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Need for growing non-*Bt* cotton refugia to overcome *Bt* resistance problem in targeted larvae of the cotton bollworms, *Helicoverpa armigera* and *Pectinophora gossypiella*

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Abstract

Background: The effectiveness of *Bacillus thuringiensis* (*Bt*) cotton against target arthropod larvae is decreasing day by day. The comparative effect of *Bt* expression among *Bt* cotton varieties and different plant parts was observed against the cotton bollworms: *Helicoverpa armigera* and *Pectinophora gossypiella* larvae.

Results: In the present study, larval mortality of *H. armigera* was higher than *P. gossypiella* among selected *Bt* cultivars. Median lethal concentration (LC_{50}) values were 8.91, 13.4, 14.0, and 36.4 for *P. gossypiella*, while 5.91, 4.04, 2.37, and 8.26 for *H. armigera* of FH-142, MNH-886, IR-3701, and FH-Lalazar, respectively. These values depicted that *P. gossypiella* had more *Bt* resistance problem than *H. armigera* larvae. The host range of both targeted insect larvae was different from each other due to the polyphagous feeding nature of the larvae of *H. armigera* that feed on different host plants, but *P. gossypiella* attacked only cotton with monophagous feeding habit. It was also notable from results that *Bt* expression in reproductive parts where the attacked pink bollworm was lower than the American bollworm, so the former had the maximum chance of resistance due to repeated exposure to *Bt*.

Conclusions: It was concluded that farmers be advised to follow the practice of growing non-*Bt* as a refuge crop to reduce the problem of *Bt* resistance in the target arthropod species.

Keywords: *Cry1Ac*, ELISA, Insecticidal protein, Herbivory behavior, Lepidopterous larvae, Refuge crop

Background

Transgenic crops are the most widespread technology of proteintic engineering due to their effective control against lepidopterous arthropods (Wu *et al.* 2005). The range of *Bt* crops planted worldwide has increased from 1.7 million hectares to 185.1 million hectares since 1996 to 2016, respectively (Huang *et al.* 2017). *Cry1Ac* insecticidal protein produced by *Cry1Ac* protein proved to be very lethal against lepidopterous insect pests. Due to various environmental and socio-economic benefits, *Bt* cotton initially available in the USA started growing in different parts of the world afterward (Sansinenea 2019).

Insecticidal crystalline protein intensities and their effectiveness depend upon the cultivar's potential to produce *Bt* protein, its growth stage, and plant part used (Olsen *et al.* 2005 and Wan *et al.* 2012). It also influences the biological parameters of feeding insect species because the life parameters of herbivores depend on the

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stage of larvae and the quantity of *Cry1Ac* ingested by it (Stewart et al., 2001).

Cultivation of non-*Bt* refuge is ignored in Pakistan; therefore, frequency of cases of evolution of *Bt* resistance is very high. The lepidopterous larvae of *Helicoverpa armigera* (Hb.) and *Pectinophora gossypiella* (Saund.) are the main insect pests that adversely affect crop productivity by feeding directly on reproductive parts of cotton. Due to the polyphagous feeding existence, *H. armigera* continues its activity in different host plants compared to the monophagous *P. gossypiella* larvae (Pogue 2004). *H. armigera* larvae feed on leaves, buds, and fruit sections, causing damage to the vegetative and reproductive stages of various crop species other than cotton (Pratissoli *et al.* 2015). *P. gossypiella* larvae feed only on the bolls internally (Mapuranga et al., 2015). Owing to continuous cultivation of *Bt* cotton and restricted feeding only on cotton, the field population of monophagous larvae has more chances of evolved resistance against the *Cry1Ac* protein (Tabashnik et al., 2013; Jin et al., 2015 and Sansinenea 2019). In this way, *P. gossypiella* seems to become a more damaging insect pest of cotton than the polyphagous *H. armigera* (Dhurua and Gujar, 2011).

In previous studies, *Cry1Ac* concentration was determined in leaf parts, and its effect on only single herbivorous insect pest larvae was observed by Kranthi *et al.* (2005). In the present research, it was determined in all vegetative and reproductive parts of the plant as well as its effect on larvae of two notorious insect pests. In Pakistan, it is also the first field report on the importance of refuge crops to mitigate *Bt* resistance problem in the targetted larvae.

Methods

The present study was carried out during the cotton growing season 2018–2019. The commercially available material for proteinral sowing consisted of five cultivars of cotton (*Gossypium hirsutum* L.) including 4 *Bt* possessing *Cry1Ac* protein, i.e., FH-142, MNH-886, IR-3701, FH-Lalazar, and one non-*Bt* cultivar, i.e., FH-4243 kept as a control for comparison throughout the experimentation. The seeds were sown on 10 May 2018 on beds, following the dibbling method (placement of seed in holes and covering with sand on raised rows). Each variety comprised of 6 rows with row to row 7.5-cm width and 75 cm length, so plot size was 3375 cm^2 for each variety wise and the total plot size of one replication of all selected 5 varieties (both *Bt* and non-*Bt*) comprised 16,875 cm^2. The experiment was laid out following Randomized Complete Block Design (RCBD) with three replications.

Procedure of *Bt* testing

Kanamycin application on leaves is one of the methods that can be used to screen out transgenic and non-transgenic plants. At 30 days after sowing, Kanamycin's 1500 ppm solution was applied to differentiate *Bt* and non-*Bt* plants. Kanamycin affected photosynthesis at the application surface of the non-*Bt* leaves but neomycin phosphotransferase protein in transgenic plants degrades the effect of kanamycin so leaves remained healthier (Duan *et al.* 2009 and Shahid *et al.* 2017). For further confirmation with ELISA, 5 representative plants from each cultivar were selected randomly and tagged. Upper, middle, lower leaves, leaf pedicel, root, and stem from *Bt* plants of each cultivar were taken to quantify Cry1Ac protein concentration at 30, 60, 90, and 120 days after sowing; however, flowers were taken from the 120 days old plants of each variety. The number of the days taken to the first flower differed with respect to selected cultivars of cotton depending upon the earliness starting from 35 to 50 days (Wrather *et al.,* 2008). Flower initiation started from 35 to 50 days depending upon the earliness behavior of the selected cultivars; therefore, *Bt* quantification was done after 60 days after sowing (Shah *et al.* 2017), when flowers were available on all selected genotypes. Sandwich-enzyme linked immunosorbent assay: quantification of Cry1Ac protein (*Bt* toxin) in all selected parts of each variety was carried out, following sandwich-ELISA ELISA as the instructions of Envirologix Inc. USA (manufacturer). Optical density reading was recorded on ELISA plate reader at 450 nm. *Bt* toxin level (μg/g) was calculated and compared to standard (1.5 μg/g recommended by EPA-USA) and according to the procedure, followed Ali et al. (2018).

Insect culture

In order to obtain a susceptible generation, 400 larvae (3rd instar and above) of both species were collected from *Gossypium arborium* (locally called Desi cotton). The reason behind the selection of *G. arborium* was that due to species difference between *G. arborium* and *hirsutum*, it does not have a chance of pollen-mediated gene flow of *Cry1Ac* to *G. arborium* and ultimately to larval population of both target species. Collected population was kept in glass Petri dishes and shifted to the laboratory. To overcome the fungal problem, field-collected population was washed with sodium hypochlorite 0.05% solution. It was separated into a single specimen due to cannibalism at larval instars of lepidopteran, especially *H. armigera.* Both species were provided by food for rearing under laboratory maintained at 28 ± 2 °C and 65 ± 5% RH. After adult emergence from pupae, both male and female moths were released for egg-laying on small cotton plants into separate cages.

Eggs were incubated for hatching and the neonates were used for further experimentation.

Determination of mortality of target insect species by Bioassays technique

A total of 50 F_1 neonate of *H. armigera* and *P. gossypiella* larvae of uniform size and age were obtained from the culture, released individually into the flowers of 5 plants of each selected variety, and covered with the help of muslin cloth under semi-natural field condition to avoid their escape. Percentage of mortality of both insect pest species larvae was recorded on all tested transgenic cultivars of cotton in each replication and it was compared to FH-4243 (non-*Bt* cultivar, kept as control, to avoid the chance of error due to the natural mortality of the targeted larvae). Mortality was recorded, using the formula given as under:

$$\text{Mortality}\% = \frac{\text{Dead larvae}}{\text{Total larvae released}} \times 100$$

Statistical analysis

Mortality parameter of *H. armigera* and *P. gossypiella* was correlated by *Bt* toxin quantification of *Cry1Ac* among selected cultivars determined in different parts and at different instars. Data were analyzed using ANOVA techniques through computer-operated Statistica package 5.5 and means were compared using Tukey's HSD test for significance. Sigmaplot 12.5 package was used for graphical presentation of results subjected to Probit analysis for resistance monitoring by using computer-operated Polo plus statistical software package.

Results

Expression of *Cry1Ac* among different cultivars

Cry1Ac concentration was quantified from the top, middle, lower leaves, and leaf pedicel of the selected cultivars. *Cry1Ac* concentration was maximum in the top leaves of IR-3701 (3.39 μg/g), followed by FH-142 (2.97 μg/g) and FH-Lalazar (2.5 μg/g). In the middle leaves, concentration was maximum in IR3701 and MNH-886 (2.91 μg/g), followed by FH-Lalazar (2.17 μg/g). In lower leaves also, the concentration was high in IR-3701 (1.62 μg/g), followed by FH-Lalazar (1.6 μg/g) and FH-142 (1.32 μg/g). However, in leaf petiole, it was the maximum in MNH-886 (0.85 μg/g), followed by IR-3701 (0.3 μg/g) and FH-142 (0.2 μg/g) (Fig. 1).

In pedicle, the highest *Bt* toxin level of *Cry1Ac* protein was quantified in IR-3701 (1.5 μg/g), followed by FH-142 (0.87 μg/g), MNH-886 (0.39 μg/g) and FH-Lalazar (0.31 μg/g) (Fig. 1). In sepals, the highest values of *Cry1Ac* proteins were recorded in IR-3701 (0.8 μg/g), followed by FH-Lalazar (0.4 μg/g), MNH-886 (0.36 μg/g), and FH-142 (0.01 μg/g). In petals, the highest concentration of *Bt* toxin *Cry1Ac* was recorded in IR-3701 (0.5 μg/g), followed by MNH-886 (0.48 μg/g), FH-Lalazar (0.2 μg/g), and FH-142 (0.13 μg/g). Pollens had the highest level of *Cry1Ac* proteinin FH-Lalazar (1.8 μg/g), followed by IR-3701 (1.1 μg/g), FH-142 (0.73 μg/g), and MNH-886 (0.4 μg/g) (Fig. 1). Stigmaa had the highest

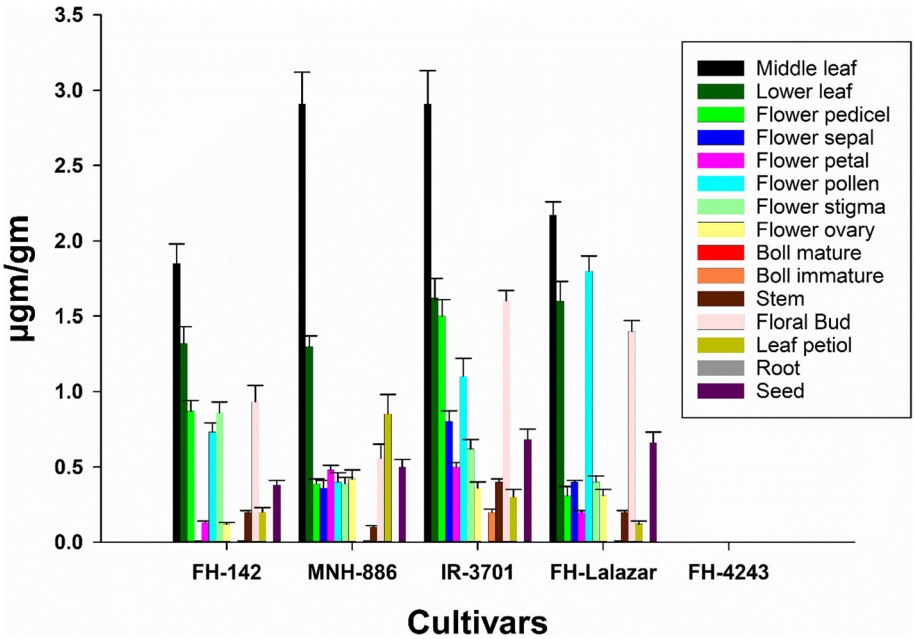

Fig. 1 *Bacillus thuringiensis* expression of *Cry1Ac* (μg/g of fresh wt.) in different parts of the plant

level of *Cry1Ac* proteinin FH-142 (0.86 μg/g), followed by IR-3701 (0.62 μg/g), FH-Lalazar (0.4 μg/g), and MNH-886 (0.39 μg/g). In the ovary, the highest concentration of *Bt* toxin was found in MNH-886 (0.42 μg/g), followed by IR-3701 (0.36 μg/g), FH-Lalazar (0.31 μg/g), and FH-142 (0.12 μg/g). In floral buds, the highest level of *Cry1Ac* protein was recorded in IR-3701 (1.6 μg/g), FH-Lalazar (1.4 μg/g), FH-142 (0.93 μg/g), and MNH-886 (0.56 μg/g) (Fig. 1).

The highest concentration of *Cry1Ac* toxin in the stem was recorded at IR-3701 (0.4 μg/g), followed by FH-Lalazar, FH-142 (0.2 μg/g), and MNH-886 (0.1 μg/g), whereas in roots, none of the selected cultivars showed expression of *Cry1Ac* protein (Fig. 1).

Cry1Ac protein was very low in both mature and immature bolls. In mature boll, the highest amount of *Cry1Ac* protein was recorded in IR-3701 (0.008 μg/g), followed by FH-142 (0.005 μg/g), FH-Lalazar (0.004 μg/g), and MNH-886 (0.002 μg/g) (Fig. 1). Immature bolls had a relatively greater amount of *Cry1Ac* toxin than that of mature bolls. In immature bolls, the highest concentration of *Cry1Ac* toxin was recorded in IR-3701 (0.03 μg/g), followed by FH-142 (0.015 μg/g), FH-Lalazar (0.012 μg/g), and MNH-886 (0.010 μg/g), (Fig. 1). The highest concentration of *Cry1Ac* toxin was quantified in the seeds of IR-3701 (0.68 μg/g), followed by FH-Lalazar (0.66 μg/g), MNH-886 (0.5 μg/g), and FH-142 (0.38 μg/g) (Fig. 1).

Expression of *Cry1Ac* at different crop stages

Cry1Ac protein differed concerning different growth stages of the cotton crop. Maximum expression of this protein was quantified at 60 days (3.37 μg/g), after sowing compared to that of 30 days (2.07 μg/g), 90 (2.17 μg/g), and 120 days (0.92 μg/g) (Fig. 2). The highest amount of *Cy1Ac* toxin quantified after 30 days of sowing was 2.4 μg/g, 2.3 μg/g, and 2.2 μg/g, after 60 days of sowing 4.5 μg/g, 3.8 μg/g, 3.0 μg/g, and 2.2 μg/g, while after 120 days post sowing 1.1 μg/g, 1.1 μg/g, 0.8 μg/g, and 0.7 μg/g by FH-142, IR-3701, FH-Lalazar, and MNH-886, respectively. However, after 90 days of sowing, the highest concentration of *Cry1Ac* protein was recorded in IR-3701 (2.8 μg/g), followed by FH-142 (2.1 μg/g), FH-Lalazar (1.9 μg/g), and MNH-886 (1.5 μg/g) (Fig. 2).

Mortality of targeted larvae

Mortality of *H. armigera* on transgenic cultivars ranged from 8 to 14%. On IR-3701, mortality was a maximum of 14.0% compared to 8.00, 9.00, and 10.0% on FH-142, FH-Lalazar, and MNH-886, respectively (Fig. 3). On FH-4243 (Non-*Bt* cultivar), there was no mortality of *H. armigera* (Fig. 3). The Scatter diagram and trend line revealed a positive linear relationship of *Cry1Ac* with percentage of mortality because scatter points were distributed very close towards the trend line's positive end. The line further explained that the critical concentration of *Cry1Ac* required to kill 50% of the target

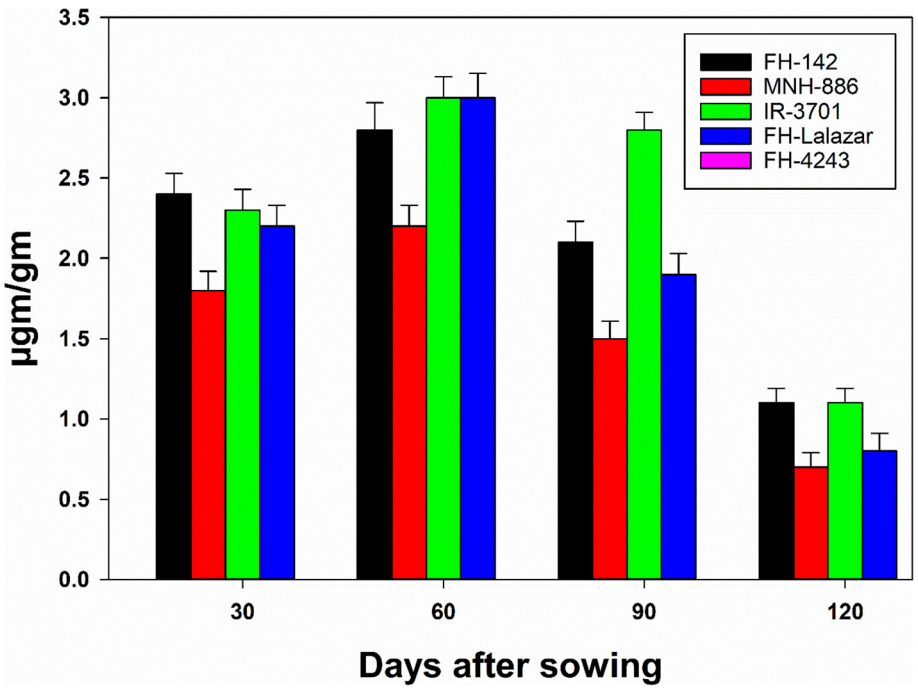

Fig. 2 *Bacillus thuringiensis* expression of *Cry1Ac* (μg/g of fresh wt.) in different cultivars of cotton at different crop stages and time intervals

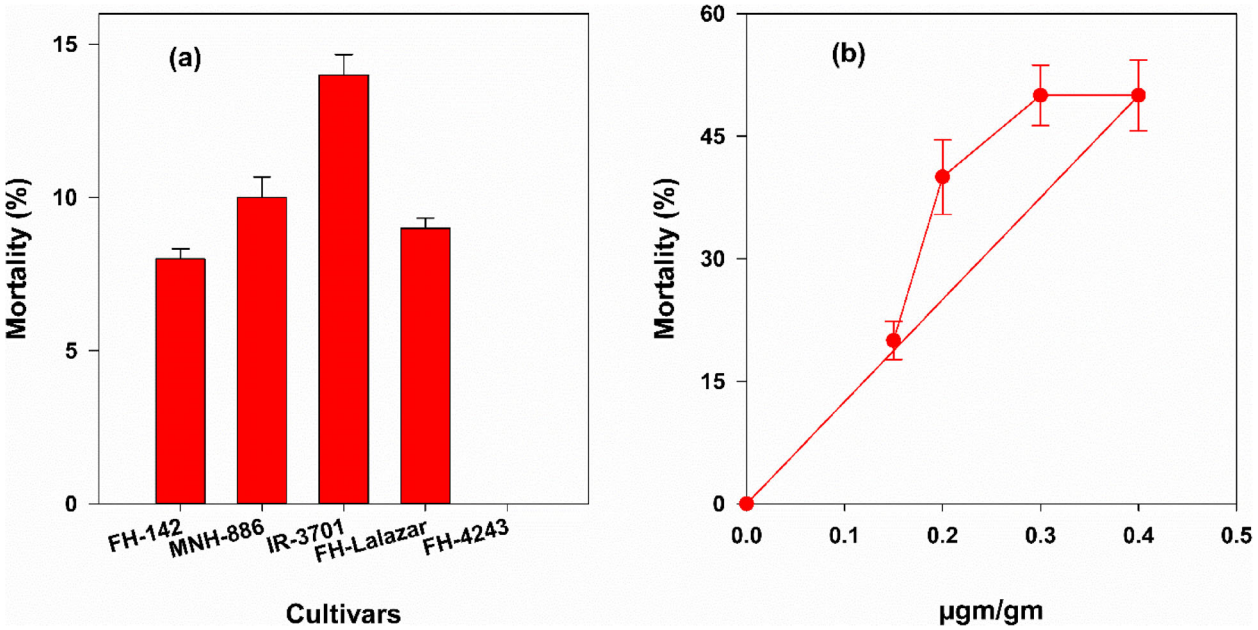

Fig. 3 **a** Mortality percentage of *Helicoverpa armigera* by feeding bolls of different cultivars after 90 days of infestation. **b** Scatter diagram indicating *Cry1Ac* concentrations inducing larval mortality of *Helicoverpa armigera*

larvae of *H. armigera* was 0.3 µg/g of fresh weight (Fig. 3). Mortality of *P. gossypiella* due to feeding on the bolls of selected *Bt* cultivars was very negligible. However, it was maximum (1.6%) on FH-142, followed by MNH-886 and IR-3701 (1.2%) as compared to 0.4% on FH-Lalazar and FH-4243 with 0% mortality (Fig. 4). Expression of *Cry1Ac*, i.e., 0.2 to 0.4 µg/g of fresh weight in the reproductive parts of transgenic cultivars of cotton induced only 4.0 to 5.0% mortality of *P. gossypiella* larvae (Fig. 4).

Among selected cultivars, the toxicity of *Cry1Ac* to *H. armigera* was similar (95% FL overlap). However, the toxicity of IR-3701 was significantly higher than all other

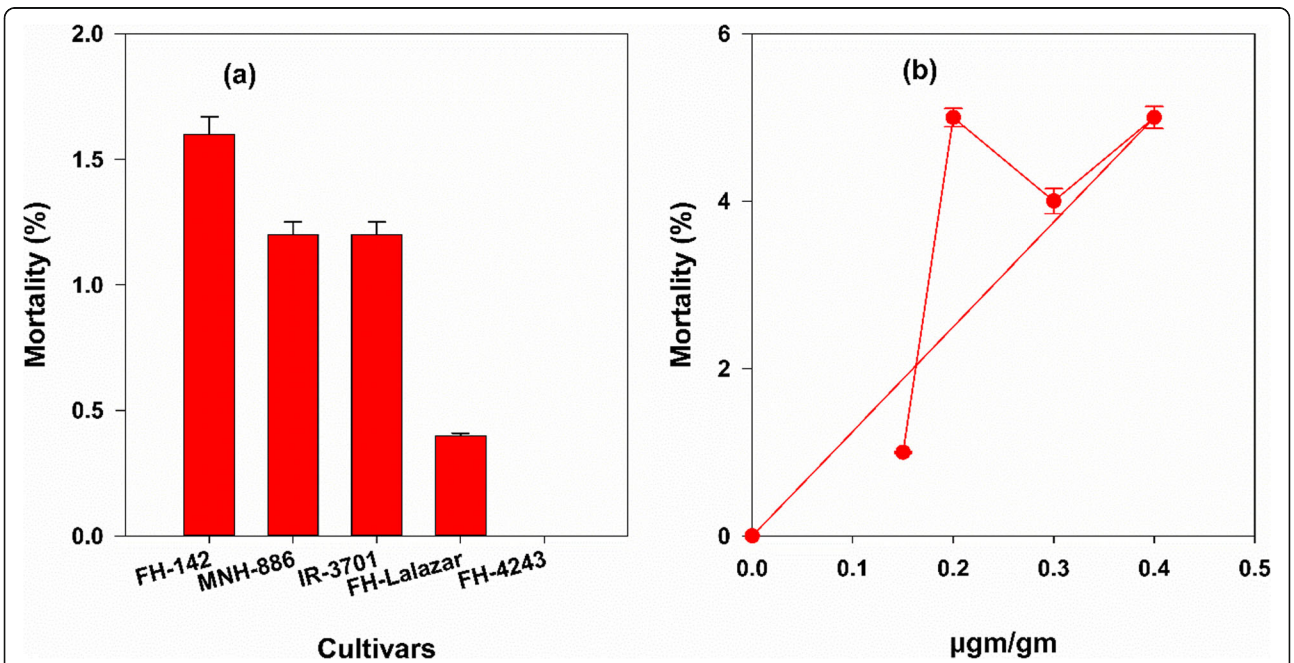

Fig. 4 **a** Mortality percentage of *Pectinophora gossypiella* by feeding bolls of different cultivars after 90 days of infestation. **b** Scatter diagram indicating *Cry1Ac* concentrations inducing larval mortality of *Pectinophora gossypiella*

cultivars. The toxicity of FH-142 and MNH-886 was almost similar to each other (95% FL overlap) and was significantly greater than FH-Lalazar. Among cultivars, the toxicity of *Cry1Ac* to *P. gossypiella* was significantly different from each other (95% FL overlap). The toxicity of *Cry1Ac* in MNH-886 and IR-3701 was similar but was significantly higher than FH-Lalazar; however, the maximum toxic effect of *Cry1Ac* was observed by FH-142 (Table 1).

Discussion

The present study results demonstrated that the highest expression of the *Cry1Ac* protein was recorded in the upper leaves of tested cultivars compared to the other plant parts, followed by middle and lower leaves. Medium expression was displayed in stem and floral parts by the *Cry1Ac* protein, whereas the least or negligible expression was recorded in bolls and roots. In selected cultivars, *Cry1Ac* concentration ranged from 2.50–3.39, 2.17–2.91, and 1.32–1.62 μg/g in upper, middle, and lower leaves, respectively. Results further exhibited that after 90 days of sowing, the *Bt* expression was maximum, however in the upper canopy, leaves expression was maximum than the middle and lower canopy leaves. Maximum *Bt* expression was reported at the seedling/young leaf stage that gradually decreased. Kranthi et al. (2005) reported the reason of this phenomenon that *Bt* crystalline proteins in old leaves were denatured.

Results further exhibited that *Bt* concentration (3.39 μg/g) in leaves of selected cultivars was very higher than the flower (1.8), seeds (0.8), stem, and roots (0.4 μg/g). Mortality of *P. gossypiella* comparatively was less than *H. armigera* might be due to the monophagous/polyphagous feeding nature and spatio-temporal expression of *Bt* protein. *H. armigera* fed on the leaves that contained higher concentration of *Cry1Ac* than reproductive parts and are killed at the initial stage. However, larvae of *P. gossypiella* very soon after their hatching from the eggs enter into the flower and bolls that possess less concentration of *Cry1Ac* than leaves might survive. The field evolved resistance had earlier been reported in *P. gossypiella* by Dhurua and Gujar (2011). They observed more susceptibility of these insects against Bollgard-II populations because of high concentration (15.4 ng mg^{-2}) of Cry2Ab2 proteinthan (0.005 ng mg^{-2}) *Cry1Ac*.

Due to continuous exposure of *Bt* cotton, different levels of toxicities of *Cry1Ac* against a population of lepidopterous larvae have been observed. These differences in toxicities against mortality gave us a clue that *H. armigera* and *P. gossypiella* posed varying resistance levels against *Bt*. LC_{50} values ranged from 8.91 to 36.4 and 2.37 to 8.26 ppm for *P. gossypiella* and *H. armigera*, respectively. It is also clear from the results that the resistance problem was more in *P. gossypiella* than *H. armigera*. The field evolved resistance in lepidopteran larvae to *Bt Cry1Ac* was reported by Tabashnik et al. (2013). Kranthi et al. (2005) identified a critical expression level of 1.9 μg/g for mortality of *H. armigera* below which this insect will develop resistance, furthermore feeding larvae can develop a high degree of resistance against *Cry1Ac*. Tabashnik et al. (2013) also reported that laboratory-selected pink bollworm population showed up to 420-fold cross-resistance to *Cry1Ac* and 240-fold resistance to Cry2Ab.

To impede the threat of *Bt* resistance in lepidopteran larvae, the production of *Bt* plants are required to ensure the guaranteed mortality of the target larvae, with concentrations high enough (LD_{99}). Better is to provide a susceptible population of *H. armigera* by cultivation of refuge crop strategy. Refuge crop limits the intensity of

Table 1 Median lethal concentration of *Bacillus thuringiensis* protein against the target insect pest species

Target larvae	Variety	LC_{50}[a] (ppm)[b]	95% FL[c]	Slope (± SE[d])	χ^{2}[e]	df[f]	P[g]
Pectinophora gossypiella	**FH-142**	8.91	4.16–12.2	0.6 (± 0.21)	1.45	3	0.83
	MNH-886	13.4	6.21–20.7	0.76 (± 0.49)	0.46	3	0.92
	IR-3701	14.0	3.20–25.3	0.70 (± 0.43)	0.87	3	0.83
	FH-Lalazar	36.4	22.04–50.7	0.65 (± 0.2)	0.39	3	0.94
Helicoverpa armigera	**FH-142**	5.91	3.26–7.2	0.8 (± 0.41)	0.45	3	0.93
	MNH-886	4.04	1.23–7.2	0.52 (± 0.22)	0.34	3	0.95
	IR-3701	2.37	0.15–4.8	1.47 (± 0.17)	3.94	3	0.27
	FH-Lalazar	8.26	4.18–12.35	0.5 ± (0.25)	0.08	3	0.97

[a]Median lethal concentration
[b]Parts per million
[c]Fiducial limit
[d]Standard error
[e]Chi-square
[f]Degree of freedom
[g]Probability
[h]Total number of insects tested in each bioassay

selection on the target pest and enhances the life span of *Bt* cotton/ transgenic technology. In the USA, the cultivation of refuge crop has been strictly supervised, where the compliance rate has been calculated 82%. In Australia, farmers have chosen to be among the 5 choices for refuge (conventional cotton spraying, unprayed traditional cotton, sorghum, maize, or pigeon pea in different areas as defined by the relative productivity of the refuge. In the same way, in South Africa, mono transgenic event containing the cry1Ab protein for regulation of maize stem borer (*H. armigera*) has been commercialized with stipulated requirement for the establishment of a 20% refuge crop. Frequency of cases of evolution of resistance is very high in the countries where non-Bt refuge has been ignored by the growers.

H. armigera fed on a variety of host plants. There was a less chance of field evolved resistance against *Bt* cotton compared to *P. gossypiella* being monophagous and having a great potential to evolve resistance against *Cry1Ac*. Farmers may be advised to follow the practice of growing non-*Bt* as refuge crops to reduce the problem of field evolved resistance in the target arthropod species. Refuge crop was very useful, and the same practice has been practiced in other countries of the world to manage field evolved resistance in lepidopterous insect species against *Bt* cotton. Wan et al. (2012) also suggested that non-*Bt* cotton as a refuge crop should be cultivated on a large scale and integrated with *Bt* cotton as a management tactic.

Conclusion

Based on the results, the increase in concentration of *Bt* protein will induce *Bt* resistance problem. It was notable that in top leaves, *Cry1Ac* expression was maximum than in other plant parts. Larval mortality in *H. armigera* was higher than *P. gossypiella* in selected *Bt* cultivars due to its polyphagous feeding nature. LC_{50} value depicted that there was more *Bt* resistance in the tested population of *P. gossypiella* than of *H. armigera* larvae. It can be suggested that growing of non-*Bt* as refuge crop will be very useful to minimize *Bt* resistance problem in *P. gossypiella*.

Abbreviations

Bt: *Bacillus thuringiensis*; LC: Lethal concentration; ELISA: Enzyme-linked immunosorbent assay; US: United States; RCBD: Randomized Complete Block Design; Ppm: Parts per million; R.H.: Relative humidity; ANOVA: Analysis of variance; HSD: Honestly significant difference; FL: Fiducial limit

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Authors' contributions

MRF and MF designed and performed the experiments. MS, MA, and ZUZ helped in the data analysis and manuscript writeup. SA and AM supervised the whole experiments and reviewed the early draft of the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

There is no conflict of interest

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