

Bollgard II[®] cotton

Technical Manual



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EXECUTIVE SUMMARY

Bollgard II cotton

INGARD cotton was the first commercially available genetically modified crop in Australia. Bollgard II is the successor to INGARD cotton. Bollgard II contains two *Bacillus thuringiensis* genes Cry1Ac and Cry2Ab which both control certain Lepidopteran larvae when they feed on it.



Integrated Pest Management

Integrated Pest Management (IPM) is the judicious use of all methods of pest control to minimise the negative affects of one form of control over another. Bollgard II cotton is a corner stone in the development of sustainable IPM in cotton. Bollgard II provides prolonged control that reduces the necessity for pesticides to be applied.

Resistance

Resistance to conventional pesticides has been a major concern in cotton, especially in Australia. This has resulted in recent years in making cotton unprofitable in some areas. The Ord River Irrigation Area in the Kimberley region of Western Australia produced cotton in the 1960's and 1970's. Due to the over-use of insecticides, the major pest, *H. armigera*, developed resistance and even the application of 28 sprays per season was not sufficient to control the pest; consequently the cotton industry withdrew ten years after it began. In Bollgard II insects with some tolerance or inbuilt resistance to one protein will generally be removed through their susceptibility to the other. There is no cross resistance between the two proteins and this will result in Bollgard II being much more resilient against resistance developing in the target pests.

The Value of Bollgard II

Bollgard II has the potential to save the environment from excessive pesticide use. It also has the potential to save the grower in terms of expenditure on pest control. Another valuable benefit is that the use of Bollgard II will increase the available area of land suitable for cotton production by opening areas of land previously too sensitive for cotton production due to the crop's requirement for pesticides.

Prudent management of Bollgard II will offer the Australian cotton industry what is probably the greatest benefit, the potential for greater sustainability. This can be achieved through placing less reliance upon traditional chemistries. Such a management policy can remove much of the selection pressure against traditional chemistries and potentially increase their effective life as pesticides for cotton. In addition, reduced use of broad-spectrum pesticides will result in increased beneficial insect activity. This will provide greater control of *Helicoverpa* spp., through predation and parasitism resulting in lower numbers exposed to the B.t. proteins. All this combined will reduce selection pressure for resistance to develop to these proteins and an increased life for the Bollgard II technology.

BOLLGARD II PROTEINS

Both of the insecticidal proteins produced by Bollgard II are encoded by genes derived from the common soil-dwelling bacterium, *Bacillus thuringiensis*.

Bacillus Thuringiensis

Bacillus thuringiensis (B.t.) is a facultative anaerobic, gram-positive bacterium that forms characteristic, crystalline proteins. These proteins are toxic for certain invertebrates, especially species of insect larvae belonging to the insect orders Coleoptera (beetles), Diptera (flies) and Lepidoptera (moths and butterflies). There are at least 67 known subspecies of B.t., which are naturally found in soil, water and on leaf surfaces.

These bacteria produce a large array of crystalline proteins, two of which are now produced by Bollgard II cotton. The currently known crystal (*cry*) gene types encode insecticidal crystal proteins (ICPs) that are specific to *Lepidoptera* (*cry1*), *Diptera* and *Lepidoptera* (*cry2*), *Coleoptera* (*cry3*), *Diptera* (*cry4*), or *Coleoptera* and *Lepidoptera* (*cry5*)¹.

Each insecticidal crystal protein has a different physical structure and possesses a unique domain (attachment site). It is these unique differences that are mainly responsible for host susceptibility and toxicity. Each protein is the product of a single gene.

History of B.t. Development

B.t. has been around in agriculture for a long time. In 1901, a Japanese bacteriologist, Ishiwata Shigentane, first isolated B.t. on infected silk worms. In 1915, German scientist, Ernst Berliner, isolated B.t. from dead Mediterranean flour moths from a grain mill in the German district of Thuringen. He named it *Bacillus thuringiensis*. In 1927 the first preparation containing B.t. was used in Germany to control *Lepidopteran* insects and in 1938 the first commercial product was launched in France under the trade name Sporeine. Twenty years later, in 1957, the Sandoz Corporation produced a large-scale B.t.-based product marketed as Thuricide and since then this has been used in commercial food production.

Currently there are numerous preparations based on B.t. used in a wide range of crops. Some of the preparations are specific, such as MVP (containing only the Cry1Ac protein) and some contain a range of B.t. proteins, such as Dipel.

1901	First isolated B.t.
1915	<i>Bacillus thuringiensis</i> recognised and named
1927	First preparation produced to control Lepidoptera
1938	First commercial product
1957	Thuricide produced as large-scale commercial agricultural product

¹ Höfte, H. and H.R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53: 242–55.

The Cry proteins

Bollgard II cotton contains the gene encoding for the Cry1Ac protein. This protein possesses specific toxicity to certain species of *Lepidoptera* that includes *H. armigera* and *H. punctigera*. Control of *Pectinophora gossypi*, rough bollworm and some other *Lepidoptera* spp. has also been seen.

Cry2Ab is another ICP from *Bacillus thuringiensis*. It differs from Cry1Ac in having different structural domains on the crystalline protein. A different receptor site on the midgut wall of target animals is required for the protein to have insecticidal effect. A single gene produces the Cry2Ab protein and this gene has been isolated and inserted into cotton already containing the cry1Ac gene (INGARD cotton) to form Bollgard II cotton.

Mode of Action on Target Insects

The ICPs have different physical structures and possess different domains (attachment sites). It is the differences in the domains that is mainly responsible for host susceptibility and toxicity.

The mode of action occurs through:

1. Ingestion of the ICP by an insect larva;
2. Dissolving the ICP in the insect midgut;
3. Activation of the ICP by protease enzymes;
4. Binding of the activated protein to specific receptors on the cell membrane in the midgut;
5. Insertion of the protein into the cell membrane and formation of a pore into the body cavity;
6. Starvation, destruction of cell tissue and septicaemia and resultant death of the insect larvae.

The efficacy of a B.t. protein in killing a pest depends on;

- the level of solubilization in the midgut (which is dependant upon the pH of the midgut);
- the conversion of the protein to the active protein by the enzymes present;
- the possession by the pest of specific membrane receptor sites which can bind with the active protein;
- Resultant formation of pores and destruction of gut-wall tissue.

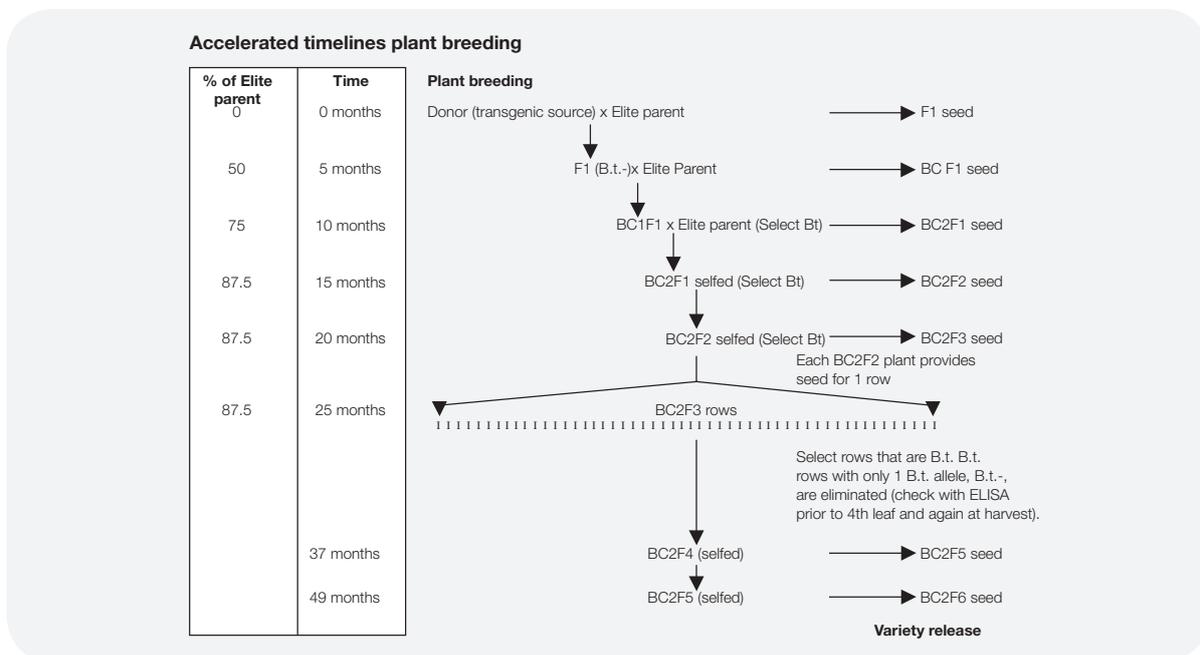
Specificity

Cry1Ac and Cry2Ab are very specific in their target range due to the pH, enzymes and receptor sites required. A detailed description of the impact on non-target organisms can be found in 'The exposure and effects of B.t. proteins on non-target organisms' within this manual.

DEVELOPING A TRANSGENIC COTTON CULTIVAR

- (a) Transgenic varieties are usually developed by crossing an elite conventional variety with a transgenic donor variety. The donor variety contains the desired protein or gene.
- (b) Subsequent generations are backcrossed to the elite variety (recurrent parent).
In this process, each generation is backcrossed with the elite cultivar for several generations to recapture the bulk of the genetics from the elite variety. Plant breeders may use a number of backcrosses to develop a new transgenic variety. Each generation is tested to ensure that it carries the desired protein (gene), and any progeny that do not carry this protein are eliminated.
- (c) At the end of the backcrossing process, the seeds are grown out and the plants are allowed to self-pollinate.
- (d) The progeny seeds are grown out as individual plants and all plants that are not homozygous for the desired gene are eliminated.
- (e) Seed from each homozygous plant are planted in progeny rows for agronomic evaluations. Each progeny row contains the unique genetics of the individual cross that resulted in the parent seed. In general, a plant breeder will have from 12 to 50 progeny rows. The schematic chart below shows the backcrossing process.

Figure 1. Backcrossing Process.



- (f) Most new, fully commercial, Bollgard II varieties will normally have 97-98%+ of the same genetic background as the elite conventional cotton parent variety.
- (g) Using the same processes as when new conventional varieties are developed, the plant breeder evaluates each progeny row and chooses those that meet the criteria established for the new transgenic variety. Plant breeders either select a single progeny row or bulk lines with similar characteristics together to form the new transgenic variety. The plant breeder may look for lines that are very similar to the parent variety or they may choose progeny lines that exhibit some improved characteristics if they exist.

The final result is that the new transgenic variety will not be identical to the recurrent parent and may have characteristics that are different enough to require changes in agronomic management practices. They are not an exact copy of the conventional (recurrent) parent. It should not be assumed that the new transgenic variety would require the same management as the recurrent parent variety.

Any new variety, whether conventional or transgenic, should be judged on its agronomic characteristics first. It is important to follow the seed company recommendations on agronomic management of the new variety.

BOLLGARD II PERFORMANCE

PERFORMANCE TRIALS

Both efficacy and crop safety trials were initiated in 1999 and continued through until late in 2001. Sites were chosen to fulfill two main criteria; firstly, the sites could be adequately managed, monitored and contained and, secondly, they represented the kind of environmental diversity in which cotton could grow in Australia. The first efficacy trials were planted in April of 1999 in the Ord River Irrigation area of northern Western Australia and crop safety trials began the following summer (1999/2000) in NSW and Queensland. Trials continued over both winter and summer seasons until 2002.

The Bollgard II variety, DP50BX, was used for all initial performance testing. DP50BX is directly derived from DP50B (INGARD cotton). However, once the insertion of the *cry2Ab* gene was made, normal sexual reproduction was used for seed bulking and therefore there is genetic variability between individuals. Comparisons were made to demonstrate that the insertion of the *cry2Ab* gene has no adverse effect on the normal growth and development of the cotton plant. The results showed some variability, which is to be expected, but the data collected across four seasons showed that the presence of the second transgene, *cry2Ab*, had no negative affect on the overall agronomic development of the Bollgard II cotton plant when compared with its INGARD cotton parental line.

Efficacy

All of the trial sets provided conclusive results confirming the greatly improved efficacy of Bollgard II over INGARD cotton.

This is a simple method of assessment: including measuring larval index, survival rate and differences in weights of surviving larvae were used as a measure to compare efficacy. In the last season of testing, the use of a quantitative bioassay protocol was included to determine the equivalent concentration of Cry1Ac in the different samples evaluated.

Crop Safety

The effect of the addition of the *cry2Ab* gene on crop safety was measured by comparing certain agronomic criteria from both the Bollgard II cotton plants and the INGARD cotton plants of the same varietal background (DP50BX and DP50B respectively). Hence these two lines were used in the comparison. If the *cry2Ab* gene has no impact on the agronomy of the plant then there should be no significant differences in any measurable agronomic characteristic in a fully sprayed situation (i.e. when plant damage by pests and resultant growth compensation are eliminated). In many instances the conventional cousin was also used as a comparison. Some variability was to be expected as the plants are not clones, but they should express the same basic varietal characteristics for which they were originally selected.

All trial sites managed included measurements of plant height and number of nodes as criteria for evaluation. Yield measurements, plant mapping and fibre quality were also taken.

Conclusions

The overall conclusion is that the presence of the second gene, *cry2Ab*, contributes to the overall insecticidal efficacy of the plant and increases the period for which it is effective in controlling *Helicoverpa* spp. Bollgard II has as good an efficacy against *Helicoverpa* spp. as INGARD cotton early in the season and greater efficacy later in the season providing extended control of *Helicoverpa* spp.

Some variability of agronomic characteristics was observed, which was to be expected. The data collected throughout the duration of trials showed that the presence of the second transgene, *cry2Ab*, had no negative affect on the overall agronomic development of the Bollgard II cotton plants when compared with both its INGARD and conventional cotton parental lines.

BENEFITS OF BOLLGARD II

INGARD technology (Cry1Ac only) was an important component of the cotton industry. Bollgard II provided us with the opportunity to make this technology a long-term sustainable benefit. Without Bollgard II, and the inclusion of the second gene, the threat of resistance developing to Cry1Ac would become a serious issue.

Commercial

1. Reduced Insecticide sprays
2. Decreased resistance development to conventional insecticides and B.t.
3. Improved pest control efficiency
4. Increased survival of beneficial insects
5. Increased biological control of secondary pests
6. Increased biological control of *Helicoverpa* spp.
7. Less machinery and labour demand
8. Allows access to marginal areas
9. Decreased compaction

Environmental

1. Reduced water, soil and air contamination
2. Reduced personnel risk
3. Increased biological diversity and survival of non-pest species
4. Improved public acceptance of cotton production

Impacts on Secondary Pests

One major concern with the introduction of Bollgard II was that the reduction in sprays for *Helicoverpa* spp. would be offset to a degree by additional sprays required to control secondary pests that would normally have been controlled by the sprays for *Helicoverpa* spp.

The beneficial insect populations in the cotton can be maintained and encouraged through the selective use of insecticides when spraying is needed. This will ensure some additional control of both heliothis and of secondary pests by beneficial insects. This will not remove the necessity for some pesticides to be applied for secondary pest control but will assist in minimising the requirement. In the 13 commercial sites monitored. There was no significant difference in the number of sprays required for secondary pests between conventional and Bollgard II cotton. The range of extremes can be seen by looking at the Warren and Emerald trial sites. At Warren there were no sprays required for secondary pests in the Bollgard II but four were required in the conventional cotton. At Emerald, five sprays were required for secondary pests on Bollgard II and none were required on the conventional cotton. The reason for the increase in sprays applied at Emerald was mainly attributed to the presence of whitefly in the Bollgard II block whilst there was none in the conventional block.

Current data suggests that similar attention will be required for the control of secondary pests on Bollgard II to that on conventional cotton. There will undoubtedly be some situations that will result in more sprays, and expense, on Bollgard II than on conventional cotton.

2004/05 BOLLGARD II COMMERCIAL EVALUATION TRIAL RESULTS

Monsanto conducted Commercial Evaluation trials during the 2004/05 season. Information was gathered from commercial growers who were independently making management choices for both their Bollgard II and conventional cotton crops. Management data was collected from Bollgard II and conventional crops. The information was used to assess the value of the Bollgard II trait.

Figure 11. Total Number of Insecticide Sprays

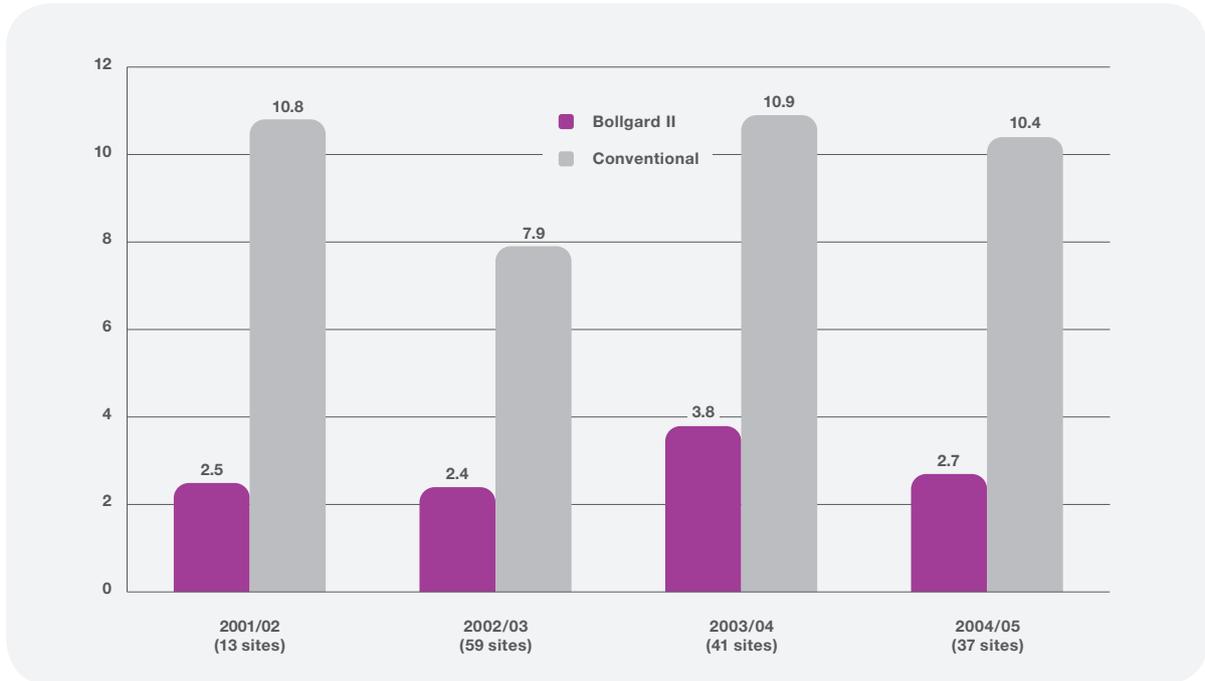


Figure 12. Total Number of Heliothis Sprays

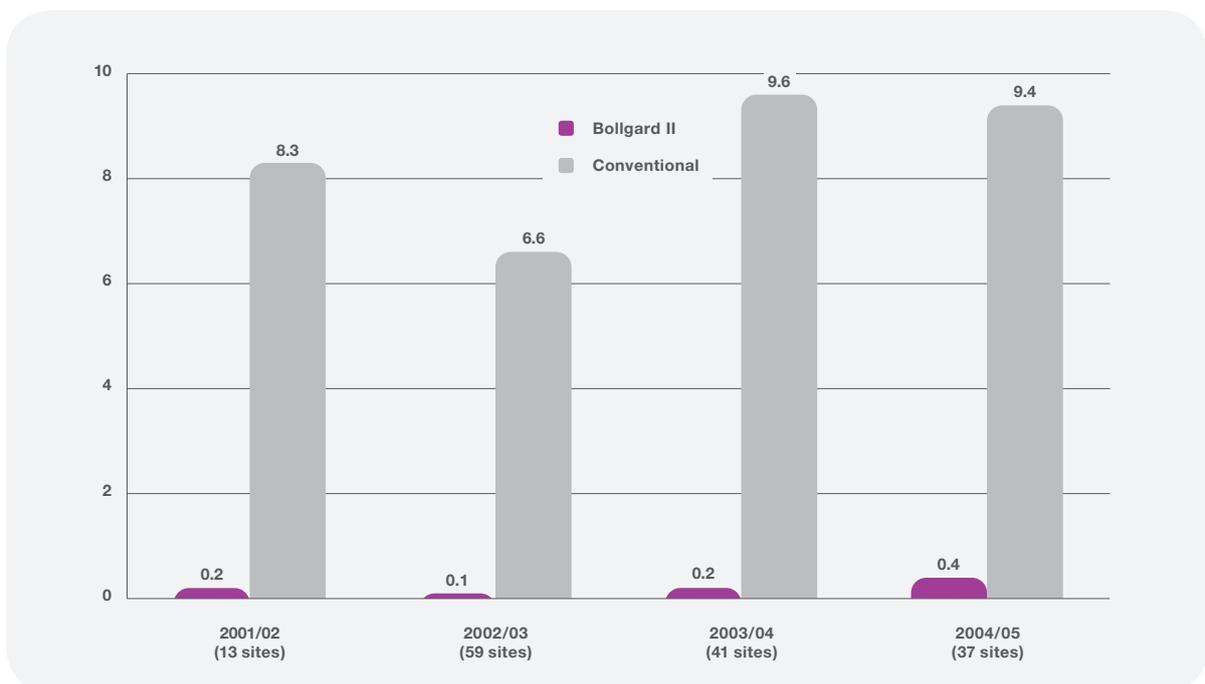


Figure 13. Mean Number of Total Secondary Pest Sprays

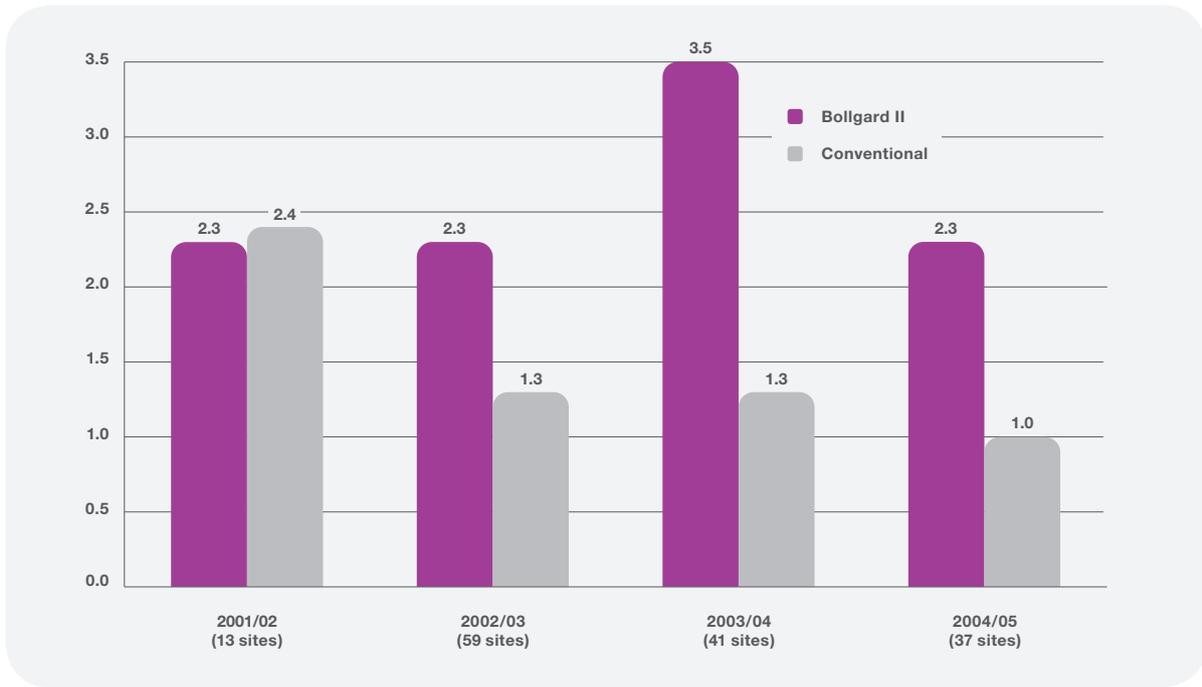


Figure 14. Cost of sprays

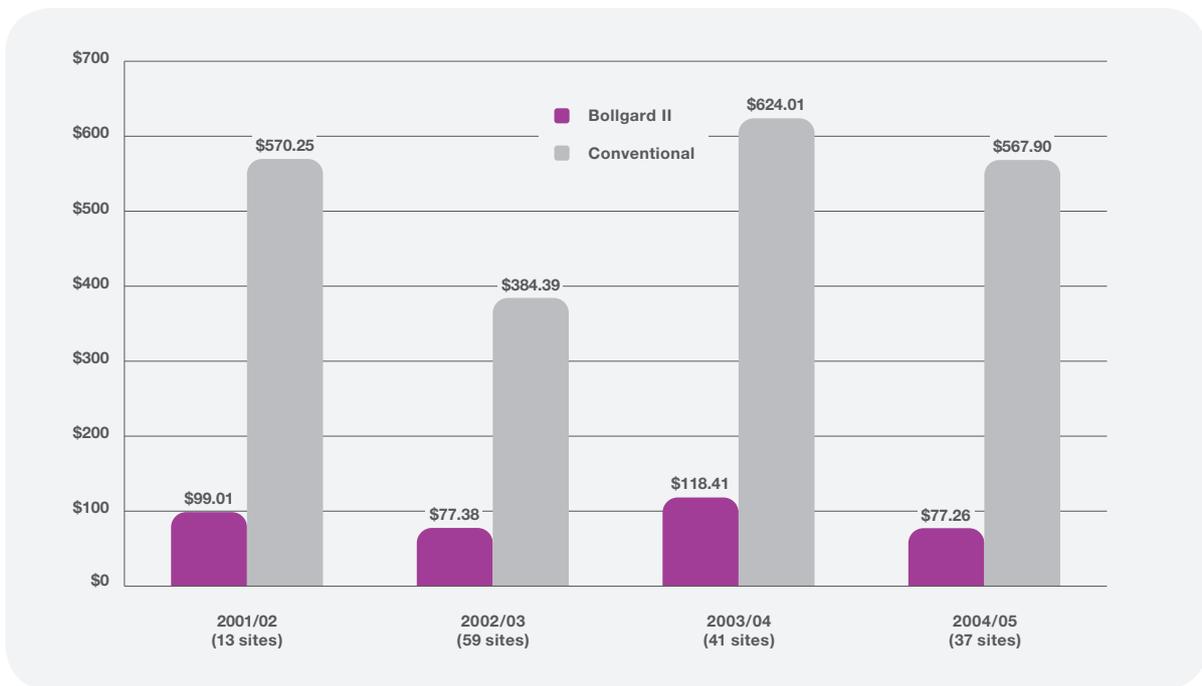
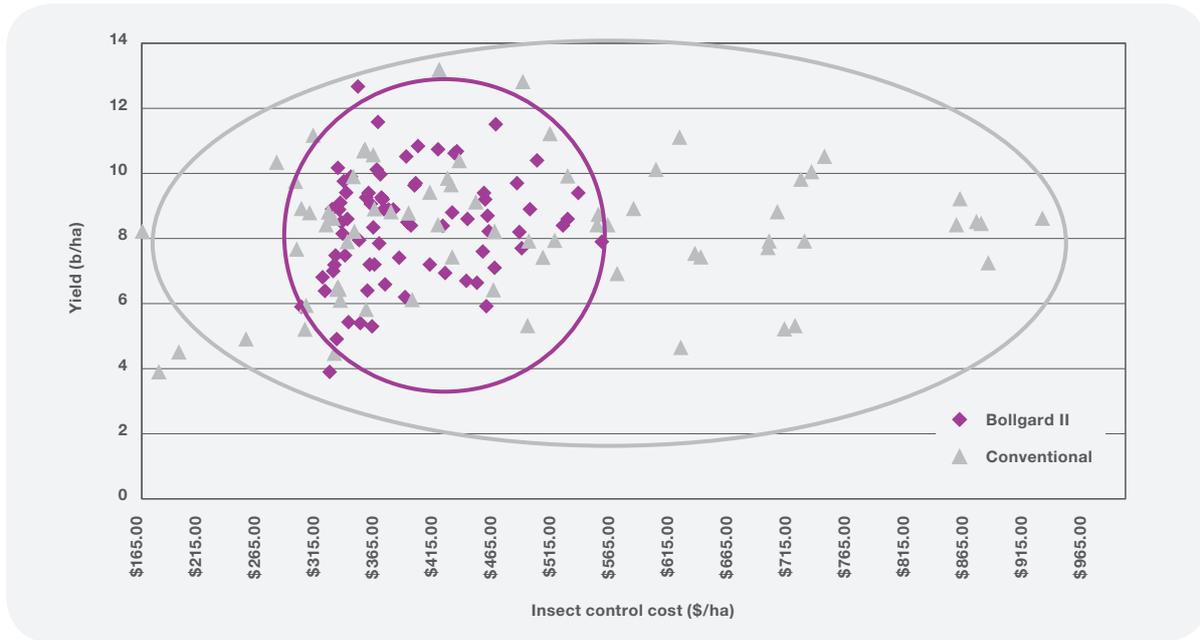
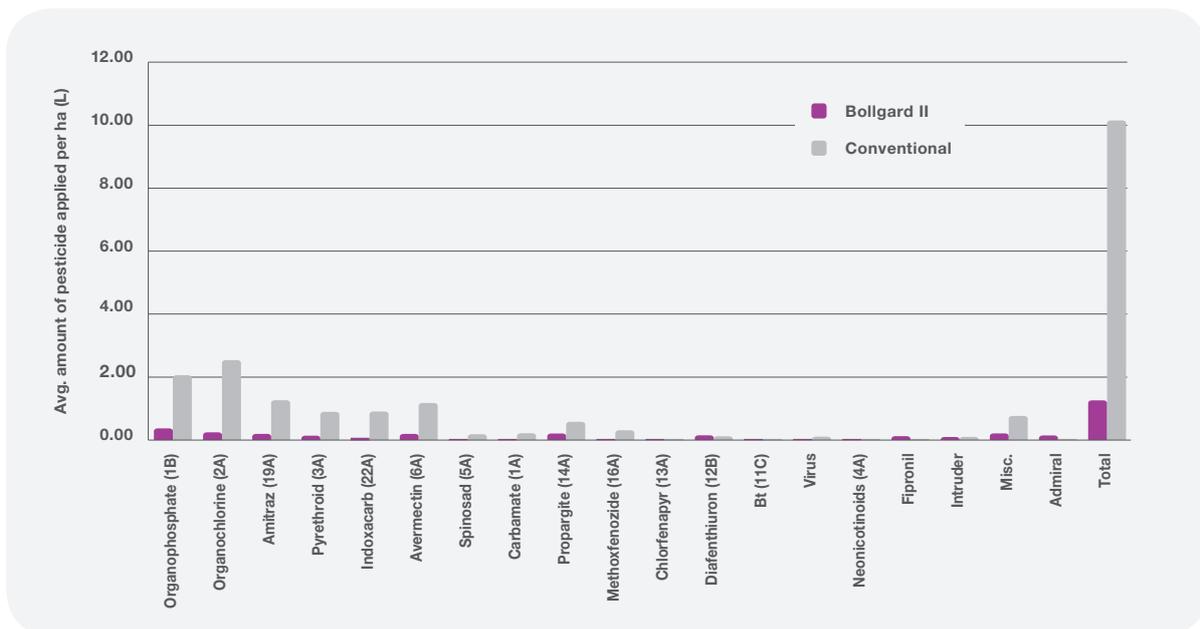


Figure 15. Total Insect Control Costs Versus Yield



The variability of cost of sprays is much greater in a conventional system to that of a Bollgard II field. The chart also indicates that yield is more consistent with Bollgard II varieties.

Figure 16. Pesticide Loading



Summary of Results

Commercial evaluation trials indicated that across all valleys there was a significant reduction in the use of pesticides used in cotton. The industry has seen an 80% reduction in the use of pesticides since the introduction of Bollgard II. On average across the cotton industry the total number of in crop pesticide sprays have been reduced by 6.1 sprays. The total number of *Helicoverpa* sprays has reduced by 9 sprays industry wide. Secondary pest sprays have increased in Bollgard II fields by 2.2 sprays. However, in conventional fields the uses of broad spectrum chemicals such as synthetic pyrethroids also control the majority of secondary pests as well but have only been recorded in the trials conducted by Monsanto to target *Helicoverpa* spp. The total average cost of pesticide use throughout the cotton industry has been \$536.64/Hectare for conventional crops over the past four seasons and Bollgard II costs have been \$93.01 (exclusive of licence fee). The variability of insecticide costs is also greatly reduced with Bollgard II compared to conventional cotton.

BOLLGARD II PERFORMANCE VARIABILITY

Since the introduction of Bollgard II cotton in Australia, it has been reported by growers and cotton consultants that there has been variability of performance (i.e. some larval survival) of Bollgard II cotton in controlling the target pests *Helicoverpa armigera* and *H. punctigera*. There has been considerable speculation as to what factors may or may not impact on the performance of Bollgard II genes and a range of studies have been undertaken to identify the causes of the observed variability.

There are a number of reasons for variability, some are manageable and some are not. Protein expression and efficacy are not the same, though they are linked.

Efficacy

Expression is a measure of the level of protein produced by the gene in plant tissue. Efficacy is a result of both the level of expression of B.t. within a variety and the level of susceptibility of *Helicoverpa* spp. to the B.t. protein. Factors that may affect the efficacy of the B.t. genes include:

- Toxicity of the proteins to the target insects
- Quantity of the protein produced by the plant
- Stability of protein production
- Period of protein production
- Uptake of protein by the target pest

Factors Influencing the Performance of Bollgard II Cotton

Any one or more of the following factors may influence the field performance of Bollgard II cotton in controlling *Helicoverpa* spp.

- Inherent plant physiology affecting rate of protein production in the plant
- Spatial distribution of protein production within the cotton plant
- Inherent genetic variability of the cotton plants
- Inherent genetic variability of *Helicoverpa* spp.
- Behavioural response of *Helicoverpa* spp. to the B.t. proteins
- External environmental conditions
- Management practices

Inherent Non-Controllable Factors

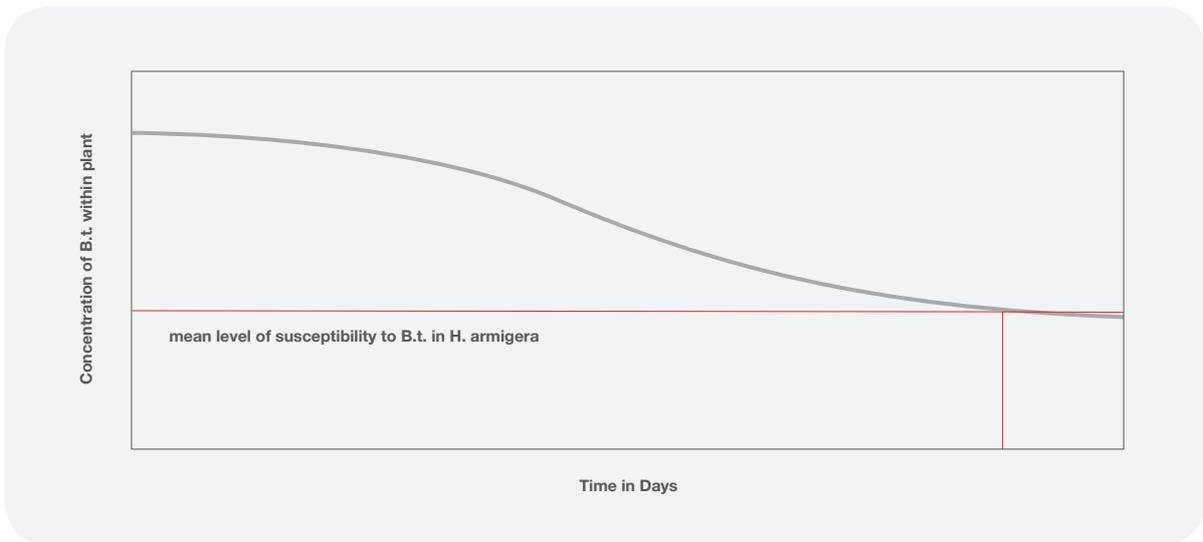
Protein Production over time

As mentioned above, the efficacy of Bollgard II cotton is directly related to the toxicity of the two proteins to the target pests and to the level of production of the proteins within the cotton plant. The toxicity does not alter and the protein is stable under field conditions. However, the level of protein production decreases throughout the season. The decrease in production does not appear to be controlled by development phases of the plant but is a gradual decline from an initial high level of protein production. Both Cry1Ac and Cry2Ab decline over the season. The initial level of production of Cry2Ab is up to eight times that of Cry1Ac though the toxicity of this protein to *Helicoverpa* spp. is considerably less than that of Cry1Ac. Combining the effects of the increased level of production of the Cry2Ab protein with the lower toxicity, the overall effect is that the toxicity of the leaf tissue of Bollgard II is two to three times greater than that of INGARD cotton leaf tissue. Although the production of protein in Bollgard II declines throughout the season, there is still a high level produced at the end of the season, which is capable of exerting significant control of *Helicoverpa* spp.

Inherent Genetic Variability Cotton Plants

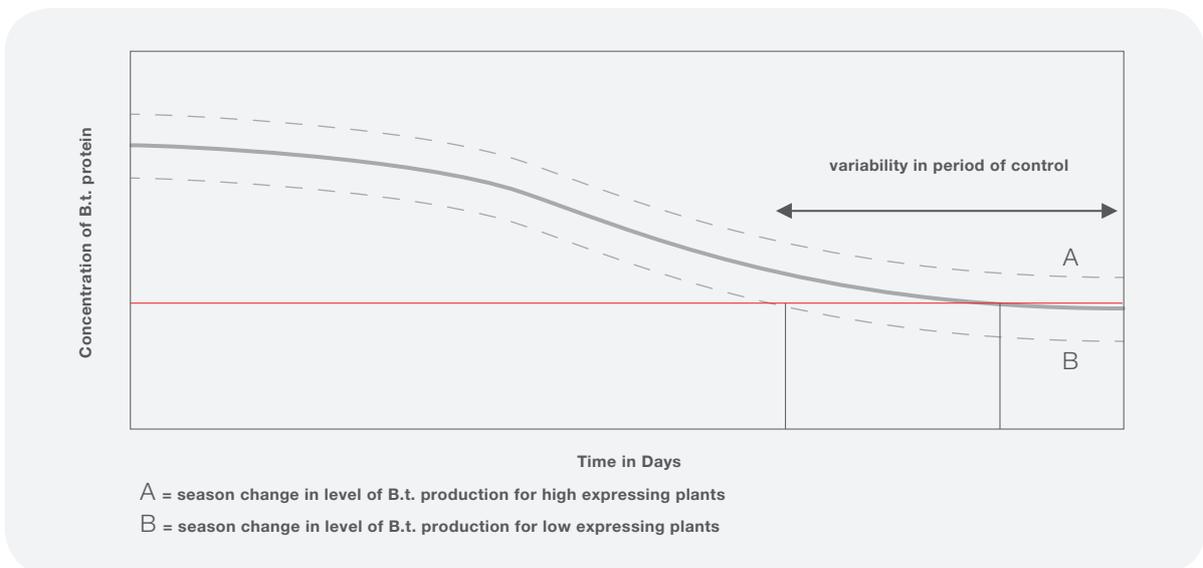
The overall development of B.t. production within the Bollgard II plant is simply illustrated in figure 5.1 with the level of B.t. declining over the life of the plant. This represents a typical population of Bollgard II cotton, but individual plants within this will vary. Cotton plants comprise a complex array of individual plant cells. Each cell has the same genetic information, but different cells, through complex switches, develop into different plant structures and have different functions. Each cell that makes up this complex organism contains in excess of 20,000 genes. One of these genes encodes for the Cry1Ac protein and another encodes for the Cry2Ab protein, which are just two out of the 20,000+ genes. Each plant, through normal reproductive gene mixing, has a unique array of these genes. Each plant is therefore slightly different from its neighbour.

Figure 17. Mean rate of B.t. production in cotton plant



Other genes can affect the genes that are responsible for producing the B.t. proteins. From this it can be understood that individual plants may be slightly different in the way the B.t. genes are expressed. Differences may occur in the length of time that the genes are expressing, the rate of production of the proteins or both. Both these characteristics may also behave differently when the other genes are affected by external conditions. The level of expression determined for a particular variety at any particular time will be an overall level for the variety. Individuals within the population will vary either side of this level.

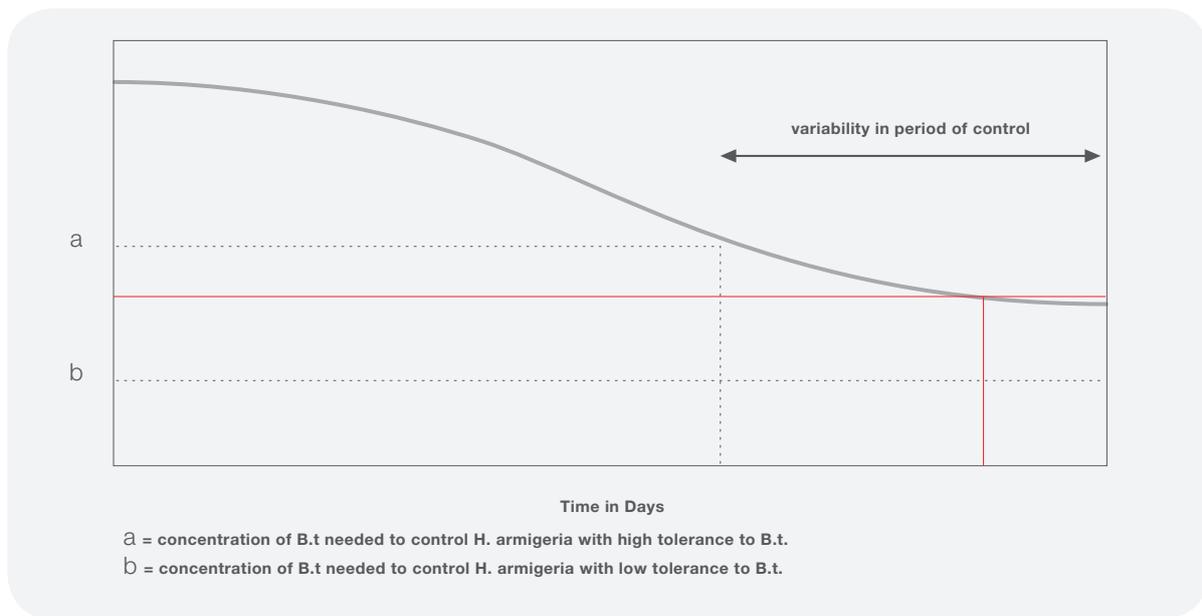
Figure 18. Variability in period of control



Inherent Genetic Variability Helicoverpa species

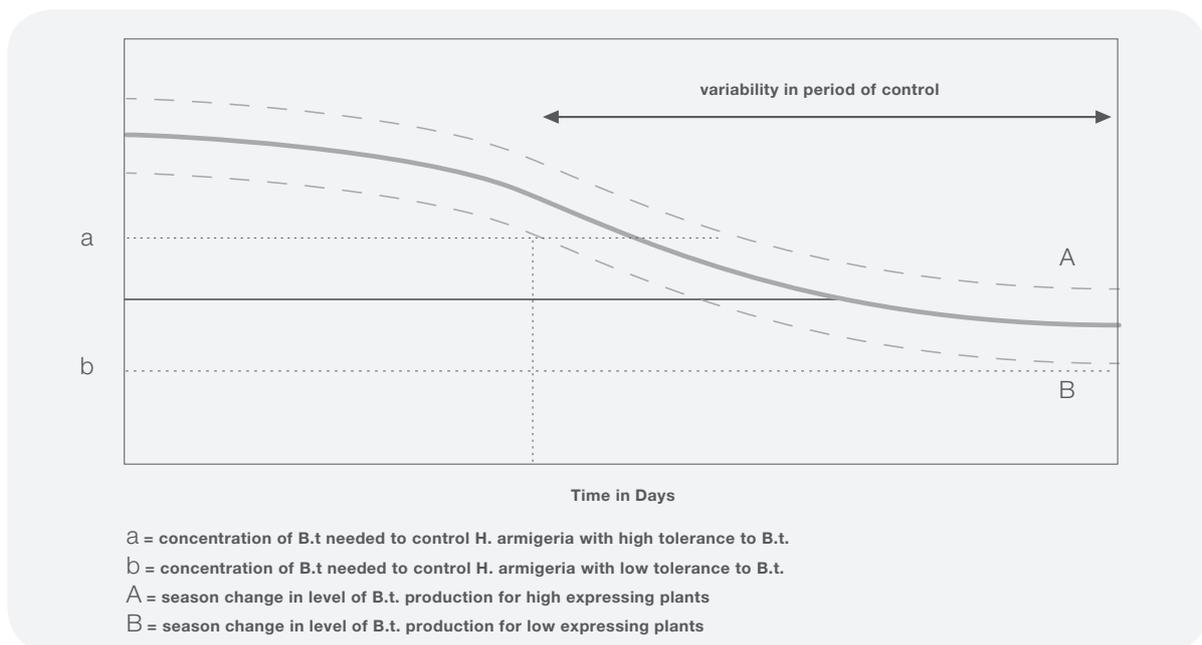
Helicoverpa spp. individuals are not clones and therefore have the same genetic variability and interaction of genes as other living organisms. It should not be unexpected that there exists a large variability in susceptibility to the B.t. proteins between individuals within and between colonies. Jenkins et al (1997)² carried out studies to show the relative dose threshold of Cry1Ac to control *H. armigera*, *H. punctigera* and *H. zea*. These studies showed that they were comparable and that the susceptibility to Cry1Ac is highly variable. Stone and Sims (1993)³ showed a 16-fold difference in susceptibility amongst different populations of *H. zea* in Southern USA. Some individuals will be very susceptible and require lower rates of protein than others for their control. These will be less likely to survive in a Bollgard II cotton field compared to those that are more tolerant towards the protein. The potential is greater for the more tolerant individuals to survive on Bollgard II cotton at an earlier stage than those that are more susceptible. This was evident in INGARD fields as considerable variability was seen on some occasions.

Figure 19. Variability of *H.armigera* in response to B.t.



In any field situation the overall efficacy is limited by the combination of the level of expression of the B.t. proteins in the plants and by the degree of susceptibility to those proteins of the target pests.

Figure 20. Typical field situation



²Jenkins, et al., 1997. Resistance of cotton with delta-endotoxin genes from *Bacillus thuringiensis* var. *kurstaki* on selected lepidopteran insects. *Agronomy Journal*, Vol. 89 (5): p.768–780.
³Stone, T. B., and S. R. Sims. 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. *J. Econ. Entomol.* 86: 989–994.

In a Bollgard II field situation, variability will still occur within the cotton plants as well as within the *Helicoverpa* populations. However, relatively little variability in efficacy is expected within Bollgard II fields due to the high levels of protein production.

Behavioural response of Helicoverpa spp. to the B.t. Proteins

Observations suggest that *H. armigera* can detect Cry1Ac at the levels found in the plant tissue during the first part of the season. In the absence of a choice situation (food source without B.t.), they will still feed on the plant tissue and die. The presence of the B.t. proteins has been observed to elicit more movement of the larvae in an attempt to find B.t.-free food.

External Semi-Controllable Factors (Environmental conditions)

- Waterlogging
- Temperature
- Light intensity
- Nutrition
- Other stress factors

Such factors may affect the production of proteins within the plant and effectively reduce the level of protection to be obtained. Ensuring the crop is healthy and does not suffer undue stress will minimise this effect on protein expression. Trials are being carried out by Monsanto to investigate the effect of environmental conditions on B.t. protein expression.

Management practices

These are controllable by the grower. Such factors as the nutritional status of the field, when to spray and what to spray can have a major influence on how well a Bollgard II variety performs. We have seen how the level of B.t. production is not fixed and that the target pest is variable in its susceptibility to B.t.. How can this variability be managed to obtain the optimum performance from Bollgard II cotton? How can spray decisions themselves affect the overall ability of a Bollgard II crop to withstand pest attack and minimise the need for spray applications?

Monitoring

The inherent variability in the level of production of the B.t. proteins between plants means that adequate monitoring is essential to truly reflect the overall condition within a field. When scouting, it is important to take a large, random sample in the field to ensure good representation of the average field situation. Looking at bad plants is misleading – there will inevitably be bad plants.

Spray Decisions

A crop is sprayed when pest numbers and potential damage caused by *Helicoverpa* spp. increases to a level where it is more economical to control them than to leave them. Industry has developed spray threshold levels based upon this.

Bollgard II Threshold

- Two consecutive checks produce more than two small larvae (>3mm) per metre,
- or one check produces one or more medium larvae (>8mm),

This is an indication that a supplementary spray may be required to achieve the best economic outcome. The crop state is also important when making a spray decision. If retention is poor, a spray decision may be reached before the threshold level as the grower may not be able to incur a low level of fruit loss. Similarly, a grower may decide to spray before the threshold level has been reached late in the season if there is a high egg lay. Expression declines with time and a very high egg lay may result in sufficient larvae surviving late in the season to merit a spray. Earlier in the season, Bollgard II should be expressing at a sufficiently high enough level to control any emerging larvae.

PEST MANAGEMENT

Direct Benefits from Bollgard II

The obvious benefit to be obtained through planting Bollgard II is the reduction in sprays required for *Helicoverpa* spp. Bollgard II effectively controls *H. armigera* and *H. punctigera* for most of the season. Even late in the season, there is still B.t. protein within Bollgard II plants and, if spraying is required, the surviving larvae will be less fit and should be more susceptible to pesticides. It may be possible to utilise single insecticides late in the season instead of tank mixes to reduce to total pesticide usage further.

Bollgard II reduces the requirement for larvicides in general. However, this is not the only value of Bollgard II in pest management. With the use of Bollgard II there is a reduced need for the application of broad-spectrum pesticides. In the absence of any pesticides other insects will be able to develop and thrive in Bollgard II cotton. This includes beneficial insects.

Indirect Benefits from Bollgard II

Increased survival and numbers of beneficial insects has two benefits to the farmer:

Firstly, beneficial insects are able to successfully control many secondary pests such as aphids and whitefly. This will reduce the requirement for additional sprays to control these pests. There will still be times when economic outbreaks do occur but by using selective pesticides targeting only the pests (i.e. not broad spectrum insecticides) the beneficial insects can still be preserved. If the beneficial insects are depleted then there will be no natural control of any secondary pests that may occur in the field and chemicals will therefore be required more frequently.

The second benefit of preserving the beneficial insects is in reducing selection pressure for resistance to B.t. proteins. Many beneficial insects feed on the eggs and small larvae of *Helicoverpa* spp. Predation and parasitism removes eggs from the field, so less *Helicoverpa* spp. will hatch and fewer individuals will feed on Bollgard II. This will effectively reduce the selection pressure for resistance in *Helicoverpa* spp. by reducing the numbers of individuals exposed to the B.t. proteins. This may also have an economic impact at the end of the season by reducing the likelihood of the need for late season *Helicoverpa* sprays as the larval numbers may be maintained below threshold levels by the beneficial insects.

Beneficial insects:

Egg parasites:	Trichogramma, Microplitis
Egg predators:	Ladybirds, Red and Blue beetles, Damsel bugs, Smudge bugs, Lacewings
Neonate predators:	Predatory bugs, Spiders

Even with the higher levels of beneficial insects in the cotton, insecticides will still be required to control sucking pests and mites both early in the season and late in the season as the presence of these pests (no longer controlled through *Helicoverpa* sprays.) may cause economic damage. Mirids, Apple Dimpling Bug, Green Vegetable Bugs and Aphids may become more of a problem and require specific spraying. In order to achieve the best value from Bollgard II technology, careful monitoring of these pests and action in a timely manner to selectively control these pests is required to minimise crop loss. The selective use of chemicals in adjacent conventional cotton is also important as drift can disrupt the beneficial levels in Bollgard fields. The judicious use of insecticides will minimise the impact of these secondary pests on yield and profitability.

How to Maximise the Insecticidal Benefits

Varieties of Bollgard II cotton may perform differently, in terms of observed efficacy. Factors include different locations, seasons, planting times and management practices. By following these suggestions the optimal value can be obtained from growing Bollgard II cotton:

- Refrain from the use of synthetic pyrethroids, broad-spectrum organophosphates and carbamates on both Bollgard II and conventional cotton where possible (Appendix 2).
- Monitor the cotton and check large random samples in the field to ensure good representation of the average field situation.
- Don't spray unless thresholds and crops dictate.
- Don't stress the cotton.

Maintaining beneficial insects will reduce selection pressure for resistance to all pesticides including Bollgard II. Maximise the potential of Bollgard II cotton by minimising the disruption to the beneficial populations; this will decrease the *Helicoverpa* pressure extending the period of control for Bollgard II cotton.

Spray Decision Examples

It is recommended to use the following thresholds for Bollgard II cotton. The industry developed spray threshold for Bollgard II cotton is 2 consecutive checks of 2 smalls (>3mm) per metre. For the following examples it is assumed that there is an egg lay of 20 eggs per metre.

Early season

Assume 5% of eggs survive to smalls,

Therefore,

5% of 20 will survive = 1 small per metre **No spray**

Late season – (IPM managed Bollgard II field)

Assume 50% eggs are parasitised or predated by beneficial insects and assume the level of larval survival, 15%.

Therefore,

50% of the 20 eggs per metre laid will survive the beneficials = 10 eggs per metre

15% of 10 will survive = 1.5 smalls per metre **No spray**

The hidden benefits of Bollgard II cotton

Managing Bollgard II facilitates the preservation and encouragement of beneficial insects, which can extend the effective period of control of Bollgard II cotton.

AGRONOMIC MANAGEMENT

Points to Watch For

The benefits of Bollgard II traits are significant, however a grower must choose a variety that is first and foremost suited to their growing region. The choice of the transgenic options should be a secondary consideration. Listed below are the key parameters in cotton agronomy and some possible areas for concern or change when growing Bollgard II cotton.

Varietal Maturity

Maturity in conventional cotton is determined to a large degree by the genetic background of the particular variety. In Bollgard II this will be the same and will depend on the characteristics of the recurrent parent used in the initial cross. There are other factors such as soil compaction, nutritional stress, water stress and fruit retention that can significantly influence maturity.

Planting Density

Planting density is generally in the range of 10–15 seeds/metre assuming an 85% germination. Normally as the planting density increases there is an associated reduction in the number of main stem fruiting branches. With the likelihood of significantly higher 1st position retention with Bollgard II, there should be no need to increase planting rates from their current levels. Planting density influences potential yield and investigations are planned to determine whether the currently accepted planting rates are still suitable for Bollgard II with its generally higher retention levels.

Fruit Retention

The largest bolls on a cotton plant are generally produced in the middle of the plant (nodes 13–18). These bolls tend to be around 12–15% larger than bolls held in the second position. The further bolls are from the main stem, the smaller they are. The reason these first position middle-plant bolls are larger is that they flower in more optimal temperatures and are less affected in their development by canopy shading and reduced leaf function and they are nourished from their subtending leaf, the main stem leaf and the second position leaf. They are the main sink of photosynthates and carbohydrates.

In conventional cotton this first position fruit is frequently missing due to insects, physiological shedding (normally towards the end of flowering) or some other form of stress. First position retention can range from 30% through to 70% in the first five (5) fruiting branches. Normally this is not a concern when the retention is being monitored, because there can be compensation through the production of outer fruit positions and fruiting branches. A second position boll takes the place of the first position boll normally, however this boll will never be as big as the first position boll would have been.

Earliness, or the number of days between planting and defoliation, is measured as the node number where 95% of the harvestable bolls are set. There is no direct relationship between the bottom five first position retention and final yield per se. There is however a maturity delay. As boll retention decreases by 20%, it takes approximately one extra node to set the crop. This can represent a delay in maturity of around five (5) or six (6) days at harvest, depending upon temperatures. The relationship between earliness (maturity) and all the first position fruit in the 95% zone is similar, providing there is compensatory growth.

Final plant height has a strong correlation with maturity. An increase in plant height of approximately 12 cms has the same effect on maturity as a 20% reduction in first position fruit retention under the same temperature regime.

Bollgard II cotton plants will hold significantly more of their first position fruit than conventional cotton plants will. They will have significantly higher first position retention on the bottom five fruiting branches, which may shorten the number of days required to make a crop. This would normally only be a concern if the variety chosen was a very 'short season' one and could not compensate to make use of favourable, late weather conditions.

Irrigation Management

The water requirement *per se* of a Bollgard II plant should not be any different to that of a conventional plant. The ability of the roots to extract moisture will be more limited by the physical properties of the soil. However, the timing of irrigation may be slightly different from conventional cotton due to the possible earlier drain on resources associated with higher retention.

Nutrition Management

Growers will need to ensure that their nutrition management is correct for their soil characteristics. The potential for premature senescence through K deficiency from rapid fruiting on crops with a heavy boll load may be a possibility. The key to managing this lies in the careful monitoring of boll load, early/mid season soil K levels and selecting more tolerant varieties.

Disease Management

Disease tolerance is a product-of-selection through plant breeding and is controlled to a large degree by the breeding process where strict selection and screening ensure that only disease tolerant varieties make it to the market place in disease-prone areas.

Seedling Emergence

There is no difference to conventional cotton plants.

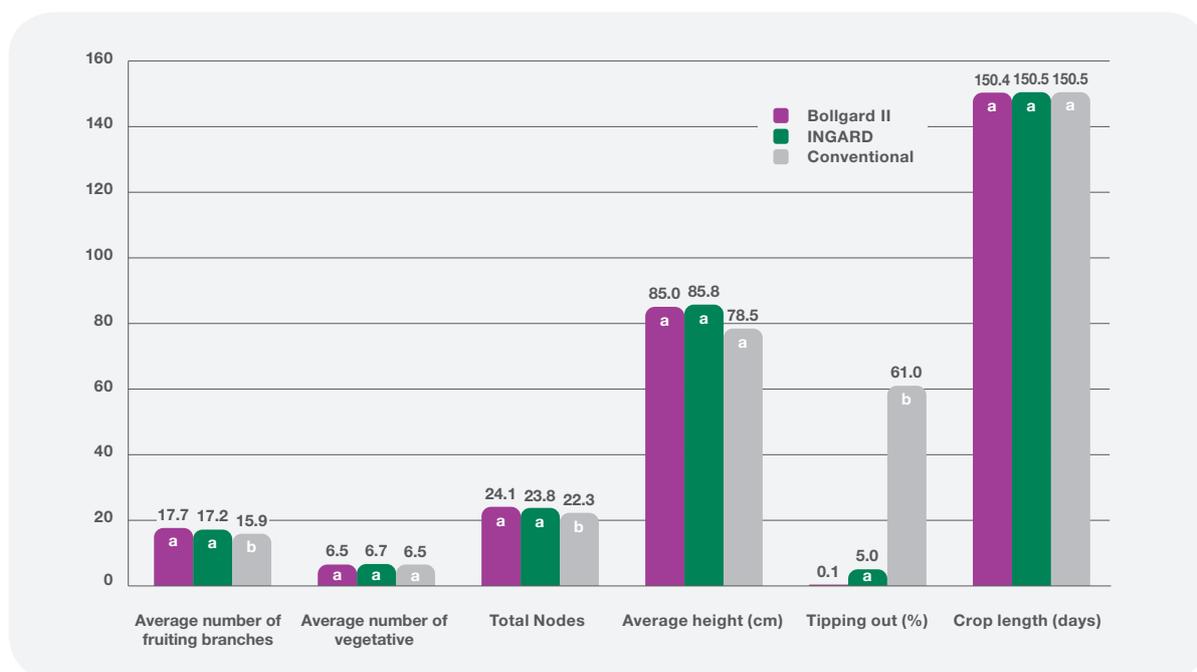
Growth Regulator Requirement

There may be a need for growth regulants in Bollgard II cotton. This obviously will depend upon the varietal selection and the individual field history and management.

Fibre Quality

By holding more of the total harvestable bolls in the first position there may well be less chance of low micronaire from the 'top crop', with the plant 'cutting out' in a more uniform fashion, provided that other agronomic influences are well managed.

Figure 21. Growth and Development 2003/04



The growth and development for Bollgard II varieties are similar to that of conventional varieties. Differentiation between a Bollgard II cotton plant and a conventional cotton plant does occur when comparing the tipping out percentages.

Figure 22. Growth and Development 2003/04

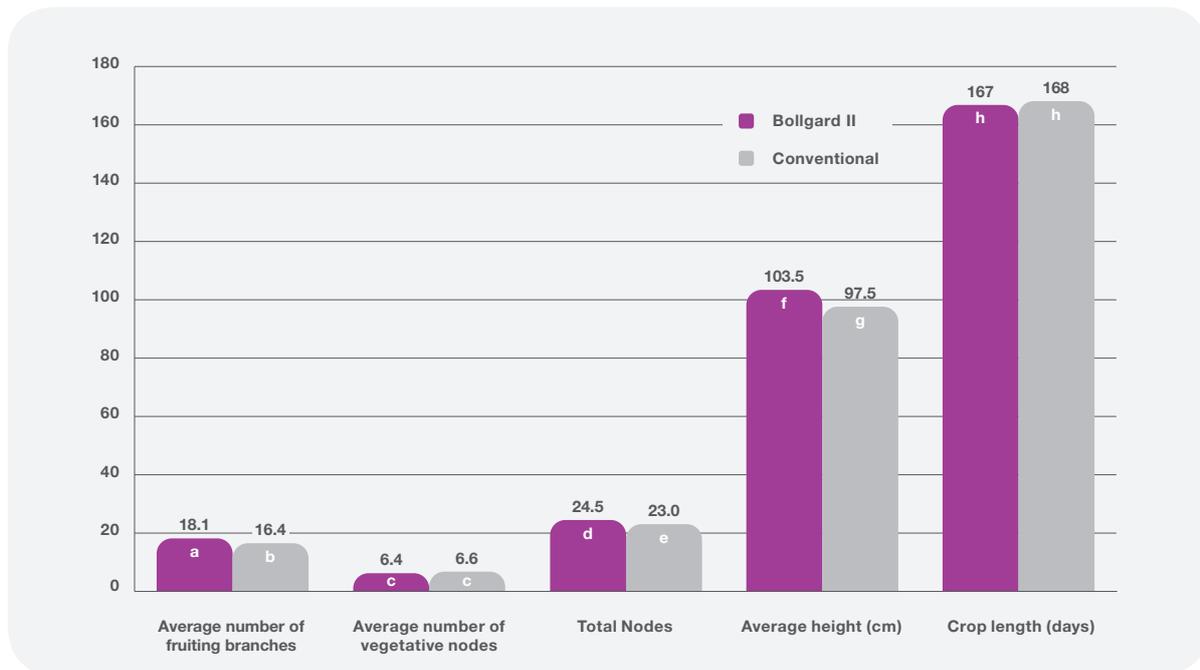


Figure 23. Fruit Retention 2002/03

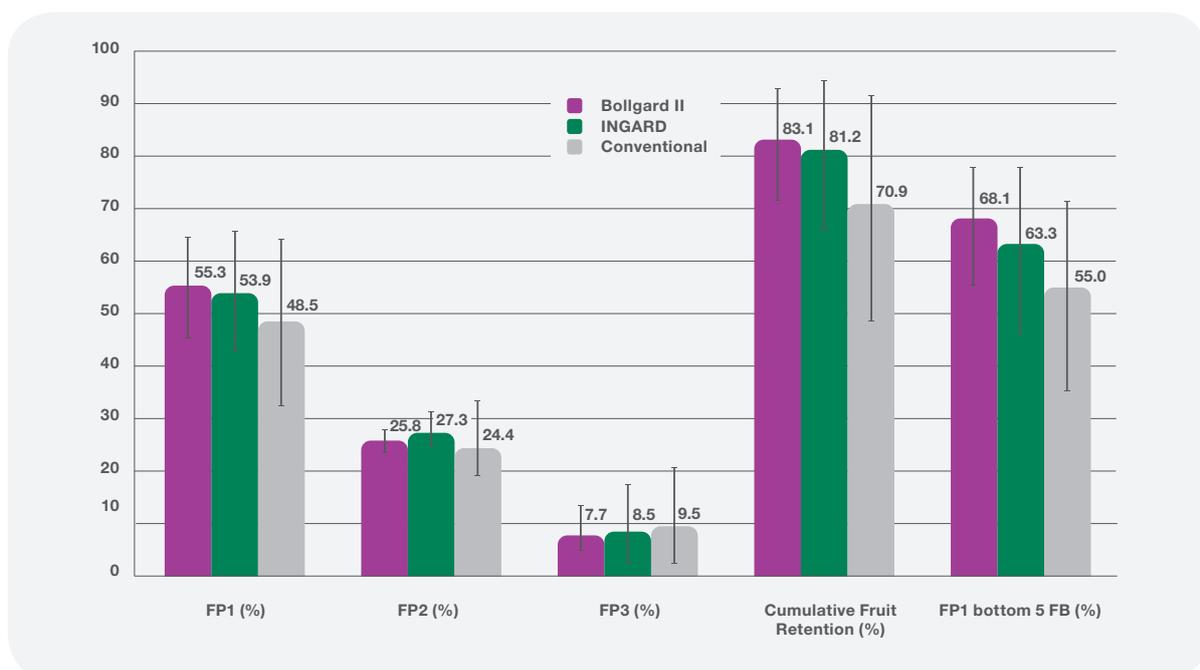


Figure 24. Fruit Retention 2003/04

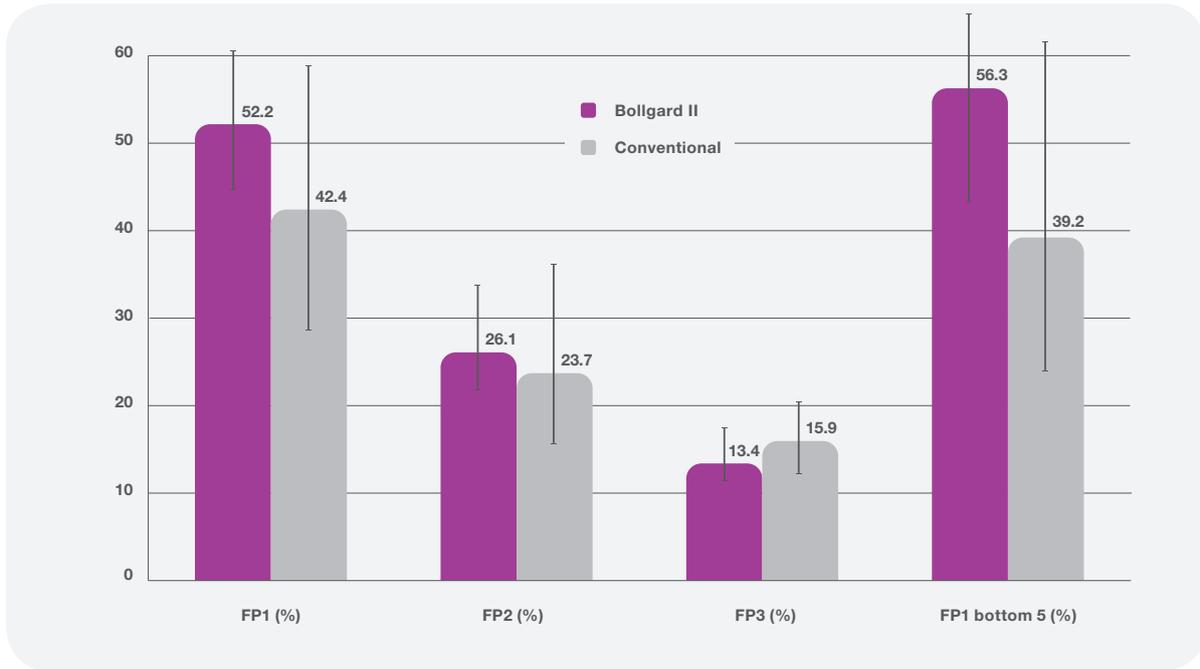


Figure 25. Fruiting Pattern 2002/03

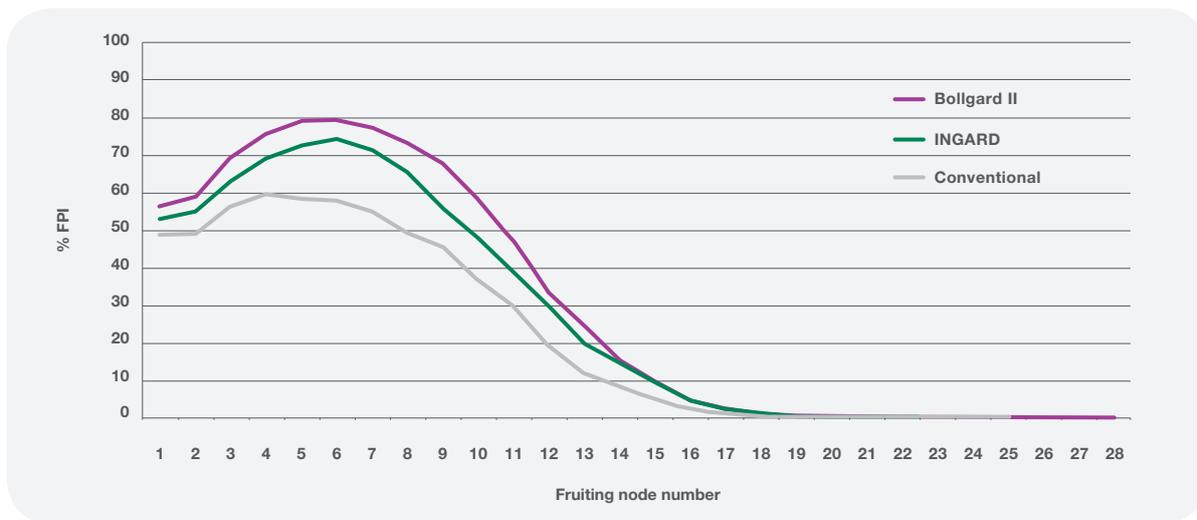


Figure 26. Fruiting Pattern 2004/05

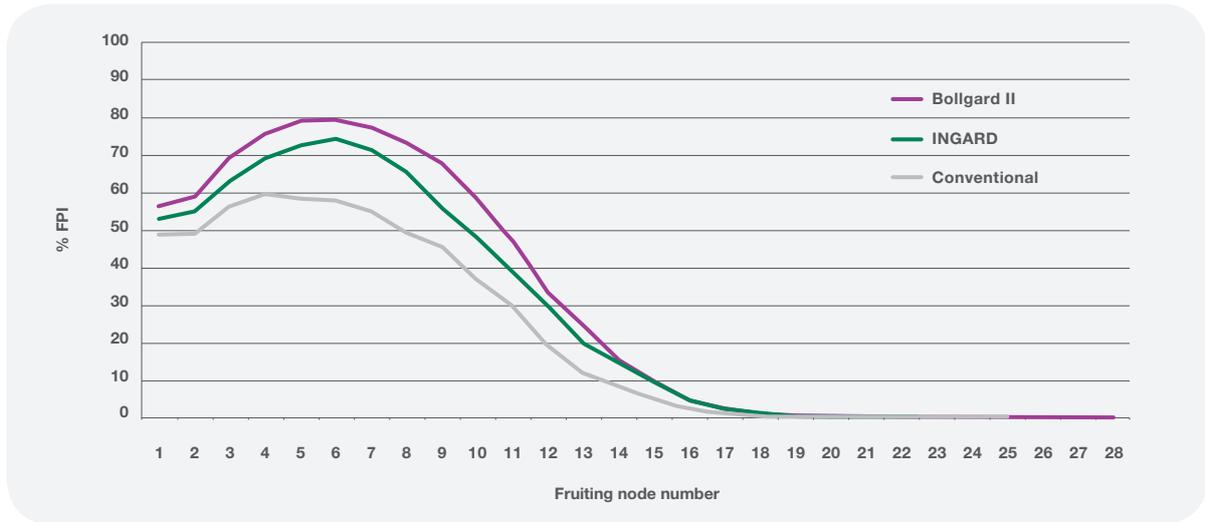
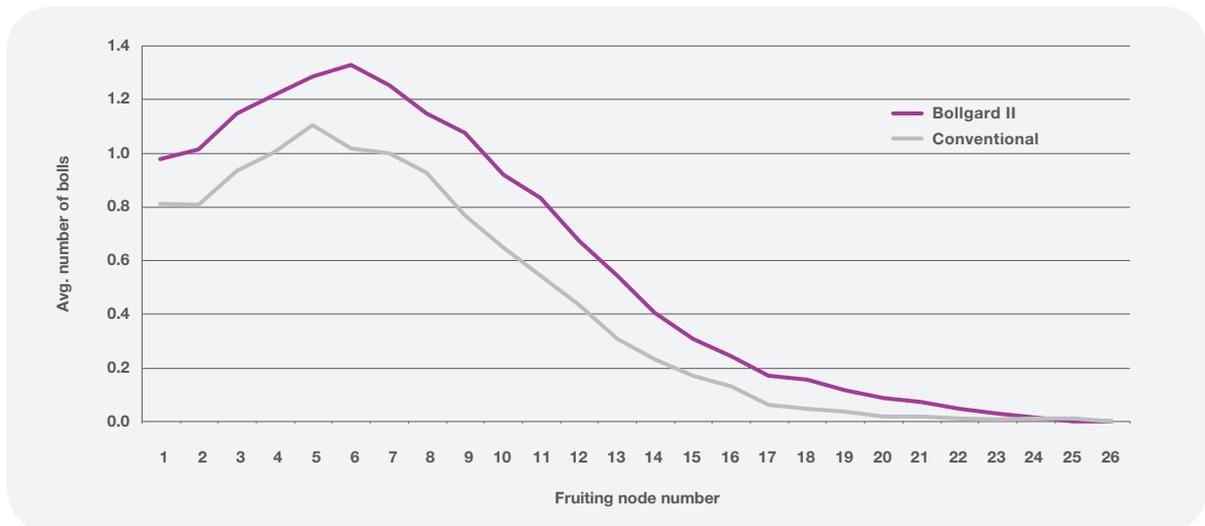


Figure 27. Total Boll Distribution 2003/04



The fruiting patterns of Bollgard II cotton plants are similar with that of conventional plants but the fruit retention is consistently higher than conventional cotton plants.

INSECT RESISTANCE

Introduction

Resistance poses a serious threat to transgenic cotton. Australian cotton growers are very familiar with the devastation that the industry can suffer from insecticide resistance, as in the Ord in the 1970s. Similarly, cotton growers are also well aware of the importance of transgenic cotton for reducing pesticide use in the industry. Unfortunately, transgenic plants are no less subject to selection for resistance than classical insecticides.

A major pest of cabbage, the diamondback moth, has already evolved resistance to *Bacillus thuringiensis* (B.t.) sprays in many cropping areas around the world. B.t.-resistant diamondback moth larvae are completely resistant to transgenic plants that carry the cry1Ac gene (incorporated in both INGARD and Bollgard II cotton). There is every reason to suspect that a few cotton bollworms carry a resistance gene that is very similar to the one found in the diamondback moth.

In contrast to B.t. sprays, the B.t. proteins are continuously expressed in transgenic plants, which means that every insect feeding on them will be selected for resistance. This persistent exposure offers the potential for even stronger selection for resistance than would come from sprays. There is also evidence that genes for resistance to B.t. may be more common than were genes for resistance to chemical insecticides, which could provide an avenue for faster resistance. It is thought that the frequency of the gene may be as common as 1 in a thousand or 1 in ten thousand. Simulation models suggest that resistance this common could be selected fast enough to cause damage in only 5 years in a single-B.t. gene transgenic plant (INGARD cotton) in the absence of careful management. Thus, resistance management is just as critical to transgenic crops as it has been for chemical insecticides.

Professor Roush believes that 'the pyramiding of protein genes offers what appears to be the most effective way to manage resistance to B.t. and other insecticidal transgenic proteins. Pyramids have the potential to greatly reduce refuge requirements for successful resistance management from perhaps 30–40% down to perhaps 10%⁴.'

Bollgard II has the advantage of having pyramided genes and produces two proteins with no cross resistance in *Helicoverpa* spp. Therefore Bollgard II has more protection against the establishment of resistance in comparison to INGARD cotton that only expressed the single B.t. protein. However, careful management is still necessary to ensure that this advantage is maintained for the future and not squandered.

Resistance Management Plan

The cotton industry has learned that resistance is a real threat and that resistance management can work well. In the case of pyrethroids, the resistance management strategy was put into place after resistance was already apparent and this severely reduced the benefit of the resistance management plan. Even so, it has been estimated that the resistance management plan for pyrethroids has extended their effective life by 3–10 years, which is an impressive achievement. In the case of transgenic crops, a comprehensive resistance management strategy has been established within the industry prior to any detected levels of resistance. This should result in greater benefits than have been achieved with pyrethroids. With Bollgard II there is the opportunity to develop a resistance management strategy before any resistance has been detected to either of the pyramided proteins. This should help delay the onset of resistance.

An effective resistance management strategy in cotton must contain three key criteria;

- (1) large refuges with no selection for B.t. resistance;
- (2) destruction of pupae under the transgenic crops to remove any selected individuals;
- (3) compliance with a resistance management strategy before any resistance has developed.

⁴ Roush, R. 1998. Two toxin strategies for management of insecticidal transgenic crops: Can pyramiding succeed where pesticide mixtures have not? *Phil. Trans. Royal Soc. Lond. B.* 353: 1777–1786.

Key Components of the RMP for Bollgard II

Key Components of the RMP for Bollgard II cotton
Refuge crops
Planting window
Spray limitations
Pupae busting/Trap crops
Control of ratoon cotton and volunteers

The refuge chosen will determine the percentage of Bollgard II cotton grown on any farm. However, due to some target pests having only cotton as their host, there will always be a requirement for some conventional cotton to be included.

The reasoning behind the specific RMP requirements

The Planting window (Dr G Fitt, CSIRO)

The planting window aims to avoid having late maturing transgenic crops. *Helicoverpa armigera* is the main concern with respect to damage to Bollgard II cotton and the potential to become resistant to the B.t. proteins. Populations of *H. armigera* increase during the growing season reaching high levels by February/March. *H. armigera* is the predominant species on cotton at this time of year. Every effort is needed to minimise exposure of crops to those high *H. armigera* populations at that time. The planting window limits the number of generations of *H. armigera* that are exposed to the B.t. proteins. The Planting Window dates accommodate the most favourable planting time in most areas and allows for some replanting.

Refugia requirement (Dr G Fitt, CSIRO)

Refuge crops are required to produce populations of moths that have not been exposed to selection with B.t. proteins. Mating of these moths with survivors from Bollgard II crops can help dilute selection for resistance and slow the rate at which resistance becomes a problem.

In comparing refuge crops for Bollgard II cotton we use unsprayed conventional cotton as the control refuge because it is attractive for a similar period to Bollgard II cotton. Modelling studies have arrived at a requirement for resistance management of a 10% refuge where the refuge has characteristics like unsprayed conventional cotton. Our research with different refuge options has compared the capacity of each crop to produce *Helicoverpa* moths over an extended period with unsprayed conventional cotton. With this information the relative areas of each refuge can be adjusted.

Therefore if 10ha of unsprayed conventional cotton is required for every 100ha of Bollgard II cotton and if a new refuge option produces twice as many moths as the unsprayed cotton then it would need 5 ha per 100 ha Bollgard II cotton to be as effective.

B.t. spray restrictions (Dr G Fitt, CSIRO)

Use of B.t. sprays may also select for B.t. resistant insects. For this reason B.t. sprays may not be used on any refuge crops where the aim is to produce unselected (or susceptible) moths.

Pupae destruction (Murray Schoenfisch)

Helicoverpa spp. larvae can form pupae that will over winter (diapause). Such pupae pose a risk for resistance development as any resistant individuals surviving the B.t. in Bollgard II cotton at the end of the season will enter diapause. There is likely to be a concentration of resistant individuals, as the susceptible ones will not have survived. In the spring the pupae emerge into adults with a higher proportion of resistant individuals than normal and resistance can begin to develop. When larvae pupate, they burrow and form open tunnels to the pupation site. These tunnels must be clear for the adults to emerge from in the spring. At this stage the *Helicoverpa* can be effectively controlled without the necessity for chemicals. Simply by destroying the emergence tunnels the adults will be unable to emerge and they will die. The best way to destroy the tunnels is to cultivate to a depth of 10cm. 95% destruction is possible with the use of a chisel plough or offset discs and chisel. Centre-busting and cultivation with lillistons will give approximately 90% control.

This provides us with an easy tool to selectively remove resistant individuals from the population and provide effective control of the development of resistance to the B.t. proteins. No control puts the technology and the sustainability of cotton at risk.

Trap crops for central Queensland

An important aspect of the RMP for Bollgard II is to reduce the survival of over wintering pupal populations of *Helicoverpa* spp. under Bollgard II crops. Destroying these populations that may contain B.t. resistant individuals will maximise the value of the in-season refuge crops and allow an even greater dilution of resistance each spring in the first generation of *Helicoverpa* spp.. This strategy can be effective in most cotton growing areas where *Helicoverpa* spp. passes the winter in diapause.

However, in central Queensland very few of the *Helicoverpa* spp. enter diapause from cotton crops. Instead, the survivors emerge and breed again on later crops. For this reason the strategy in CQ includes a late season trap crop designed to attract and concentrate the late season survivors emerging from cotton. Eggs are laid on the trap crop and larvae develop but are then destroyed by full cultivation of the trap crop. In this way the trap crop helps to keep these moths in the cotton region and serves a similar purpose to the crop residue cultivation required further south.

Resistance Monitoring

Monsanto is the provider of the biotechnology providing the cotton industry with cotton plants that can withstand attack by *Helicoverpa* spp.. A resistance management plan is in place to protect the technology from resistance and to create a sustainable cropping system. This management plan needs to be monitored to ensure that it is working well and is following the model upon which it is based. It is therefore of great importance to ensure that the levels of resistance to these crops is adequately monitored to maintain longevity of this technology and of the technologies in the future.

Resistance Monitoring Program

B.t. resistance monitoring program background

Monsanto has been collaborating with CSIRO on the B.t. Resistance Monitoring Program since 2003/04. The program was established to determine the frequency of alleles which impart resistance against the proteins Cry2Ab and Cry1Ac.

F₂ screens for Cry2Ab and Cry1Ac resistance alleles

The F₂ screen measures the frequency of resistance alleles in the population. To do this, isolated pairs of moths from the same collection are mated and their grandchildren (F₂) are exposed to a concentration of B.t. protein that is survived only by resistant insects. This test takes approximately 12 weeks to run.

F₁ screens for Cry2Ab resistance alleles

The F₁ test also measures the frequency of alleles in the population. In this test, a field -collected moth is mated with a known resistant moth and their offspring (F₁) are exposed to a dose of Cry2Ab protein that will only be survived by resistant larvae. This test takes approximately 6 weeks to run.

Monsanto and the TIMS Technical Panel meet annually to review the resistance monitoring data. Monsanto has developed with input from the TIMS Technical Panel a Cry2Ab Contingency Plan. This document will be used to determine if changes need to be made to the Bollgard II RMP.

QUALITY ASSURANCE

Introduction

The backcrossing of the Bollgard II genes into the Australian seed companies' elite cotton lines and the bulking of that seed for commercial production is a carefully managed process which has to comply with strict quality standards set by Monsanto and the seed companies. As part of the license agreement between Monsanto Australia and the seed companies producing cottonseed containing the Bollgard II technology, certain Quality Assurance testing guidelines must be adhered to. This is to minimize or prevent any quality issues in the market for growers. To this end, all lines being developed as Bollgard II or Bollgard II Roundup Ready® cotton varieties in Australia must have the following tests:

- (1) Gene Purity
- (2) Seed Lot Verification
- (3) Commercial Crop Tolerance (Roundup Ready) / Gene Equivalency (Bollgard II)
- (4) Variety Performance.

Seed Company Quality Assurance

Gene Equivalency

Bollgard II – Gene Equivalency

Prior to commercial variety approval, all new Bollgard II lines must pass the Gene Equivalency testing carried out by Monsanto in Australia. The term gene equivalence infers that the proposed new varieties express the Bt proteins, produced by the new genes, in an equivalent manner to other acceptable Bollgard II varieties/lines. The gene equivalency testing is not to determine efficacy, which is dependent upon a number of factors such as plant health and nutritional status etc. Gene equivalency is to ensure that sufficient protein is produced so that a minimum level of activity is obtained under a range of conditions, which are not significantly different from other Bollgard II varieties on the market.

THE EXPOSURE AND EFFECTS OF B.T. PROTEINS ON NON-TARGET ORGANISMS

SEE APPENDIX 2: TOXICOLOGY

Exposure and Effects of B.T. on Humans

Humans have been exposed to B.t. in their natural habitats since man first appeared, particularly from soil and water. However in the recorded scientific literature, only a few adverse effects to these environmental B.t. levels have been documented.

People are exposed to B.t. products through the manufacture and field application of these products. Agricultural and horticultural uses of B.t. can also result in dietary exposure. Agricultural uses of B.t. can result in certain B.t. levels in potable water and food. B.t. has not been reported to cause adverse effects on human health when present in drinking water or food. Human volunteers as part of a scientific study ingested and inhaled quantities of a B.t. formulation with no adverse health effects.

Owing to their specific mode of action, B.t. products are unlikely to pose any hazard to humans or other vertebrates or to the great majority of non-target invertebrates. B.t. products are registered and may be safely used for the control of insect pests in agricultural and horticultural crops. They are also safe for use in aquatic environments including drinking-water reservoirs for the control of mosquito, black fly and nuisance insect larvae.

Effects of B.T. on Non-Target Organisms

Multiple dose studies with B.t. have been conducted with mammals, birds, fish and other non-target animals to investigate the effects of dietary, dermal and inhalatory exposure to B.t., with negligible adverse effects. In rats, no toxicity or infectivity was associated with dietary exposure to B.t. (4 g/kg per day) for 3 months and the only effect observed from a 2-year study in which a commercial B.t. preparation was fed to rats at 8400 mg/kg per day in the diet, was a slight decrease in body weight of females.

B.t. has not been reported to adversely affect birds, fish or other non-target aquatic vertebrates tested in a large number of laboratory and field studies. B.t. does not adversely affect earthworms.

The B.t. proteins have generally been shown to be highly specific in their insecticidal activity for *Coleoptera*, *Diptera* and *Lepidoptera* and have demonstrated little, if any, direct toxicity to non-target arthropods. Most of the existing safety data on non-target arthropods has been generated using the B.t. proteins with activity against *Diptera* and *Lepidoptera*.

Impact of B.T. Transgenic Cottons on Abundance of Non-Target Arthropods in Australia

Three trials were conducted over the 1999 – 2001 period. One trial was conducted at the Frank Wise Institute of Tropical Agriculture, Kununurra, Western Australia to compare the differences in non-target arthropod abundance between Bollgard II and INGARD cotton. Two trials were conducted at the Dalby Agricultural College, Queensland comparing the difference in abundance of non-target arthropods between Bollgard II, INGARD and conventional cotton. All three trials were contracted to independent sources.

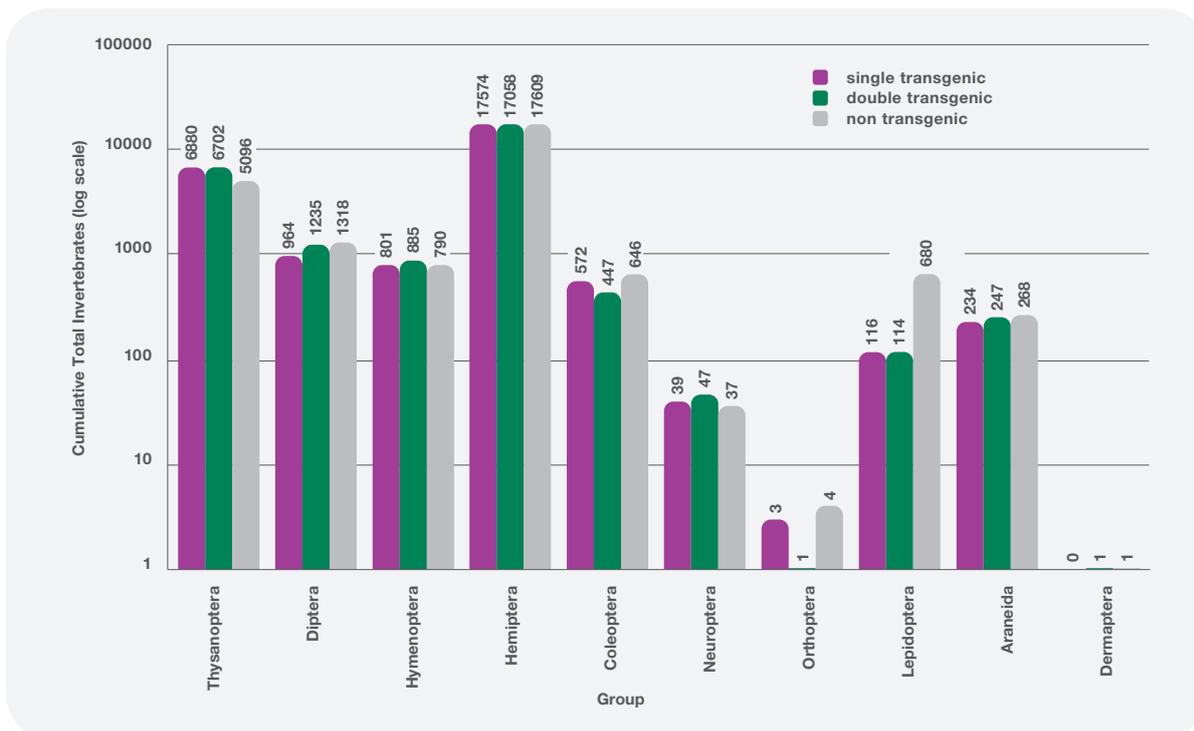
All three trials concluded that the presence of the Cry2Ab protein within the cotton plant had no measurable significant effect on the abundance of non-target arthropods within the cotton environment.

Studies on the effects of Cry2Ab on Non-Target Organisms

The experiments in Dalby were supervised by Dr David Murray, from the Queensland Department of Primary Industries. These experiments indicated that there were no differences in the abundance of individuals from different arthropod groups between each of the crop types.

1999/2000 Dr. David Murray (QDPI) made the following summary for the trial carried out by his department. "The abundance of non-target arthropods was compared on unsprayed non-B.t. cotton and two lines of unsprayed B.t.-transgenic cotton at Dalby, Queensland during 1999/2000. As determined by suction sampling and pitfall trapping, unsprayed B.t.-transgenic cottons (both single and two gene constructs) had no observable impact on the abundance of non-target arthropod groups compared to unsprayed non-B.t. cotton." In the 1999/2000 season there were no significant differences recorded for any non-target species measured in the experiments.

Figure 28. Abundance of non-target arthropods in suction samples at Dalby, 1999/2000.

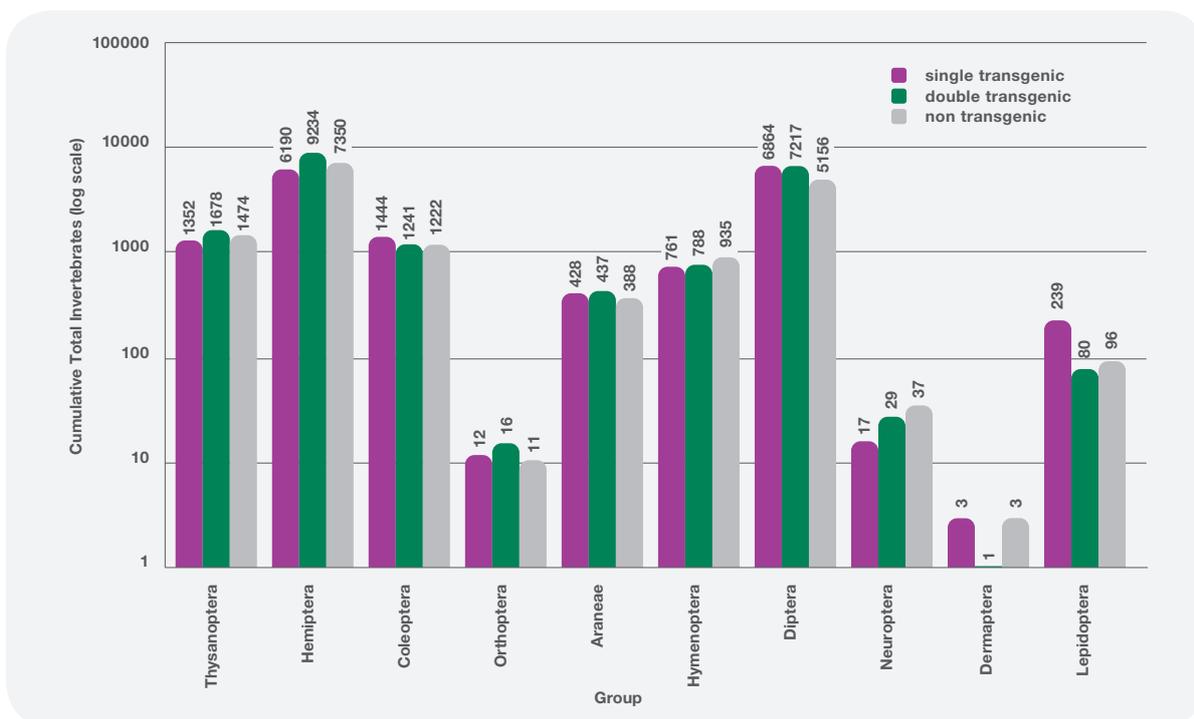


2000/2001

In 2000/2001, differences were found between the different crop types though no trend could be seen. *Hemiptera* were actually more prevalent in Bollgard II plants than in non-transgenic plants at four sample times. *Coleoptera* were less abundant in Bollgard II at only the first sample time. *Thysanoptera* were more abundant in both transgenic crops compared to the non-transgenic crop at the second sample time, but *Thysanoptera* were more abundant in the non-transgenic crop at the fourth sample time. *Diptera* were less abundant in the Bollgard II crop at two different sample times. From these differences no trend can be seen.

Dr David Murray, QDPI, summed up the 2000/2001 trial with the following: “With respect to the Environmental Impact Assessment carried out on non, single and double-transgenic cottons at Dalby during 2000/2001, as stated in the report, I am of the opinion that there were no observable treatment effects on arthropod fauna sampled during this study. This opinion is based on the data collected as part of the trial and personal observations at the site during the season.”

Figure 29. Abundance of non-target arthropods in suction samples at Dalby, 2000/01.



EXPECTATIONS FROM BOLLGARD II

Bollgard II will provide protection against *H. armigera* and *H. punctigera* however, the technology does not make cotton *Helicoverpa*-proof. High pest pressure may still necessitate sprays for *Helicoverpa* during the season and pressure at the end of the season may still require additional chemical control. Secondary pests will still be an issue but through judicious use of pesticides the impact can be minimised by encouraging and protecting beneficial insects.

- Bollgard II provides excellent insecticidal activity against *Helicoverpa* spp.
- Bollgard II is NOT *Helicoverpa*-proof!
- High pest pressure, end of season or plant stress may necessitate pesticide application.
- Secondary pests may still need controlling.
- Bollgard II will not produce higher yields or better quality cotton but should produce cotton of a similar quality and with similar yields to existing commercial cotton lines.
- Bollgard II provides the grower with a foundation for IPM and the potential to increase sustainability.

RECOMMENDATIONS FOR GROWERS AND CONSULTANTS

- Select Bollgard II varieties on their agronomic merits only and ensure that the variety is recommended for the region it is proposed for.
- Read and understand the Bollgard II label and the Resistance Management Plan prior to planting.
- Plant Bollgard II into clean fields with no cotton residues.
- Ensure the nutrient content of the soil is sufficient prior to planting. Early fruit set and high retention can stress the plant, which may cut out early if sufficient nutrients are not present.
- Ensure that irrigation is timely to prevent undue stress on the plant.
- Bollgard II cotton requires careful scouting for pests. Although *Helicoverpa* spp. may not be a serious season-long problem in Bollgard II, secondary pests can still cause severe damage if not controlled when necessary.
- Use selective insecticides as much as possible to maintain high beneficial insect numbers as this WILL assist in controlling secondary pests and will reduce the selection pressure for resistance to B.t. by removing *Helicoverpa* spp.
- *Helicoverpa* spp. may need supplementary insecticide application if pest pressure is high or if the plant is under stress. Bollgard II is NOT immune to attack by *Helicoverpa*.
- Use the Bollgard II cotton spray thresholds when making spray decisions.
- Bollgard II does not affect *Helicoverpa* eggs. Larvae must feed on the plant tissue before it will affect them. Therefore, do not make spray decisions based solely on eggs or very small (vs.) larvae.
- Plan to carry out pupae busting directly after harvesting for best results. Dry conditions can make this very difficult to achieve. If left until later it can prove to be an expensive operation if the set standards are to be obtained.

Contact your nominated Technology Service Provider, Bollgard II Cottonseed Company (Cotton Seed Distributors) or your local Monsanto Regional Business Manager for advice on growing and managing Bollgard II cotton.

Appendix 1: Bollgard II Sampling

Bollgard II cotton **must** be monitored regularly throughout the season for *Helicoverpa* spp. and other pests.

Additional *Helicoverpa* spp. control methods are required if 2 small larvae (> 3 mm long) per metre continues over 2 consecutive checks or 1 medium or large larvae are found on the first check. Eggs and larvae < 3 mm are not included in the current spray thresholds.

This threshold requires an accurate assessment of larval sizes. The abundance of beneficial insects in Bollgard II crops should be taken into consideration when considering pest control. Where possible, choose the most effective pesticide that is the least disruptive to the beneficial complex.

Appendix 2: Toxicology

Four proteins are expressed at low levels in Bollgard II cotton:

1. An insect control protein derived from the common soil bacterium, *Bacillus thuringiensis subsp. kurstaki*, the Cry1Ac delta endotoxin protein (Cry1Ac protein).
2. An insect control protein derived from the common soil bacterium, *Bacillus thuringiensis subsp. kurstaki*, the Cry2Ab delta endotoxin protein (Cry2Ab2 protein).
3. Neomycin phosphotransferase II enzyme (NPTII) protein from the nptII gene from *E. coli*.
4. β -glucuronidase (GUS) protein from the *uidA* gene from *E. coli*.

The toxicology of the naturally occurring *Bacillus thuringiensis var. kurstaki* delta endotoxin (Cry1Ac) has been previously considered and approved by the NRA. A number of insecticidal products containing this active constituent (and other sub-species of *Bacillus thuringiensis*) are currently registered for use in cotton.

Based on consideration of information relating to human safety, the Australian Health Ministers' Advisory Council has recommended that *Bacillus thuringiensis* (B.t.) be exempt from the requirement for scheduling under regulations relating to drugs and poisons.

The safety of *B.t.* to humans, other mammals, birds and fish is well substantiated.

There are no receptors for the protein delta-endotoxins of *B.t.* sub-species on the surface of mammalian intestinal cells; therefore, humans are not susceptible to these proteins. This has been confirmed in numerous safety studies carried out in laboratory animals which are traditionally surrogates for humans. The results of some of these studies have been published in scientific reviews (Ignoffo, 1973; Shaddock, 1983; Siegel and Shaddock, 1989). Results of unpublished safety studies generated by registrants of *B.t.* commercial preparations in the USA have also been summarized in the EPA Registration Standard for *B.t.* Formulations (EPA, 1988).

In published reviews and the EPA document, studies are referenced where large doses (5000 mg/kg) of *B.t.* formulations were administered as single or multiple oral doses (up to 2 years) to different laboratory animals, with no adverse effects. Avian and aquatic organisms have also been fed *B.t.* formulations, with no adverse effects.

Safety assessments of the Cry1Ac, Cry2Ab, GUS and NPTII proteins expressed in Bollgard II cotton event 15985 are summarised below. These include demonstrating the lack of similarity to known allergens and toxins, the long history of safe consumption of comparable proteins in microbial formulations, rapid digestion in simulated gastric and intestinal fluids, and the lack of acute oral toxicity in mice.

Safety of Cry1Ac and Cry2Ab proteins

Cry1Ac protein

The amino acid sequence of the Cry1Ac protein expressed in INGARD cotton has been predicted based on the nucleotide sequence of the coding region. The Cry1Ac protein produced in INGARD cotton is >99.4% identical to the protein produced by the *B. thuringiensis subsp. kurstaki* (*B.t.k*) bacterial strain.

Cry2Ab protein

Cry2Ab protein produced in Bollgard II cotton event 15985 exhibits a high degree of amino acid similarity (97%) to the Cry2A protein in sprayable microbial B.t. products. Thus, safety studies conducted with microbial B.t. products containing Cry2A proteins are relevant to the safety assessment of Cry2Ab protein.

Cry1Ac and Cry2A proteins, as components of various *B.t.* microbial products, have been tested in acute, subchronic and chronic toxicity studies with rats, rabbits, sheep and humans. The highest doses administered to animals in these studies produced no observable effects (NOEL), consistent with the absence of toxicity of other Cry proteins when fed at high doses to animals.

A safety summary of Cry1Ac and Cry2Ab is given in the table on the next page.

No Observed Effect Levels for Microbial *B.t.* Preparations Containing Cry1Ac and Cry2A Proteins

Test Substance ¹	Animal Model	NOEL ²	Reference
Acute Toxicity Studies			
Crymax	Rat	> 2.5–2.8 x 10 ⁸ CFUs/rat	Carter & Liggett, 1994
Crymax	Rat	>5050 mg/kg	EPA, 1996b
Cutlass OF	Rat	> 10 ⁸ CFUs/rat	David, 1989
Dipel	Rat	> 2670 mg/kg	EPA, 1996b
Dipel	Rat	> 3.4 x 10 ¹¹ spores/kg.	EPA, 1986
Dipel	Rat	> 4.7 x 10 ¹¹ CFUs/kg	EPA, 1986
Dipel	Rat	>5 000 mg/kg	EPA, 1986
Dipel	Rat	> 1.3 x 10 ⁹ spores/kg	McClintock <i>et al.</i> , 1995
Dipel	Rabbit	>2 x 10 ⁹ spores/animal	EPA, 1986
Subchronic Toxicity Studies			
Dipel	Rat	8400 mg/kg/day/90 days	McClintock <i>et al.</i> , 1995
Dipel	Sheep	10 ¹² spores/day/153 days	Hadley <i>et al.</i> , 1987
Chronic Toxicity Study			
Dipel	Rat	8400 mg/kg/day/2 years	McClintock <i>et al.</i> , 1995
Human Toxicity Study			
Dipel	Humans	1000 mg/day/5 days	McClintock <i>et al.</i> , 1995; EPA, 1986

¹ Crymax contains Cry2A, Cry1Ac, Cry1C
Cutlass OF contains Cry2A, Cry1Aa, Cry1Ab, Cry1Ac, Cry2B
DIPEL contains Cry2A, Cry1Aa, Cry1Ab, Cry1Ac

² These NOELs represent the highest doses tested. Doses are expressed in various units for *B.t.* microbial technical grade materials e.g., milligrams technical ingredient per kilogram body weight, or more commonly CFUs or spores per animal or kilogram body weight. It is not possible to directly compare doses on a milligram technical material per kilogram of body weight basis. This is due to the fact that colony-forming units (CFUs) or spore count can range from approximately 10⁸ to 10¹¹ per gram of technical grade *B.t.* microbial material (McClintock *et al.*, 1995). Secondly, the Cry protein content in different *B.t.* microbial preparations may vary depending on the microorganism and fermentation conditions. Cry2A protein dosages administered to animals in the referenced studies range from milligrams to grams per kilogram of body weight.

Summary of Safety of B.t.-proteins Cry1Ac and Cry2Ab

Test Organism	Test Substance	Results ¹
Allergan Homology	Cry2Ab	No homology with known protein allergen
	Cry2Ac	No homology with known protein allergens
Toxin Homology	Cry2Ab	No homology with known protein toxins or other proteins of concern to human health
	Cry2Ac	No homology with known protein toxins or other proteins of concern to human health
Digestive Fate	Cry2Ab	Half-Life <15 sec in SGF; Digested to stable tryptic core in SIF ²
	Cry2Ac	Half-Life <15 sec in SGF; Digested to stable tryptic core in SIF ²
Acute Mouse	Cry2Ab	No effects at highest dose tested, 1450 mg/kg ³
	Cry2Ac	No effects at highest dose tested, 4200 mg/kg body weight

¹ The Cry2Ab data is summarised from Leach *et al.*, 1999, Hileman and Astwood, 1999A, Hileman and Astwood, 1999B, Bechtel, 1999;

² SGF = Simulated Gastric Fluid; SIF = Simulated Intestinal Fluid

³ Limited solubility of Cry2Ab

Summary of safety of Cry1Ac and Cry2Ab

The Cry1Ac and Cry2Ab proteins have been shown to be safe for consumption by both humans and animals by the:

- low levels in cotton;
- lack of allergenic potential of Cry1Ac and Cry2Ab;
- lack of homology of Cry1Ac and Cry2Ab with any known protein toxins;
- rapid digestion of Cry1Ac and Cry2Ab in simulated gastric and intestinal fluids;
- lack of acute toxicity of Cry1Ac and Cry2Ab to mammals as determined by an acute mouse oral gavage study.

The toxicity profile for the Cry1Ac and Cry2Ab proteins indicates no risk from exposure to the Australian population. Therefore, there is a reasonable certainty that no harm will result from aggregate exposure of the Australian population, including infants and children, to the Cry1Ac and Cry2Ab protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information.

Summary of safety of NPTII protein

A safety summary of NPTII is given below (references Fuchs *et al.*, 1993a, b and c):

- The NPTII protein expressed in Bollgard cotton is chemically and functionally similar to the naturally occurring NPTII protein.
- The degradation of NPTII in digestion fluids was assessed over time by western blot analysis. The enzymatic activity of the NPTII protein was shown to be destroyed after a 2-minute incubation in simulated gastric fluid and a 15-minute incubation in simulated intestinal fluid (Fuchs *et al.*, 1993b).
- The NPTII protein caused no deleterious effects in mice when administered by gavage at dosages up to 5000 mg/kg body weight (Fuchs *et al.*, 1993b).
- The NPTII protein does not show meaningful amino acid sequence similarity when compared to known protein toxins present in protein databases.
- The NPTII protein does not show meaningful amino acid sequence similarity when compared to known protein allergins present in protein databases.
- NPTII proteins are present at low levels in Bollgard cotton plants and are not detectable in the components of cotton that are used for food.
- In addition, the NPTII protein has been approved by the United States Food and Drug Administration as a processing aid food additive for tomato, cotton and canola (Food and Drug Administration, 1994), and exempted from the requirement of a tolerance as an inert ingredient by the United States Environmental Protection Agency (EPA, 1994). These approvals included an assessment of potential allergenic effects for the NPTII protein, and both agencies concluded there were no significant concerns.

Summary of safety of the GUS protein

A safety summary of GUS protein is given below:

- Human exposure to GUS protein from cotton-derived food products would not be expected since the processing removes or denatures the protein.
- The *uidA* gene was not obtained from a source known to be allergenic. A database of protein sequences associated with allergy and coeliac disease was assembled from publicly available genetic databases (GenBank, EMBL, PIR and SwissProt) and from current literature. The amino acid sequence of the GUS protein was compared to these sequences using the sequence alignment tool FASTA. The GUS protein sequence did not share any structurally significant sequence similarity to sequences within the allergen database.
- GUS protein is present at low levels in these plants (<0.007% dry weight in the seed).
- The GUS protein degraded rapidly when added to simulated gastric and intestinal fluids, which simulate human digestion, as assessed by both western blot analysis and enzymatic activity assays. Within 15 seconds of exposure to SGF, there was no detectable GUS protein by western blot or enzymatic activity. After two hours in SIF, a very faint band was observed in the western blot and the protein had lost approximately 91% of its original enzymatic activity. Based on these results, it is concluded that the GUS protein, if ingested by humans, will readily degrade in the digestive tract (Fuchs and Astwood, 1996).
- A database of protein sequences associated with toxicity was also assembled from publicly available genetic databases (GenBank, EMBL, PIR and SwissProt). No structural homology with known toxins was observed.
- A mouse gavage study evaluating the acute administration of the GUS protein showed there were no treatment-related adverse effects in mice administered the GUS protein by oral gavage at actual dosages up to 69 mg/kg, the highest dose tested. Results demonstrated that the GUS protein is non-toxic to mice.

Safety assessments of the Cry1Ac, Cry2Ab, GUS and NPTII proteins expressed in Bollgard II cotton event 15985 include demonstrating the lack of similarity to known allergens and toxins, the long history of safe consumption of comparable proteins in microbial formulations, rapid digestion in simulated gastric and intestinal fluids, and the lack of acute oral toxicity in mice.

Appendix 3: Bollgard II Label



This cotton seed contains Bollgard II technology

ACTIVE CONSTITUENT: *Bacillus thuringiensis subsp. kurstaki* delta endotoxin as produced by the Cry1Ac and Cry2Ab genes and their controlling sequences.

FOR EXPERIMENTAL USE ONLY

THIS PRODUCT IS NOT REGISTERED

- This product should be grown in accordance with the conditions of the OGTR license for Bollgard II cotton, DIR 012/2002.
- This product should also be grown in accordance with the conditions of the APVMA permit as provided by Monsanto Australia Limited.
- This product should be grown in accordance with the directions and conditions set out in the Technology User Agreement.
- Ensure that the person making the crop management decisions has passed the Bollgard II Cotton Accreditation Program before planting and has read and fully understood the Bollgard II Cotton Technical Manual.
- Users must follow the Resistance Management Plan for BOLLGARD II® cotton as described in the TUA Terms and Conditions (and the Crop Management Plan if variety also contains the Roundup Ready and Roundup Ready® Flex technology).
- Any breach of the conditions and requirements as set out in the Technology User Agreement or the Technical Manual must be reported to Monsanto immediately on 03 9522 7122.

The Bollgard II gene in this seed is protected under Australian Patent laws and can only be used by growers who have entered into a Technology User Agreement with Monsanto Australia Ltd.

Buyers and users are deemed to have accepted all the terms and conditions as set out in the Technology User Agreement upon opening this bag of cotton seed containing the Bollgard II gene.

®Bollgard II is a registered trademark of Monsanto Technologies LLC, used under licence by Monsanto Australia Limited.

Appendix 4: Facts about GM Crops

Commonly Asked Questions About GM Foods

Are GM crops safe to use as Livestock feed?

The USFDA (United States Food and Drug Administration), the USDA (United States Department of Agriculture) and the USEPA (United States Environmental Protection Agency) verify that any human food or livestock feed derived from biotech crops is safe before it can be commercialised.

DNA and proteins are essential components of living organisms and are naturally present in all foods and feeds and they are rapidly degraded by normal digestive processes.

No transgenic DNA or protein has been detected in milk, meat, eggs or other animal tissues when poultry, lactating dairy cattle and beef cattle were fed biotech crops.

Are food products from animals fed with GM crops safe to eat?

The levels and quality of nutrients in GM crops with input traits are the same as in conventionally crops. The nutrients in animal products are the same whether livestock and poultry have been fed GM crops or not. The bottom line is that meat, milk and eggs from animals fed GM crops are safe and nutritious to eat – as safe and nutritious as animal products from livestock and poultry fed conventional feed.

“There is no credible evidence to suggest that genetic modification per se reduces the nutritional value of food”
ANZFA Assessment of Food derived from glyphosate corn Dec 2001 (p61)

“There are no health risks associated with GM foods, and those passed for use in Australia and New Zealand are as safe as conventional products” Ian Lindemayer – Managing Director ANZFA 7th ASAEN Food conference, 2000

How are GM foods determined to be safe or otherwise?

FSANZ (Food Standards Australia New Zealand) is charged with the role in both New Zealand and Australia to ensure that all foods offered for sale are safe and wholesome. FSANZ will not allow any GM food on the market in Australia or New Zealand unless it has passed a stringent scientific risk assessment carried out by FSANZ. This includes collaborating with other world food organisations.

FSANZ is required (by law) to carry out a mandatory pre-market safety assessment that looks at:

- The Nature of the Modification – looks at the origin at the introduced protein and whether there are any unintended events associated with the modification. Whether the transfer into the plant genome is stable and are stably inherited from parent to offspring over several generations.
- General Safety Issues – looks at the level of the introduced protein in the GM food and the level of ingestion that would occur.
- Toxicology Issues – looks at the toxicology and allergenicity of the introduced proteins. Also if there is any naturally occurring toxins in the GM crop due to the transfer.
- Nutritional Issues – compositional analysis of the modified food to assess if there is any significant differences in fat, ash carbohydrates, calories, amino acids, fatty acids and other measurable nutritional components of the food.

What does ‘substantial equivalence’ mean?

This concept was put in place in 1991 by consultation between the WHO and FAO. The level of key nutrients and other components in GM foods are compared to levels found in conventionally derived counterpart.

When a GM food is said to be substantially equivalent to a conventionally derived food with a history of safe use, this GM food is regarded as being as safe as its conventional counterpart.

What are Antibiotic Resistance Markers (ARMs) and what is the concern?

ARMs have been used in past genetic modifications to link genes of interest, allowing the initial selection of modified cells in the laboratory. The genes of interest contain the ARM gene, and grow in the presence of the particular antibiotic.

Some groups have raised concerns that the ingestion of these ARMs, may lead to the transfer of genes to bacteria in the gut, therefore compromising the future use of these antibiotics. Humans have been consuming genes from other creatures since the beginning of time and there has been no scientific evidence to show that we are taking up genes from plants into our chromosomes. ARMs, like all proteins, are rapidly broken down in our digestive tract. Gene transfer of ARMs from the plant genome to the gut has never been shown to occur.

“The potential for such gene transfers is effectively zero, given the complexity of the steps required”
WHO 1993 “Health Aspects of Genetically Modified Plants” – Geneva

What GM foods have to be labelled?

From December 7th 2001, all foods which contain novel DNA or protein from a genetically modified source must be labelled.

The following are exempt from labelling requirements:

- refined food where the effect of processing removes novel DNA or protein (for example oils and sugars) and processing aids and additives except where novel protein or DNA is present in the final food;
- flavours present in a concentration less than 0.1% in final food;
- food which has been packaged or manufactured prior to 7 December 2001 for a period of 12 months;
- food prepared at point of sale (i.e. restaurants).

What about the development of Resistance to crop protection traits?

The development of resistance to either the modified B.t. or EPSPS protein is being managed by the implementation of Resistance Management Plans by the growers who use these technologies. These plans are incumbent upon all users.

Quick Facts

Fewer pesticide sprays is good for the environment

Due to plant biotechnology:

Fewer kgs of pesticides are sprayed.

Overall, 20 thousand fewer tonnes of pesticides were sprayed in the USA in 2000 and nearly 2 thousand tonnes fewer in Australia (1999).

6 thousand fewer tonnes of herbicides were sprayed on canola crops in the USA in 2000.

Fewer hectares are sprayed.

Over 2 million fewer hectares of cotton and a million fewer hectares of corn were sprayed in 1998 in the USA.

Fewer spray applications are needed.

It is estimated that globally, cotton growers sprayed insecticides 15 million fewer times in 2000.

19 million fewer herbicide sprays were needed for cotton crops in 2000.

19 million fewer herbicide sprays were needed for soybean crops in 1999.

Apart from reduced environmental pollution due to less chemicals, there was a considerable saving in the amount of fossil fuels used to apply these chemicals (and the associated pollution) and in water usage.

Cotton growers conserved almost 94 million gallons of water and used nearly 2.5 million fewer gallons of fuel in 2000, because they had less need to transport and apply pesticides.

Surface and ground water quality is enhanced because fewer pesticides are found in water runoff.

Less pesticide use encourages biodiversity.

Beneficial insects can flourish, which helps control more harmful insects.

Biodiversity is increased because exposure to harmful sprays is lower.

Safety and government regulation.

The Office of the Gene Technology Regulator (OGTR) has the task is to help realise the benefits of gene technology for the Australian community, industry and the environment, whilst ensuring human safety and environmental protection.

Food Standards Australia New Zealand (FSANZ) develops and ensures uniform food standards are maintained, in a cooperative arrangement between all Australian States and Territories, and New Zealand.

National Registration Authority for Agricultural and Veterinary Chemicals (NRA) registers agricultural and veterinary chemicals and assess them for efficacy to the target species, safety to operators and others who might be exposed to the product, safety to consumers/users of treated foodstuffs and other agricultural products, safety to the environment, quality, labelling, packaging and impact on trade.

Australian Quarantine and Inspection Service (AQIS) is responsible for ensuring that products imported into Australia do not lead to the introduction, establishment and spread of pests and diseases which may endanger plants, animals and human life or health.

How plant biotechnology works

By inserting a gene from a naturally occurring plant or organism that produces a desired protein, into a different plant, researchers can cause that plant to produce that protein. This protein may have insecticidal properties as in the case of Bollgard II; it might give the plant tolerance to specific herbicide applications; it might enhance the production of certain vitamins or decrease the production of fatty acids or toxins in a plant.

What Experts Say

"Biotechnology can help us solve some of the most vexing environmental problems: it could reduce pesticide use, increase yields, improve nutritional content and use less water."

Dan Glickman, Secretary of the U.S. Department of Agriculture, speech given to the National Press Club, 7/13/1999

"We've got a product that's safe, it's good for the environment and it allows us to be even more efficient on the farm...After planting every year I wish I was 100 percent [biotech]. It's just that much easier."

Tony Anderson, Ohio grower and president of The American Soybean Association, quoted in an associated press article 7/8/2001

"Before introduction of this technology...I would typically treat approximately 50% of my corn acres for first generation South-western corn borer. By using the B.t. corn technology, we have eliminated all corn borer treatments..."

Dee Vaughan, Texas grower and board member of the National Corn Growers Association (NCGA), 4/12/2002

"The EPA believes that our regulatory system is based on the most rigorous scientific information available, is credible, is defensible, and will serve to protect the environment and public health."

Janet L Andersen, Ph.D. Biopesticides and Pollution Prevention Division (BPPD) US Environmental Protection Agency Congressional hearings on biotechnology, October 1999.

"The world also needs to begin realizing the enormous potential of biotechnology to help end hunger. The U.N. has recently reported biotechnology can dramatically improve crop yields in developing countries while using fewer pesticides and less water. We need to move forward based on sound science, to bring these benefits to the 800 million people, including 300 million children, who still suffer from hunger and malnutrition."

U.S. President Bush, speech at World Bank 17/07/2001.

The research has not found "any new risks to human health or the environment, beyond the usual uncertainties of conventional plant breeding," said the European Commission, the EU's executive branch. "Indeed, the use of more precise technology and the greater regulatory scrutiny probably make them even safer than conventional plants and foods."

The EU biosafety report summarizes 81 research projects financed by the EU over the last 15 years, at a cost of \$64 million, on genetically modified crops and products made from them. 09/10/2001

"All foods must be safe, and extensive scientific research has shown that foods derived through biotechnology are as safe as traditional foods. Furthermore, foods produced through agricultural and food biotechnology must meet the rigorous government standards designed to ensure the safety of any food marketed in the United States."

Dr Jeffrey Barach, Vice President of Special Projects National Food Processors Association (NFPA) Hearing of the Senate Agriculture, Nutrition and Forestry Committee, October 6–7 1999.

"Biotechnology has great potential to reduce our reliance on some older, more risky chemical pesticides, and lower worker and ecological risk...Our biotechnology programme is based on five important principles..."

- sound science,
- transparency in decision-making,
- consistency and fairness,
- collaboration with regulatory partners,
- and building public trust.

The EPA believes that our regulatory system is based on the most rigorous scientific information available, is credible, is defensible, and will serve to protect the environment and public health."

Janet L Andersen, Ph.D. Biopesticides and Pollution Prevention Division (BPPD) US Environmental Protection Agency Congressional hearings on biotechnology, October 1999.

"In addition to those steps that breeders usually take, for products of gene technology, companies are doing far more extensive testing than has ever been done on commercial varieties."

James H Maryanski Ph.D., Biotechnology Coordinator US Food and Drug Administration Worldnet interview, May 26 1999.

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