Review of literature
Chapter 2
REVIEW OF LITERATURE

2.1 Economic importance and biology of Heliothinae

Transgenic Bt cotton has been successfully deployed in India since 2002 and increasingly incorporated into integrated pest management programs to combat the bollworm complex comprising Cotton bollworm, *H. armigera*; Pink bollworm, *P. gossypiella*; Spotted bollworm, *Earias vittella* and Spiny bollworm, *Earias insulana*. Among the bollworms, *H. armigera* is considered as one of the major threats to the present day intensive agriculture (Ramasubramanian and Regupathy, 2003) of several states in India and other countries. *H. armigera* is reported to feed nearly 181 host plants (Manjunath *et al.*, 1989) and is considered to be an economic pest of pigeon pea, chickpea, cotton, tomato, tobacco, maize, sorghum, okra, and pearl millet. Economic losses involve both direct yield reduction and the cost of inputs for the control of the pest. *H. armigera* causes about $1 billion loss to agriculture annually (Gujar *et al.*, 2000).

*H. armigera* (Lepidoptera: Noctuidae: Heliothinae) till recently identified as genus, *Heliothis* consists of more than 75 species or subspecies. The sub-family “Heliothinae” signifies the special features of this group. This sub-family includes some of the most destructive pests of cultivated crops in all continents of the world. Out of these, three *viz.*, *H. armigera*, *H. zea* and *H. virescens* are major and four are minor in terms of global distribution (Fitt, 1989).

<table>
<thead>
<tr>
<th>Species</th>
<th>Distribution</th>
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<tr>
<td><em>Helicoverpa armigera</em> (Hübner)</td>
<td>Cape Verde Islands in Atlantic to Africa, Asia, Australia and South Pacific Island, Germany in North to New Zealand in South</td>
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<tr>
<td><em>Helicoverpa punctifera</em> (Wallengren)</td>
<td>Australia</td>
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<tr>
<td><em>Heliothis peltigera</em> (Denis &amp; Schiffermüller)</td>
<td>Europe, Africa and Asia</td>
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Helicoverpa assulta (Guenee)  
*Australia and Asia*

Heliothis viriplaca (Hufnagel)  
*South west Asia and Russia*

*Heliothis zea* (Boddie) and *Heliothis virescens* (Fabricius)  
*USA.*

The biology of *H. armigera* under Indian conditions is studied earlier (APAU, 1995). The moth emergence mostly takes place during the evening hours and mating occurs during dusk or night hours. The eggs are spherical with flattened base dome shaped appearance and having longitudinal ribs. Female moths lay eggs singly mostly on the upper surface of the terminal leaves with 2-3 days incubation period. The newly hatched larvae are translucent and yellowish brown colour and in next developmental stages the larvae are green with yellowish orange longitudinal lines on lateral sides. A full grown larva is light brown and 35 to 45 mm long with white spines like hairs on dorsal surface of the body. Larval period ranges from 11 to 20 days depending on the temperature in the environment. Pupation occurs in soil. Pupae are brown in colour with two tapering parallel spines at the posterior tip. Pupal period ranges from 8-14 days. The female moth emerges earlier than the male. Female moth has reddish brown forewings, stout 15-20 mm long with wingspan of 42 mm. The male moth has light brown colour forewings and comparatively smaller than the female (Fig. 1).

![Fig. 1. Life cycle of *H. armigera.*](Photo Courtesy Dr. Vinay Kalia)
2.2 Resistance to Bt toxin in Helicoverpa species

Insects have an ability to develop resistance to different insecticidal compounds. The non-judicious use of chemical insecticides has led to development of resistance to one or multiple insecticides in at least 17 species of insects (Bellinger, 1996; Kumar et al., 2008). *H. armigera* has a history of developing resistance to virtually all the insecticide molecules used against it (Kranthi et al., 2005). Existence of resistance to pyrethroids, organophosphates, carbamates and cyclodiienes is also reported by Dhingra et al. (1988), McCaffery et al. (1989), Armes et al. (1992, 1994) and Kranthi et al. (2001a).

A number of commercial Bt formulations have been used for a long time as effective biopesticides to food crops, ornamentals, forest trees and stored grains (Meadows, 1993). Bt toxins are highly toxic and specific to insects and hence ideally suited for incorporation into integrated pest management (IPM) programs (Nester et al., 2002). In spite of these, Bt based formulations have several disadvantages (McGaughey and Whalon, 1992). The biopesticide needs to be applied repeatedly and it is effective only against immature stages of target insects feeding on exposed plant surfaces. These limitations are overcome by expression of Bt genes in transgenic plants and thereby reducing the use of synthetic insecticides (Krattiger, 1997).

The most serious threat to the durability of this novel insect control technology is the potential of insect populations to develop resistance to Bt Cry proteins (Férré and Van Rie, 2002). Extensive studies on the susceptibility of heliothines to the Cry1Ac toxin have shown the ability of *H. armigera* to develop resistance under selection pressure in Australia, India and China (Akhurst and James, 1999; Daly and Olsen, 2000; Kranthi et al., 2000; Liang et al., 2000; Akhurst et al., 2003; Chandrashekar and Gujar, 2004; Wu and Guo, 2004). Selection experiments on laboratory and field-collected heliothines have been performed by using a variety of Bt products such as formulated spore crystal mixtures, encapsulated cells expressing Cry proteins, Cry toxins and materials derived from Cry proteins (Gould et al., 1995).

Gould et al. (1992) found that *H. virescens* developed resistance to Cry1Ac in response to selection under laboratory condition and showed cross resistance to
Cry1Ab and Cry1Aa. Further, Gould et al. (1995) reported that field collected strains of *H. virescens* developed >500-fold resistance to Cry1Ac after 19 generation of selection. They found a drastic increase in resistance between 12th and 19th generation of selection after exposing a maximum of 1760 neonates for 30 generations.

Lee et al. (1995) reported development of 10,000-fold resistance to Cry1Ac in *H. virescens* under laboratory conditions, which also showed cross resistance to Cry1Ab. Luttrell et al. (1999) observed a 10-fold resistance within two generations of selection and 100-fold resistance after eight generations to Cry1Ac in *H. zea*. Zhao et al. (1997) reported resistance ratio of 5.5 (LD$_{50}$) and 13.6 (LD$_{90}$) for *H. armigera* after 11 generations of rearing on Bt transgenic tobacco plants.

Kranthi et al. (2000) reported 76-fold resistance to Cry1Ac in *H. armigera* after 10 generations of selection pressure under laboratory conditions. In contrast, Akhurst et al. (2003) did not find any response in three strains of *H. armigera* after 7-11 generations of selection to Cry1Ac protoxin. In the composite strain obtained after combining the three strains, resistance to Cry1Ac was detected after 16 generations of selection. Resistance ratio was maximum at the 21st generation (300-fold) and later declined to 57 and 111-fold.

Chandrashekar and Gujar (2004) reported development of 31-fold Cry1Ac resistance in *H. armigera* over six generations under laboratory conditions. Wu and Guo (2004) observed slow resistance development in *H. armigera* reaching only 6-fold after 15 generations of selection and found a rapid increase in resistance level from 16-fold in F$_{30}$ to 106-fold in F$_{44}$ generation.

Liang et al. (2008) observed resistance levels of 170.0, 209.6 and 2893.3-fold, on selection of field population of *H. armigera* in the 16th (BtR-F16), 34th (BtR-F34) and 87th (BtR-F87) generation with artificial diet containing Cry1Ac toxin, respectively.

Rapid response to laboratory selection shows that many pests naturally harbor genetic variation in susceptibility to Bt toxins and thus have the potential to evolve resistance to Bt crops in the field (Gould, 1998; Tabashnik, 1994; Tabashnik et al.,
In order to delay the development of resistance, resistance management programs need to be implemented. For resistance management programmes to be effective, monitoring, surveillance and early detection of resistance are important prerequisites (Kranthi et al., 2005). This programme involves the detection of changes in the susceptibility of the insects that may result from repeated and prolonged exposure to Bt Cry toxins.

2.3 Baseline susceptibility studies

The baseline susceptibility of different populations of *H. armigera* will help in providing a database for developing transgenic crops with the right kind and amount of Cry toxin expression, and would also serve to monitor spatial and temporal development of resistance in target insect species, which is a primary regulatory requirement for transgenic crop technology. This necessitates a study on the toxicity of Bt and its Cry toxins against *H. armigera* in order to rationalize its use.

Several resistance monitoring techniques are used, and the most widely used is a diagnostic or discriminating dose incorporated into an artificial diet (Bates et al., 2005). Stone and Sims (1993) evaluated the geographical variability of *H. virescens* and *H. zea* from several southern states, Hawaii and the Virgin Islands. They found that the LC$_{50}$ values for Bt product Dipel® ranged from 9.30-33.70 µg/ ml for *H. virescens* and 25.0-393.7 µg/ ml for *H. zea*. While, the LC$_{50}$ values for purified Cry1Ac ranged from 0.04-0.32 µg/ ml and 0.45-7.39 µg/ ml for *H. virescens* and *H. zea* respectively.

Luttrell et al. (1999) studied the susceptibility of populations of *H. virescens*, *H. zea*, *Psuedoplusia includens*, *S. exigua* and *S. frugiperda* collected from different cotton belts in USA to purified endotoxin and commercial formulation of Bt. These studies conducted during 1992 and 1993 before commercial deployment of Bt cotton showed wide range of variation in LC$_{50}$’s among populations of *H. zea* and *S. exigua* than other species. Greenplate et al. (1998) recorded a 16-fold difference in LC$_{50}$ values of Cry1Ac toxin to *H. zea* populations from different parts of USA.

Wu et al. (1999) established a baseline susceptibility data by studying the geographical variability of *H. armigera* populations collected from 5 cotton growing
areas of China to Bt toxin Cry1Ac. They found that the LC$_{50}$ to third instar larvae ranged from 0.09-9.073 µg/ml and IC$_{50}$ for growth reduction ranged from 0.011-0.057 µg/ml. Further, Wu et al. (2002) reported the sensitivities of *H. armigera* to Cry1Ac during 1998-2000 in China. The IC$_{50}$ values ranged from 0.020-0.105 µg/ml in 1998; 0.016-0.099 µg/ml in 1999 and 0.016-0.080 µg/ml. Their diagnostic concentration studies on field populations of *H. armigera* sampled over a period of three years suggested susceptibility to Cry1Ac protein.

Gujar et al. (2000) extensively studied the geographical variation in susceptibility of populations of *H. armigera* collected from India to Bt var. *kurstaki* HD-1 and HD-73 spore crystal complex. The populations showed wide variation in their susceptibility to both the Bt strains. HD-73 was found more toxic than HD-1. They also recorded a considerable amount of temporal variation in susceptibility to HD-1 strain in population collected from Delhi.

Siegfried et al. (2000) determined the susceptibility to Bt Cry1Ab toxin for 12 field populations of *H. zea* from the United States and found the differences between LC$_{50}$ and EC$_{50}$ values to range between 18- and 36-fold, indicating significant effects of this toxin on growth and development of *H. zea* at concentrations much lower than those that caused mortality.

Hardee et al. (2001) reported that the susceptibility of *H. virescens* remained unchanged during the period 1996-1998. They also found significant increase in percentage of larval survival after 5-day on Cry1Ac treated diet from 1997-1998.

Chandrashekar et al. (2005) showed differences in the susceptibility of neonates of *H. armigera* populations from nine locations in India to Bt var. *kurstaki* strains (HD-1, 14.1 and HD-73, 5.7-fold) and individual Cry toxins (Cry1Aa, 10.5; Cry1Ab, 12.8; Cry1Ac, 16.2). Kalia et al. (2006) studied the temporal susceptibility of neonates of the *H. armigera* to Bt var. *kurstaki* HD-73 or its Cry1Ac toxin and observed a wide variation of LC$_{50}$ which in turn reflected a wide genetic variation.

Ali et al. (2006) studied the susceptibility of *H. zea* to Cry1Ac during 2000-2006. They found a variation of 12-fold in LC$_{50}$ estimates of five laboratory, seven
laboratory-cross and 10 field populations of *H. virescens*. Similarly they also observed a variation of over 130-fold in five laboratory, nine laboratory-cross and 57 field populations of *H. zea*.

Gujar *et al.* (2008) found significant differences in the toxicity of different Cry1Ac proteins namely, 0.158–5.42 µg/ g for BGSC Cry1Ac (23 populations), 0.076–5.76 µg/ g for JK Cry1Ac (24 populations) and 0.0085–0.822 µg/ g for MVP Cry1Ac (21 populations), suggesting a need of evolving a consensus on Cry1Ac resistance monitoring of the larvae of *H. armigera*.

Brévault *et al.* (2009) established the baseline susceptibility data of populations of *H. armigera* collected in West Africa (2006-2008) to Cry1Ac and Cry2Ab2 proteins. Dose-response curves were used to estimate mortality (LC$_{50}$), growth inhibition (IC$_{50}$), and stunting (EC$_{50}$) of larvae. For each of these parameters, susceptibility was found to vary 44, 23, and 37-fold for Cry1Ac; 10, 40 and 25-fold for Cry2Ab2; and 37, 11, and 9-fold for the mixture of both proteins. They also found that Cry1Ac was two- to three-fold more toxic than Cry2Ab however did not detect cross-resistance among populations.

Kranthi *et al.* (2009) studied the susceptibility of *H. armigera* to Cry2Ab toxin before (2004-2005) and after (2007-2008) the introduction of Bollgard-II® cotton in India. They found that the LC$_{50}$ values ranged from 6.0 to 28.6 µg Cry2Ab/ ml of diet in 2004–05 (25 districts) and from 2.46 to 34.7 µg Cry2Ab/ ml of diet in 2007–08 (21 districts). The IC$_{50}$ range in 2004–05 and 2007–08 was from 0.31 to 2.3 µg/ ml and 0.10–3.4 µg/ ml of diet, respectively. These studies indicated insignificant changes in baseline after two years of Bollgard®-II cultivation in India.

Yenagi *et al.* (2010) studied the geographical variability in susceptibility of *H. armigera* to Bt toxin across northern Karnataka cotton ecosystem and found population from Raichur and Haveri as most tolerant (resistance fold 5.56 and 4.77 respectively); and strain from Dharwad as most susceptible (LC$_{50}$, 0.149). Similarly, Jayaprakash *et al.* (2013) studied the baseline susceptibility of *H. armigera* from major cotton growing areas across Tamil Nadu with RCH-2 (Bollgard®-II) Bt cotton
and found that the populations Kovilpatti, Theni and Madurai were highly susceptible to leaves, squares and bolls of 60, 90, 120 and 150-day-old plants.

Discriminating dose bioassays are most useful when resistance is common or conferred by a dominant allele (resistance allele frequency >1 per cent) (Andow and Alstad 1998). Typically, discriminating dose assays are based on 100-300 larvae to detect resistance at a frequency of 1-3 per cent (Roush and Miller 1986). This method is less useful for detecting the resistant alleles that are highly recessive or at low frequencies. Alternately, the F$_2$ screen may be a useful monitoring technique especially for the detection of rare recessive resistant alleles (Andow and Alstad, 1998). The technique also allows fewer samples to be collected to detect potential susceptibility shifts than the discriminating dose assay. The F$_2$ screen is conducted by sampling mated females from natural populations, rearing the progeny of each female as an isofemale line and sib-mating the F$_1$ larvae using an appropriate screening procedure such as a discriminating concentration assay or Bt crop, and performing statistical analysis.

Blanco et al. (2008) used the F$_2$ screen method to test the assumptions and theoretical predictions related to detection of recessive Bt-resistant alleles in field populations. They were able to detect Cry1Ac-resistant homozygous larvae but the proportion of resistant larvae was lower than the theoretical expectation of 6.25 per cent. Their studies also indicated that F$_1$ sib-mating density and F$_2$ neonate screening are important for the successful implementation of this method. Further in 2009, they used second generation F$_2$ of 1001 single-pair families from 7 geographical regions in North America and found resistant allele frequencies ranging from 0.0036-0.0263 and did not detect any major resistant alleles.

Liu et al. (2010) used the F$_2$ screening procedure and documented a case of field-evolved resistance in *H. armigera* after several years of intensive planting of Bt cotton in Qiuxian area (Hebei, China). Field survey showed an increased trend of egg populations of *H. armigera* on Bt cotton in this area from 2003 to 2007. The resistance allele frequency in the population of *H. armigera* collected during 2007 was estimated to be 0.075 (95 per cent CI: 0.053–0.100), which was 12 times greater
than that estimated 9 years ago. Dose–response bioassay with the field population collected from the same area showed a significant resistance level (11-fold) to Cry1Ac toxin compared to a laboratory susceptible strain. Zhang et al. (2011) detected increases in the frequency of resistance to Cry1Ac in populations of *H. armigera*, from northern China. Further, Jin et al. (2013) reported that the percentage of individuals resistant to a diagnostic concentration of Cry1Ac was significantly higher in two populations from different provinces of northern China (1.4 per cent and 2.3 per cent).

The evolution of resistance to Bt Cry toxins in *H. armigera* as evident from laboratory selection experiments and field documentation reports (Liu et al., 2010; Jin et al., 2013; Tabashnik et al., 2013) provide an early warning of resistance that could become a serious problem. However, detection of resistant individuals in the field or laboratory is not equivalent to claiming field-evolved resistance; further analysis are necessary to demonstrate genetic changes in susceptibility directly because of the insecticide (Sumerford et al., 2013). Managing the evolution of resistance to Bt Cry toxins therefore requires extensive knowledge about the mechanism of resistance as well as the genetics of resistance genes.

### 2.4 Inheritance of Cry toxin resistance

One of the best strategies proposed to delay the evolution of Bt resistance is the high-dose and refugia strategy. This strategy aims at maximum mortality of genetically heterozygous and homozygous susceptible insects on the assumption of resistance trait being recessive. The random mating between the surviving homozygous resistant individuals with susceptible insects emerging from the non-Bt refuge crop would produce the susceptible heterozygous progeny, thereby diluting resistance alleles in the succeeding generations. However, if resistant trait is dominant or semi-dominant in their inheritance, there is likelihood of these resistant insects multiplying to a level causing failure of even Bt crop (Nair et al., 2010).

A variation in the mode of inheritance to Bt Cry toxins has been reported in heliothines. Sims and Stone (1991) found that the resistance in *H. virescens* to Bt var. *kurstaki* HD-1 was autosomally inherited, incompletely dominant and controlled by
several genetic factors. Gould et al. (1995) observed that the genetic basis of resistance to the Cry1A toxin-complex in *H. virescens* is partially recessive and due to a single locus or a set of tightly linked loci. They also reported as high as $1.5 \times 10^{-3}$ frequency of alleles for resistance to Bt toxin in field population of *H. virescens* and emphasized need for caution in deploying transgenic cotton to control insect pests.

Mahon et al. (2007) established that resistance to Cry2Ab toxin in SP15 strains of *H. armigera* was recessive and due to a single autosomal gene. Liang et al. (2008) found that inheritance of Cry1Ac resistance trait in *H. armigera* at three resistant levels viz., 170.0-fold (BtR-F16); 209.6-fold (BtR-F34) and 2893.3-fold (BtR-F87) was autosomal and incompletely recessive. However, as the resistance developed the recessivity was found to increase from -0.21 to -0.25 in BtR-F16 to -0.44 and -0.43 in BtR-F87. Moreover, they also found that the gene number in relation to the resistance changed as the resistant level increased; resistance was primarily monogenic in BtR-F16 strain and polygenic in BtR-F34 and BtR-F87 strains.

In contrast to these, resistance was reported to be completely dominant in BKBT strain of *H. armigera* originating from Cote d’Ivoire showing 160-fold resistance to Cry1Ac (Uraichuen, 2002). Kranthi et al. (2006) studied the mode of inheritance to Cry1Ac in two resistant strain RES-Ac (93-fold) and RES-Bt (205-fold) strains of *H. armigera*. The dose mortality relationship among LC$_{50}$ values of the homozygous susceptible, resistant and heterozygous individuals indicated that resistance was autosomal and inherited as a semi-dominant trait. The estimate of dominance (D$_{LC}$) for reciprocal crosses was expressed at 0.42/0.43 for RES-Ac and 0.57/0.54 for the RES-Bt strains. The values obtained for the minimum number of independently segregating genes were 0.53 for the RES-Ac and 1.1 for the RES-Bt strains, indicating a single gene governed resistance.

Wu et al. (2009) reported dominant resistance to activated Cry1Ac in BKBT strain of *H. armigera* derived through 30 generations of selection with activated Bt δ-endotoxin Cry1Ac.

Alvi et al. (2012) found that selection of the field collected population of *H.*
armigera with Cry1Ac in 2010 for five generations increased resistance ratio to 5440-fold. The LC\textsubscript{50} of Cry1Ac for Cry1Ac-SEL population was over 3000-fold which was significantly higher than LC\textsubscript{50} of Cry1Ac for Lab-PK. The degree of dominance ($D_{LC}$) for the reciprocal crosses was 0.60 and 0.57 indicating incomplete dominance of resistance to Cry1Ac in Cry1Ac-SEL population. Resistance to Cry1Ac was found to be due to a single factor and was not sex linked.

Jin et al. (2013) isolated two Cry1Ac resistant strains, AY2 from Anyang and QX7 from Qiuxian showing 1200-fold and 460-fold. Both strains, showed dominant resistance to a diagnostic concentration of Cry1Ac in diet (dominance value, $h$ was 0.85 for AY2 and 0.83 for QX7). In both the strains dominance decreased as concentration increased, with the lowest dominance value ($h$) seen at the highest concentration tested against each strain (0.51 at 32 µg Cry1Ac/ cm\textsuperscript{2} diet for AY2 and 0.62 at 16 µg Cry1Ac/ cm\textsuperscript{2} diet for QX7). Similarly, in both the strains AY2 ($h$, 0.94) and QX7 ($h$, 0.97) inheritance of resistance to Bt cotton leaves was also found to be dominant.

2.5 Mechanism of Bt resistance in Helicoverpa spp.

Comparative physiological and biochemical studies of susceptible and resistant insect species provide insights into some of the biological changes that might confer Bt toxin resistance to a susceptible species (Van Rie et al., 1990a; McGaughey and Whalon, 1992; Tabashnik, 1994; Gould et al., 1995). The mode of action of Cry toxins involves a complex multistep process. These include the solubilization of the crystal to release Cry proteins in their protoxin form, activation of protoxin form to their active form by midgut proteases, binding of a protein to midgut receptor and pore formation (Whalon and Wingerd, 2003) (Fig. 2). Resistance to Cry toxin is attained through alteration in one or more steps of the process.

Midgut protease has an important role in determining the toxicity of Bt; the inactive protoxin gets cleaved and is converted into active toxin in alkaline pH of the insect midgut, due to action of midgut protease enzymes (Whalon and Wingerd, 2003). In Plodia interpunctella and H. virescens, resistance to Cry1A was shown to be caused due to defects in midgut protease activities that affected the activation of
Cry1A protoxins (Forcada et al., 1996; Oppert et al., 1997). Forcada et al. (1996, 1999) found specific proteolytic bands in the gut extracts of the resistant strain of *H. virescens* which were absent from the susceptible strain, thereby demonstrating a relation between resistance and the composition of the insect midgut proteolytic extracts. Further, in 1996 they investigated the change in midgut proteolytic activity of resistant *H. virescens* and reported that *in-vitro* processing of Cry1Ab toxin was different for resistant and susceptible strains. The protoxin was found to degrade more slowly and active toxin was degraded more quickly in the resistant strain compared to the susceptible strain of *H. virescens*.

![Mode of action of Bacillus thuringiensis](Photo_Courtesy_Dr_Vinay_Kalia)

Fig. 2. Mode of action of *Bacillus thuringiensis*.

Johnston et al. (1991) purified the serine protease from alimentary tract of larvae of *H. armigera* and using ion-exchange chromatography they showed that
protease activity was largely associated with a 24 kDa polypeptide. High level of carboxypeptidal activity has been reported in midgut extracts of *H. armigera* with MW of 46.6 kDa (Bown et al., 1998).

Meenakshisundaram and Gujar (1998a) purified and characterized alkaline proteases from midguts of *Achaea janata, H. armigera, Plutella xylostella, Spilosoma obliqua* and *S. litura*. The molecular weight of purified protease was found to be 30 kDa and 29 kDa for *H. armigera* and *A. janata* respectively, whereas *P. xylostella* and *S. obliqua* each showed two major bands of 56, 21 and 40, 29 kDa respectively. Further, Meenakshisundaram and Gujar (1998b) studied the proteolytic digestion of Bt var. *kurstaki* δ-endotoxin with purified midgut protease and reported that δ-endotoxin gets cleaved into 11 fragments in *A. janata*, 15 fragments in *H. armigera*, 18 in *P. xylostella*, 12 in *S. obliqua* and 4 in *S. litura*. The common fragments of 60.5 and 15.25 kDa were observed in the proteolytic processing of δ-endotoxin by midgut protease of all insects.

Shao et al. (1998) reported strong casein hydrolyzing activity from midgut juice of *H. armigera* and found that proteases were of trypsin, chymotrypsin and elastase in nature. They also observed an increase in the mortality of larvae to 70 per cent on addition of serine protease inhibitors with protoxin. Similarly, MacIntosh et al. (1990) also reported the enhancement of activity of Bt against *H. zea* and *H. virescens* due to addition of serine protease inhibitors.

Karim et al. (1999) reported that Bt toxins Cry1Aa, Cry1Ab, Cry1Ac and Cry2A are stable to *in-vitro* processing by midgut juice of *H. armigera*. Chandrashekar and Gujar (2004) showed that proteases of resistant *H. armigera* insects degraded Cry1Ac faster than those of susceptible insects leading to the unavailability of about 58 kDa for binding and perforation of midgut epithelial membrane of target insects.

Karumbaiah et al. (2007) found that the gut extracts of the resistant strains of *H. virescens*, YHD2-B and CXC processed Cry1Ac and Cry2Aa protoxin slower than extracts from susceptible YDK larvae. Further, the casein zymogram analysis of gut extracts showed qualitative and quantitative differences in caseinolytic activities.
among all strains, but the overall caseinolytic activity of YHD2-B gut extract was lower. They proposed that the alterations in midgut serine protease activities contribute to resistance in strains of *H. virescens*.

Xu and Wu (2008) found multiple Cry1Ac-binding proteins in the midgut of susceptible *H. armigera*, but found only the Ha_BtR as a putative functional receptor of Cry1Ac. They showed that the binding pattern of Cry1Ac to midgut BBMVs of *H. armigera* was altered quantitatively and qualitatively by knockout of Ha_BtR. They did not exclude the possible involvement of other receptor proteins in the intoxication process *in-vivo*.

The best characterized mechanism of resistance is the alteration of binding of Cry proteins to their midgut receptors (Whalon and Wingerd, 2003). The major candidates for targets of Cry1Ac are alkaline phosphatase, aminopeptidase N (APN) and cadherins, all of which bind Cry1A toxins in Lepidoptera. Alkaline phosphatase (HvALP) was found as a potential receptor and tested for its utility as a marker for resistance to Cry1Ac in *H. virescens* (Jurat-Fuentes and Adang, 2004, 2006). Of the different aminopeptidase N that have been reported to bind to Cry toxins, only two have shown to mediate toxin susceptibility (Gill and Ellar, 2002; Pigott and Ellar, 2007). A 170 kDa aminopeptidase N purified fraction was considered functional receptor of *H. virescens* for Cry1Aa, Cry1Ab, Cry1Ac toxins (Luo *et al.*, 1997). Similarly four aminopeptidase N (APN) isoforms, TnAPN1, TnAPN2, TnAPN3 and TnAPN4, were identified in *Trichoplusia ni* (Wang *et al.*, 2005).

Ning *et al.* (2010) characterized a Cry1Ac toxin binding alkaline phosphatase and their role as receptors in *H. armigera*. Jurat-Fuentes *et al.* (2011) through proteomic and genomic detection found reduced levels of midgut membrane-bound alkaline phosphatase (mALP) as a common feature in strains of Cry-resistant *H. virescens*, *H. armigera* and *S. frugiperda* as compared to susceptible larvae.

Caccia *et al.* (2012) suggested binding of Cry1Ac toxin to ALP receptor in the larval midgut lumen of resistant larvae as one of the mechanism of resistance to Cry1A toxins in *H. zea*.
Among the Cry-toxin binding molecules cadherin, a cell adhesion protein was first reported as binding to Cry1A toxins in *Manduca sexta* (Vadlamudi et al., 1993, 1995; Hua et al., 2004), *B. mori* (Nagamatsu et al., 1998 a,b), *H. virescens* (Gahan et al., 2001), and *P. gossypiella* (Morin et al., 2003). A cadherin-like protein present in the midgut epithelial cells of Lepidoptera is associated with insect resistance to Bt Cry toxins. Cadherins are a superfamily of transmembrane glycoproteins that are responsible for maintaining the integrity of selective cell-cell recognition and adhesion properties (Wang et al., 2005).

Bel and Escriche (2006) described the genes that encode the cadherin-like proteins in *Ostrinia nubilalis*, *H. armigera*, and *B. mori*, and showed that genes were found to encompass 19.6 kb, 20.0 kb and 41.8 kb of genomic DNA, respectively, and were all composed of 35 exons that are linked by 34 introns.

In three lepidopteran species, *H. virescens* (Gahan et al., 2001), *P. gossypiella* (Morin et al., 2003), and *H. armigera* (Xu et al., 2005; Yang et al., 2006), resistance is caused by mutations in a toxin-binding 12-cadherin-domain protein expressed in the larval midgut. The BtR4 cDNA of *H. virescens* (Gahan et al., 2001) has been reported to encode a predicted 1732- amino acid and pro-protein (HevCaLP) with a 22 amino acid trans-endoplasmic reticulum signal peptide, 11 predicted cadherin repeats, a hydrophobic transmembrane domain and a cytoplasmic domain at the C-terminus. These mutations in all the three species interrupt the primary sequence of the protein and prevent its normal localization in the membrane, presumably removing a major toxic binding target of the Cry1A toxins.

Heckel et al. (1997) identified a linkage group with a major effect on resistance Cry1Ac endotoxin in the tobacco budworm. Gahan et al. (2001) showed that a mutated cadherin allele was responsible for 40–80 per cent of Cry1Ac resistance levels in *H. virescens* strain YHD2. Resistance was due to retrotransposon mediated insertion in HevCaLP, a *H. virescens* homologue of the BtR175 cadherin receptor. However, additional mutations linked to resistance in this strain affected the production of a glycosyl-phosphatidyl-inositol (GPI) anchored alkaline phosphatase (Jurat-Fuentes and Adang 2004, 2006). *S. exigua* strain that is resistant to Cry1C was
shown to lack the mRNA transcript encoding a GPI-anchored aminopeptidase N1 (Herrero et al., 2005).

In the Cry 1Ac resistant pink bollworm strains (AZP-R), resistance was attributed to three different alleles \( (r_1, r_2, r_3) \) with deleted or truncated cadherin receptors (Morin et al., 2003; Tabashnik et al., 2004).

In a laboratory-selected strain (GYBT) of \( H. \) armigera, a deletion mutation of the cadherin gene \( Ha_BtR \) was genetically-linked with high levels of Cry1Ac resistance (Xu et al., 2005). Introgression of this \( Ha_BtR \) deletion allele \( (r_1) \) into the susceptible SCD strain was found to enable the SCD strain to obtain 438-fold resistance to Cry1Ac (Yang et al., 2009).

Yang et al. (2006) reported a deletion from exon 8 to exon 25 to be responsible for truncated protein in Cry1Ac resistant GYBt insect strain. Further, in 2007 they identified two new cadherin alleles created by insertion of long repeat of transposons at same position in exon 8. All three alleles were found to be associated with a mutation in exon 8 of \( Ha_BtR \), which they regarded as a hot spot for mutation. Another allele from a Chinese population has been reported to have a large deletion involving several exons from genomic DNA sequences (GenBank Accession no. AY714875; cf. Gahan et al., 2007).

Wu et al. (2009) used a backcross approach to map dominant resistance to Cry1Ac in BKBT strain of \( H. \) armigera. They used one hundred and forty-seven informative amplified fragment length polymorphism (AFLP) DNA markers covering all 31 linkage groups of \( H. \) armigera. They found that five AFLP markers linked to Cry1Ac resistance in the BKBT strain were on the same autosomal linkage group and reported this as the only linkage group contributing to dominant Cry1Ac resistance in the BKBT strain.

Zhao et al. (2010) identified five new resistance alleles of \( Ha_BtR \) and showed that mutational diversity of \( Ha_BtR \) could impair DNA screening for Cry1Ac resistance allele frequency in the field.

Zhao (2010) studied the effects of RNAi-mediated silencing of an APN gene \( Haapn1 \) and a cadherin gene \( Ha_BtR \) on Cry1Ac toxicity of \( H. \) armigera and
confirmed that both Haapn1 and Ha_BtR are functional receptors of Cry1Ac in *H. armigera*, and both of them are involved in intoxication of Cry1Ac.

*Zhang et al.* (2012) showed that in Northern China most common resistance alleles in field populations of *H. armigera* had recessive cadherin mutations. They also found that in one of the field-selected cadherin resistance allele conferred nonrecessive resistance and detected nonrecessive resistance that was not genetically linked with the cadherin locus.

Resistance to Bt Cry toxins have also been reported to be associated with elevated esterase levels. *In-vitro* studies demonstrated that resistant strain esterase could bind to Cry1Ac protoxin and activated toxin. *In-vivo* studies showed that Cry1Ac-resistant larvae fed on Cry1Ac transgenic cotton or Cry1Ac-treated artificial diet had lower esterase activity than non-Cry1Ac-fed larvae, thereby proposing a resistance mechanism in which esterase sequesters Cry1Ac (*Gunning et al.*, 2005).

ABC transporters are likely to play a role in detoxification and defense response for xenobiotic resistance in insects (*Labbe et al.*, 2011). *Gahan et al.* (2010) found an inactivating mutation of the ABC transporter ABCC2 that is genetically linked to Cry1Ac resistance and is correlated with loss of Cry1Ac binding to membrane vesicles in *H. virescens*.

**2.6 Effect of Bt toxin on fitness of Helicoverpa spp.**

The evolution of resistance is influenced by several factors *viz.*, the inheritance of resistance, initial frequency of resistance alleles, life-history traits, behavior, multitrophic interactions, pest management practices, population dynamics, and fitness costs (*Bottrell et al.*, 1998; *Gould*, 1998; *Oppert et al.*, 2000; *Tabashnik et al.*, 2008). While, fitness is defined as the ability of an individual of a certain genotype to survive and reproduce relative to other individuals of the same species; fitness cost is defined a trade-off in which alleles conferring higher fitness in one environment (*e.g.*, presence of Bt) reduce fitness in an alternative environment (*e.g.*, absence of Bt)(*Gassmann et al.*, 2009).

Comparative studies on fitness traits *viz.*, survival, development time, size,
mass etc. between conspecific Bt resistant and susceptible are reported in heliothines (Akhurst et al., 2003; Bird and Akhurst, 2007; Jackson et al., 2007). Feeding on treated diet plants resulted in increased larval duration and decrease in pupal weights when compared to the control in *H. virescens* (Dulmidge et al., 1978). Bell and Romine (1986) reported that sublethal concentrations of Bt affected larval weight of *H. virescens* and *H. zea*. Herbert and Harper (1987) found that the fourth instar larvae of *H. zea* intoxicated with β-endotoxin consumed less amount of untreated groundnut leaves compared to control insects. Meyers et al. (1997) noticed that mean weight of *H. zea* larvae feeding on Bt transgenic cotton has half of the mean weight of larvae feeding on non-transgenic cotton.

When resistant insects grow on the non-Bt refuge crops, a physiological trade-off that occurs between resistance development and the other biological attributes helps in delaying the evolution of resistance under field conditions (Lenormand et al., 1998). Occurrence of fitness costs in response to development of Bt resistance has been reported in several insect species under laboratory conditions on artificial diet (Gassmann et al., 2009) (Table 1).

Bird and Akhurst (2004) found that the fitness cost of resistance in *H. armigera* occurred on transgenic cotton, but was not evident on artificial diet. Further, Bird and Akhurst (2005) also reported a significantly faster development of resistant strain ISOC4 on Bt cotton compared with their resistant counterparts on non-Bt cotton.

Fitness costs have been reported in *H. zea* (Anilkumar et al., 2008a) and *S. littoralis* (Müller-Cohn et al., 1996). Anilkumar et al. (2008a) observed that the resistant strains produced higher percentage of normal adults when exposed to toxin in selection experiments than when reared on untreated diet.

Liang et al. (2008) found that the fitness costs associated with the resistance on artificial diet and non-Bt cotton, and the relative fitness in resistant *H. armigera* reduced as the resistance levels increased. They observed that the relative fitness of *H. armigera*, measured as a ratio of $R_0$ (the net replacement rate) of resistant strain divided by $R_0$ of the susceptible strain, decreased with an
increase of the resistance levels, with ratios of 0.79, 0.64 and 0.59 in their respective BtR-F16, BtR-F34 and BtR-F87 strains. The differences between resistant and susceptible *H. armigera* on artificial diets were observed in terms of fecundity, larval development and pupal weight. They observed reduced fecundity, delayed larval development and decreased pupal weight in the resistant strains.

Table 1: Review on studies on fitness costs of Bt Cry toxin in resistant lepidopterans (Gassmann et al., 2009).

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Insect</th>
<th>Biological Traits Studied</th>
<th>Observations</th>
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</table>
| 1     | *P. gossypiella* | • Survival  
• Development Time  
• Adult Fertility | • Reduced survival on non-Bt cotton by 51.3 per cent in two resistant strains relative to susceptible strain.  
• No difference in development time.  
• Negative effects on embryogenesis, adult fertility, reduced ability of neonates to enter cotton bolls.  
• Fitness costs recessive in related strains and dominant and unrelated strains. |
| 2     | *H. zea* | Pupation | Increased pupal mortality on Bt corn. |
| 3     | *H. zea* | Pupation | Increased pupal mortality on Bt cotton compared to non-Bt cotton. |
| 4     | *H. armigera* | • Survival  
• Dominance of fitness cost | • Survival to pupation of resistant strain on Bt cotton was 54-51 per cent lower than on non-Bt cotton.  
• Reduction in survival of post diapausal adult from resistant and F<sub>1</sub> indicating non recessive overwintering cost.  
• Fitness cost was recessive. |
| 5     | *H. zea* | • Pupation  
• Mating ability  
• Sex ratio | • Increased pupal mortality, male biased sex ratio and decreased mating ability of moths compared with susceptible strain.  
• Resistant strain produced higher per cent normal adults when exposed to toxin than reared on untreated diet |

Sedaratian *et al.* (2013) studied the sublethal effects of Bt var. *kurstaki* on the biology and development of *H. armigera* under laboratory conditions and found that duration of the different life stages in treated neonates was significantly affected by
sublethal treatments. Fecundity was also negatively affected in female moths developed from Bt var. *kurstaki* treated neonates, with the rate of egg hatchability reaching zero in the LC$_{25}$. Further, sublethal Bt var. *kurstaki* concentrations were found to reduce the net reproduction rate ($R_0$); significantly lower the intrinsic and finite rates of increase ($r_m$ and $\lambda$, respectively) compared with control insects, consequently increasing the mean generation time (T) and doubling time (DT).

### 2.7 Population dynamics of *H. armigera*

*H. armigera* adults are highly mobile, with movement occurring on several spatial scales: between fields, between areas within a region, and between regions (Farrow and Daly, 1987). These extensive movements contribute to the complexity of *H. armigera* population dynamics from diverse origins (Fitt *et al.*, 1995). Hence, there is need to understand the population dynamics at both the regional level and local scales in understanding the evolutionary mechanism of insecticide resistance. Measures of gene flow are an important component in understanding the genetic structuring within a species and can be obtained directly by measuring dispersal and breeding contribution, or inferred by measuring the allele frequencies (Slatkin, 1987; Zhou *et al.*, 2000).

Molecular approaches used to measure the gene flow in *H. armigera* in Australia include isozyme studies (Daly and Gregg, 1985); mitochondrial DNA variation (Mckechnie *et al.* 1993) and a sodium channel gene (Stokes *et al.*, 1997). Zhou *et al.* (2000) studied the population structure of *H. armigera* in Eastern Mediterranean using RAPD analysis. They reported low genetic distance and high gene flow resulting in high similarity among the Israeli and Turkish moths of *H. armigera*.

Han and Caprio (2004) studied the spatial and temporal variation in the genetic structure of local populations of *H. virescens* using 11 polymorphic allozyme and 36 RAPD markers. They found that genetic variance increased progressively through the season, peaking in the second generation and decreasing in the latter part of the summer. They also found that these changes were related with changes in the gene flow rates and influenced by phonological changes in the primary host plants.
Fakrudin et al. (2004) using RAPD markers studied the genetic structure of *H. armigera* populations occurring in cotton ecosystems in South India. They found polymorphism between samples from geographical locations and within the location. They found two major groups each comprising six populations and concluded that factors such as wind, monsoon dynamics, topological barriers and cropping patterns could play a role in isolation and intermixing of populations.

Gao et al. (2009) evaluated the impact of Bt cotton planting on seasonal population patterns of *H. armigera* and found a negative correlation between moth densities and planting years of Bt cotton in high and low Bt density areas.

Microsatellite technology has been recently used for estimating *H. armigera* population dynamics and migration over several years (2001-2004) in seven major agricultural growing regions of eastern Australia (Scott et al., 2003, 2005a,b); in Murrumbidgee Valley in southern Australia (Scott et al., 2006) and in the Victorian populations from south eastern Australia (Endersby et al., 2007). Microsatellite markers for *H. armigera* have been developed by Tan et al. (2001); Ji et al. (2003, 2005) and Scott et al. (2004). All these studies on population structure helps in understanding the evolutionary mechanism of resistance. Scott et al. (2005b) in cropping region of Australia identified genetic structuring, migration events and significant population genotype changes over a 38- month sample period from 1999-2003 using microsatellite markers. In contrast Endersby et al. (2007) did not find genetic structure among samples from different locations or time in Australia. They found all samples to deviate from Hardy-Weinberg equilibrium suggesting high frequency of null alleles.

However, several technical limitations of application of microsatellites markers have been identified. These include the high frequency of null alleles, occurrence of size homoplasy, presence of multiple copies of flanking sequence in the genome and the lack of PCR amplification robustness between populations. In view of this most recently exon-primed intron-crossing (EPIC) nuclear DNA markers have been developed (Tay et al., 2008). Polymorphic EPIC markers based on single copy
nuclear genes are robust both for use as markers in evolutionary and population genetic studies (He and Haymer, 1997; Garrick and Sunnucks, 2006) and for gene mapping purposes (Lee, 2006; Yasukochi et al., 2006).

Behere et al. (2013) studied the population genetic structure of *H. armigera* using five EPIC-PCR markers. The genetic analysis of the 26 populations showed significant genetic differentiation within India especially in cotton-feeding populations in the 2006–2007 cropping season. In contrast, the overall pair-wise $F_{ST}$ estimates from populations feeding on food crops indicated no significant population substructure irrespective of cropping seasons.

### 2.8 Resistance management strategies for sustainability of Bt transgenic plants

Bt transgenic plants are vulnerable to adaptation of the pests. Hence resistance management becomes inevitable for sustainability of such transgenic plants. Several resistance management strategies have been proposed (Tabashnik, 1994; Gould, 1998) which can delay the adaptation of insects to transgenic insecticidal cultivars.

Strategies for resistance management involve:

1. Refugia approach
2. Ultra high dose approach
3. Low dose of toxins in concert with natural enemies
4. Tissue/time dependent expression of toxins
5. Combination of toxin/stacking of genes

#### 2.8.1 Refugia approach

This involves growing of transgenic insecticidal cultivars in special mixture of transgenic and non-transgenic plants. Providing of untreated refugia is known to reduce adaptation of insects to transgenic plants (Gould et al., 1991a). When only transgenic plants are grown, the survival of resistant larvae will be much higher than that of susceptible ones. In refugia management, the delay of evolution of resistance was achieved by providing susceptible insects for mating with resistant insects. This
will reduce the degree to which resistant insects can pass on phenotypic trait to its offsprings. The refuge strategy is expected to work best with recessive inheritance of resistance and random mating between susceptible insects emerging from refuges and resistant insects from Bt crops (Gould, 1998).

The refugia crops are likely to sustain heavy damage. A refugia kept completely free from pesticides must be 20 per cent of transgenic plot. If insecticides have to be sprayed on refugia it should be 40 per cent of transgenic plot. Refuge strategy differs in various countries even for the same crop. For cotton producing only Cry1Ac, the minimum percentage of non-Bt cotton required on each farm in Australia was 70 per cent from 1996 to 2003 (Downes et al., 2012) versus 4 per cent in the United States (US EPA, 1998, 2001). While, for two-toxin cotton, Australia requires 10 per cent non-Bt cotton or the equivalent in terms of other non-Bt crop host plants on each farm (Cotton, CRC, 2009), whereas the United States has eliminated refuge requirements in most regions (US EPA, 2007).

In China, where resistance appears to be closer to recessive trait than it is in India (Liang et al., 2000, 2008) the smaller field size (mean < 0.5ha) and the presence of alternative host crops during the cotton season is a source of susceptible \textit{H. armigera}. Therefore, structured non-Bt cotton refuges are not recommended for Chinese cotton growers (Wu and Guo, 2005).

In India it is recommended by the Government of India to have five border rows or 20 per cent of the cotton area (whichever is the greater) as non-Bt to serve as a refuge for resistance management (http://www.envfor.nic.in/divisions/csurv/geac/geac_home.html).

2.8.2 Ultra-high dose approach

This strategy involves, using ‘high-dose’ Bt plants that can kill 95 per cent of the heterozygotes for Bt resistance thereby, preventing heterozygous insects on Bt plants from transmitting the resistance alleles to the next generation. Secondly, it requires the planting of specified proportion of non-Bt variety of the crop to serve as a refuge for hosting susceptible insects. Bt-susceptible insects emerging from refuge
areas should mate with the rare potentially resistant homozygous individuals that might emerge from the Bt crop. If the frequency of resistance is low enough, typically ca. 0.001, most offspring will be heterozygous and thus be killed by the high-dose Bt plants (Huang et al., 2011).

Mathematical modeling has shown that the ‘high-dose/ refuge’ IRM strategy should delay resistance development in target pest populations (Alstad and Andow, 1995; Onstad and Gould, 1998; US EPA, 2001; Caprio et al., 2004; Tyutyunov et al., 2008).

2.8.3 Low-dose of toxins that can act in concert with natural enemies

A partially resistant crop that causes low pest mortality but alters the behavior and slows down the growth of immature stages can enhance the efficacy of natural enemies (Bottrell et al., 1998). Outcome of such strategy depends on the details of ecological interactions between the pest and its natural enemies. The model examining low dose approach found that the interactions between the pest and natural enemies would either increase or decrease or have no effect on the rate of pest adaptation (Gould et al., 1991b). Hence, effect of such strategy on the rate of pest adaptation must be examined more.

2.8.4 Tissue/ time dependent expression of toxins

Reduction in yield due to pests depends upon pest population density as well as when and where on the plant insect feeds. Expression of toxin coding genes could be limited to vulnerable parts (Gould, 1988). If pest causes no damage when it feeds on non-economic parts, the production of the toxin can be limited only to economic and vulnerable parts. Such strategy can be more advantageous when vulnerable/ economic tissue is present only once in a growing season viz., cotton bolls. The use of selective promoters was recognized as potentially effective to reduce selection for resistance to Bt crops (Tabashnik et al., 1994) but it limits owing to the available technology.

2.8.5 Combination of toxin/ stacking of toxin

This technique involves inserting genes for production of two or more