Efficacy of Bt Cotton Plants in Australia: What is Going On?

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ABSTRACT

We report on the patterns and causes of variable efficacy of Bt cotton plants for control of Helicoverpa spp. in Australia. Initially, it was hoped that these plants would be efficacious against their target pests throughout the cotton growing cycle. Spatial and temporal changes in efficacy have been followed in field plots of cotton using whole leaf bioassays and changes in Bt concentration and efficacy have been quantified using a variety of new bioassay approaches. In the laboratory, we have measured the response of the plants to a variety of stresses, including extremes of water availability and temperature. A consistent decline in plant efficacy was observed from mid-summer and, in the field, some crops require alternative treatments to control Helicoverpa from peak flowering onwards. By late season, larvae can develop and survive to pupation on Bt cotton. Further, some plants have variable efficacy in early season, even before squaring has commenced. There are many possible causes for the seasonal decline and variability. At this stage, we have evidence for a decline in the amount of Bt protein present and also for possible interference by plant factors in the availability of the Bt protein to the insect.

Introduction

Transgenic cotton was introduced into Australia in spring 1996 for the control of the major lepidopteran pests, *Helicoverpa armigera* (Hübner) and *H. punctigera* (Wallengren). This cotton, called INGARD®, was to form the platform from which the Australian cotton industry would move further towards integrated pest management with a reduced dependence on pesticides. Certainly, in the first two seasons of commercial use, there has been a significant reduction in pesticide usage on Bt cotton of about 60% in most cotton regions (Fitt *et al.*, 1998). However, variation in efficacy of the plants in controlling *Helicoverpa* spp. has been observed in space and time. In collaboration with others, we have sought to document and understand the pattern and causes of this variation.

INGARD® cotton has been genetically engineered to express the Cry1Ac toxin protein from the bacterium, *Bacillus thuringiensis*. While the promoter for the inserted gene is a constitutive one, plants expressing the insecticidal protein do not have uniform efficacy over the growing season. Field trials by Fitt *et al.* (1994), before commercial release of the Bt cotton, revealed that plant efficacy declined over the growing season. They detected survival of hatchling larvae from mid-summer when these larvae were fed in the laboratory on leaves from field-grown plants. Efficacy had declined sufficiently by the time the crops started to senesce that pupae could be found in the field under transgenic cotton plants. More recently, variation in efficacy of some field-grown cotton has also been detected early in the growing season (Fitt, 1998). The early season problems are particularly worrying because to date it is not clear what factors are involved, although there are many hypotheses (see below).

Changes in efficacy have had consequences on both pest and management for *Helicoverpa* pests. Some Bt cotton fields have required applications of insecticide in late spring or early summer for control, of *Heliothines*, in addition to the late season applications recommended in the resistance management strategy. Problems with efficacy could also increase the possibility of, or rate of development of, resistance to Bt crystal proteins in *H. armigera* (Daly, 1994).

There are many possible causes for the decline in efficacy of Bt cotton plants, but they fall into two groups:

* the expression of the Cry1Ac gene may be reduced — by down-regulation of the promoter, by silencing of the gene, or by post-transcriptional events, or
* the quantity of Cry1Ac protein may be reduced — by greater turnover of the protein or by sequestration of the protein, or by dilution due to plant growth or aging (Daly, 1994).

Evidence to date suggests that more than one factor is involved in the seasonal change: Bt levels are expected to decline as the protein content of the leaves decreases but we also know from glasshouse experiments that the growth stage of the plant is having a major influence on efficacy (Daly and Olsen, unpublished) and that Bt concentrations are also declining relative to total protein (Holt, 1998). In addition, preliminary experiments have shown that Cry1Ac transcript levels

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are unstable in both immature and mature plants. Stress may also be a factor. Some secondary products, such as phenolics and orthoquinones that are produced or increased by plants during periods of physiological stress or physical damage, do seem to alter the efficacy of Bt crystal proteins against Noctuid larvae (Sivamani *et al.*, 1992; Navon *et al.*, 1993; Gibson *et al.*, 1995); the latter compound possibly acts on Bt protein itself (Ludlum *et al.*, 1991).

We have conducted a range of field and laboratory experiments to examine the patterns and causes of the variation in field performance of Bt cotton plants. Here we report on the observed changes and investigate a number of causes.

Field Data

The field efficacy of cotton plants in *Helicoverpa* spp. control was measured in three ways in replicated trials. The same plots of cotton were used in each method. Further details are available in Fitt *et al.* (1998). All results reported refer to the transformation events, MON757 (varieties Sicala V2i and Siokra VI5i, Cotton Seed Distributors) and MON531 (variety Nucotn 37, Deltapine Australia).

(1) *Whole leaf assays*. Leaves were collected from unsprayed plots of Bt cotton from the third node (numbered from the top of the plant). Neonate larvae, susceptible to Bt, were fed on the leaves for 5 days. Percentage mortality of larvae and growth stage of survivors were measured.

(2) *Field counts of* Helicoverpa *spp*. Weekly counts were made in the same plots of cotton to determine field densities of eggs, larvae and pupae.

(3) *Diet incorporation of leaf material.* Bt cotton leaves from the 3rd node were incorporated into modified *Heliothine* diet in a serial dilution. This enabled changes in efficacy to be quantified. Only plants from MON757 were used.

These three sets of data all show a consistent decline in efficacy of cotton plants from mid to late summer, although the actual date at which the decline is detected is not the same. Figure 1 illustrates the results of the whole leaf assays for 1996/97, averaged over the three cotton varieties. Overall, plants had high efficacy until late December/early January. Efficacy rapidly declined over the following few weeks, during the period of peak squaring and flowering and remained low until plant senescence. This general trend is similar to that observed in all field grown crops since 1992/93 although the rate of decline varies considerably. An early, and unexpected, drop in efficacy was also observed in late November 1996 which coincided with reports from some commercial farms that larvae were surviving beyond first instar.

Despite the changes in efficacy detected in bioassays, field counts of larvae and pupae indicate that significant survival of larvae beyond 1st instar did not occur in the field until late January/early February. Bioassays have been shown to overestimate likely field survival by about 30% (Fitt, 1998) due to the range of other factors which impinge on larval development in the field. This, combined with the reduced growth rates of larvae, even if they do survive, means that many crops have not required insecticide treatments until late season, although this is dependent on the abundance of *Helicoverpa*.

Diet incorporation assays, using Bt leaf powders, do detect a measurable change in efficacy of Bt cotton at the same time as the whole leaf bioassay. The change in LD_{50} from the beginning of the growing season to early January was about 4-fold. However, the greatest change was not detected until late in the season, in early March, when the change in LD_{50} was 110-fold. No such major change was evident in the whole leaf bioassays which, by that time, often show consistently high survival. Even so, the development rate of surviving larvae is significantly delayed throughout growth of the plants.

Changes in Bt protein concentration

Total protein in cotton leaves declines as a new leaf unfurls, and also as the plant senesces (Thompson *et al.*, 1976). Young leaves on mature plants are initiated with less protein compared to leaves at the same node on younger plants. This developmental change in protein concentration could directly influence the dynamics of Bt production and be contributing to a decline in efficacy.

Holt (1998) used an ELISA-based assay to measure the amount of soluble Cry1Ac protein from Australian-grown cultivars of cotton. Plant samples from Bt plants used for whole leaf bioassays above were used in her analyses. She observed a highly significant relationship between bioassay survival and estimated Bt protein concentration, expressed as the percentage of total protein in the tissue (r_2=0.77, Fitt *et al.*, 1998). This suggests that the seasonal decline in soluble Bt protein is over and above the decline in total protein.

Plant Age

Olsen and Daly (unpublished) have examined the impact of plant background on the efficacy of Bt cotton plants. They collected leaves from immature (pre-squaring) and 'mature' (here defined as from first flower) plants grown in the glasshouse. In the first experiment, they performed a series of bioassays in which Bt leaves, either immature or mature, were added in serial dilutions to either immature or mature non-Bt leaves (all four combinations). In the second experiment, they added Cry1Ac toxin (formulated product MVP®, Mycogen Corporation, USA) in a serial dilution to non-Bt leaves, either immature or

mature. In the third experiment, they dipped disks of non-Bt leaves into a serial dilution of MVP®. In all bioassays, neonate larvae were fed on the Bt/leaf mix and mortality was recorded after 8 days.

Olsen and Daly (unpublished) observed reduced efficacy of the Bt whenever it was placed into plant material collected from leaves from mature compared with immature plants. Mature Bt plants were 49-fold less efficacious when mixed in with mature non-transgenic leaf material than when the same Bt plant material was mixed with immature non-transgenic leaf material. The same trend was observed for immature Bt plants but the effect was smaller. MVP® toxin had reduced efficacy when added to mature non-transgenic leaves (164-fold) or when mature leaves were dipped into MVP® solution (ca. 700-fold), compared with similar bioassays using immature non-transgenic leaves.

From these results, Olsen and Daly (unpublished) proposed that plant factors must be responsible in part for the decline in efficacy of Bt plants over the growing season.

Tannins

Olsen *et al.* (1998) looked at the possible role of condensed plant tannins (proanthocyanidins) in an antagonistic effect on the efficacy of Cry1Ac protein. Condensed tannins are known to increase in concentration during the growth phase of the cotton and a number of authors have reported the possibility of tannins interfering in the performance of Cry1Ac against insects.

Olsen *et al.* (1998) grew three cultivars of cotton in full sun: two commercial cultivars (Siokra V15 and Sicala V2) and one non-commercial, high tannin cultivar (HT 35-14-3), to obtain maximum expression of the condensed tannins. The experiments were repeated in growth rooms. Leaf material was collected from 3rd node leaves and used in diet incorporation bioassays in two sets of experiments (1) with added Cry1Ac toxin, as MVP®, and (2) the Cry1Ac toxin plus polyethylene glycol (PEG), 3% by weight of leaf. Condensed tannins bind to PEG in preference to plant proteins. Neonate larvae were fed for 8 days on the leaf material and mortality was recorded.

Olsen *et al.* (1998) observed that efficacy of mature plants from the high tannin line grown outside, was 950-fold lower compared with plants grown in growth rooms. The difference was lower for the two commercial cultivars (ca. 55-fold). After the addition of PEG to the samples grown outside, there was no difference in efficacy between plants grown inside and outside. This suggests that tannins produced in lines grown outside is interfering with the action of the Cry1Ac protein.

Similar results were observed when PEG was added to cotton collected from commercially grown cotton. Some 'reversion' of efficacy was observed with the addition of PEG, but efficacy still remained very low on cotton collected after mid-summer. Altogether, these results show that condensed tannins may be having an antagonistic effect on the efficacy of Cry1Ac toxin against *H. armigera* but that any effect can only be partly accounting for the seasonal decline performance of Bt cotton.

Conclusions

Our results clearly show that the efficacy of Bt cotton plants in killing *H. armigera* larvae declines during the growing season. We report on a number of causes such as changes in plant protein concentration the age of the plant and tannin levelsin plant tissue. This is not an exhaustive list of possible causes. Experiments are under way by other Australian colleagues to investigate stress as a possible trigger for changes in efficacy. Expression of the Cry1Ac gene is also being examined.

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Figure 1. Seasonal pattern of neonate survival in a laboratory bioassay of leaves from 3 transgenic Bt cotton varieties in the 1996/97 growing season. Two varieties (Sicala V2i and Siokra V15i) expressed the MON757 event while the third (Nucotn 37) expressed the MON531 event. Mortality is corrected for control mortality. Points are mean and SE.