

# FINAL REPORT

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## **Efficacy of Bt Cotton Plants and Causes of Variation in Performance**

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**CSE 74C**

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CSIRO

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## Part 1 - Summary Project Details

# REPORTS

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## Aims

This project had three principal objectives:-

1. Study the effects of environmental factors on the performance of transgenic Bt cotton plants under controlled, insecticide-free conditions.
2. Test the efficiency of the Bt ELISA technique to predict field efficacy of Bt cotton plants.
3. To determine whether changes in efficacy of transgenic Bt cotton in response to developmental age or environmental stress is associated with a change in the level of *Cry1Ac* transcripts.

## Background

The efficacy of Bt cotton plants against field populations of *Helicoverpa armigera* has not been consistent over the growing season. A repeatable late-season reduction in efficacy has been observed and variation in efficacy has also been recorded early in the season (Fitt *et al.*, 1998). The early season problems are particularly worrying because the cause is unclear, although hypotheses abound.

The variation in efficacy observed could reflect changes at the level of the genome. The unexpected inactivation of transgenes leading to the loss of a newly introduced trait has been well documented (reviewed in Stam *et al.*, 1997). Transgene silencing in response to environmental stresses, such as high light intensity or elevated temperatures, has been reported both for plants growing in the field and in experimental plants growing in controlled environments (Meyer *et al.*, 1992; Walter *et al.*, 1992). An understanding of the molecular basis for both the developmental (late season) and early season decline in efficacy of Bt transgenic cotton will provide a basis for making decisions concerning the management of the current Bt cotton lines and the development of new lines with insertions of the Bt transgene.

The apparent instability of efficacy has consequences for the management of heliothine pests. On occasions, Bt cotton fields have unexpectedly required applications of insecticide for heliothine control in late spring or early summer in addition to the late season applications recommended as part of the resistance management strategy. Furthermore, reductions in efficacy could impact on the rate of evolution of resistance to Bt in *H. armigera* (Daly, 1994).

Evidence to date suggests that more than one factor is involved in the seasonal change; for example Bt levels are expected to decline as protein content of the leaves decreases (G. Constable, pers. comm.). In addition, we have learned from glasshouse experiments that the growth stage plants have reached when efficacy is examined is important (Olsen and Daly, 2000) and that as the plant matures, Bt concentrations decline relative to the total protein in the plant (H. Holt, 1998).

The role of environmental conditions is less clear. A number of researchers at ACRI and in CSIRO Canberra have examined the impact of one environmental stress, namely waterlogging, on efficacy of Bt plants, with mixed success. Our preliminary experiments on



plant stress conducted under controlled conditions indicated that waterlogging, insect damage, low light intensity or high UV radiation are all capable of inducing significant changes in the efficacy of Bt cotton (see Progress report, February 97). The impact of stress on Bt efficacy is not totally unexpected, at least in hindsight, as it is known that secondary products (phenolics and orthoquinones - Dixon and Paiva, 1995) are induced or increase in concentration during periods of physiological stress or physical damage. Such secondary products have been implicated as affecting the efficacy of Bt against noctuid larvae (Arteel and Lindroth, 1992; Sivamani *et al.* 1992; Navone *et al.*, 1993; Gibson *et al.*, 1995), with orthoquinones possibly acting on Bt itself (Ludlum *et al.*, 1991).

It is difficult, if not impossible, in the field or even a glasshouse, to isolate and test individual stress parameters, because other factors such as light intensity, water and nitrogen availability or insect, wind and hail damage cannot be controlled. Thus, experiments to test the interaction between efficacy and the environment must be carried out under controlled conditions such as a phytotron or growth room, where conditions to support normal plant growth can be provided and parameters (light, temperature etc) altered as required. This proposal focused on the impact of environmental stress on the efficacy of Bt plants to control *H. armigera* under controlled conditions. The work was performed in collaboration with Dr Greg Constable who assisted with the planning of initial experiments to ensure that they were relevant to field conditions and current Bt cotton varieties.

This project combined the areas of expertise of two groups, plant efficacy assays and Bt analysis at Entomology (Dr Daly and Dr Mahon) and plant mRNA studies at Plant Industry (Dr Finnegan), to provide a unique understanding of the variations that occur in Bt cotton plants in response to environmental stress. In addition, mRNA levels were also investigated in relation to plant stage. The effect of plant stage on efficacy was previously investigated by Dr Daly/Dr Mahon's group (CRDC Project CSE 53C).

### **Summary of Achievements against objectives**

#### **1. Investigate stress factors individually on one Bt cultivar.**

Three environmental factors were investigated. Temperature was found to affect efficacy, whether plants were grown or germinated at different temperatures or exposed to a change in temperature for a limited time. Damage caused by chewing insects produced a dramatic increase in the efficacy of presquare Bt cotton. Interestingly, plants did not respond in a similar fashion to sucking (aphid) insect damage. A reduction in light intensity did not influence efficacy. Importantly, where plants responded to any of the various treatments, the change in efficacy appeared to be mediated through the physiological background of the plant rather than changes in the concentration of Bt toxin.

#### **2. Investigate combinations of different stress factors and sequential periods of stress.**

Experiments to investigate the effect of sequential damage in presquare Bt plants were begun, however this aspect of the project was soon abandoned, as the period required for the treatments was such that the plants began to square, and this change induced a marked reduction in efficacy that masked any impact of the treatments.

#### **3. Quantify inter-plant variation in efficacy in response to stress.**

In all experiments, plants and leaves were assayed separately. There was no significant difference between plants that could be attributed to environmental stress. Likewise, intra-



plant differences were found to be a function of plant stage rather than treatment of the plants. Plants show a reduction in efficacy when they start to produce squares, with the youngest leaf having the lowest efficacy.

#### **4. Test variations in efficacy in response to stress between commercially available Bt cultivars.**

Combinations of five commercially available Bt cotton varieties were used in the experiments. They included the 757 and 531 'Events' and both normal and okra leaf varieties. All varieties responded similarly when exposed to environmental stress.

#### **5. Establish if performance is related to expression by measuring mRNA levels in the leaves.**

The decline in efficacy of transgenic Bt cotton that occurs after the onset of squaring is associated with a dramatic decrease in the level of *Cry1Ac* transcripts. This decline seems to be due to reduced transcription from the 35S promoter in cotton plants that have initiated flowering as other transgenes under the control of the 35S promoter show a similar decrease in expression at this stage in development. In contrast, although a marked change in efficacy in response to temperature stress and to insect damage was observed, this was not correlated with a change in the level of *Cry1Ac* expression in transgenic Bt cotton. These observations suggest that the change in efficacy is probably due to an alteration in other metabolites that affect the efficacy of the Bt toxin, or to plant secondary compounds that affect the normal [natural?] insecticidal properties of the plant.

### **Relevance to the Corporations three outputs**

Our research has contributed in a similar way to two of the corporation's outputs, "sustainability of natural resources" and "profitability and competitiveness". Through identifying and understanding the causes of variation in the efficacy of Bt cotton plants, particularly the role of plant background and changes in the levels of Bt toxin and mRNA, improvements can be made in any new Bt varieties to make them more "efficient". This will result in reduced dependence on pesticides, leading to both an economic and environmental advantage. The information on the effect of insect damage and temperature on the efficacy of presquare Bt cotton plants, has significance in the prediction of potential resistance problems and therefore will assist to fine-tune resistance management strategies, resulting in a more selective and reduced use of pesticides.

### **Methodology**

**Insects.** Neonate larvae of *H. armigera* were used in all bioassays. The general laboratory strain used (GR) is susceptible to *Cry1Ac* protein toxin. Susceptibility was monitored regularly by an egg treatment method, described in the Final Report for CRDC Project NCQ 1C, 1995.

**Plants.** Experiments were conducted in conditions appropriate for cotton plants within plant growth rooms, or in the Phytotron at CSIRO Plant Industry. In growth rooms, plants were grown in 15 cm pots with a 50:50 mixture of sand and potting mix. Four weeks after planting and subsequently at fortnightly intervals, plants received a solution of 1 g/litre of soluble plant fertiliser containing 27% total nitrogen, at a rate of ca. 500 ml per pot. Up to three presquare, or two fruiting plants were grown in a single pot.



Two plant “stages” were used in bioassays: ‘presquare’ - four to eight weeks after planting and before the appearance of squares, and ‘fruiting’ - six to twelve weeks after planting when squares, flowers, or bolls were present. Six to ten plants were used per treatment/variety at each sampling date. At least two leaves were sampled per plant. Nodes were counted from the base of the plant, starting at the first true leaves.

**Bioassays.** Two bioassay methods were used, ‘leaf disc’ and ‘diet incorporation.’ The methods have been described in the Final Report of CRDC Project NCQ 1C, December 1995 and in Olsen and Daly, 2000. The leaf disc assay was used routinely in all experiments with individual leaves being fed to eight neonates. Diet incorporation assays were performed on a portion of the plant material to generate LC<sub>50</sub>s. Between six and eight plants were assayed per treatment.

**Bt ELISA.** Kits from Agdia Incorporated (USA) were used to measure Bt toxin levels. Protein levels were measured using a standard protein assay (Biorad).

#### **RNA extraction and Northern analysis.**

RNA was isolated from individual leaves according to Jacobsen-Lyon *et al.*, (1995) with the modifications of Townsend and Llewellyn (in press). RNA gel blot assays for *Cry1Ac*, *NptII* and *GUS* expression were done using 10 µg total RNA as described in Sheldon *et al.*, (1999). After washing to remove unhybridized probe, the filters were exposed for 1 – 2 hours on a Phosphor-Imager screen and then scanned using the Imagequant software. Filters were washed again in 2 x SSC (3 x 10 minutes) then treated with ribonuclease A (2µg/ml) in 2 x SSC for 15 minutes at room temperature. The filters were then washed in 0.1 x SSC, 0.1% SDS at 65°C for 15 minutes and exposed to a Phosphor-Imager screen for 24 – 72 hours.

**Data analysis.** LC<sub>50</sub> estimates and F values were calculated for the response of larvae to Bt toxin in the leaves of Bt cotton plants, using the logit analysis of GLIM version 3.77 (Payne 1985).

## **Results**

### **1. Individual environmental stress factors.**

#### **a) Temperature.**

Three responses to temperature were explored. Plants were grown or germinated at different temperatures or were exposed to a change of temperature for a limited period.

#### **1. Plants grown at different temperatures.**

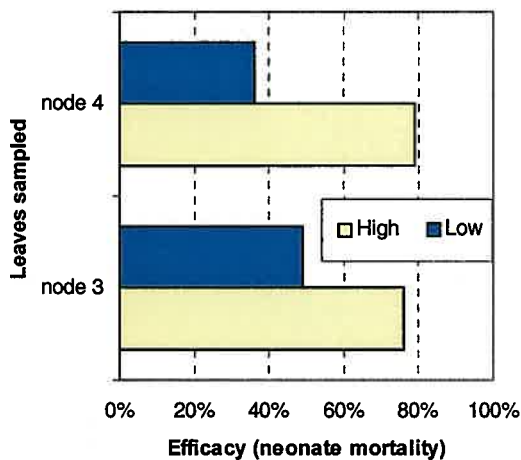
Two Bt varieties were used, Siokra V-15i and Sicot 289i. Two experiments were conducted in growth rooms that provided two temperature ranges: ‘hot’ 22-32°C and ‘cold’ 14-24°C. Plants were sampled at the six-node stage (early presquare). Leaves were sampled at three nodes in the first experiment and two in the second experiment.

When assayed using the leaf disc method, plants grown at the warmer temperature regime induced significantly more mortality ( $F_{1,135} = 54.73$ ,  $P < 0.001$ ) (Figure 1). This was true for all three nodes sampled. Total protein assays and ELISA found that neither protein nor Bt concentrations in plants from the two treatments differed significantly ( $F_{1,10} = 1.77$ ,  $P > 0.05$

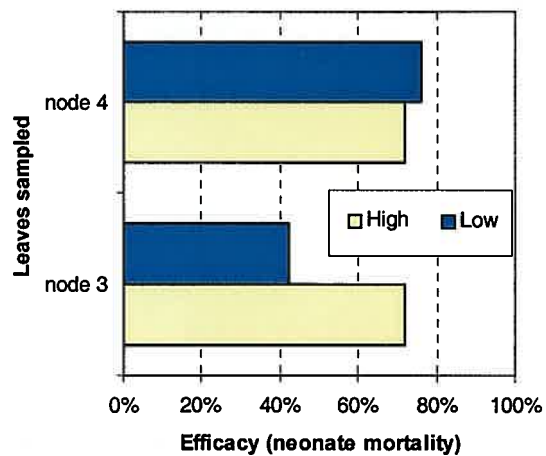
and  $F_{1,10} = 0.4, P > 0.05$  respectively). When assays were performed using the diet incorporation technique, the  $LD_{50}$ s for leaf material were indistinguishable, with  $LD_{50}$ s of 0.17% (% of Bt leaf in diet) with 95% CIs of 0.14, 0.2%.

The most interesting outcome from these experiments was that while efficacy varied when plants were grown under different temperature regimes, Bt levels did not change. Thus it is proposed that a change in the physiological background of the plants was induced when grown at different temperatures and this was detectable in leaf disc assays. It is further proposed that the failure of the diet incorporation assay technique to detect these changes was brought about by the treatment required to prepare the material for assay, (freezing and grinding of the leaf material) which nullify the changes in the physiological background.

**Figure 1: Differences in the efficacy of leaves from Bt cotton plants grown at high or low temperatures.**



**Figure 2: Differences in the efficacy of leaves from Bt cotton plants germinated at high or low temperatures.**



## 2. Plants germinated at different temperatures.

Two Bt varieties, Siokra V-15i and Sicot 289i, were used in a single germination experiment performed in growth rooms. Plants were germinated at either a high (22-32°C), or low temperature range (14-24°C) until the two node stage, then all plants were moved to medium temperature range (18-28°C). Plants were sampled at the six-node stage (early presquare). Plants germinated at the warmer temperature regime induced significantly more mortality at the lowest node sampled (node 3) ( $F_{1,37} = 8.22, P < 0.01$ ) (Figure 2) but not other nodes.

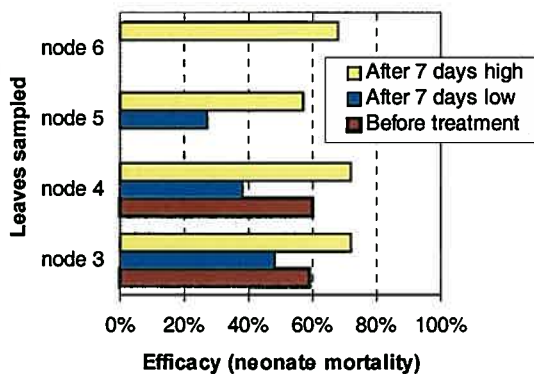
## 3 Plants exposed to change of temperature for seven days.

In preliminary experiments, it was observed that the growth-stage that plants had reached determined the response to short periods of temperature stress. Therefore two sets of experiments were performed, one on early presquare plants, the other on late presquare plants.

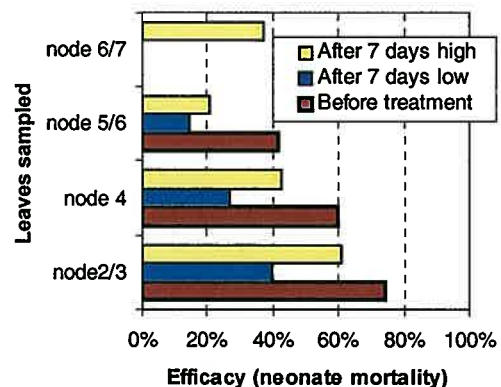
**i) Early presquare plants:** Only one Bt variety (V-15i) was examined. The first experiment was run in the Phytotron in collaboration with Dr Greg Constable. There were three temperature regimes: high (22-36 °C), medium (16-28°C) and low (10-20°C). Plants were grown at the medium temperature range then exposed to the higher or lower temperatures for seven days. Plants were sampled before and immediately after treatment.

A similar experiment was conducted in growth rooms set at three temperature ranges: high (22-32°C), medium (18-28°C) and low (14-24°C). Plants were grown at the medium temperature range then exposed to the higher or lower temperatures for seven days. Plants were sampled before and immediately after treatment. Leaf disc assays detected significant changes in efficacy in response to the changed temperature in the sampling period immediately after treatment, ( $F_{1,167} = 40.92, P > 0.001$ ) (Figure 3) with warm conditions enhancing and cool conditions decreasing levels of mortality. This was true at all nodes sampled. ELISA and protein analyses showed no difference in protein or Bt levels between treatments, (protein-  $F_{1,10} = 4.39, P > 0.05$ ; Bt -  $F_{1,10} = 4, P > 0.05$ ).

**Figure 3: Changes in the efficacy of early presquare Bt cotton plants, exposed to high or low temperatures for seven days.**



**Figure 4: Changes in the efficacy of late presquare Bt cotton plants, exposed to high or low temperatures for seven days.**



**ii) Late presquare plants:** These experiments employed two Bt varieties, Siokra V-15i and NuCOTN. Two experiments were conducted in growth rooms that provided two temperature ranges: high (22-32°C) and low (14-24°C) and plants were sampled before and immediately after treatment. These plants were close to squaring and responded differently to early presquare plants, however an effect of the change in temperature regime on efficacy was still significant, ( $F_{1,234} = 18.36, P < 0.001$ ) (Figure 4) and again warm conditions enhanced and cool conditions decreased the level of mortality. Unlike the less mature plants, the effect was not observed at all nodes. Changes were observed at the lower nodes while the younger leaves (nodes 5/6) did not exhibit significant changes.

Diet incorporation bioassays performed on material from plants exposed to a change in temperature did not detect significant changes in the  $LC_{50}$  for leaves from either treatment, ( $F_{1,111} = 1.3, P > 0.05$ ). This suggests that temperature differences induced a change in the physiological background of the plant rather than in the level of Bt toxin. As discussed above, it is assumed that these background effects could not be detected using this bioassay technique.

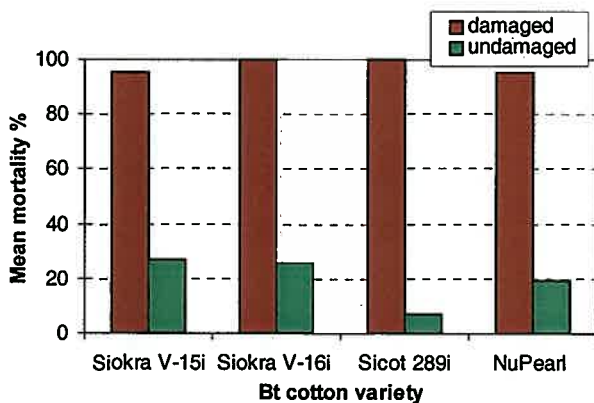
### b) Insect damage.

The effect of chewing insects (*H. armigera* larvae) and sucking insects (*Aphis gossypii*) on the efficacy of Bt plants was investigated. Both presquare and fruiting plants were challenged with *H. armigera* larvae and presquare plants were challenged with aphids.

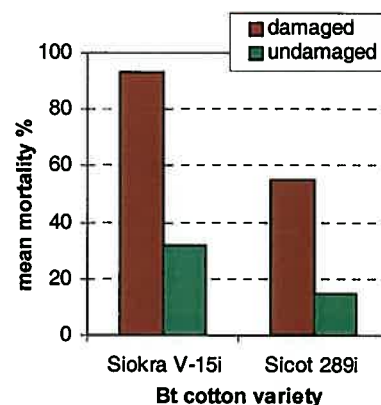
#### 1. Chewing insects (*H. armigera* larvae).

**i) Presquare plants:** Two experiments were conducted, the first with Siokra V-15i alone, and the second with three varieties, Siokra V-16i, Sicot 289i and NuPearl. Plants were infested with larvae for seven days. The leaves were then harvested and used in leaf disc or diet incorporation assays. Leaf disc assays showed a highly significant increase in efficacy of damaged compared to undamaged leaves ( $F_{1,118} = 361.24, P < 0.001$ ) (Figure 5). ELISA and whole protein analyses of the leaves detected significant changes in protein levels after larval feeding, a decrease in total protein ( $F_{1,22} = 33.09, P < 0.001$ ) and an increase in Bt toxin as % of protein ( $F_{1,22} = 13.45, P < 0.01$ ). Despite these changes, the actual concentration of Bt toxin did not change significantly ( $F_{1,22} = 2.89, P > 0.05$ ). Diet incorporation bioassays performed on the same material, did not detect changes in the  $LC_{50}$  for leaves from either treatment ( $F_{1,61} = 1.14, P > 0.05$ ).

**Figure 5: Changes in the efficacy of four varieties of presquare Bt cotton after damage by *H. armigera* larvae.**



**Figure 6: Changes in the efficacy of two varieties of fruiting Bt cotton after damage by *H. armigera* larvae.**



**ii) Fruiting plants:** Two experiments were conducted when plants had reached squaring/flowering stage. Siokra V-15i was tested in the first experiment and both Sicot 289i and Siokra V-15i in the other. As with the experiments on younger plants, damaged plants exhibited significantly enhanced efficacy when assayed using the leaf disc assay ( $F_{1,49} = 87.55, P < 0.001$ ) (Figure 6).

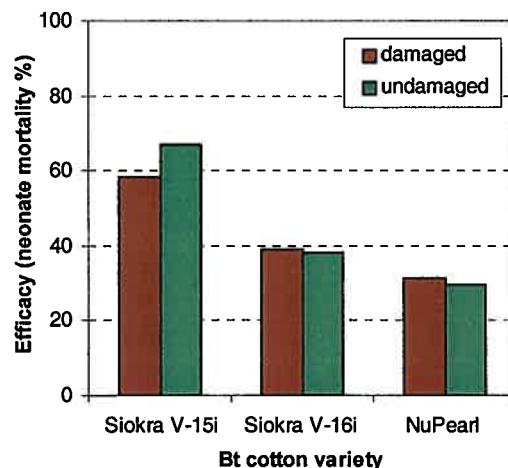
Interestingly, chewing damage on conventional varieties also affected the growth rate of *H. armigera* larvae. When controls for the chewing insect damage experiments, (conventional cotton plants) were fed to *H. armigera* larvae in leaf disc assays, larvae fed damaged leaves were markedly smaller (mean weight 1.2 mg, se 0.25) than those fed undamaged leaves (mean 14.8 mg, se 3.27). These results suggest that changed (enhanced) efficacy observed in

Bt plants ‘stressed’ through chewing insect damage, is induced by a similar mechanism to that observed for temperature stresses, and is not mediated through changes in the amount of Bt present, but rather are due to physiological changes within the plant.

## 2. Sucking insects (*Aphis gossypii*).

For these experiments, three varieties of Bt plants were infested with aphids for ten days with control and infested plants maintained in separate growth rooms. The experiment was repeated, but on this occasion the control and infested plants were housed in the alternative growth room. In these experiments, despite high infestation rates, aphid damage failed to induce detectable changes in efficacy (Figure 7).

**Figure 7: The response of three varieties of Bt cotton to damage by aphids.**



### c) Shade.

A single experiment was conducted in the Phytotron in collaboration with Dr Greg Constable, using Siokra V-15i. A “shade” treatment was induced by a 60% reduction in photosynthetically active radiation. Plants were sampled before treatment, immediately after and a further 5 times, at 10 day intervals. Shade appeared to induce no measurable change in efficacy in the sample period immediately after treatment, ( $F_{1,75} = 0.21, P > 0.05$ ). Efficacy could not be evaluated in later samples as the plants appeared to be affected by handling they had already received.

## 2. Combination / sequential factors.

An attempt was made to investigate the effect of sequential damage/stress on presquare Bt plants, however this approach was soon abandoned as at the end of two periods of treatment, plants had initiated squaring, and as mentioned above, that change signals a marked decline in efficacy. Any effect of the treatments was overwhelmed by the change associated with squaring.



### **3. *Inter- and intra- plant differences related to stress.***

In all experiments, leaves from individual plants were sampled and assayed separately in order to enable an assessment of inter- and intra-plant variability in response to environmental stress. Significant differences in efficacy were consistently observed between nodes of the same plant when approaching squaring. In general, all leaves tested at nodes existing at the time the treatment took place, responded in the same way. The one exception to this situation was for the youngest leaf in the experiment where late presquare plants were exposed to a change of temperature. Plants show a reduction in efficacy when they start to produce squares, with the youngest leaf having the lowest efficacy. Late presquare plants exposed to higher temperatures initiated squaring during the experiment, so the efficacy of the youngest leaf drops markedly in response to this development of the plant, overriding the effect of the treatment. While in leaf disc assays, leaves from the lower portion of plants tended to be more efficacious than younger leaves, in diet incorporation assays the trend was not as clear and on some occasions the situation was reversed.

In general, little inter-plant variability in the response to treatments was detected. In three out of the five groups of plants exposed to higher temperatures for a limited period, a significant difference in efficacy between plants was observed but this is again probably related to small differences in the developmental stage as the plants approached squaring.

### **4. *Variation in response between commercial Bt cultivars.***

The Bt expressing variety Siokra V-15i was adopted as the standard variety and it was employed in all experiments investigating single environmental factors. V-15i contains the 757 Event. Experiments investigating the effect of temperature and insect damage also included at least one of the commercially available varieties containing the 531 Event, NuCOTN, Siokra V-16i, Sicot 289i and NuPearl. Varieties with normal and okra leaves were also employed. As expected, the overall efficacy of the varieties differed. The experiments where the differences were significant were those which evaluated the effect of germination temperature; the effect of late presquare plants when exposed to a change in temperature, and experiments examining the effect of chewing larvae on fruiting plants. The differences observed were both in the initial level of efficacy and the magnitude of response by the variety. However, where a significant response to treatment was observed, it was consistent for all varieties tested in that experiment.

### **5. *The relationship between performance and expression.***

*a. The developmental decrease in efficacy of transgenic Bt cotton is associated with a reduction in CryIAC transcripts in plants grown under both field and controlled environment conditions.*

Preliminary data using RNA isolated from leaves harvested from a pool of plants grown in the field demonstrated that the reduction in efficacy seen at the time of squaring was correlated with a decrease in the level of *CryIAC* transcripts (Finnegan, unpublished). We have now extended this analysis by measuring *CryIAC* transcripts in leaves harvested from individual plants grown under controlled conditions. We found that plants from five cultivars (V-15i, V-16i, Sicot 289i, NuCOTN and NuPearl) demonstrate a similar decline in efficacy after the onset of squaring, under these conditions. In general, this decrease in efficacy was

associated with a decline in the level of transcripts of the 35S::*Cry1Ac* transgene, suggesting that the loss of efficacy can be attributed to changes in *Cry1Ac* expression. The decline in transcripts post squaring was not unique to *Cry1Ac* as the level of transcripts from the linked 35S::*NptII* selectable marker also declined in many plants at this same stage in development (Figure 8a).

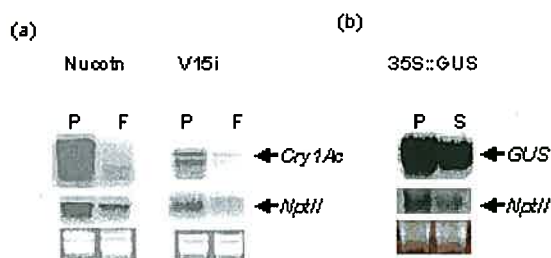
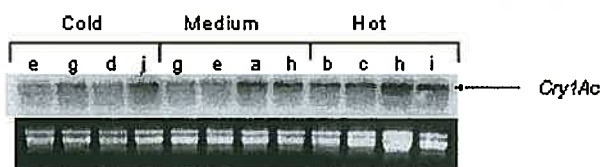


Figure 8 Developmental decline in transcript levels of 3 independent transgenes each under the control of the Cauliflower mosaic virus 35S promoter. (P = presquaring; S = squaring; F = fruiting)

These observations suggest that the expression of transgenes under the control of the 35S promoter decreases in post-squaring cotton. This is supported by data from an independent transgenic line containing a 35S::*GUS* transgene whose expression also declined after the onset of squaring (Figure 8 b).

*b. Cry1Ac transcript levels are not affected by the temperature at which plants are grown.* Leaf material for this analysis was the same as that used in experiments in section 1.a.1. The level of *Cry1Ac* transcripts was compared in RNA isolated from the equivalent leaves on plants grown at each temperature. The efficacy of these plants varied, but we found no correlation between the level of *Cry1Ac* transcripts and efficacy (Figure 9).



Temp	Cold				Medium				Hot			
Plant	e	g	d	j	g	e	a	h	b	c	h	i
Kill %	50	60	20	20	30	30	80	30	30	50	70	70

Figure 9 *Cry1Ac* expression does not correlate with bioefficacy of *Bt* toxin in plants grown at different temperatures. *Cry1Ac* expression in individual leaves harvested from plants grown at low, medium or high temperatures.

The temperature at which plants were grown did not affect the expression of the *Cry1Ac* transgene. Similarly, expression of the 35S::*NptII* transgene was unaffected by the growth temperature.

*c. Cry1Ac transcript levels are not affected by a short temperature stress.*

Leaf material for this analysis was the same as that used in experiments in section 1.3. RNA was isolated from individual leaves and the level of *Cry1Ac* transcripts measured.

Initially, we compared the level of *Cry1Ac* transcript in leaves that expanded under the new growth conditions with that in leaves that had developed prior to the change in temperature.

We found no difference in expression of *Cry1Ac* following the change in growth temperature (Figure 10).

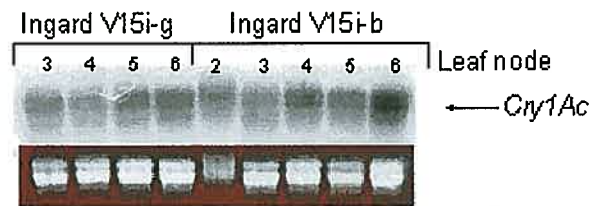


Figure 10 *Cry1Ac* expression is not altered by temperature stress. *Cry1Ac* expression in individual leaves harvested from 2 Ingard plants following the shift from control to high temperatures. The leaf at node 6 expanded under the new growth conditions

We next compared the level of *Cry1Ac* expression in plants that had experienced a change in growth conditions and that showed variable efficacy. As for the plants grown continuously at different temperatures, we found no correlation between efficacy and the level of *Cry1Ac* transcripts.

Taken together, these data suggest that the changes in efficacy associated with environmental stresses, such as temperature extremes, are not caused by a change in *Cry1Ac* expression. It is concluded that environmental conditions influence the production of other metabolites that affect efficacy either by interacting directly with the *Cry1Ac* toxin, or by directly affecting caterpillar viability. The production of these metabolites could be the major cause of alterations in efficacy of Bt transgenic cotton that has been subjected to environmental stresses.

*d. Insect damage does not alter the expression of the Cry1Ac transgene*

Northern analyses of RNA isolated from insect damaged leaves taken from cultivars V-15i, V-16i or NuPearl has shown that there is no change in the level of *Cry1Ac* transcripts in response to aphid damage (Figure 11) which was expected given the absence in changes when such leaves were used in leaf disc assays.

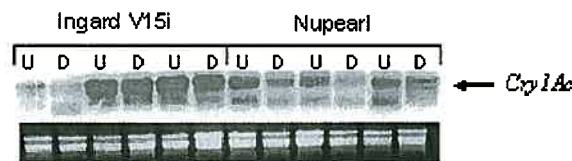


Figure 11 *Cry1Ac* expression is not affected by damage due to sucking insects. *Cry1Ac* in individual leaves harvested from plants that were damaged by aphids (D) or undamaged controls (U)

In sections 1.B we have shown that transgenic Bt cotton plants that have been damaged by a chewing insect pest (but not by a sucking insect) show enhanced efficacy in the fully expanded, but undamaged leaves. Our data on the response of plants to damage by caterpillars is less clear as we were unable to isolate good quality RNA from these plants. However, the available data suggest that there is no change in *Cry1Ac* expression in response to this challenge.

## Conclusion and discussion

This study examined the impact of environmental factors on the efficacy of Bt plants. Such effects were clearly shown. Temperature at germination, growth at high or low temperature or short periods at high or low temperatures all influenced the survival of *H. armigera* larvae on Bt plants. It is clear that under certain environmental conditions, eg cool germination and cool growing season, the efficacy of Bt plants can be markedly reduced. The ELISA and RNA analyses provide evidence that the titre of Bt and the expression of the *Cry1Ac* transgene are not changed, thus we must look for different mechanisms to explain the changes in efficacy.

Plants produce an array of chemicals, some of which appear to be specifically targeted to limit insect or herbivore damage. Plants are also capable of responding to stress through the production of additional quantities of these secondary compounds or perhaps a unique set of such compounds. Our studies have demonstrated that cotton plants respond to environmental conditions by modifying their metabolism, (presumably through the modifying the production of secondary compounds, however, interaction of metabolites with Bt is also possible) and that these compounds could impact on the survival of *Helicoverpa armigera*. What was unknown prior to this study was the linkage between environmentally induced impacts on insect survival and the impacts on survival through the toxic effects of the Bt toxin produced by Bt plants. Did a stress response stimulate (or reduce) Bt toxin production



as well as the production of chemicals that brought about the response, or was the stress response independent of the production of Bt toxin? Our data conclusively support the latter of these alternatives. Whenever we were able to stimulate a response that was detectable through enhanced or reduced mortality in *H. armigera*, (eg temperature change or damage by chewing larvae) the ELISA analyses showed that titre of Bt toxin in the leaves remained unchanged, and the mRNA analyses showed that mRNA production also was unaffected. Thus, Bt production did not appear to be altered by environmental factors or stress, and the overwhelming feature of the Bt toxin production profile is a decline post-squaring. At the mRNA level, other mRNA products under the influence of the 35S promoter also decline at this stage.

An important outcome of this work is the appreciation of the importance of the promoter in post squaring cotton and this has initiated efforts to seek new promoters that will induce a more consistent production of Bt throughout the life of the cotton plant. A second important outcome relates to the value of ELISA tests on Bt varieties to predict plant efficacy against *H. armigera*. In general, the test is predictive of efficacy, as it detects the major change in Bt titre associated with plant stage (Holt, 1998). However, it failed to detect the quite marked changes in efficacy that we have discussed above that were associated with the plant response to environmental stress. Similarly, *CryIAC* transcript levels do not predict changes in efficacy in response to environmental or physical stresses.

In the studies reported here, we have concentrated on evaluating the interaction of stress and mortality induced while Bt levels are high. It is known that field-grown cotton is generally less favourable for *H. armigera* survival than glasshouse-grown plants. We have shown that quite mild "stresses" on what are otherwise cosseted plants, are capable of inducing a response from cotton that causes marked impacts on insect survival. Plants in the field are exposed to far greater temperature range and environmental assaults (eg wind and hail damage; waterlogging, nutritional stresses, insect damage and root damage) and that such plants are perhaps in a continual state of stress. We predict, on the basis of our work on plants grown under controlled conditions, that under some conditions (for example high temps and insect damage) there will be an additive effect, between Bt toxin and the plant metabolites on *H. armigera* survival. Importantly, this additive effect will probably sustain efficacy for a period after Bt toxin levels begin to decline. As our study did not deal with field grown Bt plants, we are unable to assess the duration of the period where Bt alone is not toxic to *H. armigera*, but Bt levels supplemented with the effect of the plant responses renders the crop toxic. Whatever the duration of the additive effect of the two mechanisms, it is likely to play a role in reducing the period that insects survive in Bt crops. This has two consequences. Firstly, it will reduce the duration of the season that synthetic insecticides are required and, secondly, it will delay the period during which individuals carrying resistant alleles (to Bt) might be advantaged and thus increase in frequency. It would be of considerable interest to evaluate the impact of these non-Bt responses of plants to strains of *H. armigera* that are resistant to Bt as it may be that through enhancing the existing defensive mechanisms possessed by plants, existing strategies designed to slow the rate of evolution of resistance to Bt toxins could be supplemented.

This collaborative work is being prepared for publication in an international journal.



## Dissemination of results

September 1998, Dr Daly presented a talk "Efficacy of Bt cotton plants in Australia – what is going on?" at the 2<sup>nd</sup> World Cotton Conference, Athens, Greece.

July 1999, Dr Daly presented a poster on "Seasonal Changes in the Efficacy of Transgenic Bt Cotton" at the XIVth International Plant Protection Congress in Jerusalem, Israel and also at the Australian Entomological Society Conference in Canberra in September 1999.

April 2000, Dr Daly presented a talk on the "Bioefficacy of Bt Cotton" at Rothamsted Agricultural Station, UK and also at CAB International, Silwood Park, UK.

August, 2000 Dr Daly presented a poster "Bt Cotton: Relationship Between Bioefficacy, Gene Expression And Protein" at the International Congress of Entomology in Brazil and at the ACGRA Cotton Conference in Brisbane.

November 2001, Ms Olsen presented a poster "Bt Cotton, The Effect of Two Environmental Factors, Temperature and Insect Damage, on the Efficacy of Presquare Plants" at the 4<sup>th</sup> Pacific Rim Conference on the Biotechnology of *Bacillus thuringiensis* and its Environmental Impact, in Canberra.

In June 2001, Dr Mahon discussed the work performed in this and other projects (CSE 89C) in a presentation to Cotton consultants AGM.

## Publications

**Fitt, G. P., J. C. Daly, C. L. Mares, and K. Olsen. 1998.** Changing efficacy of transgenic Bt Cotton – patterns and consequences, pp.189-196. *In* M. P. Zalucki, R.A.I. Drew, and G. G. White [eds.], Pest Management – Future Consequences, Vol 1. University of Queensland Printery, Brisbane, Australia.

**Olsen, K. M., J. C. Daly, and G. J. Tanner. 1998.** The effect of cotton condensed tannin on the efficacy of the Cry1Ac  $\delta$ -endotoxin of *Bacillus thuringiensis*, pp. 337-342. *In* Proceedings, 9th Australian Cotton Conference, 12-14 August 1998, Broadbeach, Queensland. Australian Cotton Growers' Research Association, Wee Waa, NSW, Australia.

**Daly, J.C., and G.P. Fitt. 1998.** Efficacy of Bt cotton plants in Australia – what is going on? *In* Proceedings of the 2<sup>nd</sup> World Cotton Conference, Athens, Greece, September 1998.

**Olsen, K.M., and J.C. Daly. 2000.** Plant-Toxin Interactions in Transgenic Bt Cotton and their Effect on Mortality of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 93 (4): 1293-1299.

**Roush, R.T., G.P. Fitt, N.W. Forrester, and J.C. Daly. 1998.** Resistance management for insectidal transgenic crops: theory and practice, pp 247-257. *In* M. P. Zalucki, R.A.I. Drew, and G. G. White [eds.], Pest Management – Future Consequences, Vol 1. University of Queensland Printery, Brisbane, Australia.

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## Summary

The efficacy of Bt cotton plants against field populations of *Helicoverpa armigera* has not been consistent over the growing season. Any reduction in efficacy has detrimental effects, because it increases both the use of synthetic insecticides and the opportunities offered to *H. armigera* to evolve resistance to Bt. Changes in efficacy could be due to changes at the level of the genome or in the physiological makeup of the plant and may be induced by environmental conditions. This project aimed to provide an understanding of the effect of environmental stress on early season cotton, by simultaneously monitoring efficacy, Bt concentration and *CryIAC* mRNA production of plants grown under controlled environmental conditions.

Three environmental factors were investigated. Temperature was found to affect efficacy, whether plants were grown or germinated at different temperatures or exposed to a change in temperature for a short period. Damage caused by chewing insects produced a dramatic increase in the efficacy of presquare Bt cotton. Sucking (aphid) insect damage did not induce changes in efficacy nor did a reduction in light intensity. Commercially available Bt varieties responded similarly when exposed to environmental stress.

Importantly, where plants responded to a treatment, change in efficacy appeared to be mediated through modification of the physiological background of the plant rather than changes in the level of *CryIAC* expression or in the concentration of Bt toxin. These observations are evidence that the change in efficacy is due to an alteration in other components of the plant, perhaps metabolites that interact with Bt toxin directly, or plant secondary compounds that affect the normal insecticidal properties of the plant. Secondary plant products are induced or increase in concentration during periods of physiological stress or physical damage. Such secondary products have been implicated as affecting the efficacy of Bt against noctuid larvae.

In contrast to the changes in efficacy in response to the environmental challenges we employed, the decline in efficacy of transgenic Bt cotton that occurs after the onset of squaring is associated with a dramatic decrease in the level of *CryIAC* transcripts. This decline seems to be due to reduced transcription from the 35S promoter in cotton plants that have initiated flowering as other transgenes under the control of the 35S promoter show a similar decrease in expression at this stage in development. The ELISA technique detects this change in Bt titre, however the quite marked changes in efficacy we have ascribed to the response of the plants, are superimposed on the efficacy due to Bt alone.

This study examined the impact of environmental factors on the efficacy of Bt plants. Such effects were clearly shown. It would be of considerable interest to evaluate the impact of the non-Bt responses of plants to strains of *H. armigera* that are resistant to Bt as it may be that through enhancing the existing defensive mechanisms possessed by plants, existing strategies designed to slow the rate of evolution of resistance to Bt toxins could be supplemented. The information from this study could also assist with the prediction of potential resistance problems and therefore will assist in fine-tune resistance management strategies.