera* were obtained from Entomological Research Institute, Ayub Agricultural Research Institute, Faisalabad, and reared in the laboratory for a minimum of one generation to eliminate parasitoids, and minimize pathogens. Bioassays on first three instars larvae of *H. armigera* were conducted separately. Five different Bt cotton cultivars (FH-Lalazar, FH-142, VH-259, CIM-602 and BH-178) and one non-Bt cultivar (CIM-608) (cultivar leaves, squares, flowers and bolls) were collected at 15 days intervals. Leaves were placed in petri plates while other fruiting parts were placed in small plastic cups. Petri plates and plastic cups contained 0.5% solidified agar solution at the bottom for maintenance of moisture. The lid of the plates/cups were closed after releasing larvae on leaf, square, flower and boll. Treatments were arranged in a Complete Randomized Design and in each treatment, 5 larvae were released in each replication and treatments were replicated four times. The mortality of the larvae was recorded at 24 h interval until seven days and percent mortality was calculated. Larval mortality on Bt cotton was corrected with mortality in the control treatment (non-Bt).

Diet Incorporation Bioassay

First three instars of *H. armigera* were exposed to a range of serially diluted (0.25, 0.50, 1, 2, 4 ug/mL) with distilled water of Cry1Ac protein and mixed with the freshly prepared synthetic diet (artificial wheat germ diet) (1:9) at a suitable temperature (27±2°C) in the laboratory to determine mortality of the larvae after feeding on mixed diet. The range of these dilution was based on preliminary bioassay (data not shown). Prepared material was transferred into Petri-dishes. For controls, diet was mixed with distilled water only. Larvae were released in each petri plate individually and these plates were wrapped in black paper to avoid cannibalism. All plated were maintained at 27°C, 70% RH in an incubator for 7 days. Experiment was set according to Complete Randomized Design with four replications. Twenty larvae were used in each replication one per petri plate and larval mortality was recorded after 7 days of larval release.

Statistical Analysis

The obtained data from the first experiment was analyzed by Minitab 16 software using analysis of variance (ANOVA) with factorial design. Similarly, mean percent mortality data of diet incorporation experiment were analyzed using one way ANOVA. Probit analysis was used to determine LC_{50} with Polo plus software. Treatment means were separated using Tukey's HSD test at 5% level of significance.

Results

Mortality of Bollworm on Bt and non-Bt cotton Components

Leaf bioassay: During the two years (2014-2015), all the 1st instar larvae died (100% mortality) while mortality on non-Bt cultivar was low (8-9%) after feeding on leaves. A significant difference in mortality was observed in between Bt and non-Bt cultivars (data not shown). The highest mortality (100%) of 2nd instar during 2014 on leaves of FH-142 and CIM-602 was observed followed by BH-178 (98.75%), FH-Lalazar (95.63%) and VH-259 (93.75%), while non-Bt (CIM-608) had 3.75% mortality. The mortality was significantly different in FH-142 and VH-259. Similar results were obtained in 2015 (Fig. 1). Mortality of the 3rd instar was significantly lower compared to 2nd instar after feeding on leaves of different Bt cultivars. Among Bt cotton cultivars maximum mortality of 3rd instar (95%) was observed on CIM 602 which had non-significant difference with FH-142 (93.13%), BH-178 (89.38%) and FH-Lalazar (87.63%). There was significantly lower mortality after feeding on VH-259 (81.25%) compared to CIM 602, CIM 608 (non Bt) and FH 142. During 2015, similar mortality of 3rd instar larvae was observed (Fig. 1).

Square bioassay: During the years 2014-2015, results of bioassay showed that all the 1st instar larvae were dead after feeding on squares, while on non-Bt cultivar low level mortality (4-5%) was observed (data not shown). During the two years of experiment, 2nd instar after feeding on squares among Bt cotton cultivar CIM-602 showed (97.50%) and (96.67%) highest mean mortality and lowest on VH-259 which was non-significantly different from all other tested Bt varieties but significantly different from non-Bt CIM-608.

Table 1: List of cotton cultivars used in the studies

Sr. No	Name of variety	Bt or non-B*t*	Name of the Station
1	FH- Lalazar	Bt	Cotton Research station-AARI, Faisalabad
2	FH-142	Bt	Cotton Research station-AARI, Faisalabad
3	VH-259	Bt	Cotton Research Station, Vehari
4	CIM-602	Bt	Central Cotton Research Institute, Multan
5	BH-178	Bt	Cotton Research Station, Bahawalpur
6	CIM-608	Non-Bt	Central Cotton Research Institute, Multan

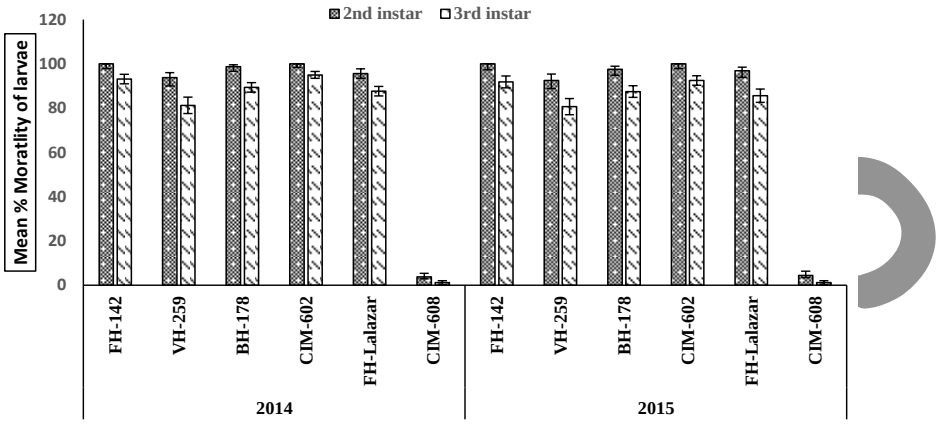

Fig. 1: Mean percent mortality of 2nd and 3rd Instar of *H. armigera* after feeding on leaves of different cotton cultivars during 2014-2015

Similar results of 2nd instar were obtained in the following year (Fig. 2). Mortality of 3rd instar larvae was more on Bt cotton cultivar CIM-602 (92.50%) in 2014 and (86.33%) in 2015 which was non-significantly different from other varieties except non-Bt control during 2014-2015. Overall 2nd and 3rd instar showed less mortality on squares as compared to leaves (Fig. 2).

Flower bioassay: Bioassay results indicates that during both the years, all the 1st instar larvae were died after feeding on flowers of all Bt cultivars as in case of leaves and squares. However, significantly lower mortality was observed after feeding on flowers from non-Bt control (data not shown). The mortality of 2nd instar larvae after feeding on flower of all Bt varieties was non-significantly different from one another during both years of experiment. Highest mortality was on FH-142 and CIM-602 and lowest was on VH-259, mortality was non-significantly difference among all Bt varieties. But mortality after feeding on flowers of non-Bt variety was significantly lower as compared to all Bt varieties. While 3rd instar showed highest mean mortality (87.00%) on CIM-602 and lowest (75.00%) on VH-259. Mortality was significantly different in Bt and non-Bt cultivars. Comparatively less mortality was observed in 2nd and 3rd instar after feeding on flower as compared to other fruiting parts leaves and squares (Fig. 3).

Boll bioassay: All 1st larvae were dead after feeding on bolls of Bt varieties during both year of experiments. But mortality of 1st instar larvae when fed on bolls of non-Bt cultivars was significantly lower (11%) (Data not shown). When bioassay was conducted on 2nd instar *H. armigera* on bolls which were excised from Bt and non-Bt varieties, highest mortality (96.25%) was observed in (FH-142) and lowest was (86.00%) in VH-259. Similar results were obtained in the subsequent year. But this mortality was significant lower when larvae were fed on bolls of non-Bt variety. In case of 3rd instar larvae when fed on bolls of Bt and non-Bt varieties lowest mortality was (71.25%) on VH-259 and highest was (81%) in FH-142. Overall non-significantly difference in mortality was observed among Bt cultivars but mortality was significantly different in Bt and non-Bt cultivars (Fig. 4).

Diet incorporation bioassay: Results of bioassays at different concentration of Cry1Ac against different instars of *H. armigera* showed that the LC_{50} of Cry1Ac against 1st, 2nd and 3rd instar of *H. armigera* were 0.34 (0.25-0.43), 0.77 (0.54-1.04) and 1.37 (1-1.99) ug/mL respectively. The values in parenthesis are upper and lower fiducial limits at 95%. LC_{50} values showed that Cry1Ac was more toxic to 1st instar larvae followed by 2nd and 3rd instar. Similarly, mean percent mortality of *H. armigera* at different concentration was observed. Minimum mean percent mortality of 1st, 2nd and 3rd instar was recorded at lower most concentration (0.25 ug/mL) of Cry1Ac. While the highest recorded mortality was (97.50), (75.00) and (65.00) at maximum concentration (4.00 ug/mL) respectively. Mortality increases with increase in concentration. Mortality results also showed that there was more mortality of 1st instar at each single dose followed by 2nd and 3rd instar (Fig. 5).

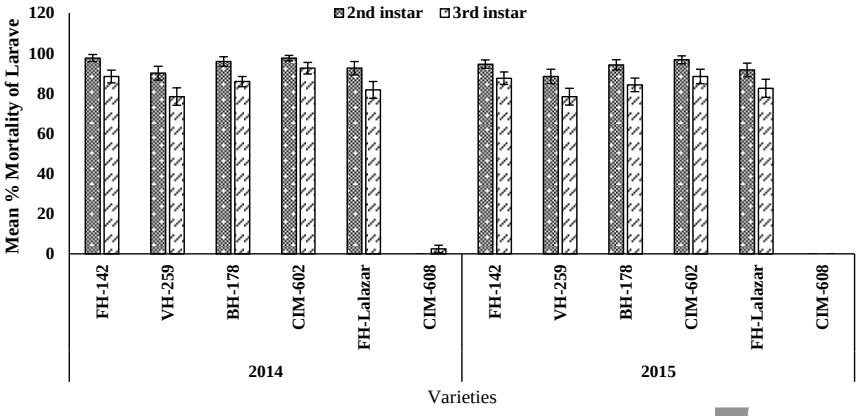

Fig. 2: Mean percent mortality of 2nd and 3rd Instar of *H. armigera* after feeding on squares of different cotton cultivars during 2014-2015

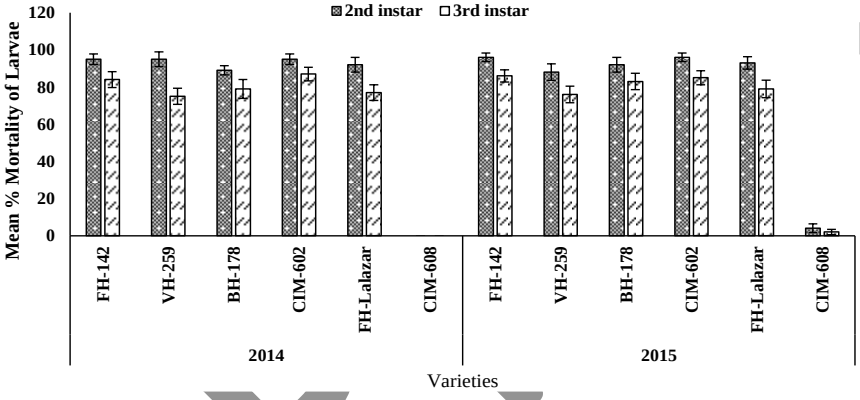

Fig. 3: Mean percent mortality of 2nd and 3rd Instar of *H. armigera* after feeding on flowers of different cotton cultivars during 2014-2015

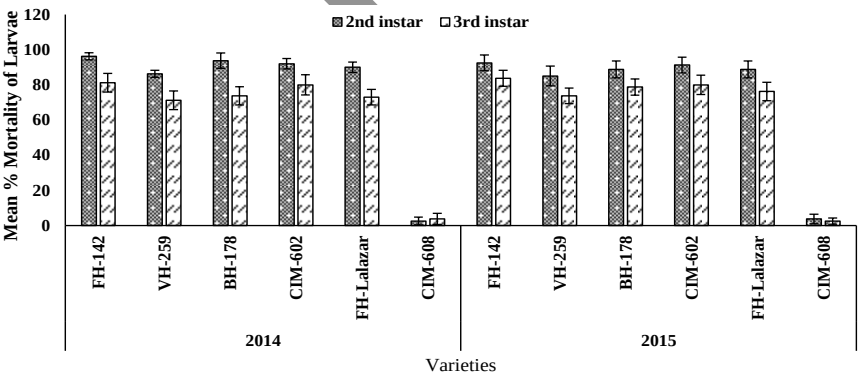

Fig. 4: Mean percent mortality of 2nd and 3rd Instar of *H. armigera* after feeding on bolls of different cotton cultivars during 2014-2015

Discussion

The sustainability and effectiveness of Bt-cotton has long been correlated to the quantity of toxin (Cry1Ac one of toxin) expressed in the cotton plant parts. It is considered very crucial for its effectiveness against cotton bollworm especially *H. armigera* as it directly affect survival of the bollworm (*H. armigera*) when tested in laboratory conditions (Adamczyk and Sumerford, 2001; Kranthi *et al*., 2005; Ullah *et al*., 2014). We observed that there was significant more mortality of *H. armigera* after feeding on cotton leaves which were detached from the Bt cotton plants.

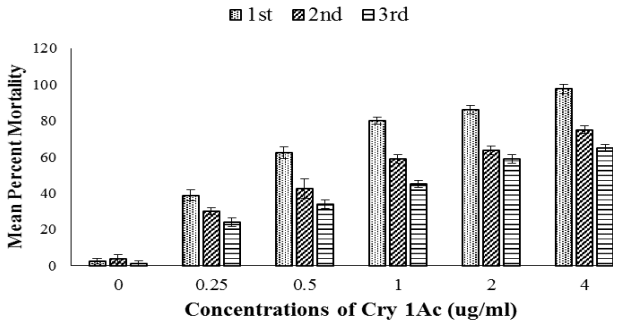

Fig. 5: Mean percent mortality of different instars of *H. armigera* after feeding on diet having different concentrations of Cry1Ac

Thus through this study we can make a logic that there is a correlation between the Cry1Ac expression in Bt cotton plant parts and mortality of *H. armigera* (Kranthi *et al*., 2005). As there was more survival of *H. armigera* on plant parts during late season of crop compared to earlier stages. Our results showed dissimilarity to the results of Wan *et al*., (2005), who observed an increase in expression of Bt toxin in plant parts at the later stages of crop in one of their tested varieties. Nevertheless, there may be several other factors which are important to consider for the expression of Bt toxin in plant parts e.g. metabolic changes in the plant in response to growth and reproduction, environmental factors, location and time of planting, availability of nitrogen or water (Benedıct *et al*., 1996; Fitt, 1998; Olsen *et al*., 2005). Though, evidence for plant and environmental effects is limited or circumstantial.

Results showed that there was complete mortality of 1st instar larvae of *H. armigera* after feeding on leaves and fruiting parts of Bt plants and there was no significant effect of time at which plant parts were detached from plant and fed to 1st instar larvae. Mortality of subsequent instars was lower compared to 1st instar. Similar results were also observed in many other studies in which there was significant more mortality of early instars compared to later. There was significant effect of time at which plant parts were excised on the mortality of *H. armigera* (Fitt, 1998; Zhao *et al*., 1998; Henneberry *et al*., 2001; Murugan *et al*., 2003; Kuijun *et al*., 2004; Mahalakshmi and Prasad, 2013). Results also showed that the expression of Bt protein was variable in different plant parts as there was significant more mortality of 1st and 2nd instar *H. armigera* after feeding on leaves compared to other fruiting parts of Bt plants at earlier stages of crop but this was not true for later instar. It means Cry 1Ac was more expressed in leaves compared to other parts (Kuijun *et al*., 2004; Kranthi *et al*., 2005). Srinivasan and Uthamasamy (2006) also observed that the expression of toxicity of Bt protein in cotton to different instars of *H. armigera* differ with different types of cotton parts; leaves were found to express higher toxicity than square and bolls. In comparison between different cotton cultivars, there was more mortality of *H. armigera* after feeding on plant parts from CIM 602 and FH 142 as compared to other Bt cultivars.

Feeding of *H. armigera* on the synthetic diet mixed with series of concentrations of Cry 1Ac revealed a wide range of biological activity (Gore *et al*., 2005). Results of present study showed that Cry1Ac was more toxic to first instar larvae followed by 2nd and 3rd instar of *H. armigera*. With the increasing concentrations of Cry1Ac there was increase in mortality of larvae. These results are in contrast with Gujar *et al*., (2007) they observed that there was lower mortality of 1st instar larvae of *H. armigera* at the maximum concentration of Cry1Ac. However, little is known whether bollworms in general avoid Cry1Ac at concentrations that produce low levels of mortality (Singh *et al*., 2008). So the Bt toxicity necessitate an additional research to find out the behavioral response of *H. armigera* larvae when feeding on diet containing Bt toxin and on Bt plant parts.

Conclusion

The current study showed that there is variability in expression of the CrylAc in plant parts of Bt cultivars during the whole season of crop growth. This variable expression of transgene would not only increase the production cost but also instigate resistance in target pests against a transgenic crop. Selection of Bt varieties for approval of general cultivation should be done on the basis of quantitative level of Cry1Ac toxin in bolls during the whole crop season. Biotechnology efforts should emphasize on developing Bt cotton varieties with tissue specific promoters to increase the toxicity of Cry1Ac in fruiting parts instead of leaves and there should be effective level of Bt toxin in the later season.

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