

## Survival and reproduction of natural populations of *Helicoverpa armigera* on *Bt*-cotton hybrids in Raichur, India

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**Transgenic *Bt*-cotton is commercially cultivated on the rationale that it produces toxins that defend the plants primarily from caterpillars damaging cotton bolls. From the context of crop protection, it is important that these bollworms remain susceptible to the toxins, so that their populations are under check. However, if certain individuals are able to survive and breed on the transgenics, they can build populations resistant to the toxins. In one such instance we discovered individuals of *Helicoverpa armigera*, the most prominent among bollworms in India, surviving on commercial *Bt*-cotton hybrids containing single (*Cry1Ac*) and double (*Cry1Ac* and *Cry2Ab*) genes in experimental plots of the University of Agricultural Sciences, Raichur campus, India. Analyses of various biological parameters measured through laboratory breeding on the respective hybrids revealed that these surviving individuals could not only complete their life cycle but also reproduce. A proportion of individuals of the succeeding generation were also able to complete their life cycle on the transgenic commercial hybrids. Interestingly, many of the biological parameters of the bollworm across *Bt* and non-*Bt* hybrids were mostly comparable. These results not only validate the occurrence of natural populations of *H. armigera* on *Bt*-cotton hybrids, but also provide evidence for its survival and successful reproduction in India.**

**Keywords:** *Bt*-cotton, *Helicoverpa armigera*, reproduction, resistance, survival.

WHILE considering the extent of problems caused by herbivorous insects to cotton cultivation in India, bollworms in general and *Helicoverpa armigera* (Hübner) (Lepidoptera, Noctuidae) in particular, stand out. During the 80s and 90s of the last century, there have been widespread and regular outbreaks of *H. armigera*<sup>1,2</sup>, leading to steady increase in pesticide application and increase in the cost of crop production. This was attributed to the decline in area and production of cotton during the time. Therefore, an opportunity was available for introducing transgenic cotton hybrids containing gene(s) that rendered the plants resistant to bollworms. Commercial cultivation of trans-

genic cotton thus began in India in 2002. Statistics show that, since the introduction of the transgenics, the area under cotton has steadily increased from 7.7 m ha in 2002 to 9.4 m ha in 2008, production has increased from 2.3 mt in 2002 to 5.4 mt in 2008 with a jump in productivity from 302 to 567 kg/ha (ref. 3). Concurrently, during these years pesticide consumption witnessed a decline by almost 50% principally due to reduction in the number of applications against bollworms<sup>4</sup>.

A feature of transgenic cotton under commercial cultivation in India is that it is claimed to be resistant to most of the herbivorous lepidopterans (moths). Several *Cry* genes, principally derived from the soil bacterium *Bacillus thuringiensis*, have been introgressed into the genome of the cotton plants (hereafter referred as *Bt*-cotton); these genes are responsible for producing proteins that are toxic to lepidopterans. It is now established that there is considerable variation in the quantum of such toxins produced in different parts of the cotton plant and at different times of the growing season<sup>5</sup>. Such variation has been speculated to cause differential survival of the target herbivores<sup>5</sup>, which can gradually lead to resistance in the herbivores to toxins produced by the transgenes. Although live individuals of *H. armigera* have been discovered feeding on *Bt*-cotton in India<sup>6</sup>, the ability of these individuals to complete their life cycle and successfully reproduce using the same host has not been demonstrated. Successful reproduction in surviving individuals could potentially lead to the development of insect populations that are resistant to *Bt*-cotton, which could play against the advantages that *Bt*-cotton offers. In this communication we report the natural occurrence of *H. armigera* on *Bt*-cotton in the experimental plots laid in the University of Agricultural Sciences (UAS) Campus, Raichur, India, which is situated in the northeastern dry zone (Zone II) of Karnataka at 16°11'N lat. and 77°20'E long. with an altitude of 389 m amsl. Further, we show that these individuals are able to complete their life cycle and reproduce on commercial *Bt*-cotton hybrids.

The experimental plots at UAS, Raichur campus used for the present study consisted of one block of 0.5 acre of the hybrid NCS-145 and one block of 0.25 acre each of the hybrids MRC-6918, MRC-7918 and MRC-7918 (non-*Bt*). The hybrids NCS-145 and MRC-7918 contain both *Cry1Ac* and *Cry2Ab* genes (hereafter referred as BG-II (Bollgard<sup>TM</sup>-II, Event MON 15985) hybrids), whereas the hybrid MRC-6918 contains only *Cry1Ac* gene (hereafter referred as BG-I (Bollgard<sup>TM</sup>-I, Event MON 531) hybrid). MRC-7918 (non-*Bt*) is similar to MRC-7918 in all aspects, except that the former lacks *Cry* genes. Sowing was taken up simultaneously for all the above hybrids on 18 August 2009. The blocks were regularly irrigated and provided with recommended fertilizers; there were no insecticide applications against bollworms at any stage. However, 5–7 rounds of systemic insecticides were sprayed against various sucking pests based on their

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economic threshold levels (ETL). There were no perceptible problems for the growth of the plants.

During boll formation at 90 days after sowing, the plants were physically screened for the presence of *H. armigera* larvae. A total of 133 larvae found feeding on the bolls of both BG-I and BG-II hybrids were collected. Among them, 20 were collected on BG-II and 113 on BG-I hybrids. We emphasize here that this was not a quantitative survey and no inference may be drawn on the relative occurrence of *H. armigera* on the different hybrids. A quantitative survey in this regard was not possible as a substantial number of larvae was mechanically collected from the *Bt* hybrids at various times by other researchers for different purposes and data were unavailable to the present authors. The extent of boll damage to the plants from which larvae were collected was recorded. The presence of *Cry* toxins in various parts of the plants, viz. leaves, flowers and bolls from which larvae were collected was confirmed using Design *Bt* expression kit<sup>®</sup>. All the tested plant parts of the *Bt* hybrids showed presence of toxins with double bands on the test strips (Figure 1). All tested plants were tagged and their leaves and bolls were used for rearing the first generation ( $F_0$ ) of *H. armigera*. Larvae collected were between second and fifth instar (there are five instars in *H. armigera*). Individuals were isolated from each other and laboratory-reared in plastic boxes (15 cm diameter  $\times$  7 cm height) with a perforated lid. Food was replaced every alternate day. For the sake of mating and egg-laying, male and female pupae were separated and a pair of newly emerged male and female moths was released in a plastic box (15 cm diameter  $\times$  20 cm height) with black muslin cloth placed inside. Honey solution (10%) mixed with yeast was soaked in a cotton wad and provided as food for the moths. Eggs laid on the muslin cloth were collected every day and placed in an incubator set at 28°C and 80% rela-

tive humidity. Completion of the larval stage, pupal duration, pupal weight, survival to adulthood, number of eggs laid by each female (fecundity) and hatchability were recorded while rearing the larvae in the laboratory. Concurrently, 50 larvae from MRC-7918 (non-*Bt*) were collected from the adjacent block and reared in the laboratory. This served as the control. The earlier mentioned parameters were recorded from among individuals from the control and compared with those of individuals from *Bt* hybrids. Differences across the *Bt* hybrids and control with respect to proportion of individuals pupated, moth emergence proportion and sex ratio were tested for significance using the chi-square test. Weight of pupa, pupal period, fecundity and adult duration were subjected to one-way ANOVA [SPSS (15 version) statistical package] with three treatments and a control, and each larva collected acted as a replication. Data on proportion boll damage and proportion hatching were arc-sin transformed before subjecting them to one-way ANOVA.

As a surrogate for direct population counts on non-*Bt* and different *Bt* hybrids, the extent of boll damage due to *H. armigera* was recorded from plants from which larvae were collected and tested for the presence of toxins. Total number of bolls and bolls damaged by *H. armigera* (boll with a characteristic hole or larva (Figure 2) and faecal pellets collected at the base of the sepals were the criteria used for considering a boll to be damaged by *H. armigera*) were recorded to calculate proportion of boll damage. Boll damage of 4.9% and 4.3% was recorded on the BG-II hybrids NCS-145 and MRC-7918 respectively. BG-I hybrid MRC-6918 recorded 8.6% boll damage, which was significantly higher than that of the BG-II hybrids (One-way ANOVA;  $P < 0.05$ ). However, all the *Bt* hybrids recorded significantly less boll damage compared to the non-*Bt* hybrid (30.9%). Higher boll damage among non-*Bt* plants suggests that the *Bt* hybrids offer



**Figure 1.** Design *Bt* expression strips<sup>®</sup> exhibiting double bands indicating the presence of *Cry* toxins in different plant parts of *Bt* hybrids.



**Figure 2.** Larva of *Helicoverpa armigera* feeding on boll of *Bt* hybrid.

# RESEARCH COMMUNICATIONS

**Table 1.** Per cent boll damage and developmental biology of field-collected populations of *Helicoverpa armigera* on *Bt* and non-*Bt* hybrids

Parameters observed	Cotton hybrid			
	NCS-145(BG-II)	MRC-7918(BG-II)	MRC-6918(BG-I)	MRC-7918 (non- <i>Bt</i> )
Number of larvae collected	12	8	113	50
Instar of larvae collected	II to V	II to V	II to V	II to V
Extent of boll damage (%) <sup>#</sup>	4.9 (12.79) <sup>a</sup>	4.3 (11.97) <sup>a</sup>	8.6(17.05) <sup>b</sup>	30.9 (33.71) <sup>c</sup>
Number pupated	9	8	61	35
Pupation (%)	75 ( $\chi^2 = 0.72$ )	100 ( $\chi^2 = 25.7^*$ )	54.04 ( $\chi^2 = 7.28^*$ )	70
Average weight of pupa (mg)	206 $\pm$ 46.12 <sup>a</sup>	178.12 $\pm$ 37.07 <sup>b</sup>	203.83 $\pm$ 30.03 <sup>a</sup>	222.51 $\pm$ 29.49 <sup>a</sup>
Pupal period (days)	14.5 $\pm$ 0.52 <sup>b</sup>	15.24 $\pm$ 1.03 <sup>a</sup>	14.2 $\pm$ 1.48 <sup>b</sup>	14.2 $\pm$ 1.35 <sup>b</sup>
Moth emergence (%)	66.67 ( $\chi^2 = 8.45^*$ )	75 ( $\chi^2 = 2.68$ )	83.60 ( $\chi^2 = 0.10$ )	85.71
Moth appearance	Normal	Normal	Normal	Normal
Sex ratio (♀ : ♂)	3 : 3 ( $\chi^2 = 1.51$ )	2 : 4 ( $\chi^2 = 0.02$ )	20 : 31 ( $\chi^2 = 0.23$ )	9 : 21
Fecundity	325.4 $\pm$ 45 <sup>b</sup>	307.5 $\pm$ 81.31 <sup>b</sup>	353.3 $\pm$ 23.68 <sup>b</sup>	402.7 $\pm$ 67.02 <sup>a</sup>
Hatchability (%)	85.2(67.37) <sup>a</sup>	90.1(71.56) <sup>a</sup>	87.1(68.95) <sup>a</sup>	92.6(74.21) <sup>b</sup>
Adult duration (days)	10.6 $\pm$ 3.2 <sup>a</sup>	6.2 $\pm$ 1.47 <sup>c</sup>	6.5 $\pm$ 1.09 <sup>c</sup>	8.1 $\pm$ 1.49 <sup>b</sup>

<sup>#</sup>Figures in parentheses are 'arc-sin' transformed values. Alphabets mentioned as superscripts may be compared along the rows; differing alphabets indicate that the values differ significantly (one-way ANOVA; post-hoc *t*-test;  $\alpha = 0.05$ ). In the cases where chi-square test has been adopted, *Bt* hybrids have been individually compared with the non-*Bt* hybrid;  $\chi^2$  values are given in parentheses and significantly differing values ( $P < 0.05$ ) have been highlighted by \*.

certain protection against boll worms. However, up to 8.6% boll damage in BG-I is considerable as it falls within the suggested economic threshold of 5–10% recommended for non-*Bt* hybrids<sup>7</sup>. However, as the larvae were being mechanically but unsystematically thinned, we do not elaborate on this aspect here. For this reason, it is important that the above data should not be interpreted as BG-II being superior to BG-I. As the focus of this communication is largely restricted to comparisons across the hybrids with regard to the measured aspects of developmental and reproductive biology, we restrict further discussions to these aspects alone.

Results on the developmental biology from the  $F_0$  generation do not clearly suggest any particular pattern. All the larvae collected on MRC-7918 successfully pupated, whereas 75% of the larvae collected on NCS-145 and 70% on non-*Bt* hybrid pupated. Per cent pupation was lowest (54.04) among larvae collected on BG-I. Pupal weight, recorded as average weight of all male and female pupae together for a hybrid, was lowest (178.12  $\pm$  37.07 mg,  $P < 0.05$ ) on MRC-7918. It ranged between 203.83  $\pm$  30.03 and 222.51  $\pm$  29.49 mg for the others, which was not significantly different from each other. With the exception of NCS-145, there was no significant difference across the hybrids with respect to extent of moth emergence (66.67% in NCS-145, 75% in MRC-7918, 83.6% in MRC-6918 and 85.71% in the non-*Bt* hybrid). The external appearance of all the emerged moths was normal. Sex-ratio differences were non-significant among the individuals of *Bt* and non-*Bt* hybrids. Adult longevity was highest in NCS-145 (10.6  $\pm$  3.2 days) followed by non-*Bt* (8.1  $\pm$  1.49 days), MRC-6918 (6.5  $\pm$  1.09 days) and MRC-7918 (6.2  $\pm$  1.47 days) (Table 1). It is important to observe that there is no particular pattern in these results; they are variable across the hybrids with respect to each

of the parameters. The trends obtained cannot be attributed to either the presence or absence of *Cry* genes in the hybrids. In other words, it can be inferred that the performance of individuals of *H. armigera* on the *Bt* hybrids is 'comparable' to those on the non-*Bt* hybrid. The take-home message is that *H. armigera* can survive and complete a generation on the commercial *Bt* hybrids available in India.

With respect to some of the reproductive characteristics, fecundity was highest (402.7  $\pm$  67.02 eggs/female) in non-*Bt* hybrid followed by BG-I (353.3  $\pm$  23.68 eggs/female), and BG-II hybrids (NCS-145 (325.4  $\pm$  45 eggs/female) and MRC-7918 (307.5  $\pm$  81.31 eggs/female)). Hatchability was also highest in non-*Bt* (92.67%) followed by MRC-7918, MRC-6918 and NCS-145 hybrids (90.1%, 87.1% and 85.2% respectively) (Table 1). Although these results suggest that fecundity and hatchability are higher among individuals on non-*Bt* hybrid, it is interesting to note that the performance of those on *Bt* hybrids is also quite appreciable. It also suggests that increased diversity of toxins (BG-II hybrids) did not have a greater influence on the measured aspects of biology of *H. armigera*. On the whole, *H. armigera* is not only able to survive on the *Bt* hybrids, but also reproduce successfully.

We further enquired whether the next generation ( $F_1$ ) was able to successfully complete its development on particular hybrids. A pair of male and female adults of the  $F_0$  generation obtained from each hybrid (BG-I, BG-II (NCS-145 only) and non-*Bt* hybrids) were released in a plastic box for mating and egg-laying, as described earlier. The resultant progeny was reared on leaves of the relevant hybrids in the laboratory (tagged plants were used for obtaining leaves). Various parameters like survival to adulthood, larval duration, pupal duration, pupal

**Table 2.** Developmental biology of *F*<sub>1</sub> generation of *Helicoverpa armigera* on different *Bt* and non-*Bt* hybrids

Parameters observed	Cotton hybrid		
	MRC-6918 (BG-I)	NCS-145 (BG-II)	Non- <i>Bt</i> (MRC-7918)
Number of larvae reared	150	150	150
Number of larvae survived to adulthood	38	22	38
Survivability (%)	25 ( $\chi^2 = 0$ )	15 ( $\chi^2 = 8.0^*$ )	25
Larval duration (days)	21.36 $\pm$ 2.08 <sup>a</sup>	21.15 $\pm$ 2.19 <sup>a</sup>	20.45 $\pm$ 1.67 <sup>b</sup>
Pupal weight (mg)	216.23 $\pm$ 23.31 <sup>a</sup>	212.16 $\pm$ 22.15 <sup>a</sup>	248.58 $\pm$ 34.94 <sup>b</sup>
Pupal duration (days)	15.25 $\pm$ 1.46 <sup>a</sup>	14.17 $\pm$ 1.16 <sup>b</sup>	14.45 $\pm$ 1.25 <sup>b</sup>
Sex ratio (♀ : ♂)	15 : 23 ( $\chi^2 = 0.01$ )	8 : 14 ( $\chi^2 = 0.06$ )	16 : 22

Alphabets mentioned as superscripts may be compared along the rows; differing alphabets indicate that the values differ significantly (one-way ANOVA; post-hoc *t*-test;  $\alpha = 0.05$ ). In the cases where chi-square test has been adopted, *Bt* hybrids have been individually compared with the non-*Bt* hybrid;  $\chi^2$  values are given in parentheses and significantly differing values ( $P < 0.05$ ) have been highlighted by \*.

weight and sex ratio were recorded. Data on larval duration, pupal duration and pupal weight were subjected to one-way ANOVA [SPSS (ver. 15) statistical package] with two treatments and a control, and each larva from the respective hybrid served as a replication. Survival and sex ratio were subjected to chi-square test. Leaves used for larval rearing were randomly checked for the presence of *Cry* toxin using Design *Bt* expression kit<sup>®</sup>.

To begin with, 150 neonate larvae obtained from a particular hybrid were reared in individual glass vials (25 ml capacity) on leaves of the same hybrid. In the *F*<sub>1</sub>, survival was low in all the hybrids, including non-*Bt*. Only 25% of larvae survived to form adults on MRC-6918 and non-*Bt* hybrids, whereas populations of NCS-145 recorded only 15% survival which was significantly lower ( $\chi^2 = 8.0$ ;  $P < 0.05$ ). Generally, survival is low for larvae of *H. armigera* when reared in the laboratory. Larvae reared on *Bt* hybrids took significantly more time to complete their larval period compared to non-*Bt* (one-way ANOVA;  $f = 2.208$ ;  $P < 0.05$ ). Individuals on BG-I took 21.36  $\pm$  2.08 days followed by 21.15  $\pm$  2.19 days on BG-II; larvae on non-*Bt* took 20.45  $\pm$  1.67 days to complete the larval period. Pupal weight was significantly lower in BG-I and BG-II (216.23  $\pm$  23.31 and 212.16  $\pm$  22.15 mg respectively) compared to non-*Bt* (248.58  $\pm$  34.94 mg). Pupal period was higher (15.25  $\pm$  1.46 days) in BG-I compared to BG-II (14.17  $\pm$  1.16 days) and non-*Bt* (14.45  $\pm$  1.25 days). Sex ratio in all the populations was on par with each other (Table 2). In spite of marginally prolonged developmental duration and lower body weight for individuals on *Bt* hybrids, it is noteworthy that they are able to survive to reach adulthood.

Natural populations of *Helicoverpa zea* (Boddie), a close relative of *H. armigera* were reported<sup>8</sup> from Mississippi and Arkansas, USA, surviving on BG-I causing 'unacceptable level of boll damage' during 2002. Larval survival on *Bt* cotton leaves relative to non-*Bt* cotton leaves was higher in these two locations than for a susceptible laboratory strain. The reason for their low susceptibility to *Bt* cotton was attributed to the develop-

ment of a resistant trait which is 'heritable' and 'associated with measurable increase in survival on *Bt* plant tissue'. From India it has been reported<sup>5</sup> that survival of *H. armigera* was possible on *Bt* hybrids due to temporal and intra-plant variability of *Cry*1Ac expression; toxin expression in boll-rind, square bud and ovary was inadequate to confer full protection to the fruiting parts. Another report<sup>9</sup> shows 50% reduction in *H. armigera* larval population in *Bt*-MECH-162 compared to non-*Bt* MECH-162, which indicates larval survival on *Bt* hybrids. Up to 9% boll damage has been registered on *Bt*-MECH-162 in Guntur region, Andhra Pradesh<sup>6</sup>. These studies along with the present study claim that *H. armigera* can occur on *Bt* hybrids and cause some damage. The present study also demonstrates that naturally occurring individuals can complete their generation and those that mate among themselves are able to continue their generations. This shows that there is a distinct possibility of discovering potentially resistant populations of *H. armigera* to the *Cry* toxins in the near future. To our knowledge this is perhaps the first demonstration of natural populations of *H. armigera* being capable of surviving and breeding on commercial *Bt* hybrids in India.

Two more points are worth considering here. First, there is a possibility that adults emerging from *Bt* hybrids may mate with those from non-*Bt* crops, thereby reducing the probability of building resistant populations. However, there is also a considerable probability that some of the individuals arising from *Bt* hybrids may mate among themselves, and we show that progeny from such matings may be able to survive on *Cry* toxins. The second point is that we have not estimated the extent of resistance that the sampled populations may have already developed. However, this does not disregard the fact that populations from which individuals of *H. armigera* were sampled are capable of surviving and breeding on commercial *Bt* hybrids.

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