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DISCLAIMER All the information in this technical manual is provided for general information only and no reader should act upon any material contained in this manual without considering their individual situations.
EXECUTIVE SUMMARY

The evolution of Bollgard technology in Australia

INGARD cotton was the first commercially grown genetically modified crop in Australia. INGARD was succeeded by Bollgard II which contained two *Bacillus thuringiensis* (Bt) genes *cry1Ac* and *cry2Ab*. Bollgard 3 contains both *cry1Ac* and *cry2Ab* along with another Bt gene, *vip3A*. These genes code for proteins that control certain species of Lepidopteran pests when ingested.

Integrated Pest Management

Integrated Pest Management (IPM) is the judicious use of pest control methods to minimise the negative effects of one form of control over another. Bollgard 3 provides prolonged control therefore reducing the need for pesticides and making it the cornerstone of cotton IPM.

Resistance

Historically, resistance to conventional insecticides has been a major concern in Australian cotton production.

In Bollgard 3, insects with some tolerance or inbuilt resistance to one protein will generally be removed through their susceptibility to the other two. There is no cross resistance between the three proteins, and this results in Bollgard 3 being more resilient to resistance developing in the target pests.

The value of Bollgard 3

The use of Bt technology in Australia has decreased pesticide use in cotton over many years. The introduction of Bollgard 3 will continue this trend.

Prudent management of Bollgard 3 will offer the Australian cotton industry the potential for greater sustainability through less reliance on 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 Bt proteins. Together this will reduce selection pressure for resistance to develop to these proteins, and an increased life for the Bollgard 3 technology.
BOLLGARD 3 PROTEINS

All three of the insecticidal proteins produced by Bollgard 3 are encoded by genes derived from the common soil-dwelling bacterium, *Bacillus thuringiensis*.

**Bacillus thuringiensis**

*Bacillus thuringiensis* (Bt) 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 Bt, 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 3 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) (Höfte and Whiteley, 1989).

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.

The third protein, Vip3A, is secreted by Bt during the vegetative stage of growth rather than during sporulation (when the cry genes are expressed). Like the cry genes, vip3A shows activity against lepidopteran larvae.

**History of Bt development**

In 1901, a Japanese bacteriologist, Ishiwata Shigetane, first isolated Bt on infected silk worms. In 1915 German scientist, Ernst Berliner, isolated Bt 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 Bt was used in Germany to control *Lepidoptera* 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 Bt-based product marketed as Thuricide and this has since been used in commercial food production.

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

<table>
<thead>
<tr>
<th>Year</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1901</td>
<td>Bt first isolated</td>
</tr>
<tr>
<td>1915</td>
<td><em>Bacillus thuringiensis</em> recognised and named</td>
</tr>
<tr>
<td>1927</td>
<td>First preparation produced to control <em>Lepidoptera</em></td>
</tr>
<tr>
<td>1938</td>
<td>First commercial product</td>
</tr>
<tr>
<td>1957</td>
<td>Thuricide produced as large-scale commercial agricultural product</td>
</tr>
</tbody>
</table>

**The Bt proteins**

Bollgard 3 cotton contains the gene encoding for the Cry1Ac protein, which is an insecticidal crystal protein (ICP). This protein possesses specific toxicity to certain species of *Lepidoptera* that includes *H. armigera* and *H. punctigera*. Control of *Pectinophora gossypiella*, rough bollworm and some other *Lepidoptera* species has also been seen.

Cry2Ab is another ICP from *Bacillus thuringiensis*. It varies from Cry1Ac, with 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.

Vip3A is a vegetative insecticidal protein. It also binds to the midgut of the target species, but has a different mode of action/receptor site to the Cry1Ac and Cry2Ab proteins.
Mode of action on target insects

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

The mode of action occurs through:

1. Ingestion of the ICP/VIP by an insect larva;
2. Dissolution of the ICP/VIP in the insect midgut;
3. Activation of the ICP/VIP 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, septicemia and resultant death of the insect larvae.

The efficacy of a Bt protein in killing a pest depends on:

- the level of solubilisation in the midgut (which is dependent on the pH of the midgut);
- the conversion of the protein to the active protein by enzymes;
- the pest’s specific membrane receptor sites which can bind with the active protein;
- resultant formation of pores and destruction of gut-wall tissue.

Specificity

Cry1Ac, Cry2Ab and Vip3A are very specific in their target range due to the pH, enzymes and receptor sites required. A detailed description and explanation of the impact on non-target organisms can be found in ‘The exposure and effects of Bt proteins on non-target organisms’ section within this manual.
DEVELOPING A TRANSGENIC COTTON CULTIVAR

1. 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.

2. 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.

3. 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.

4. At the end of the backcrossing process, the seeds are grown out and the plants are allowed to self-pollinate.

5. The progeny seeds are grown out as individual plants and all plants that are not homozygous for the desired gene are eliminated. Seed from each homozygous plant is 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.

6. Most new, fully commercial Bollgard 3 varieties will normally have 97-98%+ of the same genetic background as the elite conventional cotton parent variety.

7. 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 is not an exact copy of the conventional (recurrent) parent. It may have different characteristics that require changes in agronomic practices.

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

Figure 1. Backcrossing Process

ACCELERATED TIMELINES PLANT BREEDING

<table>
<thead>
<tr>
<th>% OF ELITE PARENT</th>
<th>TIME</th>
<th>PLANT BREEDING</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 months</td>
<td>F1 (Bt-) x Elite Parent</td>
</tr>
<tr>
<td>50</td>
<td>5 months</td>
<td>BC1F1 x Elite Parent</td>
</tr>
<tr>
<td>75</td>
<td>10 months</td>
<td>BC1F1 x Elite Parent (Select Bt)</td>
</tr>
<tr>
<td>87.5</td>
<td>15 months</td>
<td>BC2F1 x selfed (Select Bt)</td>
</tr>
<tr>
<td>87.5</td>
<td>20 months</td>
<td>BC2F2 x selfed (Select Bt)</td>
</tr>
<tr>
<td>87.5</td>
<td>25 months</td>
<td>Each BC2F2 plant provides seed for 1 row</td>
</tr>
<tr>
<td></td>
<td>37 months</td>
<td>Select rows that are Bt rows with only 1 Bt allele, Bt-, are eliminated</td>
</tr>
<tr>
<td></td>
<td>49 months</td>
<td>(check with ELISA prior to 4th leaf and again at harvest)</td>
</tr>
</tbody>
</table>

VARIETY RELEASE
BOLLGARD 3 PERFORMANCE

Performance trials

Australian efficacy and crop safety trials were initiated in 2010 and continued through until 2014. The sites chosen represented the environmental diversity in which cotton can grow in Australia. The first efficacy trials were planted in October 2010 at Narrabri, New South Wales, and crop safety trials began the following summer (2011/2012) in New South Wales, Queensland and Western Australia (the Ord). Trials continued over both winter and summer seasons until 2014.

The Bollgard 3 variety, 71B3F, was used for all initial performance testing. 71B3F is directly derived from 71BRF (Bollgard II cotton). However, once the vip3A gene was inserted, normal sexual reproduction was used for seed bulking; therefore there is genetic variability between individuals. Comparisons were made to ensure that the insertion of the vip3A gene had no adverse effect on the normal growth and development of the cotton plant. The results showed some variability, which was to be expected, but the data collected across two seasons showed that the presence of the third transgene, vip3A, had no negative effect on the overall agronomic development of the Bollgard 3 cotton plant when compared with its Bollgard II parental line.

EFFICACY

The efficacy of Bollgard 3 compared to Bollgard II and non-Bt conventional cotton was measured in laboratory bioassays and in the field. Over four seasons, weekly evaluations of the trial sites recorded damage to terminals, squares and bolls, as well as the abundance of Helicoverpa spp. The results from the sites across all the regions demonstrated that Bollgard 3 is as efficacious as Bollgard II in controlling Helicoverpa spp.

CROP SAFETY

The effect of the addition of the vip3A gene on crop safety was measured by comparing agronomic criteria from Bollgard 3 and Bollgard II cotton plants of the same varietal background (71B3F and 71BRF respectively). All trial site appraisals included plant height measurements and number of nodes as criteria for evaluation. Yield measurements, plant mapping and fibre quality data was also obtained.

Results showed there were no significant differences in any measurable agronomic characteristic in a fully sprayed situation (i.e when plant damage by pests and resultant growth compensation is eliminated). While some variability was observed, as expected, the plants expressed the same basic varietal characteristics for which they were originally selected. Therefore the vip3A gene had no impact on the agronomy of the plant.

CONCLUSIONS

The overall conclusion is that the presence of the third gene, vip3A, contributes to the overall insecticidal efficacy of the plant. Bollgard 3 is as efficacious against Helicoverpa spp as Bollgard II cotton throughout the season.

The presence of the third transgene, vip3A, had no negative effect on the overall agronomic development of the Bollgard 3 cotton plants when compared with both Bollgard II and conventional cotton parental lines.
Benefits of Bollgard 3

The evolution of Bollgard technology has increased its durability and Bollgard 3 extends this benefit; three modes of action will delay the evolution of resistance to any of the Bt proteins and make it more difficult for *Helicoverpa* spp. to develop cross resistance. The introduction of Bollgard 3 will provide a more robust technology and reduce the likelihood of resistance developing.

**PRODUCTION**

1. Less restrictive planting windows;
2. Reduced refuge area requirement;
3. Reduced pupae busting requirements;
4. Reduced insecticide sprays;
5. Decreased resistance development to conventional insecticides and Bt;
6. Increased survival of beneficial insects;
7. Increased biological control of secondary pests;
8. Increased biological control of *Helicoverpa* spp.;
9. Less machinery and labour demand.

**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**

When spraying is necessary, beneficial insect populations in cotton can be maintained and encouraged through the selective use of insecticides. This will enable some additional control of both *Helicoverpa* and of secondary pests by beneficial insects. However, it will not completely remove the need for some pesticide application to control secondary pests.

Current data suggests that the control of secondary pests in Bollgard 3 crops will require similar attention to those in conventional cotton. There will undoubtedly be some situations that will result in more sprays, and therefore expense, on Bollgard 3 than on conventional cotton.
Since the introduction of Bollgard II cotton in Australia, growers and consultants have reported variability of performance (i.e. some larval survival) of Bollgard II cotton in controlling the target pests *Helicoverpa armigera* and *H. punctigera*. This may also occur in Bollgard 3.

There are a number of reasons for variability, some are manageable while others are not.

**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 Bt within a variety and the level of susceptibility of *Helicoverpa* spp. to the Bt protein. Factors that that may affect the efficacy of the Bt 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 3 cotton**

Any one or more of the following factors may influence the field performance of Bollgard 3 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 plant;
- Inherent genetic variability of *Helicoverpa* spp.
- Behavioural response of *Helicoverpa* spp. to the Bt proteins;
- External environmental conditions;
- Management practices.

**Inherent non-controllable factors**

**PROTEIN PRODUCTION OVER TIME**

The efficacy of Bollgard 3 cotton is directly related to the toxicity of the three 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 rather a gradual decline. Despite this, there is still a high level produced at the end of the season, capable of exerting significant control of *Helicoverpa* spp.

**INHERENT GENETIC VARIABILITY: COTTON PLANTS**

The overall development of Bt production within the Bollgard 3 plant is illustrated in figure 2. This represents a typical population of Bollgard 3 cotton, but individual plants within this will vary. Cotton plants comprise a complex array of individual plant cells. Every 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, another encodes for the Cry2Ab protein, and another for the Vip3A protein. Each plant, through normal reproductive gene mixing, has a unique array of these genes. Each plant is therefore slightly different from its neighbour.
Other genes can affect those responsible for producing the Bt proteins, meaning that individual plants may be slightly different in the way the Bt 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.

**INHERENT GENETIC VARIABILITY: HELICOVERPA SPECIES**

*Helicoverpa* spp. individuals are not clones - they have the same genetic variability and interaction of genes as other living organisms. A large variability in susceptibility to the Bt proteins exists between individuals within and between colonies. Jenkins et al. (1997) studied 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) 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 3 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 3 cotton at an earlier life stage than those more susceptible.
In any field situation, the overall efficacy is limited by two things: the level of expression of the Bt proteins in the plants and the degree of susceptibility to those proteins of the target pests.

In a Bollgard 3 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 3 fields due to the high levels of protein production.
BEHAVIOURAL RESPONSE OF *HELICOVERPA* SPP. TO THE BT 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 (i.e., a food source without Bt), they will still feed on the plant tissue and die. The presence of the Bt proteins has been observed to elicit more movement of the larvae in an attempt to find Bt-free food.

Monitoring

The inherent variability in the level of production of the Bt 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.

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. The cotton industry has developed spray thresholds with the deletion of levels based upon this.

BOLLGARD 3 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 made before the threshold level is reached, as the grower may not be able to incur a low level of fruit loss. Similarly, a grower may decide to spray late in the season, before the threshold level has been reached, 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 warrant a spray. Earlier in the season, Bollgard 3 should be expressing at a sufficiently high enough level to control any emerging larvae.

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, reducing the level of protection obtained. Ensuring the crop is healthy and does not suffer undue stress will minimise this effect on protein expression.

Management practices

Agronomic practices implemented by the grower include managing the nutritional status of the field, and making timely, informed spray decisions. These can both have a major influence on how well a Bollgard 3 variety performs.
PEST MANAGEMENT

Direct benefits from Bollgard 3 cotton

The major benefit of planting Bollgard 3 is the reduced number of insecticide applications required for Helicoverpa spp. control. Bollgard 3 effectively controls *H. armigera* and *H. punctigera* for most of the season.

Bollgard 3 reduces the requirement for larvicides in general. However, this is not the only pest management benefit Bollgard 3 offers. There is also 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 3 cotton - including beneficial insects.

Indirect benefits from Bollgard 3

Increased survival and numbers of beneficial insects has two benefits to the grower.

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

The second benefit of preserving the beneficial insects is in reducing selection pressure for resistance to Bt proteins. Many beneficial insects feed on the eggs and small larvae of Helicoverpa spp. Predation and parasitism removes eggs from the field, so fewer Helicoverpa spp. will hatch and fewer individuals will feed on Bollgard 3.

This may also have an economic impact by reducing the need for late season Helicoverpa sprays, as beneficial insects may maintain larval numbers below threshold levels.

How to maximise the insecticidal benefits

In terms of observed efficacy, varieties of Bollgard 3 cotton may perform differently. Factors include differing locations, seasons, planting times and management practices. Adhering to these guidelines will help ensure you gain optimal value from Bollgard 3 cotton:

- Where possible, refrain from the use of synthetic pyrethroids, broad-spectrum organophosphates and carbamates on both Bollgard 3 and conventional cotton;
- 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;
- Maintain beneficial insect populations;
- Don’t stress the cotton.

Even with the higher levels of beneficial insects in the cotton, insecticides will still be required to control sucking pests and mites both early and late in the season. The presence of these pests (no longer controlled through Helicoverpa sprays) may cause economic damage. In order to achieve the best value from Bollgard 3 technology, careful monitoring of these pests and timely action to selectively control them is a necessity. The selective use of chemicals in adjacent conventional cotton is also important, as spray drift can disrupt the levels of beneficial insects in Bollgard fields. The judicious use of insecticides will minimise the impact of these secondary pests on yield and profitability.

**Beneficial insects**

**Egg parasites:** Trichogramma, Microplitis

**Egg predators:** Ladybirds, Red and Blue beetles, Damsel bugs, Smudge bugs, Lacewings

**Neonate predators:** Predatory bugs, Spiders

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14
Spray decision examples

The following thresholds were developed for Bollgard II, and will be maintained for Bollgard 3. The spray thresholds are: Two consecutive checks of 2 smalls (>3mm) per metre or; One check produces one or more medium larvae (>8mm). For the following examples it is assumed that there is an egg lay of 20 eggs per metre.

**Spray decision examples**

**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 3 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**

While the benefits of Bollgard 3 traits are significant, a grower must first and foremost choose a variety that is suited to their growing region. Agronomically, the effect of the addition of a third insecticidal protein for control of *Helicoverpa* spp. is minimal, and the choice of transgenic options should be a secondary consideration to varietal characteristics. Below are the key parameters in cotton agronomy and some possible areas for concern or change when growing Bollgard 3 cotton.

**Varietal maturity**

Maturity in conventional cotton is determined to a large degree by the genetic background of the particular variety. In Bollgard 3 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. As there is no significant change in fruit retention or distribution between Bollgard II and Bollgard 3 varieties, there should be no need to increase planting rates from their current levels. However, growers should contact their seed distribution representative for variety-specific information.

**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 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 develop when conditions are optimal in terms of temperature, shading from leaves and neighbouring plants and leaf functioning. First position bolls are the preferential sink for photosynthates, carbohydrates and nutrients from the mainstem and first position leaves. They are preferentially supplied with these resources for growth over bolls in the second, third or fourth positions.

In conventional cotton, this first position fruit is frequently aborted due to insect damage, 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 and is supplied with resources accordingly. However, this boll will never be as big as the first position boll would have been. This loss of first position fruit can result in a significant yield penalty.

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 present 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 centimetres has the same effect on maturity as a 20% reduction in first position fruit retention under the same temperature regime.

Bollgard 3 cotton plants will hold significantly more of their first position fruit than conventional cotton plants. 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. Usually this would 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.

Trials carried out by Monsanto throughout the Australian cotton growing regions showed no difference in the retention rate or fruiting pattern between Bollgard II and Bollgard 3 cotton, and so the management strategies should not be significantly different between the two technologies.

Nutrition management

Growers will need to ensure that their nutrition management is correct for their soil characteristics and crop rotation. Knowledge of the historical yields from each field will give a good indication of the nutrient removal from the system, and nutritional inputs should be added accordingly. The nutrient demand from Bollgard 3 crops will depend on their yield. The high boll retention and fruit load on the plant will create a period of peak nutrient demand during boll development through to cut out. During this period nutrient supply from the soil is critical, especially for N and K.

Disease management

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

Seedling emergence

Emergence and early season vigour is a varietal characteristic and will not be affected by the addition of insecticidal genes in Bollgard 3 plants. Early season characteristics of each variety should be considered by growers when selecting a variety suited to their farm and growing conditions.

Growth regulator requirement

There may be a need for growth regulants in Bollgard 3 cotton. This 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 be less chance of low micronaire from the ‘top crop’, with the plant cutting out in a more uniform fashion. This is on the proviso that other agronomic factors are well managed. Trials by Monsanto showed no difference in fibre quality between Bollgard II and Bollgard 3 plants with the same breeding history, and there is no reason to assume there will be changes to fibre quality transitioning from Bollgard II to Bollgard 3.
Figure 6: Growth and Development 2011/12

- Plant height
  - 120
  - 110
  - 100
  - 80
  - 60
  - 40
  - 20
  - 0

- Nodes
  - 111.6
  - 108.1
  - 102.3

Figure 7: Fruit Retention 2011/12

- Total Bolls
  - Total Retention Rate (%)
  - 50
  - 40
  - 30
  - 20
  - 10
  - 0
  - Conventional: 17.8
  - Bollgard II: 27.8
  - Bollgard 3: 6.6

- Retention Rate (%)
  - Conventional: 0.5
  - Bollgard II: 1.2
  - Bollgard 3: 0.4

- 1st Position Retention rate (%)
  - Conventional: 0.3
  - Bollgard II: 0.8
  - Bollgard 3: 0.4

Figure 8: Fruiting pattern 2011/12 (% retention of total bolls in each 5 node segment)
INSECT RESISTANCE

Introduction

Resistance poses a serious threat to transgenic cotton. Australian cotton growers are very familiar with the devastation insecticide resistance can cause, as witnessed in the Ord in the 1970s. Unfortunately, transgenic plants are no less subject to selection for resistance than classical conventional insecticides.

The diamondback moth, a major pest of cabbage, has already developed resistance to Bacillus thuringiensis (Bt) sprays in many cropping areas around the world. Bt-resistant diamondback moth larvae are completely resistant to transgenic plants that carry the cry1Ac gene (incorporated in Bollgard 3 cotton).

In contrast to Bt sprays, the Bt 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 Bt may be more common than genes for resistance to chemical insecticides. After over 10 years of resistance monitoring in Australia, we know that resistance alleles to Bt proteins are present in the Helicoverpa spp. populations. Thus, resistance management is just as critical to transgenic crops as it has been for chemical insecticides.

Professor Rick Roush believes that ‘the pyramiding of toxins offers what appears to be the most effective way to manage resistance to Bt and other insecticidal transgenic proteins. Pyramids have the potential to greatly reduce refuge requirements for successful resistance management from perhaps 30–40% to around 10%’ (Roush, 1998).

With three pyramided genes producing three proteins with no cross resistance in Helicoverpa spp. Bollgard 3 has more protection against the establishment of resistance in comparison to Bollgard II. However, careful management is still necessary to ensure that this advantage is maintained for the future.

Resistance Management Plan (RMP)

The cotton industry has taken a proactive approach to resistance by establishing a comprehensive resistance management strategy prior to any detected levels of resistance.

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

1. Refuges with no selection for Bt 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.
Key elements of the RMP for Bollgard 3

- **Planting windows/restrictions**
- **Refuge crops**
- **Pupae destruction/trap crops**

The rationale behind the specific RMP requirements

**PLANTING WINDOWS/PLANTING RESTRICTIONS**
The planting window was implemented to limit the number of *Helicoverpa* spp. generations that are exposed to the Bt proteins. When evaluating data for the Bollgard 3 RMP, Dr Geoff Baker assessed long term data to evaluate the efficacy of planting windows since the introduction of Bollgard II. This work concluded that the “current planting window (pre November 15) achieves little in limiting the exposure of *Helicoverpa* to Bt toxins in these regions. However, insufficient information is known of the population dynamics of *Helicoverpa* further north in Central Queensland to be as definitive re the need for planting windows there. Importantly, *Helicoverpa* are active all year round and cotton can be grown over a longer period in Central Queensland, thus enhancing the risks of greater exposure to Bt toxins” (Baker et al, 2014). Monsanto and the Bt Technical Panel assessed the data generated by the project and agreed with the conclusions. This data allowed for the change in planting windows in both the southern and Central Queensland growing regions in the Bollgard 3 RMP (for further details refer to Appendix 3 – Bollgard 3 Resistance Management Plan).

**REFUGE REQUIREMENT**
Refuge crops are required to produce populations of moths that have not been exposed to selection with Bt proteins. Mating of these moths with survivors from Bollgard 3 crops can help dilute selection for resistance and slow the rate at which resistance becomes a problem.

In comparing refuge crops for Bollgard 3 cotton, unsprayed conventional cotton is used as the control refuge because it is attractive for a similar period to Bollgard 3 cotton. Modeling studies for Bollgard 3 have established the need for a 5% refuge where the refuge has characteristics like unsprayed conventional cotton. 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 (for further detail on refuge requirements refer to Appendix 3).

**BT SPRAY RESTRICTIONS**
Use of Bt sprays may also select for Bt resistant insects. For this reason, Bt sprays may not be used on any refuge crops given the aim is to produce unselected (or susceptible) moths.

**PUPAE DESTRUCTION**
*Helicoverpa* spp. larvae can form pupae that will diapause over winter. Such pupae pose a risk as any individuals carrying resistance alleles at the end of the season (having survived the Bt in Bollgard 3 cotton) will enter diapause. If left to emerge as adults, any that are carrying resistance alleles may mate and resistance could 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. By destroying the emergence tunnels, the adults will be unable to surface and will die without the need for chemical control. To destroy the tunnels, the soil should be cultivated 30cm either side of the plant line to a depth of 10cm.

Some cotton crops are harvested prior to *Helicoverpa* spp. going into diapause. Such early finishing crops do not pose a risk to resistance and therefore, any Bollgard 3 cotton crop that receives its first defoliation on or before 31 March will not require pupae busting.

For further information refer to Appendix 3.
TRAP CROPS FOR CENTRAL QUEENSLAND

The pupae busting strategy is effective in most cotton growing areas where *Helicoverpa* spp. passes the winter in diapause. However, in Central Queensland, very few *Helicoverpa* spp. enter diapause from cotton crops due to climatic conditions. Instead, the survivors emerge and breed again. 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 they are then destroyed by full cultivation of the trap crop. For further detail refer to Appendix 3.

Resistance monitoring

A Resistance Management Plan (RMP) is in place to protect the technology from resistance development and to create a sustainable cropping system. This management plan needs to be monitored to ensure that it is working effectively and is following the model upon which it is based. It is therefore of great importance that the levels of resistance are adequately monitored to ensure the longevity of this and other future technologies.

F1 SCREENS FOR RESISTANCE ALLELES

The F₁ test measures the frequency of resistance 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, for example, Cry2Ab protein. Only resistant larvae would survive the test, which takes approximately six weeks to conduct.

F2 SCREENS FOR RESISTANCE ALLELES

The F₂ screen also measures the frequency of resistant alleles in the population. Isolated pairs of moths from the same collection are mated and their grandchildren (F₂) are exposed to a concentration of Bt protein that is survived only by resistant insects. This test takes approximately 12 weeks to run.

Monsanto and the Transgenic and Insect Management Strategies (TIMS) Bt Technical Panel meet annually to review the resistance monitoring data.

Resistance Monitoring Program

BT RESISTANCE MONITORING PROGRAM BACKGROUND

Monsanto has been collaborating with CSIRO on the Bt Resistance Monitoring Program since 2003/04. The program was established to determine the frequency of alleles which impart resistance against the proteins Cry2Ab, Cry1Ac and more recently Vip3A.
QUALITY ASSURANCE

Introduction

Backcrossing of Bollgard 3 genes in Australian seed companies’ elite cotton lines, and the subsequent bulking of seed for commercial production, is a carefully managed process. Strict quality standards set by Monsanto and the seed company must be adhered to throughout the process. These Quality Assurance guidelines are in place to minimise or prevent any quality issues for growers. In Australia, all lines developed as Bollgard 3 / Roundup Ready® Flex cotton varieties must have the following tests undertaken:

1. Gene Purity;
2. Seed Lot Verification;
3. Commercial Crop Tolerance (Roundup Ready Flex)/Gene Equivalency (Bollgard 3);

Gene Equivalency

BOLLGARD 3 GENE EQUIVALENCY

Prior to commercial variety approval, all new Bollgard 3 / Roundup Ready Flex lines must pass gene equivalency testing conducted 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 approved Bollgard 3 varieties.

Gene equivalency ensures that sufficient protein is produced so that a minimum level of activity is obtained under a range of conditions. Gene equivalency testing is not to determine efficacy; this is dependent upon a number of factors such as plant health and nutritional status.

THE EXPOSURE AND EFFECTS OF BT PROTEINS ON NON-TARGET ORGANISMS

SEE APPENDIX 2: TOXICOLOGY

Exposure and effects of Bt on humans

Owing to their specific mode of action, Bt products are unlikely to pose any hazard to humans or other vertebrates, or to the great majority of non-target invertebrates. Bt 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 Bt on non-target organisms

Multiple dose studies with Bt have been conducted with mammals, birds, fish and other non-target animals to investigate the effects of dietary, dermal and inhalatory exposure to Bt, with negligible adverse effects. In rats, no toxicity or infectivity was associated with dietary exposure to Bt (4 g/kg per day) for 3 months and the only effect observed from a 2-year study in which a commercial Bt preparation was fed to rats at 8400 mg/kg per day in the diet, was a slight decrease in body weight of females.
Bt 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. Bt does not adversely affect earthworms.

The Bt 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 Bt proteins with activity against **Diptera** and **Lepidoptera**.

**Impact of Bt transgenic cottons on abundance of non-target Arthropods in Australia**

The CSIRO conducted five trials in Australia over the 2010–2012 period. Two trials were conducted at the Frank Wise Institute of Tropical Agriculture, Kununurra, Western Australia to compare the differences in non-target arthropod abundance between Bollgard 3, Bollgard II and non Bt conventional cotton. Two trials were conducted at the Australian Cotton Research Institute (ACRI), and one trial at Boggabilla – both in New South Wales. All trials compared the difference in abundance of non-target arthropods in Bollgard 3, Bollgard II and non Bt conventional cotton.

All five trials concluded that the presence of the Vip3A protein within the cotton plant had no measurably significant effect on the abundance of non-target arthropods within the cotton environment (Whitehouse et al, 2014).

**Studies on the effects of Vip3A on non-target organisms**

Overall, results from the study undertaken by CSIRO indicated there was little difference in communities of invertebrates in any of the Bt cottons in the trials e.g; Bollgard 3 and Bollgard II. When comparing individual taxa responses, differences between Bt and non-Bt cotton invertebrate communities were largely driven by changes in the abundance of lepidopteran larvae. Taxa showing significant differences between crop types were several generalist predators and some pests including several spider families; many of these are more common in non-Bt cotton and this is most probably due to prey preferences. In suction samples, there were differences observed in small dipterans between Bt and non-Bt plants. It was not clear why these insects were shown to be more common on non-Bt plants, although the authors speculated these populations may have been influenced by prolonged vegetative growth caused by insect damage in non-Bt cotton (Whitehouse et al, 2014).
EXPECTATIONS OF BOLLGARD 3

Bollgard 3 will provide protection against *H. armigera* and *H. punctigera*. However, the technology does not make cotton Helicoverpa-proof. High pest pressure may still necessitate the use of 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 3 provides excellent insecticidal activity against *Helicoverpa* spp.
- Bollgard 3 is NOT Helicoverpa-proof;
- High pest pressure, timing or plant stress may necessitate pesticide application;
- Secondary pests may still need controlling;
- Bollgard 3 provides the grower with a foundation for IPM and the potential to increase sustainability.

RECOMMENDATIONS FOR GROWERS AND CONSULTANTS

- Select Bollgard 3 varieties on their agronomic merits and ensure that the variety is recommended for the region it is proposed for;
- Read and understand the Bollgard 3 label, the Technology User Agreement and the Resistance Management Plan prior to planting;
- Plant Bollgard 3 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 3 cotton requires careful scouting for pests. Although *Helicoverpa* spp. may not be a serious season-long problem in Bollgard 3, 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. This will assist in controlling secondary pests and reduce the selection pressure for resistance to Bt by removing *Helicoverpa* spp.
- Helicoverpa spp. may need supplementary insecticide application if pest pressure is high or if the plant is under stress. Bollgard 3 is NOT immune to attack by Helicoverpa;
- Use the Bollgard 3 cotton spray thresholds when making spray decisions;
- Bollgard 3 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 but if left until later, it can prove to be an expensive operation.

Contact your nominated Technology Service Provider, Bollgard 3 cotton seed company [Cotton Seed Distributors] or your local Monsanto Regional Business Manager for advice on growing and managing Bollgard 3 cotton.
APPENDIX 1: BOLLGARD 3 SAMPLING

Bollgard 3 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 are found 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 3 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

Five proteins are expressed at low levels in Bollgard 3 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 (Cry2Ab protein);

3. An insect control protein derived from the common soil bacterium, Bacillus thuringiensis subsp. kurstaki the Vip3A vegetative insecticidal protein (Vip3A protein);

4. Neomycin phosphotransferase II enzyme (NPTII) protein from the nptII gene from E. coli;

5. 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 (currently known as the APVMA). 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 (Bt) be exempt from the requirement for scheduling under regulations relating to drugs and poisons.

The safety of Bt to humans, other mammals, birds and fish is well substantiated.

There are no receptors for the protein delta-endotoxins of Bt 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; Shadduck, 1983; Siegel and Shadduck, 1989). Results of unpublished safety studies generated by registrants of Bt commercial preparations in the USA have also been summarized in the EPA Registration Standard for Bt Formulations (EPA, 1988).

In published reviews and the EPA document, studies are referenced where large doses (5000 mg/kg) of Bt 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 Bt formulations, with no adverse effects.

Safety assessments of the Cry1Ac, Cry2Ab, Vip3A, GUS and NPTII proteins expressed in Bollgard 3 cotton event 15985 x Cot102 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, Cry2Ab and Vip3A 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 (Btk) 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 Bt products. Thus, safety studies conducted with microbial Bt products containing Cry2A proteins are relevant to the safety assessment of Cry2Ab protein.

VIP3A PROTEIN

Cry1Ac and Cry2Ab proteins, as components of various Bt 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 below.

Table 1: No Observed Effect Levels for Microbial Bt Preparations Containing Cry1Ac and Cry2A Proteins

<table>
<thead>
<tr>
<th>TEST SUBSTANCE</th>
<th>ANIMAL MODEL</th>
<th>NOEL</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACUTE TOXICITY STUDIES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crymax</td>
<td>Rat</td>
<td>&gt;2.5–2.8 x 10⁸ CFUs/rat</td>
<td>Carter &amp; Liggett, 1994</td>
</tr>
<tr>
<td>Crymax</td>
<td>Rat</td>
<td>&gt;5050 mg/kg</td>
<td>EPA, 1996b</td>
</tr>
<tr>
<td>Cutlass OF</td>
<td>Rat</td>
<td>&gt;10⁸ CFUs/rat</td>
<td>David, 1989</td>
</tr>
<tr>
<td>Dipel</td>
<td>Rat</td>
<td>&gt;2670 mg/kg</td>
<td>EPA, 1996b</td>
</tr>
<tr>
<td>Dipel</td>
<td>Rat</td>
<td>&gt;3.4 x 10¹¹ spores/kg</td>
<td>EPA, 1986</td>
</tr>
<tr>
<td>Dipel</td>
<td>Rat</td>
<td>&gt;4.7 x 10¹¹ CFUs/kg</td>
<td>EPA, 1986</td>
</tr>
<tr>
<td>Dipel</td>
<td>Rat</td>
<td>&gt;5 000 mg/kg</td>
<td>EPA, 1986</td>
</tr>
<tr>
<td>Dipel</td>
<td>Rat</td>
<td>&gt;1.3 x 10¹⁰ spores/kg</td>
<td>McClintock et al., 1995</td>
</tr>
<tr>
<td>Dipel</td>
<td>Rabbit</td>
<td>&gt;2 x 10⁸ spores/animal</td>
<td>EPA, 1986</td>
</tr>
<tr>
<td><strong>SUBCHRONIC TOXICITY STUDIES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipel</td>
<td>Rat</td>
<td>8400 mg/kg/day/90 days</td>
<td>McClintock et al., 1995</td>
</tr>
<tr>
<td>Dipel</td>
<td>Sheep</td>
<td>10¹² spores/day/153 days</td>
<td>Hadley et al., 1987</td>
</tr>
<tr>
<td><strong>CHRONIC TOXICITY STUDY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipel</td>
<td>Rat</td>
<td>8400 mg/kg/day/2 years</td>
<td>McClintock et al., 1995</td>
</tr>
<tr>
<td><strong>HUMAN TOXICITY STUDY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipel</td>
<td>Humans</td>
<td>1000 mg/day/5 days</td>
<td>McClintock et al., 1995; EPA, 1986</td>
</tr>
</tbody>
</table>

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 Bt 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 Bt microbial material [McClintock et al., 1995]. Secondly, the Cry protein content in different Bt 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.
Table 2: Summary of Safety of Bt-proteins Cry1Ac, Cry2Ab and Vip3A

<table>
<thead>
<tr>
<th>TEST ORGANISM</th>
<th>TEST SUBSTANCE</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allergen Homology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry2Ab</td>
<td>No homology with known protein allergens</td>
<td></td>
</tr>
<tr>
<td>Cry1Ac</td>
<td>No homology with known protein allergens</td>
<td></td>
</tr>
<tr>
<td>Vip3A</td>
<td>No homology with known protein allergens</td>
<td></td>
</tr>
<tr>
<td><strong>Toxin Homology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry2Ab</td>
<td>No homology with known protein toxins or other proteins of concern to human health</td>
<td></td>
</tr>
<tr>
<td>Cry1Ac</td>
<td>No homology with known protein toxins or other proteins of concern to human health</td>
<td></td>
</tr>
<tr>
<td>Vip3A</td>
<td>No homology with known protein toxins or other proteins of concern to human health</td>
<td></td>
</tr>
<tr>
<td><strong>Digestive Fate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry2Ab</td>
<td>Half-Life &lt;15 sec in SGF; Digested to stable tryptic core in SIF</td>
<td></td>
</tr>
<tr>
<td>Cry1Ac</td>
<td>Half-Life &lt;15 sec in SGF; Digested to stable tryptic core in SIF</td>
<td></td>
</tr>
<tr>
<td>Vip3A</td>
<td>Half-Life 1 min in SGF; Digested to no protein detectable in SIF</td>
<td></td>
</tr>
<tr>
<td><strong>Toxin Homology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry2Ab</td>
<td>No effects at highest dose tested, 1450 mg/kg</td>
<td></td>
</tr>
<tr>
<td>Cry1Ac</td>
<td>No effects at highest dose tested, 4200 mg/kg body weight</td>
<td></td>
</tr>
<tr>
<td>Vip3A</td>
<td>No effects at highest dose tested, 5050 mg/kg body weight</td>
<td></td>
</tr>
</tbody>
</table>


Summary of safety of Cry1Ac, Cry2Ab and Vip3A proteins

The Cry1Ac, Cry2Ab and Vip3A 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, Cry2Ab and Vip3A;
- lack of homology of Cry1Ac, Cry2Ab and Vip3A with any known protein toxins;
- rapid digestion of Cry1Ac, Cry2Ab and Vip3A in simulated gastric and intestinal fluids;
- lack of acute toxicity of Cry1Ac, Cry2Ab and Vip3A to mammals as determined by an acute mouse oral gavage study.

The toxicity profile for the Cry1Ac, Cry2Ab and Vip3A 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, Cry2Ab and Vip3A proteins 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 allergens 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 allergic 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, Vip3A, GUS and NPTII proteins expressed in Bollgard 3 cotton 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 3 RESISTANCE MANAGEMENT PLAN

The addition of the Vip3A protein in Bollgard 3 increases the durability of the technology, which allowed changes to be made to the Resistance Management Plan (RMP). A Bollgard 3 RMP was developed by Monsanto, endorsed by the cotton industry’s Transgenic and Insect Management Strategies (TIMS) Committee.

THE RESISTANCE MANAGEMENT PLAN IS BASED ON THREE BASIC PRINCIPLES:

1. Minimising the exposure of Helicoverpa spp. to the Bacillus thuringiensis (Bt) proteins Cry1Ac, Cry2Ab and Vip3A;
2. Providing a population of susceptible individuals that can mate with any resistant individuals, hence diluting any potential resistance; and
3. Removing resistant individuals at the end of the cotton season.

These principles are supported through the implementation of five elements that are the key components of the Resistance Management Plan. These elements are:

- **Planting restrictions**
- **Refuge crops**
- **Control of volunteers and ratoon cotton**
- **Pupae destruction/trap crops**
- **Spray limitations**

Growers of Bollgard 3 cotton are required to practice preventative resistance management as set out below. Compliance with the Resistance Management Plan is required under the terms of the Bollgard 3 Technology User Agreement.

**Planting restrictions**

**VICTORIA, NEW SOUTH WALES AND SOUTHERN QUEENSLAND**

All Bollgard 3 crops and refuges must be planted into moisture or watered-up between August 1 and before December 31 each year, unless otherwise specified in this Resistance Management Plan.

**CENTRAL QUEENSLAND**

All Bollgard 3 crops and refuges must be planted into moisture or watered-up between August 1 and before October 31 each year, unless otherwise specified in this Resistance Management Plan.

Any Bollgard 3 crops planted into moisture or watered-up after October 31 and up to December 31 must plant additional refuge as specified in Table 3 and 4.

**Refuges**

Growers planting Bollgard 3 cotton will be required to grow a refuge crop that is capable of producing large numbers of Helicoverpa spp. moths which have not been exposed to selection with the Bt proteins Cry1Ac, Cry2Ab and Vip3A. These unselected moths are expected to dominate matings with any survivors from Bollgard 3 crops and thus help to maintain resistant alleles to the Bt proteins Cry1Ac, Cry2Ab and Vip3A at low frequencies.

All refuge options are based on the requirement of a 5% unsprayed cotton refuge or its equivalent, as determined by the relative production of Helicoverpa spp. from each of the refuge types as described in Tables 1 and 2 for irrigated and dryland production scenarios, respectively.
For each area of irrigated Bollgard 3 cotton planted, a grower is required to plant one or more of the following:

**Table 1: Irrigated Bollgard 3 cotton refuge options**

<table>
<thead>
<tr>
<th>CROP</th>
<th>CONDITIONS</th>
<th>% OF BOLLGARD 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>Irrigated, sprayed conventional cotton</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Irrigated, unsprayed conventional cotton</td>
<td>5</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>Fully irrigated, unsprayed</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**Table 2: Dryland Bollgard 3 cotton refuge options**

<table>
<thead>
<tr>
<th>CROP</th>
<th>CONDITIONS</th>
<th>% OF BOLLGARD 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>Dryland or irrigated, sprayed conventional cotton</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Dryland or irrigated, unsprayed conventional cotton</td>
<td>5</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>Dryland or fully irrigated, unsprayed. Dryland pigeon peas can only be planted with an approved plan from Monsanto Australia.</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**Table 3: Irrigated Bollgard 3 cotton refuge options for Central Queensland planted after October 31**

<table>
<thead>
<tr>
<th>CROP</th>
<th>CONDITIONS</th>
<th>% OF BOLLGARD 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>Irrigated, sprayed conventional cotton</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Irrigated, unsprayed conventional cotton</td>
<td>10</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>Fully irrigated, unsprayed</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 4: Dryland Bollgard 3 cotton refuge options for Central Queensland planted after October 31**

<table>
<thead>
<tr>
<th>CROP</th>
<th>CONDITIONS</th>
<th>% OF BOLLGARD 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>Dryland or irrigated, sprayed conventional cotton</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Dryland or irrigated, unsprayed conventional cotton</td>
<td>10</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>Dryland or fully irrigated, unsprayed. Dryland pigeon peas can only be planted with an approved plan from Monsanto Australia.</td>
<td>5</td>
</tr>
</tbody>
</table>

**Note:** Unsprayed means not sprayed with any insecticide that targets any life stage of Helicoverpa spp.

Bt products must not be applied to any refuge (including sprayed cotton).

If the viability of an unsprayed refuge is at risk due to early or late season pressure by Helicoverpa spp., or any other caterpillar species, contact Monsanto Australia immediately. With prior approval from Monsanto Australia, a non-Bt helicide can be applied.

For the purposes of this Resistance Management Plan, conventional cotton includes any cotton varieties that do not have Bt proteins in the plant that control Helicoverpa spp. larvae.
GENERAL CONDITIONS FOR ALL REFUGES

(a) Refuge crops are to be planted and managed so that they are attractive to *Helicoverpa* spp. during the growing period of the Bollgard 3 cotton varieties.

**Irrigated:** It is preferable that all refuge is planted within the 2 week period prior to planting Bollgard 3. If this is not possible, refuge planting must be completed within 3 weeks of the first day of sowing of Bollgard 3. At this time, sufficient refuge must have been planted to cover all of the Bollgard 3 cotton proposed to be planted for the season (including Bollgard 3 already planted and any that remains unplanted). If additional Bollgard 3 is planted after this date which is not already covered by refuge, additional refuge must be planted as soon as possible and no more than 2 weeks after sowing of the additional Bollgard 3.

**Dryland:** A dryland refuge must be planted within the 2 week period prior to the first day of planting Bollgard 3 cotton.

(b) Pigeon pea refuges should not be planted until the soil temperature reaches 17°C, which is a requirement for germination, and should also be planted into moisture to ensure successful germination. If soil temperatures are not suitable to allow germination of pigeon peas in line with condition (a), an alternative refuge must be planted in its place within the prescribed period (under (a) above).

(c) All refuges should preferably be planted into a fallow or rotation field that has not been planted to Bt cotton in the previous season to avoid volunteer and ratoon cotton. See Refuge Management Guide for all unsprayed refuges.

(d) Once Bollgard 3 cotton begins to flower, the corresponding refuge must not be cultivated.

(e) All refuges are to be planted within the farm unit growing Bollgard 3 cotton no more than 2 km from the associated Bollgard 3 cotton field. For any cases where it may not be possible to plant the refuge within 2 km from the associated Bollgard 3, approval must be sought from Monsanto Australia.

(f) To minimise the possibility of refuge attractiveness being affected by herbicide drift, non-herbicide tolerant refuges should be separated from herbicide tolerant Bollgard 3 cotton crops by a sufficient distance to minimise such drift, but no more than 2 km from the Bollgard 3 cotton.

(g) To account for possible insecticide drift, the options for the width of refuge crops vary according to spray regime. If any sprayed conventional cotton is grown on the same farm unit, Bollgard 3 refuge crops must be at least 48 metres wide and each refuge area must be a minimum of 2 hectares. If sprayed conventional cotton is not grown on the same farm unit, Bollgard 3 refuge crops must be at least 24 metres wide and each refuge area must be a minimum of 0.5 hectares. Different unsprayed refuge options may be planted in the same field as a single unit; however a sprayed conventional cotton refuge must not be planted in a field that is also planted to an unsprayed refuge type unless a sufficient buffer is in place to prevent insecticide drift.

(h) In all regions, destruction of refuges must only be carried out after Bollgard 3 has been harvested. In Central Queensland, soil disturbance of refuge crops must only occur when the trap crop is being destroyed (refer to section on Pupae Destruction).

(i) Refuges for dryland Bollgard 3 cotton crops must be planted in the same row configuration as the Bollgard 3 crop unless the refuge is irrigated. If an irrigated option is utilised for a dryland Bollgard 3 crop, then that refuge may be planted in a solid configuration. Dryland cotton is measured as green hectares [calculated as defined in the Technology User Agreement].
Control of volunteer and ratoon cotton

Volunteer and ratoon cotton may impose additional selection pressure on Helicoverpa spp. to develop resistance to the Bt proteins Cry1Ac, Cry2Ab and Vip3A produced by Bollgard 3 cotton.

As soon as practical after harvest, Bollgard 3 cotton crops must be destroyed by cultivation, root cutting or herbicides so that they do not continue to act as hosts for Helicoverpa spp.

Growers must ensure that volunteer and ratoon plants are removed as soon as possible from all fields, including fallow areas, Bollgard 3 crops, conventional cotton crops and all refuges. The presence of Bollgard 3 volunteers/ratoon cotton in any refuge will diminish the value of the refuge and must be removed as soon as possible.

Note: The refuge should preferably be planted into fallow or rotation fields that have not been planted to cotton in the previous season.

Pupae destruction / trap crops

VICTORIA, NEW SOUTH WALES AND SOUTHERN QUEENSLAND

To further mitigate the risk of resistance, each grower of Bollgard 3 must undertake Helicoverpa spp. pupae destruction in fields with a higher probability of carrying over wintering pupae according to the following key guidelines:

- If first defoliation of a Bollgard 3 field occurs on or before March 31, the Bollgard 3 field must be slashed or mulched and controlled to prevent regrowth within 4 weeks of harvesting.
- If first defoliation of a Bollgard 3 field occurs after March 31, the Bollgard 3 field must be slashed or mulched and controlled to prevent regrowth within 4 weeks of harvesting and pupae busting must be complete by July 31 for all valleys except for all regions including the Lachlan, Murrumbidgee, Menindee, Murray Valleys and Victoria where pupae busting must be complete by August 31.
- Ensure disturbance of the soil surface to a depth of 10 centimetres to a distance of 30 centimetres both sides of the plant line.

CENTRAL QUEENSLAND

Crop destruction

All Bollgard 3 crops must be slashed or mulched and controlled to prevent regrowth within 4 weeks of harvesting.

End of season management of refuges/trap crops

End of season pupae busting practices are not effective in the Central Queensland region as Helicoverpa spp. are less likely to diapause. A late summer trap crop (pigeon pea) must be planted for all Bollgard 3 cotton grown in Central Queensland. The planting configuration of the trap crop should be the same as that of the Bollgard 3 crop. Irrigated Bollgard 3 must have an irrigated trap crop. Table 5 shows the requirements for the late summer pigeon pea trap crop. Dryland Bollgard 3 growers who do not have any irrigated cotton on their farm should contact Monsanto Australia for alternative options.

Refuge and late summer trap crops have different purposes. Where a pigeon pea refuge is utilised, the full pigeon pea refuge area must be managed to become the late summer trap crop. If unsprayed cotton is used as the refuge, an additional area of 1% pigeon pea must be planted as the late summer trap crop. Requirements for late summer trap crops are detailed in Table 5 (right).

FAILED CROPS – ALL REGIONS

Bollgard 3 crops that will not be grown through to harvest for various reasons and are declared to, and verified by, Monsanto as failed must be destroyed within two weeks after verification, in such a way that prevents regrowth. Crops that are abandoned before February 28 should be slashed and mulched within 4 weeks.
## Table 5: Late summer pigeon pea trap crop requirements in Central Queensland

<table>
<thead>
<tr>
<th>CRITERION</th>
<th>TRAP CROP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum area &amp; dimension (Requirement)</td>
<td>A minimum trap crop of 1% of planted Bollgard 3 cotton crop is required. If sprayed conventional cotton is grown on that farm unit: the trap crop must be at least 48m x 48m. If no sprayed conventional cotton is grown on that farm unit: the trap crop must be at least 24m x 24m.</td>
</tr>
<tr>
<td>Planting time</td>
<td>The trap crop should preferably be planted 4 weeks after the associated Bollgard 3. Note: if growers choose to plant their trap crop to coincide with the planting of pigeon pea refuges, they must manage the trap crop in such a way that it remains attractive to <em>Helicoverpa</em> spp. 2–4 weeks after final defoliation.</td>
</tr>
<tr>
<td>Planting rate **</td>
<td>35kg/ha (recommended establishment greater than 4 plants per metre)</td>
</tr>
<tr>
<td>Insect control</td>
<td>The trap crop can be sprayed with virus after flowering, while avoiding insecticide spray drift, except where a pigeon pea refuge is converted to a trap crop. In this case the full 5% pigeon pea refuge area managed to become the late summer trap crop can only be sprayed with virus after the first defoliation of Bollgard 3 cotton.</td>
</tr>
<tr>
<td>Irrigation</td>
<td>The refuge/trap crop must be planted into an area where it can receive the additional irrigation required to keep the trap crop attractive to <em>Helicoverpa</em> spp. until after the cotton is defoliated.</td>
</tr>
<tr>
<td>Weed control</td>
<td>The trap crop should be kept free of weeds and particularly volunteer Bollgard 3 cotton. When using the full pigeon pea refuge area as the trap crop, weed control must not be carried out by cultivation once flowering of the associated Bollgard 3 cotton crop has commenced.</td>
</tr>
<tr>
<td>Crop destruction</td>
<td>The trap crop must be destroyed 2–4 weeks (but not before 2 weeks) after final defoliation of the Bollgard 3 cotton crop, (slash and pupae bust – full soil disturbance to a depth of 10 cm across the entire trap crop area). All Bollgard 3 and associated trap crops must be destroyed by July 31.</td>
</tr>
</tbody>
</table>

*A pigeon pea trap crop is to be planted so that it is attractive (flowering) to *Helicoverpa* spp. after the cotton crop has cut out, and as any survivors from the Bollgard 3 crop emerge. Planting pigeon pea too early (e.g. before November) or too late (e.g. mid December) is not adequate for cotton crops planted during September through to October.

** The planting rate is a recommendation based on a minimum of 85% seed germination.
Spray Limitations

Insecticide preparations containing Bt may be used on Bollgard 3 cotton throughout the season BUT NOT on any refuge crops.

An unsprayed refuge should not be planted in the same field as any crop sprayed with a rate of insecticide that is registered for Helicoverpa spp., with the exception of Bollgard 3. Sprayed crops and unsprayed refuges that are planted in adjacent fields must be separated by sufficient distance to minimise the likelihood of insecticide drift onto the unsprayed refuge.

If the viability of an unsprayed refuge is at risk due to early or late season pressure by Helicoverpa spp., or any other caterpillar species, contact Monsanto Australia immediately. With prior approval from Monsanto Australia, a non-Bt helioide can be applied.

NB: If any grower encounters problems in complying with the Resistance Management Plan please contact Monsanto Australia.

For further background information on the various components of this plan see the "Preamble to the Resistance Management Plan for Bollgard 3" in the current Cotton Pest Management Guide.

The RMP helps support long term viability of Bt technologies, by providing farm and crop management techniques to aid in managing the risk of resistance occurring. It is also a regulatory requirement that forms part of Monsanto’s product registration with the APVMA and must be complied with for all planting of Bollgard 3.

Note. Any planting of Bollgard II cotton must comply with the Bollgard II RMP.
REFERENCES


EPA. 1986. EPA Fact Sheet for Bacillus thuringiensis subsp. kurstaki, israelensis, and aizawai.

EPA. 1988. Guidance for the reregistration of pesticide products containing Bacillus thuringiensis as the active ingredient. NTIS PB 89-164198.


EPA. 1996b. EPA Pesticide Fact Sheet for Bacillus thuringiensis subsp. kurstaki Strain EG07841. September 1996.


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