1995

CRDC

FINAL REPORT

Use of Bt for the management of Heliothis in cotton

II. Potential of transgenic plants to induce resistance to Bt in Heliothis

CRDC Project Number NCQ 1C

December 1995

PRINCIPAL INVESTIGATOR

Dr Joanne Daly
CSIRO Division of Entomology
GPO Box 1700
CANBERRA ACT 2601

Use of Bt for the management of Heliothis in cotton

II. Potential of transgenic plants to induce resistance to Bt in Heliothis

Joanne Daly & Karen Olsen

CSIRO Division of Entomology
GPO Box 1700 CANBERRA ACT 2601

Ph: 06 246 4139
Fax: 06 246 4150
Email: joanned@ento.csiro.au

Cotton Research and Development Corporation
Project NCQ 1C

The information, advice and/or procedures contained in this report are provided for the sole purpose of disseminating information relating to scientific and technical matters in accordance with the functions of CSIRO under the Science and Industry Research Act 1949. To the extent permitted by law CSIRO shall not be held liable in relation to any loss or damage incurred by the use and/or reliance upon any information, advice and/or procedures contained in this report.

CONTENTS

Summary	**2**
Iutroduction	**3**
Industry Significance	3
Background	3
Objectives	**4**
Materials and Methods	**4**
Changes to Rearing Procedures	4
Development of a Resistant Strain of H. armigera	4
Development of Bioassay to Measure Relative Changes in Bt in Plants	5
Variation in Toxicity of Leaves	5
Results	**6**
Rearing Procedures	6
Development of a Resistant Strain of *H. armigera*	6
Development of Bioassay to Measure Relative Changes in Bt in Plants	8
Monitoring of Variation in Toxicity of Leaves	9
Discussion	**9**
Conclusion and Recommendations	**10**
References	**10**
Publications 1993-95	**11**
Seminars 1993-95	**12**
Videos	**12**
Appendix	**13**
1. Protocol for testing whole transgenic leaves or other plant tissue	13
2. Protocol for cotton leaf mulch bioassays using transgenic and normal cotton plant tissue	13
3. Procedures to create and screen for a Bt resistant strain of *H. armigera*	13
4. Rearing Procedures for *H. armigera*	15

Summary

Cotton plants expressing the Cry1Ac toxin from Bt are near to commercial release. These plants have been developed to aid in the control of heliothis caterpillars, the primary target of insecticide usage in cotton production. Despite initial hopes, the plants will not give season-long control of heliothis. Late in the season, heliothis grubs can survive and grow on transgenic cotton plants. Thus, in these mature plants either the amount of Bt toxin is lower than it was earlier in the season, or the Bt toxin is partially inactivated by the leaf. The decline in the efficacy of plants late season makes resistance management more difficult than if expression of the toxin was maintained at a high level.

This project examined the potential of transgenic Bt plants to select for resistance to the insect pest *Helicoverpa armigera*. We have focused on gaining an understanding of why the plants are able to control heliothis caterpillars late in the season. It is at this time that selection for resistance may well be at a maximum. At the beginning of our study nothing was known about the cause of the decline in toxin levels, nor were techniques available that would enable us to quantify those levels. Thus, we have had to focus on developing the necessary techniques to study the performance of transgenic plants grown under field conditions.

We have successfully developed a leaf bioassay test that can measure relative changes in toxicity of Bt leaves. We observed a 3-fold decline in toxicity of the plants from young plants, before budding, to mature plants with bolls. This decline is only small so it suggests that the level of Bt toxin in young cotton plants may not be much above the level to kill heliothis.

A number of additional factors were observed to affect the efficacy of the Bt. The potency of Dipel 2X™ was reduced to half when it was fed to larvae in a soy bean based diet, compared with one of chickpea. Some component of older leaves also appears to reduce the availability of Bt toxin to larvae feeding on a mixture. Preliminary experiments also indicate that stressing plants may also affect Bt levels, although further experiments are being carried out to elucidate the precise factors.

In a second phase of the project we have attempted to develop a strain of *H. armigera* resistant to Bt. We have modified and developed a technique that produces mutant *H. armigera*. These mutants have been screened for individuals carrying Bt resistant genes. One candidate resistant strain has been established. Although its level of resistance to Dipel is low, it appears to survive better on transgenic plants compared with controls. We are investigating this strain further.

Introduction

Industry Significance

Transgenic cotton plants containing the Cry1Ac toxin from *Bacillus thuringiensis* (Bt) are due for commercial release in the next two years. These plants should enable the cotton industry to reduce significantly the use of insecticides for control of heliothis caterpillars. However, efficacy of the Bt plants appears to decline from mid-summer. While expression is still high enough to control heliothis, reduced efficacy of the plants increases the chance that resistance to the Bt toxin will evolve in *Helicoverpa armigera.* Such an outbreak of resistance would reduce seriously the benefits of transgenic Bt plants to the cotton industry. However, development of resistance management strategies can proceed only with difficulty until more is known about Bt plants and the factors that affect efficacy under field conditions. This proposal seeks to elucidate these factors.

Background

Transgenic cotton plants containing the Cry1Ac toxin from *Bacillus thuringiensis* (Bt) are due for commercial release in the next two years. However, the Bt cultivars intended for release exhibit a seasonal decline in their efficacy to control heliothis (Fitt *et al.,* 1994). The decline is first detected in mid-summer, when the plants first set bolls, coincident with the appearance of significant populations of *H. armigera* in most cotton growing areas. A similar decline in efficacy is now known to occur in American cultivars of cotton (R. Deaton, pers. comm.).

Studies with insecticide resistance had shown that selection for resistance occurs when the concentration of pesticide is in a discriminating dose range between resistant and susceptible individuals (Roush & McKenzie; Roush & Daly, 1990). Unfortunately, it is not possible to know what this concentration is *a priori* because the selecting dose range will differ among resistance mechanisms, and also among life-stages (Marshall & Pree, 1986; Daly, 1993). Insecticides with long residual affects are prone to development of resistance in insects because the population is exposed to a range of insecticide concentrations. Selection may occur when the concentration is high (Daly, 1993; Follet *et al,* 1993) or low (McKenzie & Whitten, 1982) or over a wide range of concentrations (calculation in Daly, 1994 from Mable and Pree, 1993).

It was hoped that transgenic plants expressing the Bt toxins would have a constitutive high expression of the insecticidal toxin. Indeed, the promoter for the construct that was engineered into cotton, the 35s ribosomal promoter, was chosen because it would be expressed in a wide range of living tissue. The first resistance management strategies developed for transgenic plants assumed that such high, continuous expression would be achieved (Roush, 1994). Indeed, levels would be high enough to kill heterozygous resistant individuals season long.

Two recent results indicate the need to moderate our expectations of Bt plants. Firstly, *H. armigera* and *H. punctigera* are about 200 times more tolerant of Bt, applied as an insecticidal spray, than is *Heliothis virescens,* the primary target of Bt cotton in the USA (N.W. Forrester, pers. comm.; Final Report for NCQ 1C, 1992-1995). Thus, while Bt cottons have high expression of the toxin relative to the lethal dose for *Heliothis virescens* (commercial-in-confidence, G.P. Fitt, pers. comm) the level may be more marginal for the Australian heliothis. Secondly, three years of experience with field trials in Australia indicate that levels of toxin in Bt plants can decline to levels low enough in the late season for some individuals to survive to pupation (Fitt *et al.,* 1994; G.P. Fitt, pers. comm.). Thus, resistance management strategies for Australian heliothis species need to be modified to take into account the field efficacy of the plants.

Little is known about the underlying causes of the decline in efficacy of Bt cottons. Reduced toxicity of the leaves could be due to

- down regulation of the genes controlling expression of the crystal protein genes
- sequestration of the Bt toxin by plant secondary compounds, or
- mobilisation, transportation and metabolism of the Bt toxin protein by the plant.

These effects could also vary among individual plants. However, before any of these processes can be evaluated we need reliable and accurate techniques to quantify the amounts of Bt toxin present in cotton leaves and a means to assess its availability as dietary protein to *H. armigera*.

Detailed studies of resistance are limited also by the absence of strains of *H. armigera* resistant to Bt toxins. Thus, during the period 1993-95 we have given priority to developing techniques that will enable us to generate strains of *H. armigera* resistant to Bt. Field studies by Dr N. Forrester during 1992-1994 indicate that naturally occurring alleles for Bt resistance are not readily detected in field populations. Our approach has been to use ethyl methanesulphonate (EMS) to produce resistant alleles *de novo* using mid-stage male pupae of *H. armigera*. Mutagenesis using EMS has been used in adult sheep blowfly, *Lucilia cuprina* to generate mutations of resistance alleles that are similar to those found in field populations (Smyth *et al.*, 1992). We have applied the procedures developed for the sheep blowfly to *H. armigera*.

Objectives

(i) To establish a Bt resistant colony of Heliothis armigera through

- direct exposure to transgenic plants
- exposure to Bt toxin in food
- mutagenesis.

(ii) To develop resistance management strategies for the use of conventional Bt and transgenic cottons.

This report covers the period March 1993-June 1995, the duration of the current grant. The project focused primarily on developing techniques:

- to establish a resistant colony using mutagenesis, and
- developing bioassay techniques to quantify the efficacy of Bt cotton plants.

Materials and Methods

Changes to Rearing Procedures

Our laboratory cultures of *H. armigera* were prone to disease outbreaks, particularly of viral diseases and the diet was becoming contaminated with mould or bacteria. As a result, between March 1993 and December 1993, work was greatly delayed by the continuing unavailability of healthy insects. Thus, we embarked on a series of changes to our rearing that enabled us to produce virus free insects reared on mould free diet.

Traditionally, *H. armigera* is reared in Australia on soybean based diets. Singh and Rembold (1988) reported observed significant differences in the development time and survival rates of *H. armigera* reared on soybean versus chickpea diets. They concluded that trypsin inhibitor in soybeans reduced the suitability of soybean as a diet. We switched during 1994 to chickpea as a diet ingredient after our trials suggested that the colonies reared on it were healthy (see appendix). We also compared the results of bioassays that incorporated Dipel 2x™ into soybean versus chickpea diets.

Development of a Resistant Strain of H. armigera

The technique of EMS mutagenesis was adapted from that used for the sheep blowfly (Smyth *et al.*, 1992). It had not been applied to a moth species before our study. The culture used for mutagenesis was the general rearing strain (GR). This strain was highly fecund and very healthy. It is not necessary to use field collected material for mutagenesis because resistant mutants are produced *de novo*. The procedure is given in more detail in the Appendix.

Mutagenising of Pupae. Male pupae about 3 d after eclosion are placed into a vacuum flask. A dose of 0.20 ml of EMS solution is placed into the flask in a vacuum. This dose gives good pupal survival but reduces egg hatch in the next generation — an indicator that the EMS has been effective.

After emergence, adult males are crossed to untreated females from the same cohort. We chose the pupal life-stage because individuals could be sexed so that the untreated females could be used for matings with treated males; most stages of spermatogenesis were present; younger pupae were readily damaged by EMS; and individuals could be handled easily.

Screening of Progeny. We needed to develop a rapid screening procedure for progeny so that we would increase our likelihood of harvesting resistant alleles. This is necessary because EMS mutagenesis may still only generate resistant mutants at a frequency of 10^{-5} to 10^{-6}. The technique enables 10,000 to 20,000 eggs to be screened for resistance each week. Eggs are dipped in a solution of Dipel 2X (10mg/ml). This dose kills 99.84% of susceptible neonates. The treated eggs are then placed at the bottom of a dark container. Standard heliothis diet is placed at the top. Treated and control eggs are scored after six days. This method of selection minimises cannibalism by larvae and ensures that the survivors are healthy. Survivors that have normal growth are transferred to diet and then reared to adults. Male survivors are crossed to 5 susceptible females. Each female is crossed to 3 susceptible males. Eggs are collected and put aside for rearing. Surplus eggs are tested at three or four doses of Dipel 2X in diet. If insufficient eggs are available then all individuals are reared and the next generation is tested.

Development of Bioassay to Measure Relative Changes in Bt in Plants

We trialed a number of possible assay methods that would allow us to quantify the toxicity of transgenic leaves. The preferred assay needed to fulfil a number of requirements:

a) control insects had to grow as fast as insects on whole non-Bt leaves,
b) a whole dose-response curve should use no more than one leaf (to make the technique useful for monitoring field samples),
c) it had to be sensitive enough to be able to detect changes in concentration of Bt, and
d) it had to be reproducible and produce straight dose-response curves.

Diet Incorporation. We added known quantities of Bt leaves to standard heliothis diet. The leaves were frozen in liquid nitrogen, mashed in a mortar and pestle and then added to diet. Neonate larvae were bioassayed at different concentrations of Bt leaf:diet mix. Mortality of larvae was recorded after six days.

Leaf Mush Test. An alternative technique was developed in which non-Bt and Bt leaves were mixed together in known ratios and fed to larvae. Various modifications of this technique were tried including the addition of polyethylene glycol (to sequester the tannins) and fungicides. In early versions of the test, control larvae had high survival but low growth weights relative to control larvae placed on whole leaves or diet. During the final year of the study, the assay was modified further to ensure that control larvae had similar growth to those placed on whole leaves.

Bt and non-Bt leaves are snap frozen in liquid nitrogen immediately after picking. Crushed leaves are mixed in known proportions. The mush is added to an adhesive dot label, pressed onto the surface of cooled agar (prepared with 0.1% sorbic acid). Neonates are placed onto the mush, and the trays stored at 25°C, for 7 days. Mortality and growth stages of all larvae are recorded.

Variation in Toxicity of Leaves

This work is at a preliminary stage. Further studies are being undertaken in our CRDC project for 1995-98. We have used two techniques to examine factors that may determine the variation in toxicity. Experiment 1 used whole leaf bioassay using plants grown in glasshouses, experiment 2 used both leaf mix bioassay and the whole leaf bioassay.

Experiment 1. Thirty-one plants of CS 50, expressing the Cry 1Ab crystal protein, were grown in a glasshouse at 25-30°C. One leaf was taken from the third node at various times from planting. Not all plants were sampled in each period. The sampling periods were grouped into three periods: 5-7 weeks from planting, 10-11 wk, and 14-19 wk. Neonate larvae of *H. armigera* were placed on whole leaves using the whole leaf protocol of Dr G.P. Fitt.

Experiment 2. We began a series of trials in which plants are being heat and water stressed and then the leaves are assayed for toxicity. Plants of CS 50 Cry 1Ab were placed in growth cabinets 4 wk after planting, when they had 4 nodes (week 0). From week 2-5 the test group were water stresses, by only watering the plants when they wilted. Free water was given to the controls. After two weeks the test and control group were switched and the experiment continued for a further two weeks. Leaves were sampled from the plants at week 2 and week 5. Control plants were also grown in the glasshouse.

Field Trials. We began field experiments to test the feasibility of monitoring efficacy of field grown plants using the leaf mush test. Leaves were collected from a plot of CS50 (Cry1Ab) plants grown opposite the Myall Vale Research Station by Dr G.P. Fitt. The cotton plants were stripped of leaves by a hail storm on 3 January 1995. Forty plants were chosen and each fortnight 20 plants were bioassayed by Dr Fitt using the whole leaf bioassay. Twenty leaf samples from the other plants for leaf mush on 22 December 1994, 2 February and 17 March 1995. Leaves were stored in a domestic freezer. The results will be reported in Progress Report for 1995/96.

Results

Rearing Procedures

We switched during 1994 from the traditional rearing procedures, modified from Teakle & Jensen (1985), that required mass rearing of 1st-4th instar larvae and then individual rearing of larger larvae. Our new technique separates eggs before hatching into cells containing diet. Rearing containers are from Oliver Products, USA. Diet is added to 32 cell rearing trays and allowed to dry overnight under sterile conditions. Three to five eggs are placed into each cell and a sheet of perforated material is heat sealed to the tray. The larvae are then reared in these containers until pupation. Pupae are added to 10 or 20 litre plastic opaque containers and the top sealed using a fine mesh nylon fabric. The tops are replaced Monday to Friday. Eggs are bleached from the fabric onto filter paper. This new procedure has increased greatly the efficiency of rearing. We also have observed a great reduction in the incidence of viral infections and of mould contamination of diet.

We observed the dose-response of neonate larvae tested using Dipel 2X™ incorporated into diet. The LD_{50} was two fold greater when the diet was made with soyflour rather than chickpea flour.

Diet flour	LD_{50} of Dipel 2X™ (mg/ml)	95% CI	Slope
Soybean	0.10	0.08, 0.12	1.6
Chickpea	0.05	0.04, 0.06	

Thus, for larvae we now use a chickpea based diet (see Appendix).

Development of a Resistant Strain of *H. armigera.*

Between March 1994 until June 1995, we mutagenised 50-200 male pupae per week and screened 576,000 eggs from their progeny (Figures 1 & 2). From this, 284,000 neonates hatched. To date, 500 individuals have survived, 157 healthy larvae were set up for rearing and 55 possible candidate survivors have been tested further. Progeny of 23 of these 55 survivors had elevated survival and/or growth rates. Seven of these 23 have been established as lines and are undergoing further selection for Bt.

The most promising candidate resistant strain was analysed in more detail. The differences detected using Bt incorporated into diet were small (see below). The change in LD_{50} was 1.5 fold when neonates were exposed to Dipel® 2X. No difference was observed between strains when MVP® was used instead of Dipel®, perhaps as a result of the low slopes observed when using MVP® in bioassays.

Test	Line	LD50 (mg/ml)	95% CI	Slope
Dipel in Diet - Neonates	Control	0.060	0.051, 0.070	3.64
	Resistant	0.093	0.082, 0.105	
MVP in Diet - Neonates	Control	0.051	0.028, 0.094	0.68
	Resistant	0.065	0.037, 0.117	

Results for work begun after the end of this report period indicate that the strains could be more readily distinguished when larvae were exposed to Bt plants (see 1995/96 Progress Report). This strain is undergoing further selection.

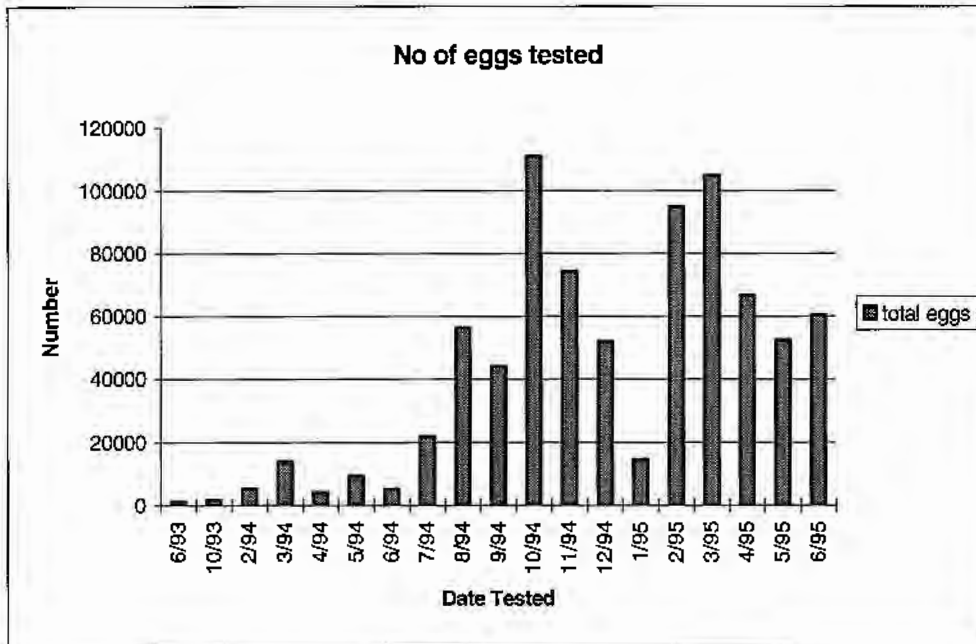

Table : The number of eggs tested with a discriminating dose of Bt after adult males had been exposed to EMS mutagenesis.

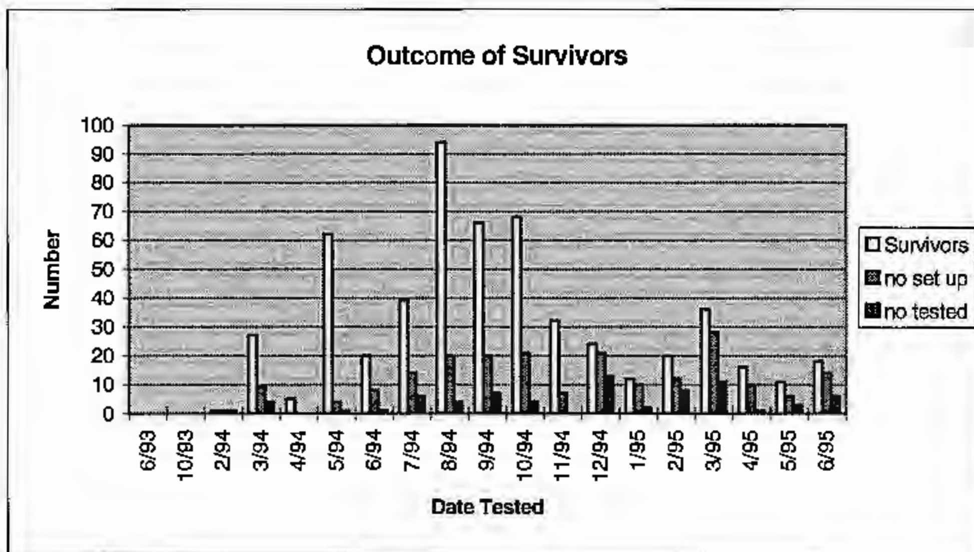

Table. The number of survivors of Bt assays above, and the number that survived to pupate and then subsequently tested for resistance.

Development of Bioassay to Measure Relative Changes in Bt in Plants

Incorporation of leaf material into Diet. Incorporation of transgenic leaves into standard diet produced unsatisfactory results because even at concentrations of 50% leaf: 50% diet, no more than 54% of neonates could be killed. Higher concentrations were not possible because too much leaf material was required to make the assay practical.

Leaf Mush Test. The leaf mush test proved a successful method of measuring relative changes in efficacy of the Bt plants over the season. A number of factors were examined for their influence on the test results, as given below.

(a) Changes with age of leaf. Preliminary analysis of bioassays with plants expressing the Cry 1Ab toxin shows that the method can detect changes in efficacy of Bt plants, although the sensitivity of the assay needs further improvements. The LD_{50} is expressed as the percentage of Bt leaves mixed with non-Bt leaves. The age of leaves is given from the time of planting the seed. Preliminary results using this test were:

Age of leaves	LD_{50}
8 wk	2%
15 wk	5.7%

This represents about a 3-fold increase in the amount of transgenic leaf material needed to kill 50% of neonates. The difference in slope between the groups of plants was not significant. These results suggest that the relative change in the amount of Bt from younger to older plants need not be very great.

However, because these experiments were undertaken using control leaves of the same age as the Bt leaves, they need repeating using only young control leaves (see b).

It is possible that the shift in LD_{50} above reflected a change in the amount of Bt available to the insect. Survival of larvae on whole leaves taken from two of the same experimental plants used above was:

Age of Plant	Plant 1	Plant 2
8 wk	5%	20%
15 wk	65%	50%

(b) Effects of the age of control leaves. Another factor observed to influence the efficacy of the leaf mush test was the age of the control leaves used in the bioassays. Differences in LD_{50}s were examined using L22 cotton variety containing the Cry1Ac toxin that was 7 wk from planting. These leaves were mixed with control leaves, 7 wk or 11 wk from planting. Results were:

Age of Control leaves	LD_{50}	95% CI	Slope
7 wk	0.56	0.34, 0.90	1.9
11 wk	2.24	1.67, 3.00	

Thus, the estimated LD_{50} increased by 2.5 fold when older leaves were used.

(c) Toxin. Preliminary experiments were carried out to compare the efficacy of cotton cultivars carrying different Bt toxins: CS50 (Cry1Ab) and L22 (Cry 1Ac). Only a single dose, 10% concentration of transgenic leaf, was used. Control leaves were all young leaves. There was no major difference in the mortality of neonates tested against each cultivar, although replicates of the experiment showed minor variation. Further tests will be carried out to quantify the differences.

(d) Polyethylene Glycol (PEG). PEG was added to the leaf mush in some trials to see if sequestration of the plant tannins by the PEG could improve the efficacy of the bioassays. One possible reason for the reduced efficacy of older plants is that the tannins sequester or bind the Bt in the cell, making it less biologically available to the insect. However, PEG made little difference to the estimates when present (LD_{50} = 2.19%) vs when absent (2.13%) from the mixed leaves. It is possible that the reaction between the tannins and the Bt in the cells, once the cells are ruptured happens too quickly for the PEG bind the tannins. Alternatively, the tannins may already be bound to the Bt in the cell.

(e) **Moisture content of the mush.** In the early stages of development of the test the mush was placed directly onto the agar base. We observed that over the test period a small amount of water accumulated around the base of the leaf mush. We found that a concentration ca. 1% of transgenic leaves (Cry1Ab) mixed with non-transgenic leaves , there was >95% mortality of neonates. Unfortunately, the control insects (placed on mashed non-transgenic plants) showed little growth so the mixture appeared to be unpalatable or was degrading during the experiment. A small adhesive dot (see appendix) placed between the mush and the agar absorbed this water. With this modification, control larvae grew as well as larvae placed on whole control leaves. However, 10% concentration of transgenic leaf was required in the mush to kill > 95% of neonates.

Monitoring of Variation in Toxicity of Leaves

Experiment 1. As expected, the efficacy of the plants declined from 1% survival (number of larvae N = 347) in the youngest plants, to 6% (N=594) in the 10 wk plants, to 23% survival (N=333) in the oldest plants. However, the reduction in efficacy was not uniform and a number of factors were detected:

a) *Inter-plant Variation.* At wk 10, 65% of the survivors (N=37) were found on leaves from 4 of the 31 plants. The other 27 plants recorded much lower survival, 3%. At week 14, 56% of survivors were from 4 out of the 27 plants tested.

b) *Intra-Plant Variation.* In the plants above that exhibited the greatest reduction in efficacy, their decline was not uniform among leaves from the same plant. Samples sizes are only small because only 10-12 larvae can be tested per leaf, but in one case, survival varied among leaves from 8 out of 10 to 1 out of 7. Even among leaves from a mature plant survival varied from 0 out of 9 to 4 out of 7 larvae. Even from leaves taken from a plant on the same day, survival varied from 0 out of 11 to 5 out of 11 larvae.

Experiment 2. There was no difference detected among test and control plants grown in the cabinets: bioassays were done at week 2, there was high survival of larvae at 10% dose, a dose that would normally kill over 90% of larvae. In contrast, the whole leaf bioassays indicated that the plants in the growth cabinets retained their toxicity - survival of larvae on stressed and unstressed plants was 3%-8% c.f. 43% of similar aged plants grown in the glasshouse.

Discussion

The focus of the last two years departed from the original objectives of the project. Originally, we intended to establish a Bt resistant colony using a variety of means and also to develop resistance management strategies using simulation models. Three issues influenced our decision to redirect our efforts:

1. Selection for resistant strains was to be done collaboratively among Dr Forrester, Dr Akhurst and Dr Daly. Our team's aim was to attempt to use mutagenesis to produce resistant *H. armigera.* However, it was evident that the methodology needed considerable development before it could be applied to *H. armigera.*

2. There was little or no quantitative measure of the amount of Bt toxin in the plant, or its availability to the insect, throughout the growing season. Thus, it was not possible to determine the selection pressure that was posed by field grown transgenic cotton. Dr Helen Holt commenced a post-doctoral fellowship to develop quantitative techniques. Our project aimed to develop bioassay methods that could assess the potential efficacy of the Bt plants.

3. Our rearing techniques for *H. armigera* needed changing to reduce the continuing problems of virus infections.

We have developed successfully a range of techniques that will allow us to address the original objectives.

Mutagenesis has not yet produced a resistant strain of *H. armigera* that appears to have all the characteristics expected of a receptor based mechanism. The level of resistance observed (2.5 fold) is low but more recent results in our project indicate that the strain can survive better on transgenic plants. We will pursue mutagenesis until June 1996 and then reappraise its success and whether or not we should terminate this approach.

Changes to the rearing techniques have implications not only for this project but other CRDC/CRC funded projects. We now can produce large numbers of virus free, or almost free, *H. armigera.* These have enabled us to undertake reproducible experiments, quite a necessity given the inter- and intra-plant variation that we observe with the transgenic plants. In addition, our team provides *H. armigera* larvae to other projects for bioassay of new transgenic plants or for virus work.

The bioassay work on the transgenic plants has been fruitful. The technique developed works well and gives reproducible results. Our preliminary studies indicate that assessing the amount of Bt toxin in cotton leaves, and its availability to insects, will be a complex task. The amount does appear to decline during the season, but other factors also appear to influence our measurements — they are sensitive also to the experimental conditions under which we do the assessment. Our early attempts to measure the Bt levels indicated that amounts of 1% of transgenic to non-transgenic leaf material was enough to kill > 95% of susceptible insects. After refining the bioassay so that control insects had normal growth, as well as survival, we found that the amount of Bt leaf material required to kill most insects was 10 times this amount, yet the only major modification was to reduce the moisture of the mixture by placing the mush onto a paper dot! More recently, we have noted that the age of the control leaves used in the mixture is also important. Efficacy of the Bt leaves was reduced when they were mixed with older control versus younger leaves. Inter- and intra-plant variation for plants at the same growth stage is also large. Thus, a single leaf from plant is not a reliable indicator of the plant's toxicity to larvae. Our current project is assessing this variation in greater detail.

The relevance of the bioassay results in the laboratory to field efficacy at this stage is not clear. But our results indicate that the Bt toxin is not a passive occupant of the leaf. Changes in the leaf, perhaps the level of plant secondary compounds, may well bind the Bt and make it less available.

The development of resistance management strategies has largely proceeded through discussion and scientific publication. Dr Daly has published three review articles that examine the issue of resistance management of Bt plants and she has given numerous seminars on this topic.

Conclusions and Recommendations

Our results indicate the need to quantify the expression and availability of Bt toxin in cotton plants. At this stage we cannot confidently assert that the reduction in efficacy in Bt plants is due solely to a reduction in toxin concentration. Sequestration of the Bt by plant secondary compounds could well play a role. The relative importance of decreased protein expression versus sequestration is an important issue, not just for resistance management, but for the general application of transgenic plants for pest management. Decreased expression may be overcome in the future by the use of different promoters. Changing the promoter may be of little benefit if sequestration is a significant problem. We need to resolve better the fate of foreign proteins in plants to facilitate the design of new transgenic cotton plants.

References

Daly, J.C. 1993. Ecology and genetics of insecticide resistance in *Helicoverpa armigera*: interactions between selection and gene flow. *Genetica* 90: 217-226.

Daly, J. C. 1994. Ecology and resistance management for Bt transgenic plants. *Biocontrol Science & Technology* 4: 563-572.

Daly, J.C. & J.H.. Fisk.1992. Inheritance of metabolic resistance to the synthetic pyrethroids in Australian *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Bulletin of Entomological Research* 82: 5-12.

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

Follet, P.A., F. Gould & G.G. Kennedy. 1993. Comparative fitness of three strains of Colorado potato beetle (Coleoptera: Chrysomelidae) in the field: spatial and temporal variation in insecticide selection. *Journal of Economic Entomology* 86: 1324-1333.

Forrester, N.W., M. Cahill, L.J. Bird & J.K.Layland. 1993 Management of pyrethroid and endosulfan resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Australia. *Bulletin of Entomological Research*, . Supplement Series No 1.

Mable, B.K. & D.J. Pree 1993. Comparison of responses of crosses of European red mites (Acari: Tetranychidae) to dicofol in laboratory and field bioassays. *Journal of Economic Entomology* 86: 275-282.

Marshall, D.B. & D.J. Pree. 1986. Effects of pyrethroid insecticides on eggs and larvae of resistant and susceptible populations of spotted tentiform leafminer. *Canadian Entomologist* 118: 1123-1130.

McCaughey, W.H. & R.W. Beeman. 1988. Resistance to *Bacillus thuringiensis*. *Science* 229: 193-195.

McKenzie, J.A. & M.J. Whitten. 1982. Selection for insecticide resistance in the Australian sheep blowfly, *Lucilia cuprina*. *Expierentia* 38: 84-85.

Navon, A., J.D. Hare & B.A. Federici. 1993 Interactions among *Heliothis virescens* larvae, cotton condensed tannin and the CryIA(c) δ-endotoxin of *Bacillus thuringiensis*. *Journal of Chemical Ecology* 19: 2485-2499.

Roush, R. 1994. Can we slow adaptation by pests to insect-resistant transgenic crops?, in *Biotechnology for Integrated Pest Management* (Persley, G. & MacIntyre, R., Eds) CAB International, Wallingfor, in press.

Roush, R.T & J.C. Daly. 1990. The role of population genetics in resistance research and management, pp 97-153. *In* R.T. Roush and B. Tabashnik, 'Pesticide Resistance in Arthropods', Chapman and Hall, New York.

Roush, R.T. & J.A. McKenzie. 1987. Ecological genetics of insecticide and acaricide resistance. *Annual Reviews of Entomology* 32: 361-380.

Smyth, K.A., A.G. Parker, J.L. Yen & J.A. McKenzie. 1992. Selection of dieldrin resistant strains of *Lucilia cuprina* (Diptera: Calliphoridae) after ethyl methanesulfonate mutagenesis of a susceptible strain. *Journal of Economic Entomology* 85: 352-358.

Tabashnik, B.E. 1994. Evolution of resistance to *Bacillus thuringiensis*. *Annual Review of Entomology* 39: 47-79.

Tabashnik, B.E., N. Finson & M.W. Johnson. 1991. Managing resistance to *Bacillus thuringiensis*: lessons ftom the Diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology* 84: 49-55.

Singh, A.K. & H. Rembold. 1988. Developmental value of chickpea, *Cicer arietinum*, soybean, *Glycine max*, and maize, *Zea mays*, flour for *Heliothis armigera* (Lep., Noctuidae) larvae. *Journal of Applied Entomology* 106: 286-296.

Teakle, R.E. & Jensen, J.M. 1985 *Heliothis punctiger*. pp 312-322 *in* Singh, P. & Moore, R.F. *(Eds)* *Handbook of insect rearing*, vol 2. Amsterdam, Elsevier.

Publications 1993-95

Daly, J.C. & J.H. Fisk. 1993. Expression of pyrethroid resistance in adult *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and selective mortality in field populations. *Bulletin of Entomological Research* 83: 23-28.

Daly, J.C., 1993. Ecology and genetics of insecticide resistance in *Helicoverpa armigera*: interactions between selection and gene flow. *Genetica* 90: 217-226.

Kennaugh, L., D. Pearce, J.C. Daly & A.A. Hobbs. 1993. A piperonyl butoxide synergizable resistance to permethrin in *Helicoverpa armigera* which is not due to increased detoxification by cytochrome P450. *Pesticide Biochemistry and Physiology* 45: 234-241

Daly, J.C. 1994. Ecology and resistance management for *Bacillus thuringiensis* transgenic plants. *Biocontrol Science and Technology* 4: 563-572.

Daly, J. & K. Paschalidis. 1994. Resistance factors - What do they mean? ACGRA Cotton Conference, Broadbeach, QLD, August 1994

Trowell, S., E.Zinkovsky, J.Daly, R.Russell & R.ffrench-Constant. 1994. DNA probes for key insecticide resistance genes in Australian H. armigera. ACGRA Cotton Conference, Broadbeach, QLD, August 1994.

Daly, J.C. & S. Trowell. 1995. Biochemical approaches to the study of population genetics: the role of selection and gene flow in the evolution of insecticide resistance, in *The Ecology of Agricultural Pests: Biochemical Approaches* (W.O.C. Symondson & J. E. Liddell) Chapman Hall, in press.

Daly, J.C. & J.H. Fisk. 1995. Decrease in tolerance to fenvalerate, in resistant *Helicoverpa armigera* after pupal diapause. *Entomologia experimentalis et applicata* 77: 217-222.

Roush, R.T. & J.C. Daly. Considerations for comparing bioassays and biochemically-based resistance monitoring methods: lessons from a study of cyclodiene resistance, *Pesticide Biochemistry and Physiology*, in press.

Pree, D.J. & J.C. Daly. Toxicity of *Bacillus thuringiensis*/endosulfan combinations to the cotton boll worm *Helicoverpa armigera* (Hubner) Lepidoptera: Noctuidae. *Pesticide Science*, submitted.

Daly, J.C. & J.H. Fisk. Sex-linked inheritance of endosulfan resistance in *Helicoverpa armigera*, submitted.

Seminars 1993-95

July 1993, 'Is it possible to manage resistance in *Helicoverpa armigera*, Australian Entomology Society Conference, Cairns, Queensland.

August 1993, invited talk 'Evolutionary biology of insecticide resistance in the moth, *Helicoverpa armigera'*, 17th International Congress of Genetics, Birmingham, U.K.

August 1993, 'Resistance management of *H. armigera* in Australia: an update', Rothamsted Agricultural Station, Harpenden, U.K. and London School of Tropical Medicine, U.K.

September 1993, 'Resistance management to Bt in Australian *Helicoverpa*', 2nd Canberra Bt Meeting, Canberra, ACT.

November 1993, invited 'Management of pyrethroid resistance in H. armigera', Genetics Department, Melbourne University, Victoria.

December 1993, invited talk 'The role of gene flow in adaptation by agricultural pests', in the symposium *Gene Flow: The Neglected Evolutionary Force*, Entomological Society of America, Indianapolos, USA.

January 1994, invited talk 'Ecology and resistance management for Bt transgenic plants', OECD Workshop on the Ecology of Transgenic Plants, Queenstown, New Zealand, January 1994.

February 1994, with Jenny Fisk and Kate Galloway, 'Managing endosulfan resistance in *Helicoverpa armigera*', World Cotton Conference, Brisbane, Queensland.

September 1994, invited talk with Stephen Trowell, 'Biochemical approaches to a study of population genetics: the role of selection and gene flow in the evolution of insecticide resistance' at symposium, The Ecology of Agricultural Pests: Biochemical approaches, University of Wales College of Cardiff, Wales, UK.

September 1994, invited talk 'Resistance management in *Helicoverpa armigera*: from insecticides to transgenic plants', Australian Entomological Society, Adelaide, South Australia.

April 1995, invited talk with Paul Wellings, 'Ecological constraints to the deployment of arthropod resistant crop plants: a cautionary tale', Nicholson Centenary Symposium, Canberra, ACT.

Videos

The Cottco Report, on 'Resistance to Bt in *H. armigera'*, 1995.

Appendix

1. Protocol for testing whole transgenic leaves or other plant tissue

Filter papers. A 1% sorbc acid solution, to prevent fungal growth, is made by boiling the solution in a microwave and allowing it to cool. 7cm hardened filter paper circles are soaked in the solution and then allowed to drain thoroughly on paper towel, so that they are just damp.

Leaves. Leaves to be tested are picked, labelled with a permanent marker and transported together in large plastic bag. Control leaves are similarly labelled. For large leaves, one filter paper circle and a size 2 dental roll are placed under a leaf.

Test Procedure. 10 neonates are placed on large leaves and together they are sealed in a partly inflated zip seal plastic bag, 22.5 x 12.5cm and 50 cm gauge. For small leaves, half a filter paper and half a roll are used in a 10 x 7.5cm plastic bag with 10 neonates. The bags are stored at 25° C and 40-50% humidity, away from direct light. They are scored after 6 days for larval mortality and/or comparative growth rate (instar and weight). All waste transgenic leaf material is autoclaved before disposal.

2. Protocol for cotton leaf mulch bioassays using transgenic and normal cotton plant tissue

Agar base. On the day before the experiment agar is made up and poured into 32-cell rearing trays. 20g agar and 1g sorbic acid per litre of cold water are mixed together and then brought to the boil. It is dispensed into the trays, using a peristaltic metering pump, with 4-5 ml per cell. The agar is allowed to cool, then covered with bench roll until the following day. A self adhesive dot label (e.g. Quik Stik™ International Ltd, Auckland, New Zealand, # MC 14 C9) is then pressed onto the agar in each cell.

Leaf material. Transgenic leaf material is collected from the plant and placed immediately into a labelled Nunc™ cryogenic tube and then into liquid nitrogen. Non-transgenic cotton leaves are collected in a plastic bag.

Leaf Mush. Normal leaves are ground-up finely in liquid nitrogen with a pestle and mortar. Each transgenic leaf sample is removed, still frozen, from its tube and ground individually in liquid nitrogen with a pestle and mortar. It is weighed into 10 ml beakers, one for each dose and the ground normal leaf is added to make up the total dose weight of 2 g. Each dose of leaf mulch is thoroughly mixed with a spatula, then pressed into a teaspoon. It is dispensed onto the sticky dots in the labelled trays by cutting off a small portion of the compacted mulch with a spatula and pressing it onto the dot.

Test. Larvae are transferred onto the mulch, 1 per cell, and the tray is heat sealed. They are stored at 25°C and 50% humidity. The tests are scored after 7 days for larval mortality and/or comparison of growth rate (instar and weight).

Cleaning. All equipment is rinsed and then soaked in a sodium hypochlorite solution overnight. All waste transgenic leaf material is autoclaved before disposal.

3. Procedures to create and screen for a Bt resistant strain of *H. armigera*

Treatment with EMS.

Male pupae are treated with ethyl methanesulphonate (EMS) 3-4 weeks after the date that they were set up as eggs. They need to be sexed carefully. A neutralising solution of 250 g sodium hydroxide and 30 ml thioglycolic acid, dissolved in 6 litres water, is made up in advance. Three pairs of gloves are worn and peeled off afrer coming into contact with EMS or EMS contaminated equipment or material. Pupae are placed in the bottom of a conical flask, up to a double layer (100-200 in a 1 litre flask). A 5 cm filter paper is folded into quarters and the point is inserted into the tip of the dropper in the stopper. The flask is stoppered and attached to the vacuum attachment of the tap that is turned on fully for 2 minutes to produce a vacuum in the flask; the pupae become motionless. The rubber hose is clamped and removed from tap, which is then turned off. The plunger of the syringe is lifted to the 1 ml mark

and inserted into the plastic cap of the opened EMS bottle. EMS is drawn up to the "0" marking on the syringe (approx. 0.2 ml). Holding the plunger firmly, the needle is removed, pushed through the rubber teat on the flask stopper and the plunger is slowly released. The syringe is removed and placed in bath. One pair of gloves is removed. After four minutes the vacuum is released by opening the clamp. After a further two minutes the pupae are removed, distributed into individual cups (one for each bucket) and all equipment immersed and left to soak in the neutralising solution, under a fume hood, for 24 hours. Another pair of gloves is removed. Male pupae are set up in their cups in buckets with a similar number of females in each and reared. A control of about 15-20 untreated pupae of each sex is also set up from the same group of pupae. The equipment is all thoroughly rinsed to remove all the neutralising solution.

Screening eggs with Dipel 2X.

This technique was developed in association with our normal rearing practices.

Collecting eggs. Treated males and untreated female adults are allowed to mate and lay eggs. Eggs on cloths are collected as with rearing. Eggs are bleached on Mondays, up to 2000 per filter paper. The eggs are rinsed well, and are evenly spread on the filter paper. Eggs from adults that were treated on different dates, with their controls are kept separate, but eggs from different days (Tuesday to Friday) from the same treatment are bleached together. Filter papers are placed in ziptop plastic bags labelled with the date of bleaching and EMS number, and left until the next day at a low temperature (6 to 18°C), exposed to natural light to break down any residual sodium hypochlorite.

Treating eggs. Eggs are treated on Tuesdays. Bags are spread out over a bench top. Filter papers with eggs are removed from bags, and placed on top to dry out for 1/2 to 3/4 hour until the edges have curled up. Dipel 2X is collected from ultrafreezer and thawed out. (It is stored in vials as 1 ml of a 100 mg/ml dose). The Dipel is diluted 1:9 with distilled water, making a 10 ml solution of 10 mg/ml. Glass petri dishes are placed next to the filter paper on each bag. 2 ml of the Dipel 2X solution (using a syringe without needle, as a measure) are dispensed into each petri dish. The filter papers are inverted a few at a time, eggs down, into the solution in the petri dish. As soon as the filter paper is entirely saturated it is turned over in the petri dish to soak any eggs on the underside. The process should take about 10 minutes. Filter papers are drained on 11 cm filter paper circles placed on each individual bag for about 5 minutes. Filter paper with the treated eggs is then placed into a plastic jar with a label copied from its bag. One filter paper is set up in each jar, or 3 to 4 in a 5 litre bucket. Two cells from the rearing trays, containing diet, are attached at the top of each jar, or 2 to 3 per bucket, using net fabric and a piece of masking tape. The top of the jar and lid is sealed with black electrical tape to stop neonates escaping. Translucent jars are covered with aluminium foil or placed in an opaque garbage bag up to their lids.

Scoring egg hatch and setting up survivors. Treated eggs are scored after 6 to 8 days, starting with the controls. The lid, cells, filter paper and inside of jar are checked for survivors; number and age are recorded. The filter paper is divided into 8 sections by drawing intersecting lines. One section is scored under the microscope for eggs hatched and not hatched. Numbers are multiplied by 8 and recorded for the total number. Survivors which are larger than those in the controls (and at least late first instar), are placed in labelled, individual 30 ml portion cups with diet and a perforated lid. The growth of survivors is monitored and those failing to thrive are discarded. Those which successfully pupate are sexed and numbered individually. Each male is put into a container with 5 susceptible females from the general rearing strain (GR) and each female survivor is placed with 3 GR males of the appropriate age. Eggs are collected as with normal rearing. They are left to hatch on filter paper in containers with food, and are set up as larvae, to ensure that sufficient numbers are carried through as a basic healthy breeding stock. Surplus F_1 or F_2 eggs are tested with MVP™. A 25 mg/ml dose is used from the stock made up and stored in the fridge. It is used the same way as Dipel 2X on eggs.

A control of about 200 to 500 eggs from the GR strain is treated at the same time. Eggs are scored after 8 to 10 days. The number and size of survivors are evaluated against the control and then kept for further selection and testing, or discarded. All equipment used with Dipel™ or MVP™ is rinsed then soaked overnight in a sodium hypochlorite solution.

4. Rearing Procedures for *H. armigera*

Diet Recipe for 2.2 litre

Chickpea flour	200 g
Wheatgerm	120 g
Yeast	120 g
Pollen	28 g
Ascorbic acid	6 g
Nipagin	6 g
Sorbic acid	2 g
Sunflower oil	20 ml
Formalin	2 ml
Agar	22 g
Water for agar	250 ml cold + 750 hot
Water for dry mix	1200 ml
Mould Control	4 ml

Mould Control (100 ml)

Phosphoric acid	4 ml
Proprionic acid	42 ml
Water	54 ml