

EFFICACY OF INGARD COTTON - PATTERNS AND CONSEQUENCES

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INTRODUCTION

The past growing season (1997/98) was the second commercial year of INGARD cotton varieties with some 60,000 ha planted across all growing regions. In the first two seasons of commercial use INGARD varieties have reduced pesticide use by 50-60% and achieved similar or better yields to conventional varieties. This represents a significant step forward in reducing pesticide use in the cotton industry. There have been several reports from CRDC and Monsanto regarding the performance of INGARD crops in relation to pesticide use and economic value to growers and I don't intend covering these issues here. Obviously the bottomline when it comes to INGARD performance is efficacy, an issue which is amenable to research. To provide most value and ease of management, transgenic cotton crops need to provide consistent and hopefully high capacity to kill the target pests quickly ie. high efficacy. Early in development of transgenic cottons the expectation was that expression of the Bt protein would be consistent throughout growth of the crop and consequently that control of the target pests would be provided almost season long. The Cry IAc gene is driven by a promoter which gives constitutive expression in all tissues in the plant, although there are significant differences between plant structures in the level of Bt protein production. However, from the very first year of small scale field trials it soon became evident that efficacy of leaves and reproductive tissues declined during plant growth (Fitt GP, Mares CL, Llewellyn DJ. 1994 *Field evaluation and potential ecological impact of transgenic cottons (Gossypium hirsutum) in Australia. Biocontrol Science and Technology 4: 535-548*) and that some larvae were able to survive beyond first instar. It was not until INGARD crops were grown on a commercial scale that the magnitude of these changes, and the variability which can occur between crops in different fields, farms and regions became fully apparent.

Given the variable performance of INGARD crops much research has been devoted to understanding the factors which influence efficacy of the plants and which result in variable performance. In this paper I will describe some of the patterns in efficacy observed and its consequences.

ASSESSING FIELD EFFICACY OF INGARD VARIETIES

In measuring the field efficacy of INGARD plants we have used three techniques:

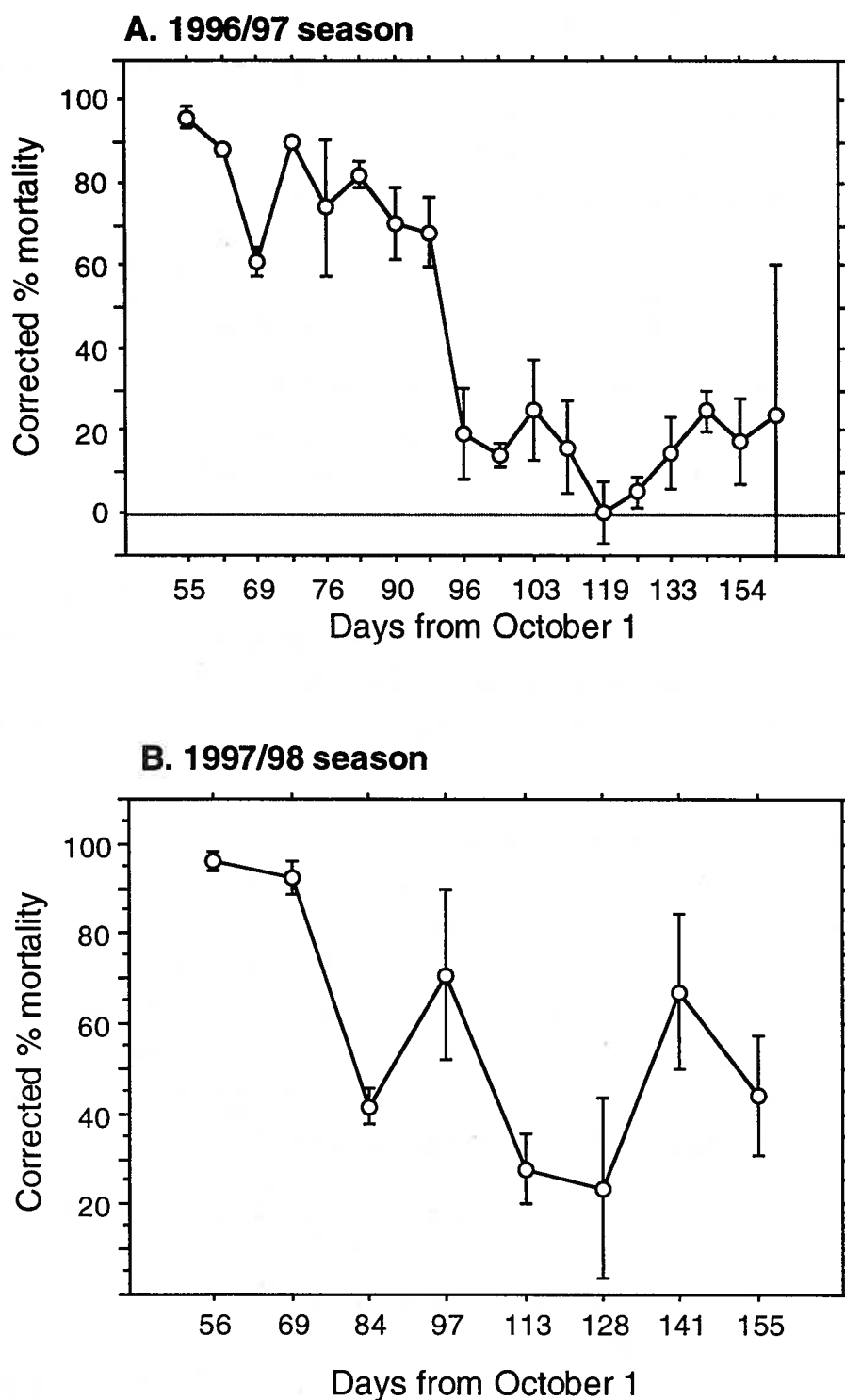
- (i) whole leaf laboratory bioassays using neonate *Helicoverpa* larvae;
- (ii) regular field counts of surviving larvae from naturally laid egg populations;
- (iii) field counts of pupal densities under INGARD and conventional plots.

Here I will only discuss the bioassays. These are conducted using laboratory cultures of *H. armigera* (and *H. punctigera*) and various tissues taken from unsprayed field plots of INGARD varieties. We routinely bioassay plants (weekly or fortnightly) during the growing season usually commencing in late November or early December when plants had just commenced squaring (flowerbud formation) but at least three weeks before first flower. In most cases we use leaves taken from specific positions on the plant to ensure a consistent age of tissue. This is usually the first fully expanded leaf at the top of the plant (designated the node 3 leaf). Leaves are placed in plastic tubs with their petioles inserted into a 1cm layer of 0.9% water agar which served to maintain turgor of the leaf. In this way leaves remain in good condition for at least the 5 day bioassay period. Five neonate larvae are added to each leaf, the containers are sealed with a fine gauze cover and then held at constant 25°C. Mortality and growth is recorded 5 days later. The instar of all surviving larvae and their weight is also recorded.

SEASONAL CHANGES IN EFFICACY

Figure 1 shows the seasonal pattern of bioassay efficacy for 3 varieties with INGARD (Siecle V2i, Siokra V15i and Nucottn 37) grown in the 1996/97 and 1997/98 seasons at one location; the Sydney University Plant Breeding Institute near Narrabri. The data shows a consistent decline in bioassay efficacy after high efficacy in the early squaring stage and with a dramatic decline around day 80-100 (after October 1) coinciding with early flowering. In 1997/98 the loss of efficacy was not as dramatic and plants appeared to stabilise at higher efficacy than in the previous year, though there was much variation. It is important to note that this data is for the young leaf at the top of the plant which we have shown has lower efficacy than older leaves further down the plant and often lower efficacy than for squares. So the pattern shown is almost the worst case scenario for possible survival on Bt plants. In addition the growth of surviving larvae on the Bt leaves was markedly reduced at all times, even at the end of the season when survival was high. Average weight of survivors on the Bt leaves was only 30-40% of that on controls after the 5 day assay and on average they were 1 instar smaller at that time. This should translate into significantly slower development and the potential for much higher mortality on Bt plants in the field than implied by Figure 1.

Figure 1A,B. Seasonal pattern of neonate survival in a laboratory bioassay of leaves from 3 transgenic Bt cotton varieties in the 1996/97 and 1997/98 growing seasons at one unsprayed site (Sydney University Plant Breeding Institute, Narrabri). Mortality is corrected for control mortality. Points are mean and standard error.



INTERPRETING BIOASSAY RESULTS - WHAT DO THEY TELL US?

A bioassay is simply a means of measuring an effect of plants or pesticides on a group of insects in a standardised way. The bioassay is not meant to measure exactly what may happen in the field; it is differences between treatments or changes over time we are interested in. The critical thing is that the bioassay is done in the same way consistently. In the case of INGARD cotton the bioassays will not reflect the performance of plants in the field exactly because:

- 1) larvae in a bioassay have no choice over where they feed; they are constrained to feed on one leaf or other structure from one part of the plant - there is significant variation in efficacy throughout the plant canopy and between plant structures.
- 2) bioassays exclude many other sources of mortality which occur in cotton fields.
- 3) efficacy of the plants changes over the season and possibly in response to short term stresses, so the bioassay provides only a snapshot of how one part of the plant is performing on a given day.

So, what does bioassay survival mean? One concern about bioassays is that we use leaves or squares which have been removed from the plants. It may be that the level of Bt protein quickly degrades in the leaves after picking and so our bioassays greatly underestimate the true efficacy of plants. We were able to show easily that this was not the case, since leaves showed the same efficacy 4 days after picking as when used on the day of picking.

Another important question is whether bioassay survival is correlated with the actual level of Bt protein in the tissues; do the seasonal changes shown in Figure 1 reflect actual changes in Bt? Plant samples from Bt plants used for bioassays in 1996/97 (Figure 1) were also analysed for Bt content by Helen Holt (this proceedings). As Figure 2 shows there is a highly significant relationship between bioassay survival and estimated Bt protein concentration expressed as the % of total protein in the tissue ($y = 84.264 - 13745.48 (\text{Bt concentration}); r^2 = 0.769$). So the bioassays are reflecting real changes in the plants related to efficacy.

We were interested then to know just how the actual level of survival measured in a bioassay relates to what happens in the field. With the assistance of summer students funded by the CRC for Sustainable Cotton Production we have conducted a series of three experiments to compare survival on Bt cotton in laboratory bioassays with survival at the same time and on the same plants in the field. In this case we constrained 5 neonate larvae in a cage on a growing plant in the field. The cages enclosed about 7 nodes at the top of the plant and larvae could feed anywhere within this on leaves, squares or the terminal. After 6 or 7 days we cut the cages from the plants and sorted through the plant material in the lab to find any surviving

larvae. These were recorded to instar and weighed just as in the laboratory bioassay. Figure 3 shows the data for three experiments completed to date over two years. There is a strong relationship between our bioassays and field survival (in cages), although the bioassays consistently overestimate the survival which actually occurs in the field. As Figure 3 shows, estimates of bioassay survival from 0% to about 30% may well mean only 2-7% survival in the field. So again the bioassays do relate to the field, but on average field survival was 30-40% lower than bioassays.

Figure 2. Relationship between bioassay survival of neonate *H. armigera* larvae on node 3 leaves and estimated content of Bt determined by Holt (this proceedings). Data for samples from one unsprayed location during the 1996/97 season.

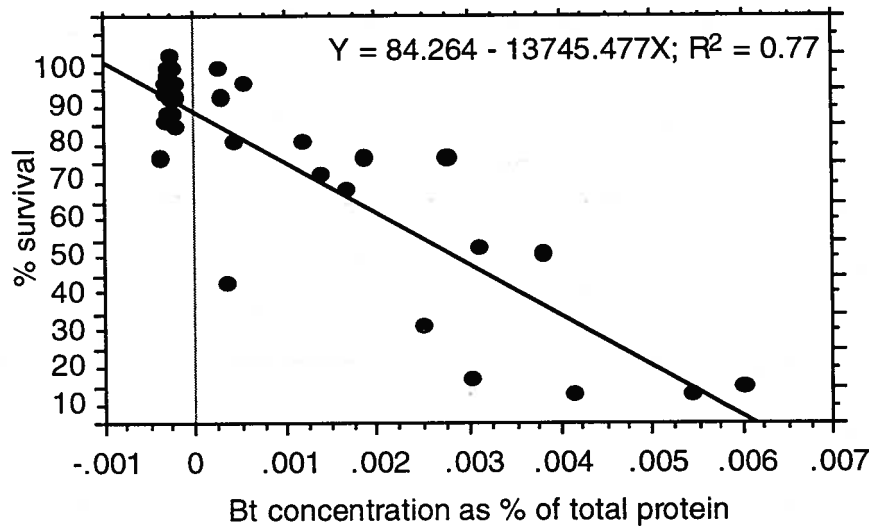
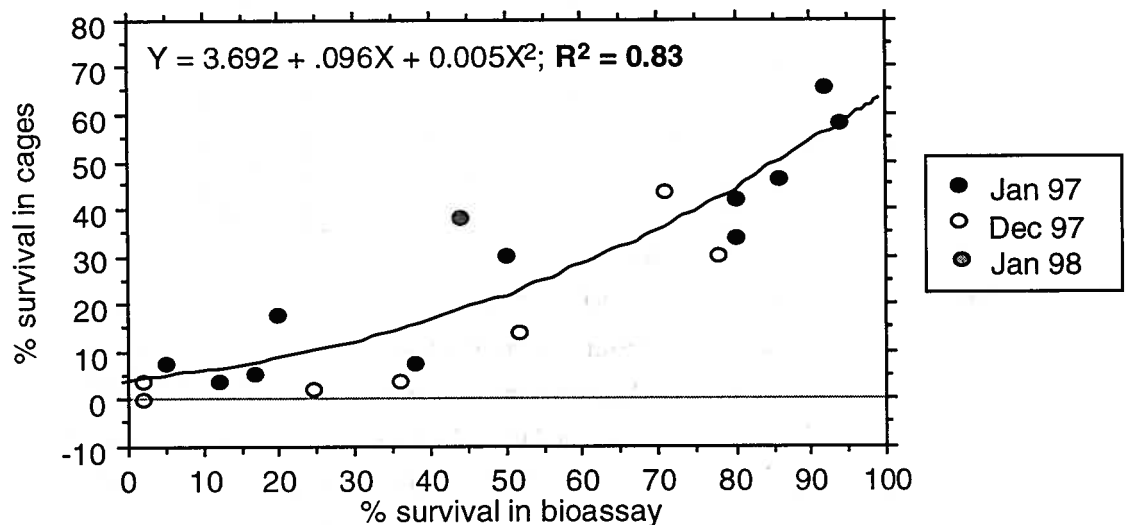


Figure 3. Relationship between estimate of *Helicoverpa* survival on node 3 leaves from INGARD plants in a lab bioassay and their survival in cages on growing plants of the same varieties in the field. Pooled data for 3 experiments in unsprayed plots of three INGARD varieties at ACRI.

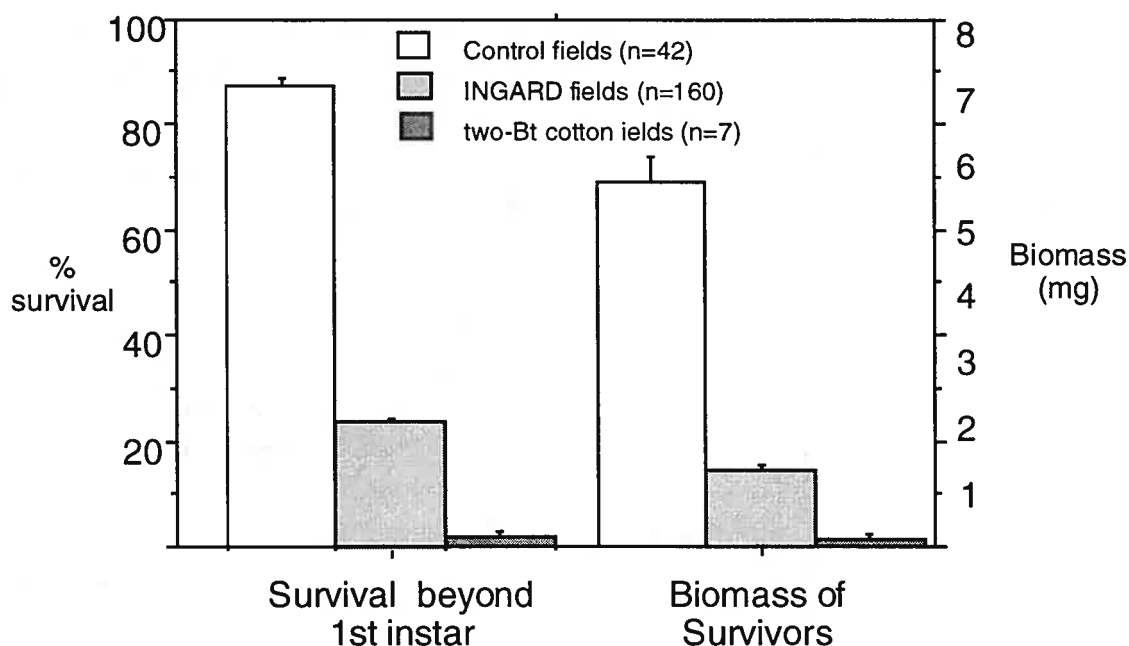


EARLY SEASON BIOASSAYS 1997/98.

With some confidence that bioassays do tell us something useful about changes and variability in the efficacy of INGARD plants we undertook a widespread survey of INGARD crops in November 1997. The aim was to assess the level of variability in efficacy present at that time when most crops were at about first square (7-10 nodes in most cases). Samples of the top fully unfurled leaf (node 3 leaf) were taken for bioassays (20 plants per field) from both INGARD and conventional fields and other samples were frozen for later analysis of other characteristics (Bt levels, nitrogen, tannins etc), which may be associated with changing Bt protein levels.

Figure 4 shows the average bioassay survival of larvae on the INGARD and control fields. Clearly there were some survivors in the bioassay leaves from INGARD fields, but many fewer than conventional fields and the INGARD survivors were much smaller. Given the relationship shown in Figure 3 the levels of bioassay survival seen in these field samples for 97/98 would probably translate to virtually no survival of larvae in the INGARD fields. In addition we collected efficacy data for 7 locations where trial plots of a two-Bt line were grown. This line expresses both the INGARD gene (CryIAC gene) plus a Cry IIA gene and clearly had much higher efficacy. We have conducted season long bioassays on two-Bt cotton lines and shown consistent high efficacy through the season, although there still a detectable decline towards the end of the growing season.

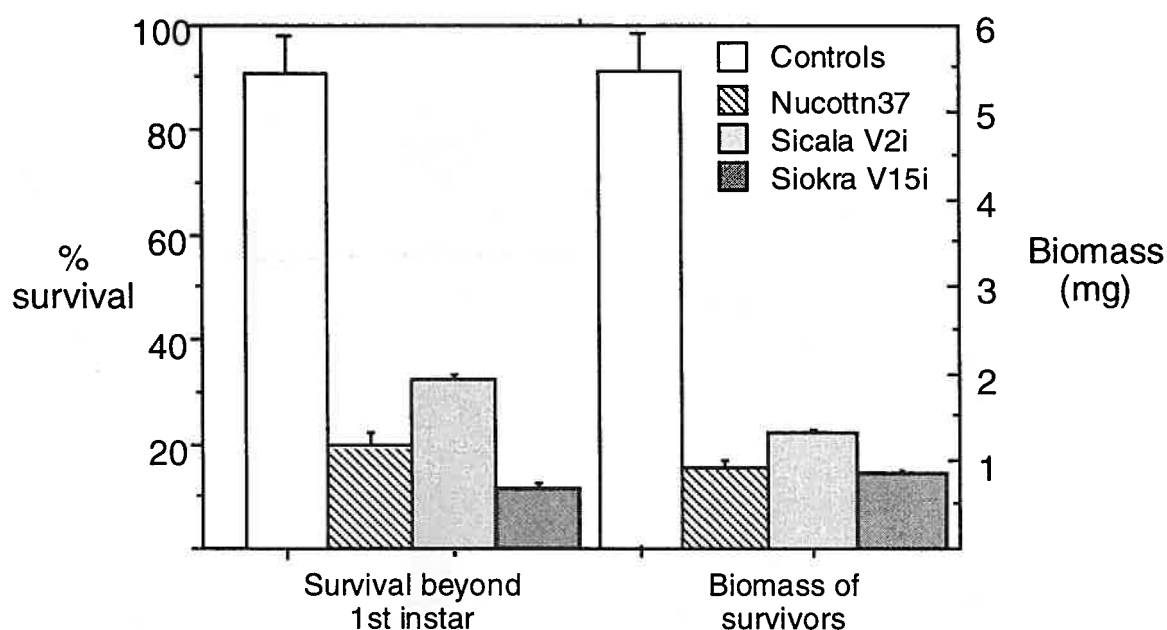
Figure 4. Average bioassay survival and biomass of survivors for 160 INGARD fields and control fields spread among all growing regions except for Central Qld. Data for 7 fields of two gene Bt cotton is also shown.



VARIABILITY BETWEEN VARIETIES

In 1997/98 the bulk of the INGARD area was comprised of three varieties; Sicala V2i, Siokra V15i and Nucottn 37. Figure 5 shows average data for crops of these varieties when assayed in late November. At this time of the season there was relatively little difference between them in efficacy or size of surviving larvae.

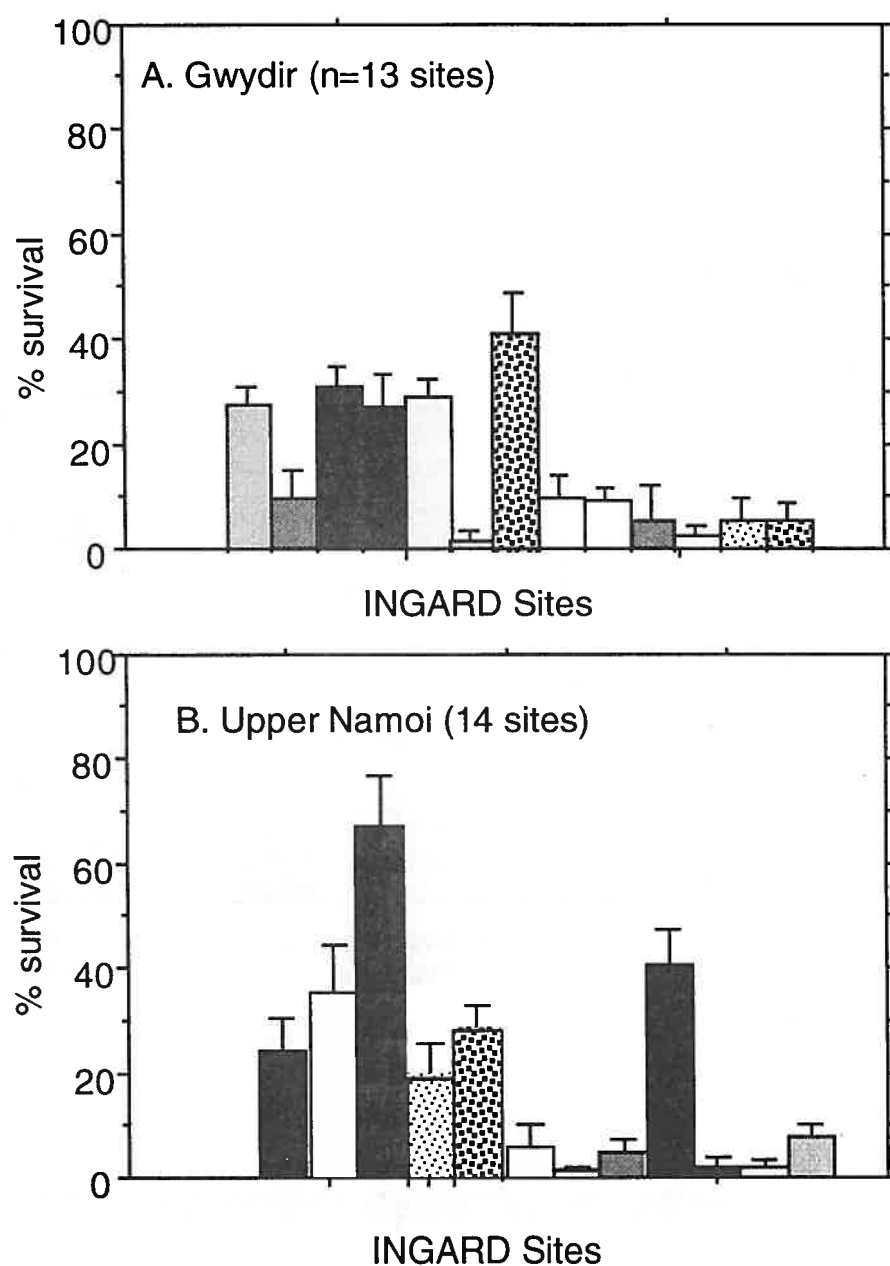
Figure 5. Average efficacy of early squaring crops of Sicala V2i, Siokra V15i and Nucottn 37. Data averaged over all crops and regions.



VARIABILITY BETWEEN INDIVIDUAL CROPS

While Figures 4 and 5 suggest reasonable levels of average efficacy the true variability present becomes apparent when data is viewed for individual crops. Figure 6 shows some examples for crops in two regions only; the Upper Namoi and Gwydir regions. Many crops had very high efficacy and at that time would have controlled larvae very well (even though *Helicoverpa* abundance was very low in most places). However, some crops showed quite low efficacy (high survival) even at that early stage of development around first square. Again note that high or low efficacy at one time does not necessarily imply continued performance at that level.

Figure 6. Variation in bioassay efficacy among sites in two regions.



UNDERSTANDING VARIATION

Trying to understand the factors leading to the variation shown between fields and regions is a major aim of this work. Some of the factors which might have contributed are discussed in the paper by Finnegan, Llewellyn and Fitt in this proceedings. In seeking to understand why some crops perform poorly we assembled a database of information on the crops surveyed. Data was received from growers for 102 INGARD sites. The information included the following:

- date of sowing
- sowing rate
- variety
- field history
- irrigation strategy at planting
- quantity and timing of fertilizer applications
- seed treatments, use of in-furrow insecticides
- weather (particularly rainfall) soon after planting
- estimates of *Helicoverpa* pressure (light, medium, high)
- numbers of pesticides applied for *Helicoverpa* pre-flowering & post-flowering.

Interpreting all this data is not yet complete, but some broad trends are apparent. Firstly there were marked differences in the abundance of *Helicoverpa* across regions with many more pesticide applications required on the Darling Downs than elsewhere (Table 1). Overall there was little difference in spray applications to the 3 main varieties with INGARD; Sicala V2i, Siokra V15i and Nucottn 37 (Table 2).

Table 1. Average spray applications for *Helicoverpa* to INGARD crops involved in early season bioassays. Crops were Sicala V2i, Siokra V15i and Nucottn 37.

Region	No. of sites	Helicoverpa Sprays before flowering	Helicoverpa sprays after flowering
Darling Downs	8	2.5	6.8
McIntyre	10	0.2	3
Mungindi	5	0	2.6
St. George	6	0.5	4.3
Gwydir	16	0.3	3.7
Lower Namoi	24	0.3	3.5
Upper Namoi	13	0	5.1
Macquarie	9	0.2	3.2

Table 2. Average number of spray applications for *Helicoverpa* to three varieties of INGARD crops involved in early season bioassays. Averages are over all regions. Because varieties were not uniformly distributed across regions, they would not necessarily have experienced similar insect pressure.

Variety	No. of sites	Helicoverpa Sprays before flowering	Helicoverpa sprays after flowering
Nucottn 37	6	0.67	4.2
Sicala V2i	52	0.44	4.0
Siokra V15i	33	0.36	3.8

Analyses in which I sought relationships between various agronomic factors and bioassay efficacy in early squaring showed:

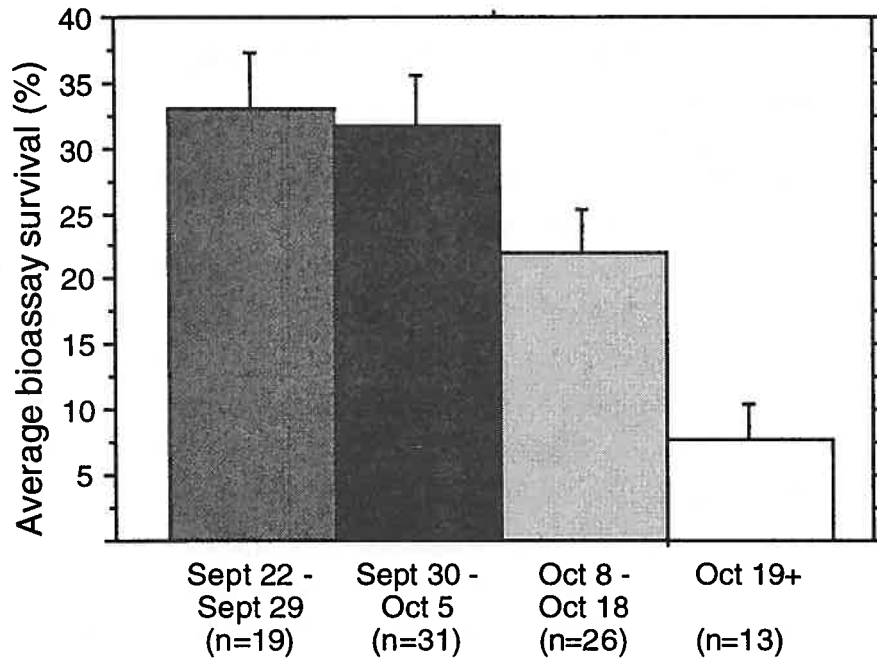
- no relationship to levels of tipping out in seedlings (average levels of 7.0-7.5%)
- no significant relationship to the use of soil insecticides, although tipping out was slightly reduced with them (9% no soil insecticide, 6.5% with Temik or Thimet)
- no relationship to the levels of applied nitrogen (range 75-300 units N)
- no simple relationship to the water regime at planting (water-up vs pre-irrigated)
- a highly significant effect of planting date ($p=0.004$); early sown crops had poorer efficacy (Figure 7)

This effect of planting date is not simply a result of early sown plants being older when surveyed. All the crops were between 7 and 10 nodes at the time of sampling, so the early sown crops had grown very slowly compared to those sown later. Presumably the earliest sown crops experienced a stress early in life which has carried through to influence efficacy at the time we sampled them. However, there remain many more questions to resolve.

Our data also contain some tantalising suggestions that rainfall soon after planting and seedling emergence may have impacted on efficacy. Three regions (Darling Downs, Gwydir and Lower Namoi) had significant rainfall within 3 weeks of planting. On average crops in these regions were sown around October 12, October 1 and October 10 received 30-35mm of rain in this period, whereas in other regions only 10-15mm was recorded. In these regions the crops which had been watered up

and then had >25mm of rain had poorer efficacy in late November than those which did not receive rain. Is this the result of early waterlogging of seedlings? We cannot yet be sure. The data serves mostly to indicate the complexities in trying to interpret the seasonal changes in efficacy of INGARD crops.

Figure 7. Average bioassay efficacy in late November/ early December 1997 of INGARD crops sown in different periods during Sept-Oct 1997. Data is averaged over all regions. Numbers of crops involved shown in parentheses. Crops were at early squaring (node 7-12) when sampled.



Following on from this work we commenced a series of studies at 6 locations to identify the spatial scale at which variation in efficacy occurs. The question was whether we see most variation between individual plants within rows, or between rows of cotton, between locations within fields or between fields; or at all scales. This work is ongoing and will be reported elsewhere.

DISCUSSION

As discussed earlier our bioassays probably do not reflect the level of survival likely to occur in a field situation. They are not done for this purpose. The bioassay results from early season this year show similar variability to last year, but do not necessarily mean that a crop will maintain high or low efficacy. What the results do show however, is the variation which is possible between INGARD crops. While we cannot be certain about the causes for this it is likely that the suitability of the environment experienced by seedlings should be added to the list of possible factors which may negatively effect efficacy. What all this really tells us is that the level of Bt we have in INGARD plants is close to the margin needed to quickly kill

Australian *Helicoverpa* larvae, and any slight variation in the environment can have dramatic effects of efficacy. Whether these are due to effects operating at the level of the Bt gene itself is not yet clear (but see Finnegan et al, this proceedings). It is worth noting that the Australian *Helicoverpa* species are much more tolerant (10 fold more) of the Cry IAc protein than the main target of BOLLGARD cotton (same Cry IAc protein) in the USA industry, *Heliothis virescens*. It is intriguing to consider that the changing patterns of efficacy and between crop variability we have observed in Australian INGARD crops would not have been evident if our species were as sensitive to Cry IAc as *H. virescens*. Studies to measure Bt protein concentration and to understand the factors underlying changing efficacy would probably not have been undertaken.

The consistent seasonal decline in efficacy, coupled with the variability of efficacy between crops has a number of implications. Firstly, they generate uncertainty for crop managers and influence the confidence they have in using INGARD cotton in IPM systems. Thresholds for Bt cotton are still being developed and validated in conjunction with expanding commercial use. Current thresholds are 2 small larvae/ metre of row for two consecutive check dates (or 1 medium larva/ metre on the first check). Refining these thresholds will allow better timing of pesticide interventions when they are necessary, but at present some pesticides are applied only after larvae have reached second or perhaps early third instar when they are difficult to control (see Gibbs this proceedings). While surviving larvae on Bt plants grow much more slowly than on conventional cotton, and many of them die eventually even if sprays are not applied, they are nevertheless causing damage and some crops have suffered damage because consultants complied with thresholds and were then unable to control larger larvae. The unfortunate consequence of this is that consultants and growers become more conservative in their use of thresholds, which may then erode the full realisation of the IPM value of INGARD cotton.

A second significant consequence of changing field efficacy is of course the selective pressure this may apply on Bt resistance. The resistance management strategy in place for Bt cotton relies on the use of refuges but these work best when the plants are highly efficacious and have the capacity to kill a high proportion of heterozygous resistant individuals. Given the level of survival seen with the first releases of Bt cotton varieties expressing a single Cry IAc gene it is clear we do not have a high dose of Bt. The means then that other components of the resistance management strategy are even more crucial; much larger refuges are the only real option to minimise the risk of Bt resistance occurring rapidly. It is for this reason that the proportion of the total cotton area which can be planted to Bt cotton has been tightly restricted to 10-20% in the first three years of commercial use. This serves to magnify the size of the total refuge which includes the refuge crops grown

specifically with the Bt cotton, the remainder conventional area of cotton (80-85% of the total) and all other *Helicoverpa* susceptible crops grown in eastern Australia. This represents a huge refuge.

Both these issues discussed above; IPM value of Bt cotton and resistance risk, highlight the imperative for Australia to develop cotton varieties with two independent Bt genes or other insecticidal proteins as quickly as possible. Field efficacy of the first lines expressing two Bt genes (Cry IAc and CryIIA) to be trialed has been very high and while agronomic issues are still being resolved these varieties are approaching the potential for commercial release (see Constable, this proceedings).

Acknowledgments

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