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FINAL REPORT

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Potential for Transgenic Plants to Select for Resistance in *Helicoverpa armigera*

CSE 53C

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CSIRO

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Report prepared by Karen Olsen and Joanne Daly

Summary

This project helped to identify the factors that affected the efficacy of transgenic Bt cotton plants to control *Helicoverpa punctigera* and *H. armigera.* This knowledge contributes the design and refinement of the resistance management strategy for the commercial use of transgenic cotton plants.

We have demonstrated, through several sets of experiments, that the plant growth stage itself has a major effect on efficacy. That is, a compound(s) in the plant appear to alter the availability of the Bt protein to the insect, after ingestion. Our results suggest that condensed tannins, accumulated in the leaves under field conditions, could decrease the efficacy of Bt. Changes in environmental conditions seemed to affect efficacy, particularly through changes in growth rate, seen in two major experiments. Thus, stresses of different kinds may impact on efficacy because generally stress alters the growth rate of the plant.

We quantified season long variation in efficacy of Bt cotton grown under field conditions. Initial bioassays, on field samples taken from individual Bt plants, over two seasons, showed that there was a significant difference in efficacy between plants. Bioassays on bulked, freeze-dried field samples of two commercial Bt varieties, over the 96/97 and 97/98 seasons, quantified changes in efficacy over the season and illustrated differences between the two varieties and the two seasons. Efficacy was measured as dropping over 50 fold for V2i by early February in both seasons and 276 fold for V15i by mid March1997, 82 fold by mid January 1998.

We selected for strains of *H. armigera* resistant to Bt. Two laboratory and two field generated resistant strains have been selected, over several generations, in the laboratory and tested with diet incorporation bioassays of Cry1Ac as MVP and Bt leaf material. Most strains showed resistance to both sources of Bt, with the field strains showing the highest resistance, of over 20 fold, compared to the susceptible strain.

Preliminary genetic studies were carried out on two strains, by Dr David Heckel, during his sabbatical, in preparation for developing a genetic map of *H. armigera* chromosomes.

USE OF BT FOR MANAGEMENT OF HELIOTHIS IN COTTON

Potential for Transgenic Plants to Select for Resistance in *Helicoverpa armigera*

Aim

To contribute to a resistance management strategy for the commercial use of transgenic cotton plants by identifying the factors that will affect the development of resistance in *H. armigera* to the crystal protein toxins expressed in these plants.

Summary of Achievements against Objectives

Objective 1. Determine the effect of environmental factors on the efficacy of Bt cotton.

- We have demonstrated, through several sets of experiments, that the plant growth stage itself has a major effect on efficacy.
- Results of experiments suggested that condensed tannins, accumulated in the leaves under field conditions, could decrease the efficacy of Bt.
- Changes in environmental conditions seemed to affect efficacy, particularly through changes in growth rate, seen in two major experiments.

Objective 2. Measure season long variation in efficacy of Bt cotton.

- Initial bioassays, on field samples taken from individual Bt plants, over two seasons, showed that there was a significant difference in efficacy among plants.
- Bioassays on bulked, freeze-dried field samples of two commercial Bt varieties, over the 96/97 and 97/98 seasons, quantified changes in efficacy over the season and illustrated differences between the two varieties and the two seasons. Efficacy was measured as dropping over 50 fold for V2i by early February in both seasons and 276 fold for V15i by mid March1997, 82 fold by mid January 1998.

Objective 3. Select for strains resistant to Bt.

- Two laboratory and two field generated resistant strains have been selected over several generations in the laboratory and tested with diet incorporation bioassays of Cry1Ac as MVP and Bt leaf material. Most strains showed resistance to both sources of Bt, with the field strains showing the highest resistance, over 20 fold, compared to the susceptible strain.

Objective 4. Elucidate the genetics of Bt resistance in H. armigera.

- Preliminary genetic studies were carried out on two strains by Dr David Heckel, during his sabbatical leave during 1196/97. This work will enable a genetic map to be developed for *H. armigera* chromosomes.

Materials and Methods

Changes to previously documented protocols for bioassays.

The whole leaf assay method was modified to reduce cannibalism. Neonates were tested individually on leaf discs placed on an agar base in rearing trays compared with the old method in which 5 neonate larvae were placed on whole leaves.

Diet incorporation was the preferred method for bioassays of Bt leaf material from 1997. Previously Bt leaves were diluted with non-transgenic cotton leaves to form a leaf mush. However, we observed that normal plants of different ages were found to have an effect on actual LD_{50} values of Bt toxin added to it. The diet provided a

neutral background to assay material from Bt plants of different stages. The diet, modified from the rearing diet, consisted of 5 g chickpea flour, 3 g stabilised wheatgerm and 6.5 g agar in 50 ml of autoclaved, filtered water. The water, at room temperature, was added to the dry ingredients and mixed. The diet was modified so that it would sustain a growth rate in the controls that was similar to that of larvae on normal cotton leaf material and retain a low level of mortality. Larval growth rates on modified diet controls were intermediate between the two types of leaf controls and mortality was consistently below 10% (Table 1).

Table 1: Controls set up with bioassays

Material used for control	Mean Mortality (%)	Number (neonates)	Number (replicates)	Mean weight survivors(mg)
immature leaf mush[a]	5.4	138	6	3.5
mature leaf mush[a]	5.0	174	6	5.9
modified diet[a]	5.6	125	4	6.6
immature whole leaf or discs[b]	3.5	90	3	7.5
mature whole leaf or discs[b]	3.5	86	3	10.0

[a] scored after 8 days; [b] after 7 days

Plant material.

A number of different Bt and normal varieties were used in experiments and in the field. The transgenic varieties that were used were: CS 50 Cry1Ab, L22 and L23 Cry1Ac, V15 Ingard and V2 Ingard. In all experiments normal cotton of the same variety as the transgenic cotton was used as the control. As far as possible, laboratory experiments used the same Bt variety as was used in the field at the time. In the experiments on plant secondary compounds additional varieties were used: high terpenoid (HG 660), high condensed tannin (HT 35-14.3) and high anthocyanin (Imperial Red).

Plants were grown in the glasshouse, growth cabinets, growth room, phytotron or outside from November to February. Plants grown in glasshouses had natural daylength, and minimum daily temperatures of 20-24°C, maximum 28-34°C. The cabinets had a 16/8 hr day/night regime and a constant temperature of 28°C. The growth room had the same light regime, linked to a 18/32°C temperature cycle. All plants were grown in 20 cm pots with a 50/50 mixture of Pryors' and potting mix. There were up to six immature or three mature plants per pot. They were fertilised fortnightly, from four weeks after planting, with a solution of 1g/l of fertiliser with 27% total nitrogen, at a rate of ca. 500 ml per pot. Plants were classified and sampled as immature, if they had true leaves but were presquare. Plants defined as mature were at the first flower stage or early boll-set.

Changes to field sampling procedures.

During the first two field seasons leaf samples were taken from individual plants, to quantify inter-plant and seasonal variations in efficacy. They were effective in showing that there is significant inter-plant variation, at least from December. Seasonal patterns in variation were more difficult to quantify. These were complicated by the length between sampling days and the scope of variations in larval mortality occurring at comparatively short intervals, sometimes accidentally, by pesticide drift. The onset of sampling was also delayed, resulting in a shortened period of sampling and the absence of early data points. From the 96/97 growing season, the problem of insecticidal spray drift was decreased by the choice of a new field site at the Plant Breeding Institute, Narrabri. Bulked samples of 3rd node leaves of V15in and V2in, collected from early in the season and at more frequent intervals, by Dr Fitt's group during 96/97 and 97/98 season, provided a better distribution of samples. This also allowed aliquots of the same samples to be tested for tannins and/or Bt levels. The leaf material from V15in and V2in and controls at PBI, was placed into liquid nitrogen in the laboratory (96/97) or in the field (97/98) before being freeze-dried, to eliminate possible variables introduced during collection.

Rearing of resistant strains.

The resistant strains were reared basically using the revised method for general stock, which continued to produce consistently high quality material for experiments. After discussions with Dr David Heckel in late 1996, the resistant strains were out-crossed and the progeny divided into five sub-cultures. These were reared and selected separately. Survivors were sexed and then males and females were rotated between sub-cultures for

each strain. This method delays the decrease of fitness associated with inbreeding, resulting from repeated selection.

Analysis of data.

The dose response of larvae to Bt toxin was analysed, and slopes and LD_{50} estimates were calculated, using the logit analysis of GLIM version 3.77 (Payne, 1985).

Results

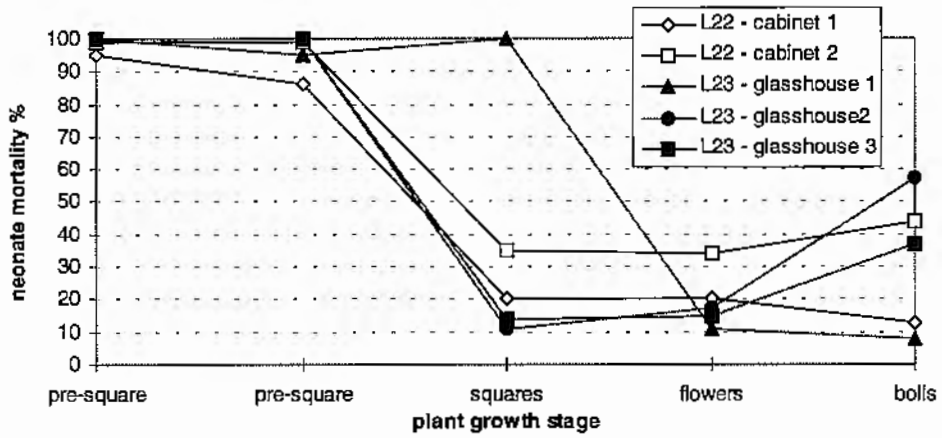
1. The Effect of Environmental Factors on the Efficacy of Bt Cotton.

The effect of plant stage.

Initial experiments included the fortnightly monitoring of changes in efficacy as plant developed under the relatively controlled conditions of growth cabinets or glasshouse. This was intended to provide a baseline of efficacy against which variations observed in the field and during experiments, could be compared.

The results illustrated in Figure 1 are consistent with those observed with field observations. Plants grown in the laboratory and field both show a drop in efficacy as the plants matured. They suggest a strong plant growth stage effect on the efficacy of the plants, which is not just a function of the plant age, since the groups of plants took from 8, up to 14 weeks, to start producing squares.

Figure 1. Results of whole leaf and leaf disc assays from serial samples of five sets of Cry1Ac Bt cotton, Siokra L22 (2 in cabinet) and Siokra L23 (3 in glasshouse).



To investigate this effect of plant stage further, experiments were designed to measure the efficacy of Bt toxin from a variety of sources when it was mixed with leaves from immature and mature normal plants. Three sources of toxin were used:

- Bt plants, in which the Bt and non-Bt leaves were mixed as a serial dilution to produce a mush
- purified Cry1Ac toxin (obtained from Dr Ray Akhurst), which was added as a serial dilution to normal leaves, and
- formulated Cry1Ac product, MVP®, as a serial dilution in water into which normal leaves were dipped, and which was also added as a serial dilution to normal leaves.

Results were also available for the efficacy of Bt leaves mixed into diet as a serial dilution.

Immature Bt plants were always significantly more efficacious than were mature Bt plants, when assayed in the same backgrounds (Table 2, part 1), although the difference was small, 2.4 to 4.1 fold. However, when we tested the Bt plants in mixtures with normal plants of different ages (Table 2, parts 1-3) the greatest difference in LD_{50} estimates was seen in immature Bt plants assayed with immature normal plants compared with assays of mature Bt with mature normal, 116 fold.

When we repeated the assays using MVP or purified toxin, mixed with mature and immature normal plants, to separate plant background and the source of the toxin, again the efficacy of the Bt toxin was significantly less, 14 and 164 fold, when added to mature versus immature normal plant leaves. The differences in efficacy were even

greater, 726 fold, when immature and mature normal plant leaves or leaf discs were dipped into solutions of MVP. These last results indicated that the effect seen with the ground leaves was not an artefact of grinding up the leaves. For all experiments there appeared to be general trend for the slope to decline as efficacy declined, although in most cases this was not significant.

Table 2. Statistical results for comparison of bioassay lines. The F-statistics are for the differences in bioassay line for immature vs mature plants.

Test	Ratio of LD_{50}	F	df	P
1. Immature vs mature Bt plant				
a. in immature control plant	2.4	8.5	1,21	< 0.01
b. in mature control plant	4.1	8.3	1,21	<0.01
c. in modified diet	3.9	19.4	1,29	<0.001
2. Immature vs mature control plants				
a. with immature Bt plants	28	90	1,21	<0.001
b. with mature Bt plants	49	37	1,21	<0.001
3. Immature Bt in immature control plants				
vs mature Bt with mature control	116	52	1,21	<0.001
4. Immature vs mature control plants				
a. with purified CryIAc toxin	164	39	1,21	<0.001
b. with MVP® in leaf mush	14	49	1,12	<0.001
c. with MVP® on leaves or discs	726	124	1,26	<0.001

From these sets of results it seems that the growth stage of cotton plants, normal or transgenic, has a major effect on the efficacy of Bt toxin, adding evidence that the background leaf itself is playing a major role in the efficacy of the transgenic plants either by enhancing and/or sequestering the Bt toxin.

The effect of plant secondary compounds.

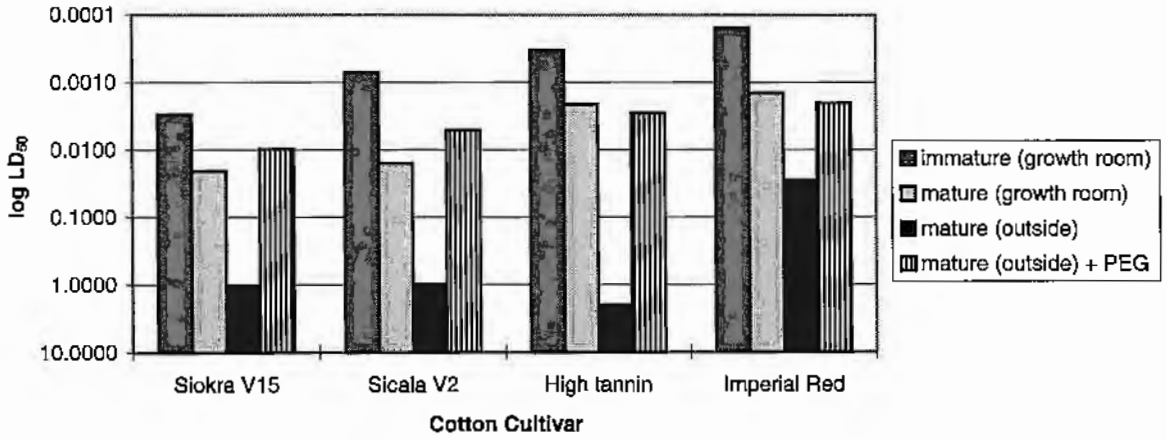
In preliminary experiments, varieties of non Bt cotton plants producing higher levels of plant secondaries, such as condensed tannins (cultivar HT 35-14.3) and terpenoids (HG 660) and 2 commercial varieties, Siokra L23 and CS 50, were grown in a glasshouse and outside during the summer months, up to the first flower stage. Leaves from the 3rd to 5th nodes of these plants were bioassayed with serial dilutions of Cry1Ac, as the formulated product MVP® or immature Bt plant leaves. There was less than a 2 fold difference in LD_{50} for bioassays of the four cultivars grown in the glasshouse, but a 2.5 fold increase in LD_{50} for bioassays using leaves from the high tannin variety and a 2 fold decrease for the high gossypol variety, compared with the commercial varieties. The experiment was repeated, but Imperial Red, a high anthocyanin variety, replaced the high gossypol variety. V2 and V15 were the commercial varieties used. Plants were grown in a growth room and outside. The leaf material was collected as above, and also from immature plants in the growth room, was bioassayed with MVP® and analysed for condensed tanniu levels by Dr Greg Tauner (Plant Industry) by the method of Li et al. (1996) (Table 3).

Table 3. Measurements of condensed tannin levels in leaves of immature plants grown in the growth room and mature plants grown outside, expressed as mg catcehin equivalents /g dry weight (G. Tanner, pers. comm).

Cotton Variety	Immature: Mean	SE	Mature: Mean	SE
Siokra V15	3.72	± 0.19	79.66	± 0.35
Sicala V2	26.07	± 0.72	83.25	± 7.66
High Tannin	39.80	± 0.39	132.95	± 1.38
Imperial Red	26.98	± 0.89	62.47	± 1.72

Comparisons of tannins levels in Table 3 and efficacy of Cry1Ac (MVP) in Figure 2 , indicate that MVP added to leaves with low tannin levels (immature), was more efficacious than MVP added to leaves with higher levels (mature, grown outside). The LD_{50} of MVP in the high tannin variety, grown outside, which had the highest tannin content, was just over 2-fold less efficacious than in the commercial varieties grown outside. The efficacy of MVP in Imperial Red, which had the lowest tannin content, was 34-fold higher.

Figure 2. Effect of cultivar, plant growth stage, growing conditions and PEG on the LD_{50} of Cry1Ac toxin (mg/ml) for *armigera* neonate larvae.



There was a substantial decrease in efficacy when comparing LD_{50}s of MVP added to leaves of mature plants which were grown inside and mature plants grown outside, from 20 fold for Imperial Red, and up to 948 fold, for HT 35-14-3, the high tannin variety. Since plants were at the same growth stage, secondary compounds (eg tannins) stimulated by the higher light levels outside, could be contributing to the decreased efficacy of MVP. Condensed tannins bind to polyethylene glycol (PEG) in preference to plant proteins (Jones and Mangan, 1977) and therefore negate the effect of the tannins. Adding PEG to these bioassays could indicate if the tannins were actively involved in the changes in the efficacy of the Bt toxin. Bioassays were, therefore, duplicated using PEG as a fixed 3% of the leaf material. There was a large increase in the efficacy when PEG was added to the ground leaf of plants grown outside, ranging from 14 fold for Imperial Red to 710 fold for the high tannin variety (Figure 2). In controls set up with leaf or diet, with and without PEG, but no MVP, mortality remained low: 1% of 172 neonates without PEG and 4% of 145 neonates with PEG. This indicates that PEG alone did not substantially increase larval mortality. Overall, therefore, our results suggest that condensed tannin have an antagonistic effect on the efficacy of Cry1Ac toxin against *H. armigera*.

The effect of stress.

Preliminary tests on the effects of mechanical (insect) damage, UV radiation, waterlogging, drought, and low light intensity on the efficacy of immature and mature Bt plants were conducted. Results which showed more than a 2 fold increase or 50% decrease in the LD50 are shown in Table 4.

Table 4. Results of preliminary tests of plant stress on the efficacy of Bt, expressed as the ratio of test LD_{50}/control LD_{50}.

Stress Test and Plant Growth Stage	Ratio	Effect on Efficacy
mechanical (insect) damage - immature plant	3.41	decrease
low light intensity - immature plant	2.46	decrease
waterlogging - immature plant	3.68	decrease
increased UV radiation - mature plant	0.45	increase
waterlogging - mature plant	0.48	increase

The effect of low light intensity (shade) and different temperature regimes were investigated in more detail in collaboration with Dr Greg Constable (Plant Industry) and Dr Helen Holt (Entomology). Experiments were carried out in the Phytotron facility at CSIRO, Plant Industry, Canberra. We monitored plant efficacy using the leaf disc method. Dr Constable will measure the nitrogen content and Dr Holt the Bt levels. The Bt variety used

was V15ingard. In the shade experiment, treated plants was subjected to one week under 70% shade cloth. The temperature regimes for the three sets of plants in the temperature experiment for the one week of treatment were: low - 10/20°C; high - 22/36°C; control - 16/28°C. A set of ten plants was sampled destructively at alternate nodes before treatment, after treatment and five times subsequently at approximately ten day intervals for each treatment and control. Before and after treatment all plants were grown under normal phytotron conditions including a 16/28°C temperature regime.

Only preliminary results are available at present as the experiments were conducted during January-May 1998. The largest effect on mortality of neonate larvae was seen in the first leaf which was fnlly expanded at the time of treatment, at the 5th node, counted from the base of the plant up (Figures 4 and 5). Although there was much inter-plant variation in efficacy in both experiments, results indicated consistently that the faster (taller) growing plants were more efficacious.

Figure 4. Efficacy of shade treated plants and controls at the 5th node, over time

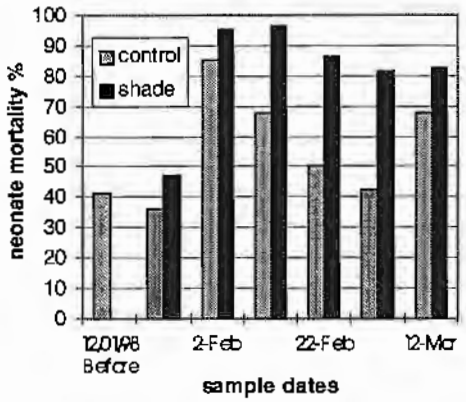
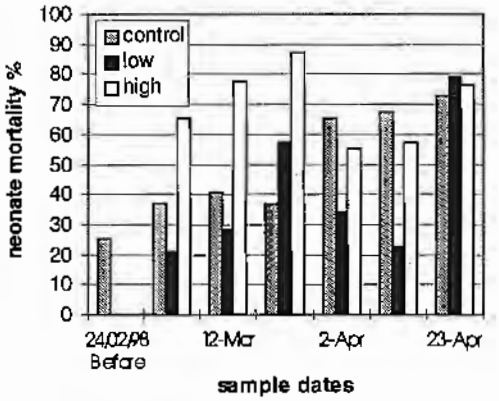


Figure 5. Efficacy of temperature treated plants and controls at the 5th node, over time.



In the temperature experiment these were the high temperature treated plants, and to a lesser extent also the shade treated plants. The slowest growing plants, after the low temperature treatment, had the lowest efficacy. The increase in efficacy seemed to be further enhanced by the staking of plants, on 22/1/98 for the shade and 12/3/98 for the temperature experiment. The staking of plants seemed to induce an increase in inter-node length in plants from all treatments. All showed higher efficacy but in the two taller groups mentioned above, this increase was sustained to the extent that it delayed the drop in efficacy associated with square production, seen in the control groups. The effect of the experiment was gradually reduced in leaves above the fifth node and was negligible by the 9th node.

While these trends were seen in these groups of plants, our preliminary experiments suggest that they may not be the same in plants at other growth stages.

2. Season Long Variation in Efficacy of Bt Cotton.

Two sets of 10 lots of CS 50 Cry1Ab plants were sampled by Dr Gary Fitt in the 94/95 season from 2 different plots and 2 sets of 10 Siokra L23 Cry1Ac plants in the 94/95 season. The 94/95 results (Table 5) showed an overall 2.4 fold drop in efficacy over the season but no significant difference between the two plots sampled. The bioassays from 95/96 showed a significant difference in efficacy between the two plots sampled ($F_{1, 215} = 9.47$, $P<0.01$). The samples from the dryland plot showed a 4.4 fold decrease in efficacy over the season, all plants exhibiting this trend with ratios varying from 1.3 to 11.7. The efficacy of plants in the irrigated plot did not show a decrease. This plot was waterlogged in Jannary and plant growth was stunted. Leaf samples taken afrer this wet period showed a 2 fold increase in efficacy, compared with those taken in December. Both the 94/95 and 95/96 collections showed significant variation between plants ($F_{8,184} = 5.91$, $P<0.01$; $F_{18, 187} = 3.36$, $P<0.01$).

Table 5. Means of LD_{50}s (% Bt leaf) for Bt plants sampled during the 94/95 and 95/96 field trials.

a. Month sampled - 94/95 (Cry1Ab)	Mean LD_{50}
December	0.67
February	0.60
March	1.47

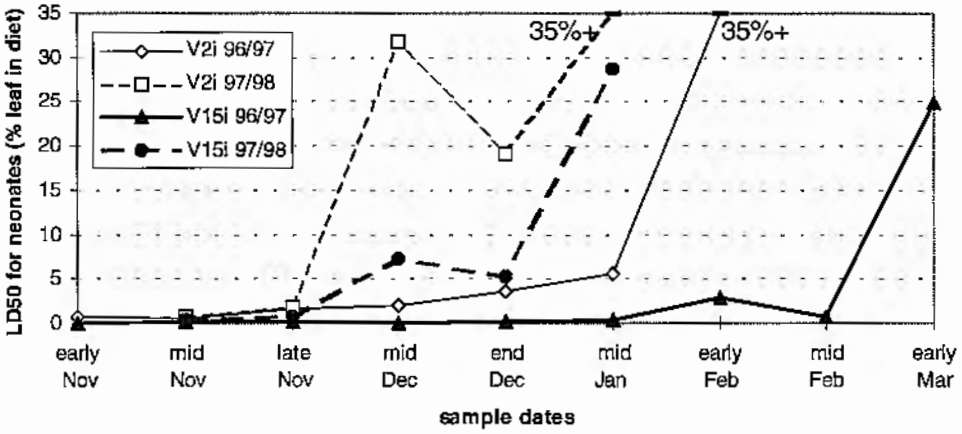
b. Month sampled - 95/96 (Cry1Ac)	Mean LD_{50}: Irrigated plot	Mean LD_{50}: Dryland plot
December	0.18	0.11
January	0.09	0.11
March	0.14	0.49

Diet incorporation bioassays on freeze-dried samples from the 96/97 field collections showed a 4-fold drop in efficacy from November to late January for V2i and a 9-fold drop from November to Mid February for V15i (Figure 5). By the next sampling date for each variety, about two weeks later, there was an additional sharp drop in efficacy that could not be accurately quantified, as the efficacy had dropped below the sensitivity of the bioassay. At that point the LD_{50} was above 25% of dried leaf material, a dose which, in practice, is already a 100% reconstitution of the leaf. V15i was more efficacious than V15i throughout the season, by 2-fold in November, increasing to 11-fold in January.

The efficacy of both varieties dropped earlier in the 97/98 season, falling by 30 fold for V2i and 21 fold for V15i by mid December. This decrease could, in part, have been the result of heavy rain following shortly after irrigation of the area, resulting in waterlogging. Efficacy increased by less than 2 fold by the end of December before dropping again sharply, in both strains, by mid January. During this season V15i again was more efficacious than V2i, by 1.9 fold in November, increasing to 4.4 fold in mid December.

Higher efficacy may have been maintained longer during the 96/97 season because plant growth was disrupted and development was delayed by a storm, which stripped plants, on 17/12/96. V15i showed a small (2 fold) increase in efficacy a week later, V2i remained the same.

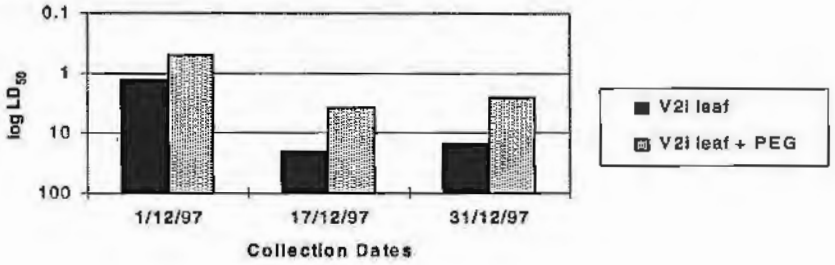
Figure 5. Seasonal variations in efficacy in the field for two commercially available Bt cotton varieties sampled during the 96/97 and 97/98 seasons.



When PEG was added to the diet incorporation bioassays of V2i field material, for three collections made in December 1997, a 2.6 to 5.8 fold increase in efficacy was seen (Figure 6). For the collection made on 16/1/98,

the efficacy of the Bt leaves dropped below the sensitivity of the bioassay (LD_{50} of above 25% leaf in diet). Adding PEG to this bioassay did not increase the efficacy above this level. These results suggest that tannin may play a part in the seasonal decline in efficacy of Bt cotton in the field, but is possibly not the only factor involved, since PEG had no effect on the sample collected in mid January. The level of available Bt itself, may have been too low.

Figure 6. Effect of PEG on the LD_{50} (% leaf in diet) of field collected Bt leaves



3. Selection for Strains Resistant to Bt.

Treatment of male pupae with EMS and routine screening of eggs continued until July 1996. Emphasis was then placed on increasing the number and fitness of four selected strains by outcrossing initially and then implementing the sub-culture / rotational method of rearing. The four strains selected were two laboratory generated strains 21A and 67A, obtained through mutagenesis of male pupae, and two field strains, Vic Rats and Rats (VR and R) from Dr Neil Forrester. The protocol for selections was done in collaboration with Dr Akhurst to increase the chance selecting for a resistant strain, and to maintain valuable strains in two rearing laboratories to ensure continuity of supply.

Our strains have been selected for resistance to Bt over 8 to 11 generations, by the egg treatment method and screening of single pair crosses with Cry1Ac, as the formulated products MVP or DiPel. Their resistance has been measured by diet incorporation bioassays of MVP and Bt leaf material.

Table 6. Ratios of LD_{50} for resistant strains/susceptible strain and growth of resistant survivors.

Strain	Ratio of LD_{50} on MVP in diet	Maximum instar of survivors at LD_{99} of susceptible strain on MVP at 10 days	Ratio of LD_{50} on Bt leaf in diet	Maximum size of survivors at LD_{99} of susceptible strain on Bt leaf at 7 days
21A	8	2nd	8	2nd
67A	5	2nd	1.2	2nd
VR	21	3rd	28	2nd
R	181	3rd	24	2nd

Bioassays carried out after 5 to 7 generations of selection, showed that, in general, the four strains have significant resistance or growth advantage with Cry1Ac, as diet incorporated leaves from immature Bt plants or MVP, compared to a susceptible strain (Table 6). The resistance of the field strains to MVP and Bt leaf is over 20 fold, the laboratory generated strains are less than 10 fold, compared to the susceptible laboratory strain. However, when tested on Bt leaf mush alone, or whole Bt leaf material, no strain has shown any level of resistance above that of the susceptible strain.

4. The Genetics of Resistance.

There was little material available of the resistant strains for genetic analysis before mid to late 1997. Preliminary genetic analysis was carried out by Dr David Heckel on VR and R, in mid 1997, during his sabbatical. All four strains needed further attention and selection to establish a healthy, homozygous colony before further genetic analysis could be done.

It was decided to use genetic linkage maps as a faster method of determining the number of mechanisms involved in Bt resistance. This would be building on experience and expertise already gained by Dr David Heckel on pyrethroid resistance in *H. armigera* and his documentation of several loci conferring resistance to Bt in *Heliothis virescens*.

Discussion

Our experiments have clearly illustrated the major effect that plant stage (immature vs mature) has on the efficacy of Bt cotton plants. This effect was supported by field material over the last two seasons.

This impact could be caused by one or more of several mechanisms.

(i) The amount of toxin expressed in the plants could be reduced because the toxin produced could simply be following the 3 to 5 fold reduction in protein synthesis seen in the leaves of cotton plants as they mature (Thompson et al, 1976). Extraction of Bt from field material by ELISA assay, however, showed a decline in Bt within total protein levels (Holt, 1998)

(ii) Bt production could also be reduced by the chemical inactivation of the Bt gene or degradation of its mRNA. Preliminary studies on cotton containing the Cry1Ac gene, indicate that levels of Bt mRNAs decline as the plants age (Finnigan et al. 1998). Murray et al. (1991) suggest that the low level of expression in Bt transgenic plants is caused by mRNA instability. In addition, auxins, hormones which promotes the growth of plant cells, particularly cell elongation, are known to increase the activity of the Ca MV 35S promoter, also used in Bt transgenic cotton (Liu and Lam 1994). Changes in auxin levels associated with growth, as seen in the stress experiments, could therefore, alter the expression of the gene directly. There is also evidence that another group of hormones, cytokinins, interact with effect of auxins on the promoter (Dominov et al., 1992). This could also explain at least some of the difference in efficacy between immature and mature Bt plants, but it cannot account for the effect of normal leaves from immature and mature plants, on the efficacy of Bt, incorporated as MVP® or the purified Cry1Ac toxin.

(iii) The effect of Bt on the insect must be influenced by the background plant material itself. The amount of Bt produced may not be significantly altered but its effect on the insect could be enhanced in immature plants or sequestered in older plants. Some secondary plant products, such as phenolics and terpenoids have been found to enhance or lower the efficacy of Bt toxin, against lepidopteran larvae (Arteel and Lindroth, 1992, Sivamani et al., 1992, Navone et al., 1993, Gibson et al., 1995, Sachs et al., 1996). We have shown that condensed tannins probably contribute to the decrease in plant efficacy. Preliminary experiments indicated that terpenoids could enhance efficacy. Although not actually measured, levels of anthocyanins were considerably higher in Imperial Red than the other varieties tested with MVP (G. Tanner, pers. com.) This may explain the substantial enhancing effect it had on the efficacy of MVP, compared to the other varieties. Levels of tannins and terpenoids change as the plant develops (Zummo et al, 1984), suggesting that they could cause variations in efficacy associated with plant development, but it is known that environmental stress alters levels of plant secondary compounds (Dixon and Paiva 1995), possibly resulting in further variations in the efficacy of Bt plants.

Overall our results suggest that plant growth stage, condensed tannins, terpenoids, anthocyanins and plant growth rate have an effect on the efficacy of Cry1Ac toxin against *H. armigera* but there is clearly still much ground that needs to be covered on causes of the variations in efficacy of Bt cotton.

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Proposal for Extension of the Results

The results have been recently published in a number of conference proceedings, including the Australian Cotton conference. They are also being prepared for publication in scientific literature. The results have been discussed widely with colleagues for integration into strategies for the deployment of Bt plants.