

Australian Cotton  
Cooperative Research Centre



**Australian Government**  
**Cotton Research and  
Development Corporation**



# Final Report

*Project 3.1.19AC (CRC 34C)*

## *Agronomic Aspects of Bt efficacy in transgenic cotton*

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**CSIRO Plant Industry**  
**Narrabri**

*A final report prepared for the Australian Cotton CRC and the  
Cotton Research and Development Corporation*



**REPORTS**

**Part 1 - Summary Details**

*Please use your TAB key to complete Parts 1 & 2.*

**CRDC Project Number:** **CRC 34C**  
**Annual Report:**  Due 30-September  
**Progress Report:**  Due 31-January  
**Final Report:**  Due 30-September 2004  
 (or within 3 months of completion of project)

**Project Title:** **Agonomic aspects of Bt efficacy in transgenic cotton**

**Project Commencement Date:** 1/7/2001      **Project Completion Date:** 30/6/2004  
**Research Program:** 4 Farming Systems

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## EXECUTIVE SUMMARY

This project aimed to identify factors that could substantially influence Bt efficacy in transgenic cotton. Cry1Ac protein concentration in cotton leaves was measured quantitatively using a commercial ELISA assay. Previous research suggested that Bt efficacy was compromised to some extent when environmental stresses were imposed on transgenic plants. Experiments were designed to investigate a range of factors that may affect Bt efficacy, including: crop nutrition, planting density, light intensity, water management, soil type, herbicides, temperature, soil fertility, growth regulators, and cotton cultivars.

*Imposing very severe agronomic or environmental stresses on transgenic cotton had the potential to substantially reduce leaf Cry1Ac protein levels, although this did not always occur. Plant health/growth must be severely impaired before substantial reductions in leaf Cry1Ac protein levels occur.*

Inadequate N nutrition reduced leaf Cry1Ac protein levels in the first year only, when N fertiliser application had a significant and positive effect on leaf Cry1Ac protein concentration. No effect was observed in the latter two years, despite there being significant responses to N fertiliser application. In some highly sodic commercial cotton fields, severe deficiencies of phosphorus and potassium were encountered that produced leaf senescence and a significant reduction in leaf Cry1Ac protein concentration. Early season zinc deficiency in other fields had no significant effect on leaf Cry1Ac protein concentration. Soil applied potassium fertilisers significantly reduced Cry1Ac protein in Bollgard II leaves.

Planting density had a small significant effect on leaf Cry1Ac protein concentration in the terminal leaves, with higher levels at higher plant density.

Herbicide application had no significant effect on leaf Cry1Ac protein concentrations. Similarly, the application of the plant growth regulator Pix® produced no significant effect on leaf Cry1Ac protein concentration.

Plants subjected to low light intensity (by shading) for one week contained slightly higher Cry1Ac protein concentrations in their leaves than plants subjected to normal light intensity.

Soil waterlogging produced no significant effect on Bt expression in two glasshouse experiments. However, in one experiment, Cry1Ac protein levels in Sicot 289i remained stable, whereas Cry1Ac protein levels in Siokra V-16i continued to decline as the soil dried out. Severe waterlogging of field-grown cotton produced a slight decline in leaf Cry1Ac protein concentration.

Imposing a period of water stress (drought) on Sicot 289RRi and Sicala V-3RRi significantly reduced the leaf Cry1Ac protein concentration.

Glasshouse experiments indicated considerable variability in Bt expression between individual plants of the same cultivar. Sicot 289i plants that expressed either high or low levels of Cry1Ac protein produced progeny with similarly high or low Cry1Ac protein levels.

The cotton industry sees transgenic Bt cotton as the basis for reducing the economic burden of *Helicoverpa* control and the environmental consequences of insecticide use. Identification of means of realising the potential of Bt cotton would assist the industry in economic terms and possibly help avoid problems of resistance to Cry1Ac genes.

This research has identified that agronomic factors have only small impacts on leaf Cry1Ac protein concentration assayed in cotton leaves. Continued research is required to assist cotton breeders to determine the efficacy of new cultivars.

## **Project CRC 34C**

### **Agronomic aspects of Bt efficacy in transgenic cotton**

#### **1. Background to the project.**

Quantitative Bt ELISA assays have only recently become available as a research tool. This tool will not only assist cotton breeders in identifying lines that produce more Cry1Ac, but indicate where agronomic and/or environmental factors impact on the levels of Cry1Ac protein in the cotton plant. For example, crop nutrition, time of sowing, soil condition, soil water management etc may impact on Bt efficacy. Environmental stresses may have a similar impact. Hence, it is possible that the productivity of some Ingard cotton crops may be substantially limited due to these factors.

This project followed on from the interim project CRC26C which addressed agronomic issues with respect to Bt efficacy. Project CSE84C also conducted Bt ELISA assays to assess cotton cultivars under varying conditions. Various projects have attempted to assess the extent to which agronomic factors influenced Bt efficacy. In Project CRC3C (Dynamics of Bt protein in Ingard cotton: mechanisms of variable efficacy against *Helicoverpa*), shade and higher temperatures were observed to increase the subsequent efficacy of Bt in cotton leaves and project CRC10C investigated physiological and agronomic factors affecting the efficacy of Bt in transgenic cotton; both of these projects were researched by Dr Philip Wright.

The cotton industry sees transgenic cotton as the basis for reducing the economic burden of *Helicoverpa* control, and as a means of reducing the impact of insecticidal sprays on the environment. If agronomic factors interfere with Bt efficacy substantially, it will be important for growers to manage their crops to avoid those conditions. Poor Bt efficacy may increase the likelihood of development of resistance to the Cry1Ac proteins and may result in the need for greater use of insecticides.

In the early years of Ingard cotton, the effectiveness of Bt was variable in some situations. This variation was attributed to soil, climate and agronomic factors, as well as variation between individual plants. This project aimed to identify those agronomic factors which most significantly impact on Bt efficacy of cotton and assist management of commercial transgenic cotton. The project would also indicate which cultivars are more robust in terms of Bt expression. Having identified factors that influence Bt efficacy, our management of transgenic cotton crops may be improved.

#### **2. Project objectives and the extent to which these have been achieved.**

The project aims to determine whether various agronomic factors affect the concentration of Cry1Ac protein in the leaves of transgenic cotton. The project will use field experiments to investigate the effects of crop nutrition, plant population, water management, soil type, light intensity, herbicides, temperature, soil fertility, growth regulators, and cotton cultivars to assess differences.

Year 1: Determine levels of Cry1Ac using quantitative ELISA assays in cotton leaf samples taken from experiments and commercial fields. Assess the impact of plant

population, nutrition, waterlogging on Bt efficiency. Determine relationships with leaf N content and Cry1Ac content. Report results.

Year 2: Determine levels of Cry1Ac in plant material collected throughout the season. Assess Bt efficacy in low fertility soils (N, P, K, Zn), cotton subjected to artificial low light conditions and temperature regimes. Report results.

Year 3: Determine levels of Cry1Ac in cotton affected by herbicides, cotton growth regulators. Assess Bt efficacy in cotton planted in sodic/saline soils. Report results. Assessment of Bt efficacy in Bollgard II cotton was included in the third year of the project.

### **3. Methodology and justify the methodology used.**

Commercial kits have recently become available which facilitate the quantitative measurement of Bt protein (Cry1Ac) in transgenic cotton. Field and glasshouse experiments were conducted at ACRI Narrabri, to investigate the effects of various management options and environmental effects on Cry1Ac protein concentration.

Fresh plant material (normally leaf tissue) was collected from field and glasshouse experiments and was analyzed for Cry1Ac concentration using quantitative ELISA assays as required by the manufacturers of the commercial kits. Samples of plant material collected and dried in previous years from nitrogen and cropping systems experiments were also analyzed using the quantitative ELISA assays.

Bioassays have been used in several projects in the past to assess the extent that agronomic factors influence Bt efficacy. A major limitation of performing bioassays on samples from field experiments is the possibility of spray drift from nearby fields.

For all experiments, Cry1Ac concentration was measured on fresh leaf tissue using the Envirologix Cry1Ab/Ac ELISA kit. Results are expressed on a fresh weight basis, as variation in leaf moisture content was not significant.

### **4. Results and Discussion.**

#### **A. Nitrogen nutrition**

Leaf samples were collected from a long-term crop rotation/nutrition experiment at ACRI on several dates during the 2001/02, 2002/03 and 2003/04 seasons.

#### **2001/02**

For the 2001/02 season, some significant differences in leaf Cry1Ac protein concentrations were associated with soil N fertility, but mainly for the youngest leaves (Table 1). Increasing N fertility tended to increase the concentration of Cry1Ac protein in the leaves. The largest differences occurred later in the season when the nitrogen deficient plants were showing symptoms. The high, medium and low nitrogen regimes were, respectively: a vetch/cotton rotation with 200 kg N/ha, cotton only with 100 kg N/ha, and cotton only with no N application. The cultivar was Sicot 289i.

<b>N applied</b>	<b>29 Jan 2002</b>	<b>12 Feb 2002</b>
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(kg/ha)	Node 8	Node 13	Node 18	Node 16	Node 21
0	480	526	435	435	341
100	709	427	418	520	379
200	735	512	485	662	360
<i>lsd 5%</i>	<i>54</i>	<i>54</i>	<i>29</i>	<i>83</i>	<i>ns</i>

**Table 1.** Concentrations (ppb) of Cry1Ac protein in the upper leaves of cotton plants growing under low, medium and high N fertility. These levels are not sufficient to control *Helicoverpa* larvae.

### 2002/03

The variety grown was 289RRi. Only upper leaves were sampled (leaf 2). On the last sample date only, there were significant differences between some treatments (Figure 2), but replications were reduced to only two because of problems with uniformity in the crop. Six treatments were sampled: cotton with nil N, 100 kg N/ha or 200 kg N/ha; and a vetch/cotton rotation with nil N, 100 kg N/ha, or 200 kg N/ha.

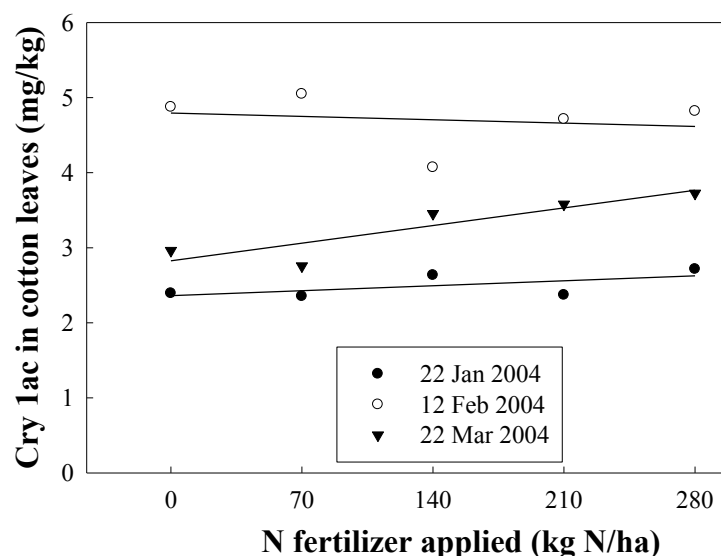
Cry1Ac protein concentration in the leaves of the 0 N treatments was lower than in the 100 kg N/ha and 200 kg N/ha treatments.

N applied (kg/ha)	Cropping system	Cry1Ac protein (ppm)
0	C-C-C	2.52
100	C-C-C	2.61
200	C-C-C	2.63
0	CvCvC	2.68
100	CvCvC	2.83
200	CvCvC	2.63
<i>lsd 5%</i>		<i>ns</i>

**Table 2.** Plants from the lowest nitrogen treatment (cotton with nil nitrogen application) had significantly lower Cry1Ac concentrations than some other treatments. Leaves were sampled on 30 Jan 2003; the cultivar was Sicot 289RRi.

### 2003/04

Sicot 289BR was grown in a N rate experiment at ACRI, Narrabri. The fifth uppermost leaf was sampled on three occasions. The first sampling was after substantial rainfall and some waterlogging on 22/1/2004. Cry1Ac protein concentrations remained high even until the last sampling on 22/3/2004, just prior to crop defoliation, which commenced on 26/3/2004 (Figure 1). No significant effect of N rate was discerned.



**Figure 1.** Cry1Ac protein concentrations in cotton leaves sampled from plots receiving various amounts of N fertilizer presowing was not significantly affected by N fertiliser application.

### **B. Plant population on leaf Cry1Ac protein concentration**

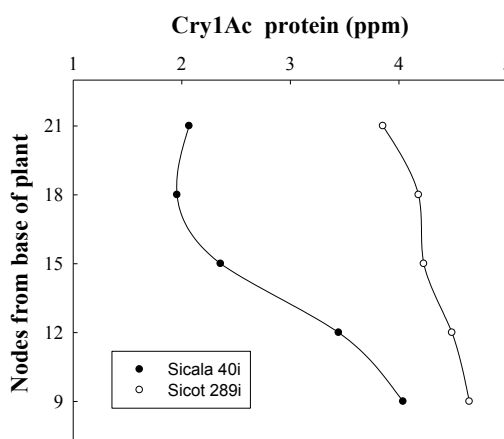
Results from previous experiments in past projects indicated that leaf Cry1Ac protein concentrations were not greatly affected by plant population, but the outcomes were inconclusive. In 2001, two cultivars (Sicot 289i and Sicala V-3RRi) were planted in a field experiment at three densities (2, 8 and 20 plants per metre). Cry1Ac protein was measured at several nodes on four occasions. Leaf Cry1Ac protein concentration in Sicot 289i was significantly increased with increasing plant density, but not in Sicala V-3RRi (Table 3).

plants /m	Sicot 289i			Sicala V-3RRi		
	node 15	node 10	node 5	node 15	node 10	node 5
2	769	779	1063	315	239	487
8	957	958	1167	347	341	652
20	1045	1118	1674	285	333	1059
<i>lsd 5%</i>	<i>158</i>	<i>ns</i>	<i>ns</i>	<i>158</i>	<i>ns</i>	<i>ns</i>

**Table 3.** Cry1Ac protein concentrations (ppb) in leaves of Sicala V-3RRi and Sicot 289i was affected by competition from adjacent cotton plants, as measured by plant density in mid-January 2002.

### **C. Plant Growth Regulator (Pix<sup>®</sup>) application**

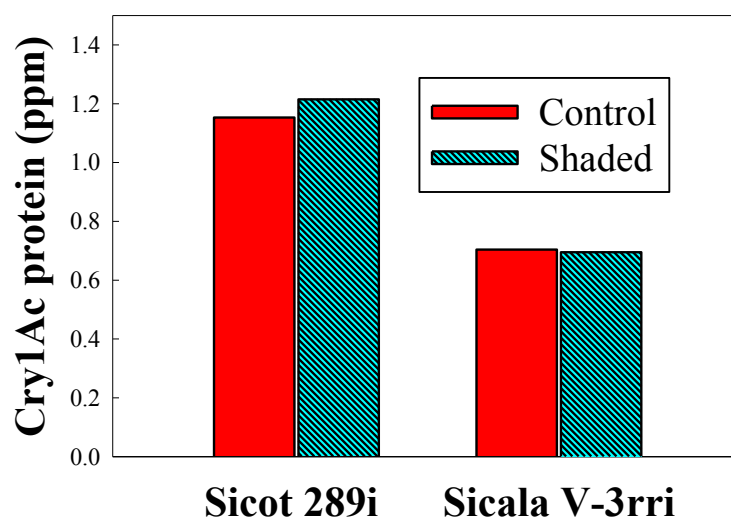
The effects of the application of a growth regulator on Cry1Ac protein levels were assed in a field experiment in 2000/01. Pix<sup>®</sup> was applied to 3 treatments; control, 600 mL at first flower, 600 mL at first flower plus 1 L at cut-out. These rates were applied to two cultivars - Sicala 40i and Sicot 289i. Pix application had no significant effect on leaf Cry1Ac concentration at the end of March 2001, but treatment means were slightly lower (2.6 and 3.6%) where Pix was applied at flowering and at flowering + cut-out, respectively. Differences between the two cultivars were highly significant, but varied throughout the leaf profile (Figure 2).



**Figure 2.** Leaf Cry1Ac concentration in Sicala 40i and Sicot 289i collected on 27 March 2001 following applications of Pix that produced no significant effect on leaf Bt protein.

#### ***D. Light intensity and shading***

A shading experiment was conducted in 2001 on field grown Sicot 289i and Sicala V-3RRi. Shade was applied on 21/12/01 using shade cloth that excluded 70% of incident light. Shade was removed on 28/12/01 and Cry1Ac protein concentrations were measured on that day and 3 weeks after the shade was removed. No shading effect was determined at any time, although Cry1Ac protein concentrations were higher in Sicot 289i than Sicala V-3RRi and higher in the lower leaves than the upper leaves (Figure 3).



**Figure 3.** Shading of cotton plants for 7 days produced no significant immediate or subsequent effect on leaf Cry1Ac protein concentration.

#### ***E. Waterlogging***

### Dr Grant Roberts' waterlogged treatment - 2004

An irrigation x cropping systems experiment was conducted by Grant Roberts and Dirk Richards during 2003/04 at ACRI, Narrabri. The 8 ML/ha irrigation treatment of their experiment was irrigated just before 100 mm rainfall on 14 Jan 2004 which produced severe waterlogging in that treatment; the other two treatments had not been irrigated just prior to the rainfall and were not as severely waterlogged, but were water-stressed prior to their first irrigation. Leaves of Sicot 289BR were sampled on 28 Jan 2004 and no significant effects of severe waterlogging were determined using the quantitative ELISA assay method (Table 4), despite there being a 9% reduction in leaf Cry1Ac concentration.

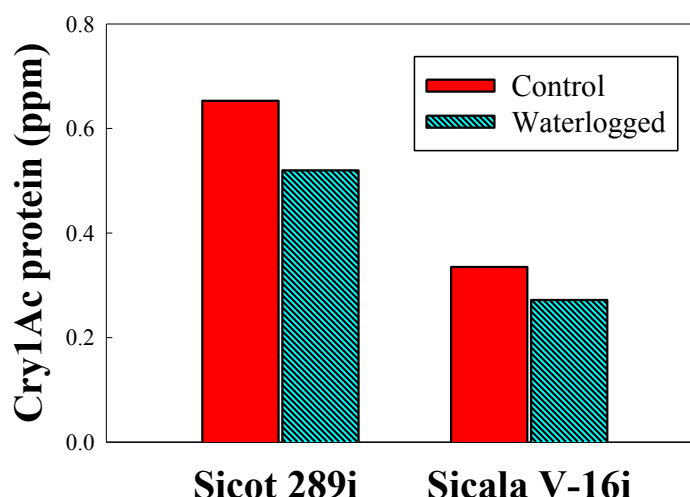
<b>Irrigation treatment</b>	<b>Cry1Ac protein (ppm)</b>
2 ML/ha	2.51
4 ML/ha	2.57
8 ML/ha	2.17

**Table 4.** Cry1Ac protein concentrations were not significantly affected by substantial waterlogging in field-grown cotton.

### **Glasshouse experiments**

#### Experiment 1

A waterlogging experiment was conducted in a glasshouse in September 2001 using varieties Sicot 289i and Siokra V-16i. Waterlogging was imposed on 20/09/01, and watering stopped on 12/10/01 (after 23 days), but soil remained waterlogged for a further seven days. There were no significant differences between treatments at each sampling time. This may be partly due to the difficulty in effectively waterlogging soil in the glasshouse environment.



**Figure 4.** Cry1Ac protein concentration in leaves of Sicot 289i and Siokra V-16i tended to be lower following a substantial period of waterlogging.

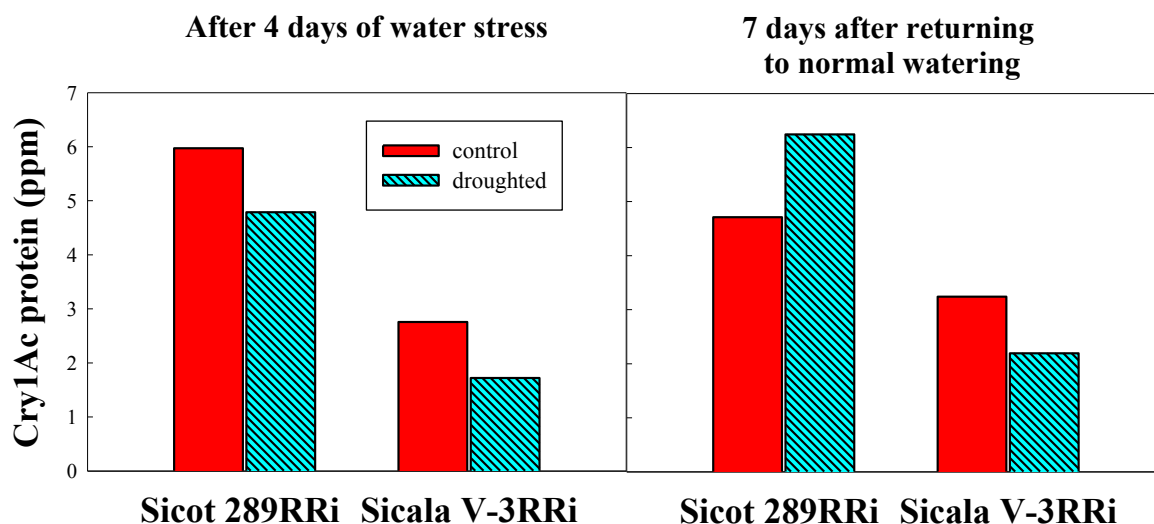
No obvious symptoms of stress appeared in the waterlogged plants until they had been waterlogged for 20 days. There was a substantial reduction in Cry1Ac protein concentration in the leaves of Siokra V-16i compared to the previous sampling date (Figure 4).

### Experiment 2

A second experiment was conducted in the glasshouse in January 2002 using Sicot 289i, with more severe waterlogging. Waterlogging was imposed for 8 days. There were no significant differences between treatments.

### F. Water stress (drought)

Cry1Ac protein concentration in upper leaves of glasshouse grown Sicot 289RRi and Sicala V-3RRi was measured after a period of water stress. The stressed plants received no water for 4 days prior to sampling on 16/09/02 – they were severely wilted. Control plants were watered normally and were not stressed. From 16/09/02 to 23/09/02 all plants were watered normally. There was significantly less Cry1Ac protein in leaves of the stressed plants compared to watered plants, and a significant difference in how the two cultivars recovered from water stress. Stressed Sicot 289RRi recovered their Cry1Ac protein levels to beyond that of the watered plants; this did not occur in the droughted Sicala V-3RRi plants. Results are expressed on a dry weight basis to correct for the difference in moisture content between wilted and non-wilted leaves (Figure 5).



**Figure 5.** The leaves of water stressed plants had lower Cry1Ac protein concentrations than non-stressed plants. Cry1Ac protein levels recovered more fully in Sicot 289RRi than in Sicala V-3RRi after water stress was removed.

### G. Herbicide application

Samples were collected on three occasions from a 2002 field experiment conducted by the farming systems scientist, looking at the effects of eight different herbicide treatments on Roundup-Ready cotton (variety Sicot 289RRi). Sample dates were 29/11/02, 04/12/02 and 17/12/02. There were no significant differences in Cry1Ac protein concentration between the treatments (Table 5).

Herbicide treatment	Cry1Ac (ppm)
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Control	2.76
Diuron	2.75
Dual	2.79
Envoke	3.06
Gesagard	2.97
Staple	2.73
Stomp	3.24
Trifluralin	3.17
<i>lsd 5%</i>	<i>ns</i>

**Table 5.** Herbicide application had no significant effect on Cry1Ac protein concentration in the leaves of Sicot 289RRi.

### **H. Soil sodicity and salinity**

In August 2002, leaf samples were collected from a glasshouse experiment on Sicot 289i plants subjected to different levels of soil sodicity. Seven treatments ranged from a soil exchangeable sodium percentage (ESP) of 2 to 22; electrical conductivity (EC) increased from 0.2 to 1.5 dS/m in these treatments as well. Plants were sampled on two dates. The seed used for this experiment was collected from a single plant of Sicot 289i in order to reduce the previously observed variability in Cry1Ac protein concentration between plants. On the second sampling date, the two highest sodium treatments had lower Cry1Ac protein concentration in the leaves than other treatments, but this was not statistically significant. Plant DM was affected when ESP was increased beyond 15 and declined rapidly with higher ESP and EC levels. Despite this, leaf Cry1Ac protein concentration remained steady at 2.1 ppm with no tendency to decline with higher salinity and sodicity.

Leaves were collected from a commercial cotton crop from areas of high and moderate soil sodicity. The leaves collected from the highly sodic areas were highly deficient in P and K and high in sodium; Cry1Ac protein concentration was 35% lower in these leaves. Thus, extreme nutrient deficiencies observed in cotton crops growing sodic and saline soils can impact on Cry1Ac protein levels in plants grown under field conditions. Application of P and K foliar and soil-applied fertilisers failed to affect leaf Cry1Ac protein concentrations in cotton grown on slightly sodic soil near Wee Waa.

#### Zinc deficiency

Leaves sampled from zinc-deficient plants early in the season had lower Cry1Ac protein concentration than those having higher zinc concentrations.

### **I. Temperature**

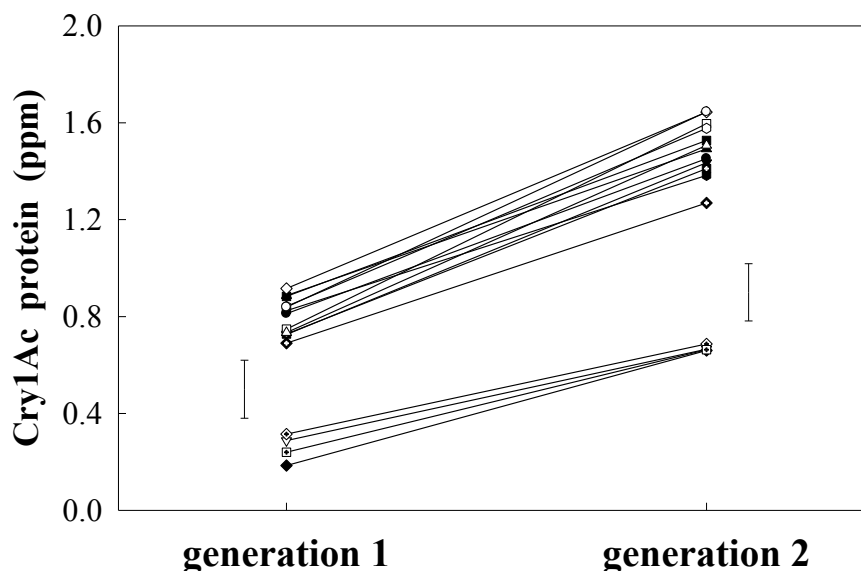
Leaf samples were collected from two glasshouse experiments conducted by Drs Milroy and Bange in which cotton plants (Sicala V-2i) were subjected to cold treatments. There were no discernible differences in leaf Cry1Ac protein concentrations between treatments and low Cry1Ac protein concentrations were measured in all treatments.

Subjecting Ingard cotton to low temperature (cold shock) had no significant effect on leaf Cry1Ac protein concentrations.

### **J. Inheritance of Bt efficacy**

It is widely accepted that there exists a large degree of variability in Bt efficacy between individual plants as well as between cotton cultivars. This became particularly evident during one glasshouse experiment with Sicot 289i when 4 of 16 plants had leaf Cry1Ac protein concentrations about 70% lower than the other 12 plants.

A glasshouse experiment was conducted to look at the inheritance of this variability. Seed was collected from Sicot 289i plants with either low or high Cry1Ac expression, and grown out to about 8-leaf stage. Leaf samples were collected on two dates, and on both occasions Cry1Ac expression was significantly lower in the plants derived from low Bt parents (Figure 6).

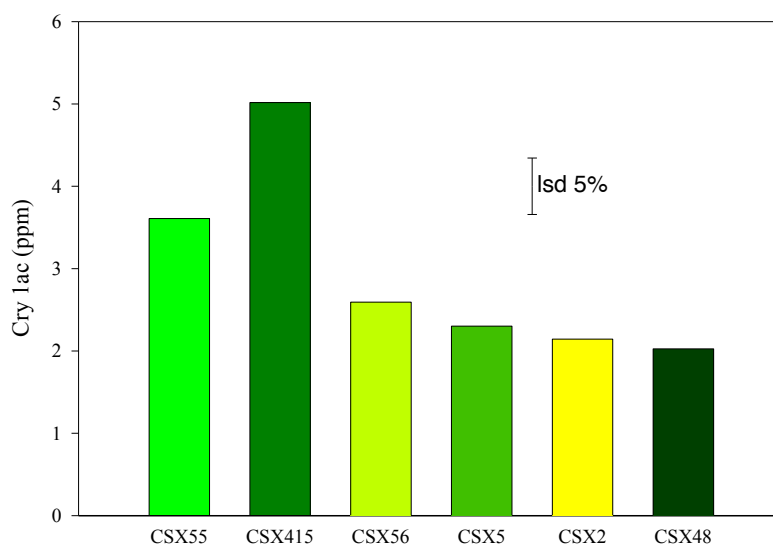


**Figure 6.** Sicot 289i plants with poor Bt expression produced progeny with poor Bt expression.

### **K. Bollgard® II**

During the 2003/04 season, some Bollgard® II cultivars showed high levels of the Cry1Ac protein in their leaves, even up to the time of crop defoliation (Figure 7).

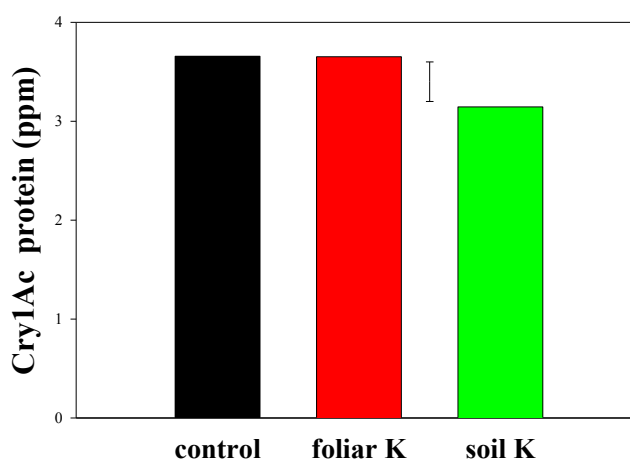
### Cry 1ac in Bollgard II cultivars - 9 March 2004



**Figure 7.** Variation in Cry1Ac protein concentrations between Bollgard® II cultivars prior to defoliation in March 2004.

### Bollgard II response to potassium (K) fertilisers

Application of potassium fertilisers to soil as either KCl or  $K_2SO_4$  reduced Cry1Ac protein concentrations at the end of the season, as depicted below in Figure 8. However, foliar application of  $KNO_3$  in three doses of 10 kg K/ha during the growing season did not reduce Cry1Ac protein concentrations in leaves of Bollgard II cotton. Lint yields were not significantly affected by K application in this experiment. However, in all treatments, leaf Cry1Ac protein concentrations were above the critical level to kill *Helicoverpa* larvae.



**Figure 8.** Cry1Ac protein concentration (26 March 2004) was significantly lower where KCl or  $K_2SO_4$  was applied to the soil.

### 5. Conclusion as to research outcomes compared with objectives. What are the “take home messages”?

- Most agronomic factors examined failed to produce significant changes in the Cry1Ac protein concentrations in cotton leaves
- Even extreme effects (eg severe nutritional deficiencies, severe drought or waterlogging) produced only small reductions in Cry1Ac protein concentrations, and normally these were not statistically significant
- Cry1Ac protein production in transgenic cotton is relatively robust, and is not compromised by adverse growing conditions

**6. How this research has addressed the Corporation's three Outputs - Economic, Environmental and Social.**

This research will aid the industry's aim to reduce pesticide use and ensure the most productive use is made of land used for cotton production. More effective use of Bt cotton will potentially improve profitability and competitiveness.

Transgenic cotton has the potential to provide a safer working and living environment by allowing for using less chemical insecticides to be used and to reduce off-farm impact of those chemicals as well.

The general public's perception of the cotton industry may be improved where they see the adoption of a more environmentally-responsible attitude by the industry which is possible through the expansion of transgenic cotton.

**7. Provide a summary of the project ensuring the following areas are addressed:**

- **technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.)**
- **other information developed from research (eg discoveries in methodology, equipment design, etc.)**

No changes to the Intellectual Property register are required.

**8. Detail a plan for the activities or other steps that may be taken:**

**(a) to further develop or to exploit the project technology.**

No further development is required. However, continued assessment of new cotton cultivars is essential to identify cotton cultivars that have higher Bt efficacy.

**(b) for the future presentation and dissemination of the project outcomes.**

An article is in preparation for publication in the Australian Cottongrower magazine to relate the results of this research to the cotton industry.

A scientific article is also in preparation to record this research and promote the value of this technology and its efficacy to the scientific community.

**(c) Future research.**

There are no plans to continue this research.

**9. List the publications arising from the research project and/or a publication plan.**

An article is in preparation for publication in the Australian Cottongrower magazine relating the results of this research to the cotton industry.

A scientific article is also in preparation to record this research and promote the value of this technology and its efficacy to the scientific community.

**10. No online resources have been developed.**

**11. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian cotton industry or the Australian community.**

These results give confidence to the industry that agronomic management provides very little scope to alter Bt efficacy in transgenic cotton. Only in the most severe instances of extreme variation in environmental or agronomic circumstance was there a significant impact on the level of Cry1Ac protein in cotton leaves. These results emphasise the robustness of the Bt technology.

Possibly, the greatest gains in improving Bt efficacy can be made with breeding more efficacious cotton cultivars. The more recently bred cultivars (including BGII) have showed enhanced levels of Bt efficacy, compared with earlier Ingard cultivars. More importantly, these levels are maintained throughout the cotton season and give adequate protection from *Helicoverpa* pests during that time. Previously, Ingard cultivars could provide protection until flowering at best, whereas recently-bred BGII cultivars can provide protection until defoliation.

This project has identified that agronomic factors generally do not impact substantially on the Cry1Ac content of Bt cotton. Our current management practices appear not to interfere with the efficacy of the Bt technology. While the costs of the technology are determined by its owners, the cotton industry may benefit from improved yields and a safer cleaner environment due to reduced pesticide use.