THE USAGE OF KARATE (Λ-CYHALOTHRIN) OVERSPRAYS IN COMBINATION WITH REFUGIA, AS A VIABLE AND SUSTAINABLE RESISTANCE MANAGEMENT STRATEGY FOR *B.T.* COTTON

J. G. Harris
Zeneca Agrochemicals, Insect Control Sector
Fernhurst, Haslemere, Surrey, U.K.
C. N. Hershey and M. J. Watkins
Zeneca Agrochemicals, Entomology Section
Jealott's Hill Research Station
Bracknell, Berkshire, U.K.

Abstract

In field trials since 1995, overspray programmes of Karate (λ-cyhalothrin) on *B.t.* cotton have demonstrated excellent control of secondary pests and lepidopteran pests such as bollworm (*Helicoverpa zea*) that are capable of survival on the transgenic plants. Laboratory studies reported here clearly demonstrate that insects such as *Heliothis virescens*, *Helicoverpa zea*, *Trichoplusia ni*, and *Spodoptera exigua* are many times more sensitive to a subsequent Karate spray treatment when they have survived prior exposure to *B.t.* As well as the inherent yield benefits from this Karate overspray approach, the enhanced insecticidal activity enables a more practical resistance management strategy for *B.t.* cotton to be envisaged. Karate oversprays, in conjunction with proper refugia, will further reduce the likelihood of *B.t.* survivors and the development of resistance. This approach is probably the best practical resistance management strategy for *B.t.* cotton.

Introduction

Transgenic cotton expressing a truncated version of the Cry1Ac gene has now been commercially available in the U.S. since 1996. The technology has been broadly accepted as providing excellent control of the tobacco budworm, *Heliothis virescens*, although control of other lepidopteran pests has been somewhat less than had initially been suggested.

Concerns about the potential of resistance development to *B.t.* cotton were being voiced early in the development process. The *B.t.* toxin produced by the plants is driven by a constitutive promoter meaning that in theory the toxin is continuously produced at high levels for the duration of the growing season. This confers a huge and uninterrupted selection pressure on the insects, unlike a traditional spray treatment where selection pressure is maintained for a few days only whilst the material dissipates. Therefore, it was clear from the start that implementing a sound resistance management plan was critical to maintaining the effectiveness of the technology (Layton, 1996).

The foundation of this resistance management strategy was the adoption of refugia, in other words adjacent areas of non-transgenic cotton which would produce high numbers of *B.t.*-susceptible insects that might dilute out any potential resistance by cross-breeding with the small number of survivors from the *B.t.* cotton. The practicality of this approach has yet to be comprehensively proven however (Harris 1997). Most of these refugia strategies are based on computer simulation models ... which are of course only as good as the parameters written into the models. Furthermore, it is far from certain how refugia requirements can be effectively enforced in practice. Most farmers operate under intense financial constraints and consequently make decisions based on immediate financial returns, not future problems. It seems very possible that unless oversprays of lepidopteran-effective insecticides with an alternative mode of action are employed, field resistance could be a real possibility within a short timescale. Indeed, some workers believe that resistance to transgenics is inevitable, it is just a question of how fast it occurs (Baum, 1996).

Field studies conducted by Zeneca and collaborators since 1995 have confirmed that specially-devised Karate (λ-cyhalothrin) overspray programmes on *B.t.* cotton give excellent control of secondary pests, including large bollworm infestations (*Helicoverpa zea*) that have been able to survive and grow through *B.t.* cotton, with consistently high yield benefits as a result. For example trials in North Carolina in 1995 & 1996 demonstrated between 337 and 525 lbs seed cotton / acre increase in yield with *B.t.* cotton oversprayed with a Karate programme compared with no Karate sprays (Mahaffey et al, 1995; Lambert et al, 1996) Likewise, in Mississippi in 1995, a Karate overspray programme on NuCOTN 33 produced an additional yield of 186 lbs lint / acre.

Apart from these obvious and clear yield benefits from the Karate overspray programme, we also observed that bollworm (*Helicoverpa zea*) populations were being hit very hard indeed by the Karate treatments, control being even higher than one would normally expect. This led us to speculate that prior exposure to a non-lethal dose of *B.t.* was weakening these insects and making them more susceptible to a subsequent Karate application. Apart from the excellent insect control achievable, this led to the concept that oversprays, combined with refugia, could provide the best practical resistance management strategy for *B.t.* cotton. By Karate reducing the number of any lepiodopteran survivors from the transgenic crop, this would enhance the effectiveness of the refugia still further as the dilution effect of an inward migration of susceptible individuals would be significantly increased.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:1217-1220 (1998)
National Cotton Council, Memphis TN

Therefore, a programme of work was conducted by Zeneca to evaluate whether prior exposure to sub-lethal rates of *B.t. cry* protein would render a range of key lepidopteran pests more susceptible to subsequent Karate applications and this work is reported herein.

Materials and Methods

Sensitising Larvae on Artificial Diet Containing *B.t.*

Bacillus thuringiensis (Kurstaki), which produces a range of Cry proteins including *Cry 1A(c)* as expressed by Bollgard transgenic cotton, was incorporated into artificial LSU insect diet at a dose sub-lethal for the species in question. (This sub-lethal dose was selected following calibration experiments which determined an LC30 dose for *B.t.* where about 70% of the larvae survived feeding on *B.t.*-incorporated diet for 3 days.) Larvae* were allowed to feed on the diet in 25-well repli-dishes (1 insect in each well). Control larvae were fed on diet treated with water. After 3 days surviving larvae were transferred to individual pots containing a 14mm leaf disc (on agar) cut from detached cotton leaves sprayed with Karate EC using a Potter tower.

The mortality rate of both control and *B.t.*-exposed larvae at each application rate of Karate was then recorded 3 or 4 days later. These Karate response results were analysed using LOGIT analysis to generate LC50 values for both the control and the *B.t.*-exposed larvae for comparison.

*Larvae used in testing :

1st instar *Helicoverpa zea*
1st instar *Heliothis virescens*
1st instar *Trichoplusia ni*
2nd instar *Spodoptera exigua*

Sensitising Larvae on Foliage Sprayed with Javelin.

11mm leaf discs were cut from cotton leaves sprayed with a sub-lethal dose of Javelin via the Potter tower and placed on agar in 25-well repli-dishes (one disc in each well). One larva* was introduced into each well and allowed to feed on the disc present. Control larvae were fed on leaf discs sprayed with water. After three days surviving larvae were transferred to foliage sprayed with Karate EC in the same way as before.

*1st instar *Helicoverpa zea*

Results

LOGIT analysis generated LC50 values for the Control and *B.t.*-exposed insects of 1.00 and 0.47 ppm Karate respectively and the 95% confidence intervals of the two values overlap by 0.07 only which strongly suggests that there is a difference in the susceptibilities of the two groups to Karate (Table 1).

LOGIT analysis of the three day results generated LC50 values for the Control and *B.t.*-exposed insects of 3.24 and 1.28 ppm Karate respectively and the 95% confidence intervals of the 2 values do not overlap at all. (Table 2).

LOGIT analysis generated LC50 values for the Control and *B.t.*-exposed insects of 0.67 and 0.26 Karate ppm respectively and the 95% confidence intervals of the two values do not overlap (Table 3).

LOGIT analysis generated LC50 values for the Control and *B.t.*-exposed insects of 4.74 and 1.72 Karate ppm respectively and the 95% confidence intervals of the two values do not overlap (Table 4).

All the results in Tables 1-5 and accompanying analyses clearly demonstrate that for each species tested the *B.t.*-exposed larvae are significantly more susceptible to Karate applications than the Control larvae.

Discussion

B.t. cotton has enjoyed strong commercial acceptance since its introduction. In 1996, about 1.8 million acres in the U.S. were planted mostly in areas where budworm (*Heliothis virescens*) infestations had traditionally been intense and difficult to control (Ripato, 1996). In 1997, some further modest growth has been achieved, with the area planted estimated at 1.8 - 2.0 million acres.

Not surprisingly, the usage of *B.t.* foliar sprays on cotton has dropped away dramatically. *B.t.* when expressed in a plant has a number of advantages compared to foliar application. Firstly, it is present in the 'activated' form in the plant whereas in foliar products the crystal has to dissolve in the gut of the insect to release the active peptides thus taking somewhat longer for feeding inhibition to be initiated. Secondly, neonates are more susceptible to *B.t.* than older instars and with a transgenic crop the insects are exposed to the *B.t.* from their first mouthful catching them at their most vulnerable stage. Thirdly, other than when the plant is maturing or under environmental stress, transgenic plants produce *B.t.* throughout most of the season and therefore there are no issues around correct application timing or U.V. degradation. All these features suggest that *B.t.* transgenic crops will tend to supplant foliar usage of *B.t.* in a number of their key markets, such as cotton, tomatoes, and potatoes.

Many such crops are currently being commercialised, and estimated dates of introduction are shown below;

Transgenics for Insect Control

Technology	Introduction	Company
Bt cotton	1996	Monsanto
Bt potatoes	1996	Monsanto
Bt maize	1996	Various
Bt tomatoes	1997	Monsanto
Bt rice	2000	Monsanto
Pyramider Bt cotton	2004 + ?	Monsanto

Commercial success inevitably leads to intensive selection pressure where development of resistance to *B.t. cry* proteins will be a competitive advantage over other insects. Gould et al (1997) conducted experiments to estimate the field frequency of *B.t.* resistant *Heliothis virescens*. They concluded that 1 in 350 individuals carried an allele for *B.t.* resistance, which is considerably higher than previous estimates which have been used to formulate the theoretical models for resistance management by refugia. The concept of a refuge strategy assumes that *B.t.* resistance will be inherited as a recessive or at worst a partially dominant trait (Fitt et al 1994). Although this has been demonstrated to be the mechanism with Diamond Back Moth (*Plutella xylostella*) field resistance to *B.t.* subsp. *kurstaki*, it is dangerous to assume that this will always prove to be the case.

Furthermore, the refugia concept relies on very high and constant expression of the insecticidal protein, sufficient to kill both susceptible and a very high proportion of heterozygous resistant individuals. However, it is becoming increasingly clear that expression levels can vary in different parts of the plant. Forrester & Bird (1996) noted in bioassay experiments that plant material from the extremes of the plant … the tip, bolls and leaves adjacent to the bolls, gave high mortality to fed insects whilst lower mortality was observed from feeding on leaf tissue in the 2nd to 4th node and in the flowers. Greenplate (1997) observed that *cry1Ac* expression levels are appreciably lower in pollen than other parts of the *B.t.* cotton plant, and attributed the high number of surviving bollworms in the U.S. in 1996 to insects feeding on pollen, which subsequently grew large enough to escape higher levels of *cry1Ac* in bolls. Expression levels also tend to decline when plants are experiencing physiological stress or plant material is senescing.

This tends to suggest that potentially rather more heterozygous-resistant insects may be capable of surviving on *B.t.* cotton than originally estimated, by a combination of higher resistance gene frequencies, lower protein expression under adverse conditions, and neonate / early instar insects feeding on lower-expressing plant parts prior to moving onto bolls.

Other than the refugia approach for resistance management, some workers have suggested that more than one insecticidally-active gene can be simultaneously engineered into plants, thereby subjecting insects to multiple modes-of-action.

This concept that genes can be ‘stacked’ or ‘pyramided’ to overcome resistance is perhaps rather simplistic. Recent work by Tabashnik (Anon, 1997) and other workers has identified the potential for cross-resistance between a range of different *B.t. cry* proteins calling into question the feasibility of stacking different *cry* proteins as a viable resistance management strategy. Genetic resistance studies have identified five separate loci or groups of loci as conferring resistance to *Cry1Ac* toxin in *Heliothis virescens* (Heckel et al 1997). In one *cry1Ac* resistant strain (CP73), there was also 50% cross-resistance to *Cry2A*, calling into question the value of developing ‘pyramided’ *B.t.* cotton expressing both *Cry1Ac* and *Cry2A* genes.

Furthermore, there are no published alternatives to *B.t. cry* proteins that can come even close to providing an equivalent level of acute insecticidal activity. Of the key areas of alternative genes being worked on, most are lectins, patatins, or enzyme inhibitors. Lectins have so far proved to be mostly coleopteran-active. Patatins (storage proteins) are also mostly coleopteran active but very high doses are required to generate a useful effect. Enzymes / inhibitors such as Cowpea Trypsin Inhibitor (CpTi) can be broad-spectrum but retard insect development rather than conferring mortality. Also, enzymes may be harder to commercially register as their effects are less insect-specific than *B.t.* Santos and co-workers (1997) recently found that when testing a stacked transgenic plant producing both *cry1Ac* and CpTi against a range of lepidopteran pests, these were less effective than plants expressing *cry1Ac* alone, suggesting some form of antagonism. They concluded that the combined use of these two genes may not be effective.

The greater the number of genes that are engineered into a transgenic plant, the more plant protein will be diverted away from creating useful yield and into manufacturing the foreign substance instead. This runs the risk of significant agronomic and yield penalties which may make the variety unattractive to the grower.

And finally, gene silencing can have a major impact on the potential of transgenics to carry functioning stacked genes. Gene silencing is a process not yet fully understood, but is a plant mechanism probably to protect against invading viruses by recognizing and methylating foreign gene promoters rendering them inoperative. If this occurs during the seed production cycle, the whole seed lot could suddenly lose the gene effect that was desired.

Although many of these hurdles are likely to be overcome in time by various mechanisms, it will be a far from trivial exercise. It simply emphasises the need to protect the existing *B.t.* technology for as long as possible as viable alternatives will be very hard to find.

The work reported here clearly demonstrates that insects which survive exposure to sub-lethal levels of *B.t.* are subsequently much more susceptible to follow-up applications of Karate. This may help to explain the clear observation of exceptional control with Karate of bollworms (*Helicoverpa zea*) in the field on *B.t.* cotton (bearing in mind though that Karate alone has always given excellent control of bollworms). These data also confirm that the same effect should be anticipated when and if budworm (*Heliothis virescens*) becomes less sensitive to *B.t.* cotton. Oversprays of Karate on *B.t.* cotton will tend to reduce the level of surviving lepidopteran pests (whatever their *B.t.* resistance status) down to very low levels indeed, vastly enhancing the dilution effect offered by the influx of susceptible individuals from the refugia. In this scenario, *B.t.* cotton, Karate, and refugia are complementary and all work in concert to offer a sound and sustainable resistance management strategy of practical benefit to cotton growers.

References

Anon (1997) Increased risk of *B.t.* resistance. *Agrow*, No.**276**, March 1997 p. 13.

Baum, J (1996) *B.t.* delta-endotoxins as malleable active ingredients for insect pest control. Presentation to SCI meeting, *Natural products as a source of crop protection agents III*, London, December 1996.

Fitt G P; Mares C L; and Llewellyn D J (1994) Field evaluation and potential ecological impact of transgenic cottons (*Gossypium hirsutum*) in Australia. *Biocontrol Science and Technology* **4**, pp 535-548

Forrester N W and Bird L J (1996) Conventional insecticide and *B.t.* transgenic resistance management in Australian cotton. *Australian Cotton Conference Proceedings*, pp 159-164.

Gould, F et al. (1997) Proc.Natl. Acad. Sci., USA **94** pp 3519-3523

Greenplate J (1997) *Proc. Society of Invertebrate Pathology* Vol. **29**, No. 2 , June 1997.

Harris, J G (1995) The efficacy of different strains of *Bacillus thuringiensis* for diamondback moth control in South East Asia, and their strategic usage to combat resistance to chemical insecticides, especially Acyl Urea compounds. In: *Bacillus thuringiensis Biotechnology and Environmental Benefits*, T-Y Feng et al (eds), Hua Shiang Yuan Publishing Co., Taipei, Taiwan, Vol. **1**, pp. 259-268.

Harris, J G (1997) Microbial insecticides - An industry perspective. 1997 BCPC *Symposium Proceedings No. **68**: Microbial Insecticides: Novelty or Necessity*?

Henkel, D G; Gahan, L G; Gould, F; and Tabashnik, B (1997) Mapping major and minor loci conferring resistance to *B.t.* toxins in Lepidoptera. *Proceedings of the second Pacific rim conference on Bacillus thuringiensis and its impact on the environment.*

Layton, B (1996) Statement in Monsanto's Bollgard Information Package, 1996.

Lambert, A L; Bradley, J R; and Van Duyn, J W (1996) *1996 Beltwide Cotton Conference Proceedngs.*

Mahaffey, J S; Bradley, J R; and Van Duyn, J W (1995) *1995 Beltwide Cotton Conference Proceedings.*

Ripato, M (1996) Personal communication, Zeneca Ag Products, Memphis, USA.

Santos M O; Adang M J; Boerma H R; and Parrott W A (1997) Testing transgenes for insect resistance using Arabidopsis. *Molecular Breeding,* Vol. **3**, 3, pp183-194.

Tabashnik, B E; Finson, N; Johnson, M W; and Moar, W J (1993) Resistance to toxins from *Bacillus thuringiensis* subsp. *kurstaki* in the Diamondback moth (Lepidoptera: Plutellidae). *Appl. Environ. Microbiol.* Vol. **59**, pp. 1332-1335.

Table 1. 1st instar *Helicoverpa zea* larvae sensitised on diet containing *B.t.* at 4ppm. Assessment of mortality made 4 days after treatment with Karate EC.

Application rate Karate (ppm)	% mortality	
	Control insects	*B.t.* exposed insects
2.5	95	90
0.83	35	80
0.28	0	35
0.09	0	10
0.03	0	15
0.01	5	15
0 (water)	0	10
Untreated	5	5

Table 2. 1st instar *Heliothis virescens* larvae sensitised on diet containing *B.t.* at 0.2ppm. Assessment of mortality made 3 and 4 days after treatment with Karate EC.

Application rate Karate (ppm)	% Mortality			
	3 dat.		4 dat.	
	Control insects	*Bt* exposed insects	Control insects	*Bt* exposed insects
5	65	85	70	90
2.5	45	65	45	65
1.25	10	60	20	60
0.63	15	45	20	45
0.31	0	20	0	25
0.16	0	15	5	30
0 (water)	0	10	0	10
untreated	5	5	5	15

Table 3. 1st instar *Trichoplusia ni* larvae sensitised on diet containing *B.t.* at 3ppm. Assessment of mortality made 3 days after treatment with Karate EC.

Application rate Karate (ppm)	% mortality	
	Control insects	*B.t.* exposed insects
2	90	90
1	70	95
0.5	40	75
0.25	5	60
0.125	0	25
0.063	5	10
0 (water)	0	5
Untreated	5	0

Table 4. 2nd instar *Spodoptera exigua* larvae sensitised on diet containing *B.t.* at 10ppm. Assessment of mortality made 3 days after treatment with Karate EC.

Application rate Karate (ppm)	% Mortality	
	Control insects	*B.t.*-exposed insects
20	80	95
10	60	80
5	50	85
2.5	45	55
1.25	20	55
0.63	15	40
0 (water)	0	15
Untreated	0	10

Table 5. 1st instar *Helicoverpa zea* larvae sensitised on cotton leaves sprayed with Javelin at 0.06 BIU/Ha. Assessment of mortality made 4 days after treatment with Karate EC.

Application rate Karate (ppm)	% Mortality	
	Control insects	Javelin insects
2.5	45	85
0.83	50	45
0.28	0	10
0.09	5	20
0.03	0	15
0.01	0	25
0 (water)	0	5
Untreated	5	15