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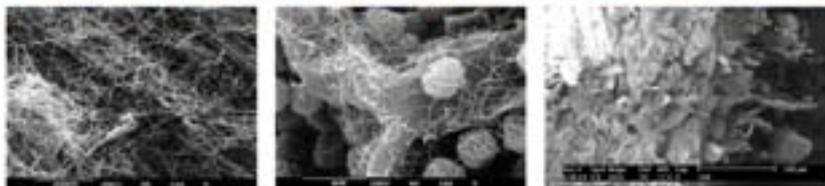
CSIRO LAND and WATER

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## Ecological impacts of GM cotton on soil biodiversity

*Below ground production of Bt by GM cotton and Bt cotton impacts on soil biological processes*

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**Final report for a project funded by  
Australian Government  
Department of the Environment and Heritage**

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This project is part of the CSIRO program investigating the  
'Ecological Impacts of Genetically Modified Organisms'

August 2004

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## **CSIRO Project Title: Ecological impacts of GM cotton on soil biodiversity**

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### **Summary**

This research programme focuses on the impacts of genetically modified (GM) cotton crops (*Bacillus thuringiensis* cotton; Bt cotton for short) on soil biodiversity and ecosystem function. The experimental work was based upon the need to establish the risk of production and release of Bt toxin by below ground plant parts of cotton and its potential persistence in the soil where Bt cotton crops are grown in Australia. In addition, the potential impacts of new gene products may affect key soil biological processes essential for a number of ecosystem functions.

Genetic modification of organisms (plants, microbes and animals) to incorporate useful traits is a powerful technology for the future development of sustainable agricultural systems. Transgenic cotton varieties modified to express the Cry1Ac insecticidal toxin (Bt cotton) that is toxic to some insect pests are now grown in Australia. However, little experimental data (especially quantitative) is available on the environmental consequences of sustained expression and/or presence of Bt toxin in various parts of Bt cotton plants. In addition very little is known about the potential for the persistence of Bt toxin released from Bt cotton plants in Australian cotton soils.

Soil biota mediate or regulate a variety of functions essential for plant growth and productivity, soil resource structure, and ecosystem health. Soil biota are diverse in terms of their physiological nature, size and environmental requirements. The composition and metabolic capabilities of the soil microbial and faunal communities underpin the occurrence and rates of many soil processes. Microbial-faunal (micro-, meso- and macrofauna) interactions play a critical role in a variety of biological functions both in the rhizosphere (the zone directly surrounding and influenced by roots) and the soil near decomposing plant residues. Plant residues are the primary source of metabolic energy (carbon) in Australian soils and the majority of biota populations and biota-mediated processes are concentrated in the rhizosphere and near crop residues. Therefore any change to the quality of crop residues and rhizosphere inputs will potentially modify the dynamics of soil biota composition and activity (Gupta *et al.*, 1998 and 1999). Genetically modified plants, through (1) the products of introduced genes, (2) modified rhizosphere chemistry, or (3) altered crop residue quality,

have the potential to significantly change the microbial dynamics, soil biodiversity and essential ecosystem functions such as nutrient mineralisation, disease incidence, carbon turnover and plant growth. While reduced pesticide use associated with Bt cotton varieties is clearly beneficial, very little is known on the potential non-target effects of Bt cotton plants on the functional groups of soil biota and associated biological processes that are critical for sustained cotton productivity and essential for ecosystem health.

It is known that the Bt cotton varieties produce Bt toxin in above ground plant parts such as leaves (particularly young leaves), flower buds etc. but no information is available on the production of Bt toxin in the below ground plant parts. In addition it has been assumed that “during the life of the plant the Bt-endotoxin in Ingard cotton is enclosed within plant cells and it would only enter the soil environment after the above ground plant material is ploughed in” (NRA, 1996).

In this project we measured the levels of Bt toxin production in different plant parts of cotton, especially below ground parts, and also evaluated the mechanisms through which the Bt toxin enters the soil environment. We found that in controlled environments (glasshouse and growth chamber) and field experiments, Bt cotton varieties expressed *Bt* genes and produced measurable amounts of Bt toxin in different parts of the cotton root system (tap, secondary and fine roots, and root hairs). Our results indicate that the levels of Bt toxin in roots are similar to those observed in leaves whereas the levels of Bt toxin in stems were the lowest. For example, Bt toxin levels in the leaves of cotton variety Sicot 289i ranged from 2,900 to 20,300 ppb and in the roots from 4,900 to 18,700 ppb.

The general decrease over time of Bt toxin levels in leaves is generally accepted (especially in Ingard varieties) to be due to the ageing of the various plant tissues and gradual breakdown of Bt toxin within these tissues. However, we found that as the plants grew older, the levels of Bt toxin in roots of 8-week old Bt cotton (Sicot 289i) were higher (4,900 and 7,000 ppb dry weight in taproot and fine roots, respectively) than that in leaves (2,900 ppb dry weight). We also found that in most situations Bt toxin levels in the fine roots were higher than other parts of the root system and plant-related reductions in this part of the root system were smaller compared to other plant parts. This higher level of Bt toxin below ground can be attributed to the continued growth of new root systems through the later stages of the cotton season.

We observed the presence of Bt toxin in the roots of Bt cotton varieties grown in three different soils (Avon, SA, Waikerie, SA and Narrabri, NSW). The results show that Bt toxin was produced in every major part of Bt cotton plants (leaves, stems, and roots), that root Bt toxin production was comparable (or higher in the later stages of cotton plant) to that in cotton

leaves and that the above observations held true for all three soil types. In these experiments we did not find any detectable levels of Bt toxin in the conventional non-Bt cotton plants.

We also found that the roots of Bt cotton varieties release Bt toxin, both in vitro (solution culture) and by soil-grown plants, through presumably *passive* release from the roots or as cell lysates, and the levels of release (cell-free) of Bt toxin from roots were significantly increased (>6-fold) following any damage to root system (eg fine roots). The non-Bt cotton cultivars, as expected, released no detectable Bt toxin. We found Bt toxin release from plants that were 2 to 12 weeks old and found no evidence for the presence of Bt toxin from roots of non-Bt cotton varieties.

Root hairs and sloughed epidermal cells contribute a significant amount of root material in the rhizosphere of actively growing plants. We found that the sloughed epidermal cells and fine-root hair fragments from Bt cotton (Sicot 289i) plants contained large concentrations of Bt toxin (eg 1317 ppb/g wet weight) whereas non-Bt control (Sicot 189) cells/fine-root hairs showed no Bt toxin. Thus, our results suggest that Bt toxin has the potential to enter the soil system throughout the Bt cotton growing season, through both a root release process and root turnover. Levels of Bt toxin entering the soil system could therefore be significantly higher than previously suggested on the basis of contributions of Bt toxin to soil from above-ground cotton material only (NRA, 1996)\*.

Unlike the Bt toxin from leaves and other above ground plant parts, which may enter soil only after defoliation (leaves) and cotton harvest (stems), roots with Bt toxin are in constant contact with the soil system (including soil biota) and Bt toxin levels in fine roots were found to be as high as that in younger leaves. In view of the results reported above (large concentrations of Bt toxin in Bt cotton roots and demonstrated root release), more detailed investigations on the environmental fate of the root-derived Bt toxin, binding to soil components and build up, and movement beyond the rhizosphere and root zone, are warranted. Results from our initial work found detectable levels of Bt toxin in the rhizosphere of Bt cotton varieties by using both immunological tests and insect bioassays.

Leaf material, in general, constitutes a major component of the easily decomposable part of crop residues and therefore supports larger populations of soil biota and higher levels of biological activity. Results from leaf decomposition experiment have shown that detectable levels of Bt toxin were observed in decomposing leaves throughout an 8-week field incubation experiment. The implications of the presence of Bt toxin for the composition of soil biota (soil fauna and microflora) during the main period of leaf material decomposition are unknown. Therefore, there is a clear need for further detailed investigations on the impacts

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\* A more recent assessment by the Australian Pesticides and Veterinary Medicines Authority, for Bollgard II cotton, does consider the contribution of Bt toxin from root sources (APVMA 2003).

of both leaf- and root-derived Bt toxins on soil biodiversity and associated biological functions.

Microbial growth indicators measured in this study (decomposition rates, substrate induced respiration, and respiration quotients) suggest that microbial population growth on Bt cotton leaf litter might be different than for non-Bt varieties. Microscopic examination revealed an apparent increase in fungi and fungal spores on the Bt cotton residues compared to the non-Bt residues. Experiments did not indicate whether these changes were likely to be detrimental, neutral or beneficial in an agricultural situation. These experiments need to be repeated over multiple seasons before firm conclusions can be drawn.

In summary, our work clearly demonstrates the evidence for avenues, other than through leaves, for Bt toxin to enter the soil system throughout the cotton growing season. This is contrary to the previous assumption that “it only enters after the above ground plant material is ploughed in” (NRA 1996). Our results indicate that rhizosphere-inhabiting soil biota are continuously exposed to Bt toxin produced in the roots (through root releases and root turnover) throughout the growing season and then further exposed as crop residues decompose after harvest.

In Australian soils, the rhizosphere environment is one of two key zones where the majority of soil biota reside, the other zone being the soil around decomposing crop residues. Populations of different groups of microbiota are generally higher (>10-fold) in rhizosphere soils compared to that in bulk soil, and rhizosphere biological activity accounts for >60% of overall soil biological activity. Implications of our results on Bt toxin production and release below ground by Bt cotton varieties are that the input from Bt cotton roots has previously been significantly underestimated and the impacts of this hitherto unknown, toxin input are yet to be fully investigated.

We have shown that different plant parts of Bt cotton (leaves, stubble and roots) contain large concentrations of Bt toxin and therefore have the potential to be a reservoir of Bt toxin in agricultural fields of Australia. Our findings showing large concentrations of Bt toxin (above soil background) in decomposing Bt cotton leaf residues even after the decomposition of >40% of leaf residue indicate that Bt toxin from dead leaves is not easily degraded by soil microorganisms, which one would expect for such a protein substance. If more Bt toxin enters the soil environment than is degraded by microbes, eaten by insect larvae or inactivated by sunlight there is potential for the toxin to accumulate if it is bound and protected by soil particles (clays, minerals and humic acids). Could accumulation of active Bt toxin constitute a hazard to non-target organisms and impact the biodiversity and functionality of the organisms inhabiting the soil?

Soil fungi associated with decomposing crop residues could be either non-pathogenic species or species that cause soilborne plant diseases. Crop residues are the primary source of carbon for soil biota populations in Australian soils hence the composition of biota associated has a great significance in regulating the essential biological functions in the ecosystem. Observations on differences in microbial populations, including a possible increase of fungi on decomposing Bt-cotton, require further investigation over more than one season before differences can be confirmed. If the observed trend is real, its significance is not yet clear since the changes to soil biota could be detrimental, neutral or beneficial to agricultural soil ecosystems.

Finally, consideration of the environmental fate of Bt toxin from Bt crops has sometimes focussed only on the expression of the *Bt* gene in above-ground plant parts, but our results suggest further investigation into the environmental fate of the root-released Bt toxin in soil is required, both during the cotton season and following the harvest of the cotton crop.

This project is based with the CSIRO Land and Water group in Adelaide with collaborative links for fieldwork with researchers at Australian Cotton Research Institute (ACRI) research farm in Narrabri, NSW (Mr. Grant Roberts, CSIRO Plant Industry).

*Note:* the material presented in this document is in preparation for publication in the scientific literature.

## Background

Genetic modification of organisms (plants, microbes and animals) to incorporate useful traits is a powerful technology for the future development of sustainable agricultural systems. Plants have been genetically modified to resist insect and fungal pathogens (to reduce reliance on pesticides), withstand specific herbicide application (better weed management) or environmental conditions (eg waterlogging), to improve crop quality (nutritional value), for bioremediation (phytoremediation) and for biomolecule production (molecular farming for medicinal products). Similarly, microorganisms have been modified either to improve performance (eg increased biocontrol), to perform multiple tasks to help crop productivity (eg decomposition of crop residues and N<sub>2</sub> fixation), or for bioremediation and biomolecule production.

The genetic modification of plants will be vital to the Australian cotton industry if it is to reduce its dependence on broad-spectrum synthetic pesticides or to combat weeds, pests and diseases. Cotton varieties genetically modified to express the Bt *CryIAc* (Ingard) insecticidal toxin gene have been available for commercial use in Australia for more than seven years. New, two-gene Bt cotton varieties (based on Ingard and RoundupReady/Ingard (RRi) cottons) have also been developed for Australian growers to overcome problems of insect resistance and to improve weed management. While reduced pesticide/herbicide use associated with Bt and RoundupReady cotton is clearly beneficial, very little is known about potential non-target effects of Bt cotton plants on the functional groups of biota and biological processes that are critical for plant health, cotton productivity and essential ecosystem functions including ecosystem health.

Pre-release evaluation of GM plant varieties is generally concentrated on the genetic stability of gene insertions and agronomic aspects of GM varieties. However, comparatively little experimental (especially quantitative) data are available on:

- 1) ***Environmental consequences of the introduced gene function*** and associated changes in management practices/farming systems on essential ecosystem functions.
- 2) ***The fate of the products of engineered genes from genetically modified organisms*** (GMOs) eg persistence in the environment and gene transfer to other organisms. ***This needs to be an essential part of the risk assessment of any GMO release.***

Soil biota communities are among the most diverse groups of earth's biota. Soil organisms regulate a number of processes in terrestrial ecosystems that are not only critical for productivity but are also essential for maintenance of ecosystem health (Brussard *et al.*, 1997). Microorganisms and microbial activity have a key role in stable aggregate formation.

Water-stable aggregates are essential for good soil structure in all types of soils. Good soil structure is necessary to reduce soil erosion. Very few biological processes are mediated by individual species of biota therefore the successful functioning of most ecosystem processes requires a balance of biota interactions in the complex soil biota community (detritus food-web). The availability of energy (carbon), the most important regulating factor of biological activity in soils, affects the composition of the soil biota community and food web structure (Elliott and Coleman, 1988; Coleman and Crossley, 1995). In addition, the number of trophic levels in a terrestrial food-web community and the stability of this complex community depend upon the amount and quality of carbon input and the level and type of disturbance (eg tillage, GM crops and use of agrochemicals).

Plant residues are the primary source of carbon in Australian soils and the majority of biota populations are concentrated near crop residues and in the plant root rhizosphere (Gupta *et al.*, 2000). Therefore, any change to the quality of crop residue and rhizosphere inputs will potentially modify the dynamics of the soil biota composition and activity. Soil organisms perform a number of key functions essential to plants, such as decomposition, nutrient cycling, disease regulation, agrochemical degradation, and the development and maintenance of physio-chemical properties of soil. Microbial-faunal (microfauna, mesofauna and macrofauna) interactions play a critical role in a variety of biological functions both in the rhizosphere and near decomposing residues (Coleman and Crossley, 1995; Gupta and Yeates, 1997).

The rhizosphere (the zone directly surrounding and influenced by plant roots) contains a large majority of the soil's biota populations (>10-fold of that in the bulk soil) and the plant-microbe interaction in the rhizosphere is one of the major factors regulating the health and growth of plants. It is also widely acknowledged that root exudates govern which organisms reside in the rhizosphere (Lynch, 1994; Bardgett *et al.*, 1999). Therefore any change to the quality of rhizosphere exudates will potentially modify the dynamics of the soil biota composition (biodiversity) and activity and may cause changes to both deleterious and beneficial microflora and microfauna.

GM plants, through (1) the products of introduced genes, (2) modified rhizosphere chemistry, or (3) altered crop residue quality, have the potential to significantly change the microbial dynamics and essential ecosystem functions such as nutrient mineralisation, disease incidence, carbon turnover and plant growth (Gupta *et al.*, 2000). For example, a decrease in specific microbial populations would lead to a decrease in decomposition processes and have secondary effects on plant pathogen survival and build up, as well as soil organic matter level and composition (Termorshuizen and Lotz, 2002). Similarly, loss of particular trophic groups of fauna will cause a loss of specific pathways within nutrient cycling processes thus affecting important biogeochemical pathways. However, little experimental data are available on the

consequences of plant-microbe-soil interactions due to the sustained expression and/or presence of Bt toxin in the rhizosphere. The well-justified caution that has been shown in Australia with regards to the introduction of Bt cotton and the development of insect resistance should also be applied to the preservation of soil sustainability to protect the soil's biological function and diversity.

There is no ongoing research on the impact of Bt cotton and herbicide-tolerant GM canola on soil biological processes in Australia (databases searched: ARRIP (Australian Rural Research in Progress) and Streamline at <http://WWW.infoscan.com.au>). In an international workshop on 'Ecological impacts of transgenic crops' held at the University of California (March 2-4, 2000), it was concluded that "research in this area of effects on soil biology is quite limited ..... and studies should evaluate the effects on biological communities and the ecological processes they mediate". Gupta *et al.* (1998) have found significant changes in the composition of bacteria in the rhizosphere of Ingard cotton compared to that of its non-GM parent variety. Limited research in Europe and North America suggests significant effects of GM crops on specific soil biota. Stotzky (2000) in a recent review recommends a thorough evaluation of the persistence of GM products such as Bt toxins in soil and their effects on the inhabitants of soil and other habitats. Due to the differences in soil and climatic conditions, and the biota composition, the evaluation of GM plant effects on soil biodiversity under Australian conditions is necessary.

A flow chart for a CSIRO research proposal to evaluate the ecological impacts for successful integration of GM plants in Australian agriculture is given in Appendix 1. The work was proposed in two parts, Parts 1 and 2:

Part 1: The stability and persistence of insecticidal proteins and/or foreign DNA from genetically modified organisms (GMOs) for example, Bt toxin from Bt cotton.

Part 2: Data on the environmental impacts, *in situ*, of genetically modified plants (Bt cotton and herbicide tolerant cotton and canola) on soil biodiversity and essential ecosystem functions.

### **Rationale for this project**

GM cotton varieties that produce insecticidal products have already been released for use in Australia and the highly bioactive substances, such as *Bacillus thuringiensis* proteins (eg Bt toxins such as Cry1Ac in Ingard cotton), may become ubiquitous in agroecosystems. Research from overseas has shown that these toxins may persist in agroecosystems for many months (>200 days) and soil components (eg clays and humic substances) could have a significant impact on the biotoxicity of these insecticidal proteins (Tapp and Stotzky, 1998).

These toxins may be released into soil either from growing roots or through dead crop residues. There is no quantitative information on the levels of Bt toxins in different plant parts of Bt cotton or if toxins could be released by Bt cotton into soil environments. In addition, there is no information on the persistence and bioactivity of Bt toxin in Australian environments (different soil types and environments). This information is not only necessary for the development of management options for sustainable agricultural (cotton) production but also to predict the potential for the movement of bound toxin outside fields into other natural ecosystems, ie both terrestrial and aquatic ecosystems.

#### Project Outputs:

This project's outputs relate to Part 1 of the CSIRO proposal. The stability and persistence of the insecticidal protein, Bt toxin from Bt cotton, will be determined by:

Objective A. Measuring the persistence and biological activity of Bt toxins from root exudates and decomposing cotton residues as influenced by soil components (clays and organic matter).

Objective B. Assessing the ecological impacts of Bt toxin on one of the key biological processes essential for ecosystem function (eg cotton residue decomposition).

#### ***A Measuring the persistence and biological activity of Bt toxins from root exudates and decomposing cotton residues as influenced by soil components (clays and organic matter)***

Initially our aim was to determine the levels of Bt toxin in different parts of Bt cotton plants (eg roots, root exudates) and in the rhizosphere.

### **1. Bt toxin presence in cotton plant tissues, root exudates and rhizosphere soil**

#### *1.1 Cotton plant growth conditions – glasshouses and growth chambers*

In order to test various Bt cotton plant tissues, root exudates and rhizosphere soils for the presence of Bt toxin, both Bt and non-Bt cotton varieties were grown in controlled environment conditions (glasshouses and growth chambers). Glasshouses were maintained at 26°C and cotton plants (Sicot 189, Sicot 289i (289i) and Sicot 289RRi (289RRi)) were grown initially in 1kg (soil) pots and later in waterproof PVC pots (2.7kg soil). Five plants were grown per pot and in three replicates. Surface soils (10cm depth), collected from long-term farming system trials at Avon and Waikerie in SA (see Appendix 2 for soil characteristics),

were used in all the controlled environment experiments. Soil moisture was maintained at approximately 17% w/w and 12% w/w for Avon and Waikerie soils respectively, which is equivalent to soil moisture at field capacity. All the pots were incubated in temperature-controlled (26°C) water baths. Approximately 20ml of modified Hoagland's solution (Gupta Vadakattu, laboratory method – unpublished) were added weekly to provide supplementary nutrients. In later experiments, cotton plants were grown in 2.7kg pots to provide greater room for root growth particularly when grown for more than 6 weeks (Photo A and B, f 3). Extra lighting was provided to enhance plant growth (12 hour day/night cycle). Cotton plants were also grown in 1kg pots in temperature-controlled cabinets.

### *1.2 Quantitative Bt toxin detection methodology*

In all experiments we measured the levels of Bt toxin using the 'Envirologix Cry1Ab/Cry1Ac ELISA Kit'. This ELISA kit was designed for the quantitative detection of Bt toxin in leaf tissue by using specific polyclonal antibodies to the Cry1Ab and Cry1Ac proteins. These antibodies are enzyme-labelled and therefore results are visualised with a colour development step whereby the colour level is proportional to the Cry1Ab/Cry1Ac concentration in the sample extract (Photo 1, Appendix 4). Prior to this detection process (colour development) the Bt toxin must be released (extracted) from the plant/soil matrix into a detection buffer.

Our preliminary experiments with the ELISA kit and various cotton plant varieties used a number of methods for the extraction of Bt toxin from different plant tissues. Attempts to grind fresh root material in liquid nitrogen alone, prior to extraction, encountered variability problems in relation to sample weights. Analyses of fresh plant samples from 1kg pots used a wet extraction/hand grind method in extraction buffer with and without sand granules (500µm diameter) in microcentrifuge tubes using plastic pestles (Envirologix, cat. # ACCO2). All the fresh plant samples in later experiments ie fresh samples from 2.7kg PVC pots, were processed using a Biospec Bead Beater that involved rapid shaking of a 2ml microcentrifuge tube containing the sample and small chrome beads (6.4mm diameter) with extraction buffer.

A summary of results from the different preliminary experiments for Bt toxin levels in different Bt cotton varieties are given in Table 1. Results from 1kg pots indicate that Bt cotton leaves, in general, produced the greatest amount of Bt toxin (1,815–22,656 ppb/g dry weight (wt)) with the stems and roots producing a lower amount (248–3,406 ppb/g dry wt). Bt toxin levels in roots from 2.7kg PVC pots were greater than levels in the leaves. This could be attributed to the greater volume of soil available for root growth and the improved extraction

methods (ie bead beating method) as it was more efficient in extracting the Bt toxin compared to the hand-grinding method. Hand grinding of roots was found to be inefficient and difficult in comparison to other plant tissues. In addition, roots in 2.7kg PVC pots may not have been constrained for resources after 3–4 weeks of growth (ie conditions reflecting closer to field situations). All further experiments were conducted in 2.7kg PVC pots.

**Table 1.** Summary of preliminary findings of Bt toxin (Cry1Ac) levels in various parts of different Bt cotton plant varieties.

Cotton variety	Leaves	Stems	Roots
	----- ppb/gram dry weight -----		
<i>Conventional cotton</i> (1kg pots) <sup>§</sup>			
V2 (4 wks)	0	0	0
Sicot 189 (8 wks)	0	0	0
<i>GM cotton</i> (1kg pots) <sup>§</sup>			
V2i (4 wks)	21,134-22,656	2,547-3,340	896-1,729
Sicot 289i (8 wks)	1,815-2,046	452-2,069	1,189-3,371
Sicot 289Ri (8 wks)	-	248-531	2,046-3,406
<i>Conventional cotton</i> (2.7kg pots) <sup>#</sup>			
Sicot 189 (9 wks)	0	0	0
<i>GM cotton</i> (2.7kg pots) <sup>#</sup>			
Sicot 289i (9 wks)	2,340.9 ± 322.5*	-	4,277.9 ± 19.9

<sup>§</sup> Extraction method used in the ELISA test was ‘hand grinding with pestle and tube’ of fresh material

<sup>#</sup> Extraction method used in the ELISA test was ‘bead beater, 1x1min, steel, 6mm’ of fresh material

\* ± Standard error of the mean

In order to further improve the efficiency of Bt toxin extraction and measurement from different plant parts, the bead beating method was further developed. Firstly, a number of different tissue preparations (ie fresh, oven-dried (60°C), freeze-dried, or snap frozen (liquid nitrogen) and stored at –80°C), were evaluated prior to use in the ELISA test kit. These samples were representative of the range of samples expected from the field and laboratory experiments. It was found that although fresh plant material consistently gave the highest results, snap-frozen samples gave the highest Bt toxin yield from the various methods of storage (drying, snap freezing and freeze drying).

**Table 2.** Effects of sample preparation and storage on Bt toxin levels<sup>2</sup>

Variety <sup>1</sup>	Sample preparation	Method of extraction	Type of beads	Bt toxin level
189	0.02g leaf (60°C)	bead beater 3 x 1min	steel 2 x 6mm	0
<b>Storage method</b>				
289i	0.02g leaf (60°C)	Hand grind (pestle and tube)	NA	1423± 25
289i	0.02g leaf (60°C)-snap frozen	bead beater 3 x 1min	steel 2 x 6mm	2331± 36
289i	0.02g leaf (60°C)-freeze dried	bead beater 3 x 1min	steel 2 x 6mm	1337± 75
289i	0.02g leaf (60°C)-oven-dried	bead beater 3 x 1min	steel 2 x 6mm	1163±236
<b>Time of bead beating</b>				
289i	0.02g leaf (60°C)-snap frozen	bead beater 1 x 1 min	steel 2 x 6mm	3082
289i	0.02g leaf (60°C)-snap frozen	bead beater 2 x 1 min	steel 2 x 6mm	2076
289i	0.02g leaf (60°C)-snap frozen	bead beater 3 x 1 min	steel 2 x 6mm	219
<b>Type of beads</b>				
289i	0.02g leaf (60°C)-snap frozen	bead beater 1 x 30 sec	steel 2 x 6mm	2432 ± 39
289i	0.02g leaf (60°C)-snap frozen	bead beater 1 x 1 min	steel 2 x 6mm	3920 ± 10
289i	0.02g leaf (60°C)-snap frozen	bead beater 1 x 30 sec	steel 10 x 2mm	3557 ± 37
289i	0.02g leaf (60°C)-snap frozen	bead beater 1 x 1 min	steel 10 x 2mm	3421 ± 62
289i	0.02g leaf (60°C)-snap frozen	bead beater 1 x 30 sec	glass 10 x 2.5mm	2305 ± 144
289i	0.02g leaf (60°C)-snap frozen	bead beater 1 x 1 min	glass 10 x 2.5mm	2336 ± 114

<sup>1</sup> Varieties 189 and 289i are Sicot 189 and Sicot 289i respectively

<sup>2</sup> Average (duplicates) ppb Cry1Ac/g dry weight as determined by ELISA ± standard error of the mean

The 60°C oven-dried leaves used for development of this method were chosen as a similar type of sample was used in litter-bag decomposition experiments (details in sections 2 and 3). Because the levels of Bt toxin in different plant parts were unknown, we evaluated appropriate sample sizes (0.1g, 0.05g, 0.02g and 0.01g) for various cotton plant parts required for the ELISA test to give readable and reliable colour development. A sample size of 0.02g was determined to give consistently good Bt toxin yields so that quantitative results were produced from the ELISA tests. We also evaluated other aspects of Bt toxin extraction and standardised the time of bead beating and the size and the type of beads (eg glass beads vs. steel beads) that gave maximum yield for different plant parts. Results for Bt toxin

measurements using various sample preparation, and extraction methods are summarised in Table 2.

The most effective storage method was determined to be the snap freezing of samples and storage at  $-80^{\circ}\text{C}$ . The most efficient Bt toxin extraction method was determined to be: a 0.02g sample of plant tissue; 600 $\mu\text{l}$  of Envirologix extraction buffer; bead beating for one minute with two steel (6mm) beads. These parameters were used for all subsequent extractions. However, some minor variations in the amount of plant tissue used in the bead-beating step were adapted to ensure accurate pipetting and that Bt toxin levels were in the readable range of the ELISA detection system.

Initially we encountered difficulties with the ELISA and Bt toxin detection in root exudates from nutrient solution-grown plants and plants grown in soil (details in section 1.4). This was due to the incompatibility between the nutrient exudation buffers and the ELISA kit procedure, giving rise to a number of false positives and inaccurate colour development. We also found that the nutrient and salt matrix of the soil and clay experiments (as per reported in literature) required modifications to be compatible with the ELISA test kit. Therefore separate methods were developed for the detection of Bt toxin in root exudates and soils (details given in later sections).

### *1.3 Bt toxin levels in glasshouse-grown and field-grown cotton plants*

Bt cotton plants grown in the glasshouse and under field conditions provided above- and below-ground materials for Bt toxin analyses in different plant parts. Conventional (Sicot 189) and their GM counterparts 289i (Ingard) and 289RRi (RoundupReady/Ingard) varieties were planted in 2.7kg Avon soil pots and grown under glasshouse conditions as described previously. The same cotton varieties were also planted in fields at Narrabri (NSW) during the first week of October 2001. This was to ensure that observations made in controlled (glasshouse) condition experiments were comparable to the field situation. Plants were harvested from the glasshouse experiments at 2, 4 and 8 weeks post germination and various plant tissue (leaf, stem, tap root, secondary- and fine-root) samples taken and snap frozen prior to using the ELISA tests. Similarly, cotton plants harvested from the experimental field at Narrabri at 8, 12, and 16 weeks, were transported in a 'cooler' to the Adelaide laboratory where different plant parts were separated and snap frozen prior to Bt toxin analyses.

**Table 3.** Bt toxin levels<sup>3</sup> in cotton plants from glasshouse experiments

Variety	Tissue type	Week 2 <sup>1</sup>	Week 4	Week 8
<b>Conventional cotton</b>				
Sicot 189	leaf	0	0	0
Sicot 189	stem	0	0	0
Sicot 189	Bulk <sup>2</sup> root	0	0	0
Sicot 189	fine root	0	0	0
<b>GM cotton</b>				
Sicot 289i	leaf	20,287.3 ± 8,144.5	15,428.8 ± 3,927.7	2,876.6 ± 485.3
Sicot 289i	stem	5,668.7 ± 1,688.3	4,855.5 ± 777.9	1,034.4 ± 132.0
Sicot 289i	bulk root	17,041.3 ± 1,623.4	18,723.0 ± 5,984.5	4,924.8 ± 1,630.7
Sicot 289i	fine root	15,530.9 ± 1,751.6	12,230.0 ± 467.8	7,082.3 ± 1,590.0
<b>GM cotton</b>				
Sicot 289RRi	leaf	13,267.4 ± 4,637.7	11,416.8 ± 6,047.3	5,832.3 ± 2,299.6
Sicot 289RRi	stem	4,265.5 ± 1,182.3	3,133.0 ± 3,832.7	725.2 ± 299.0
Sicot 289RRi	bulk root	9,641.8 ± 1,124.9	13,070.9 ± 1,227.6	3,536.8 ± 233.0
Sicot 289RRi	fine root	7,567.5 ± 1,129.5	26,009.1 ± 4,435.2	10,553.1 ± 1,426.8

<sup>1</sup> Weeks post germination

<sup>2</sup> Bulk root is a pooled sample of all root types (tap, secondary and fine)

<sup>3</sup> ppb of Cry1Ac (dry weight basis) as determined by ELISA, data are the average of duplicates of three replicates ± standard error of the mean

Results from ELISA analyses of glasshouse-grown cotton plants are shown in Table 3. It is evident that measurable concentrations of Bt toxin were present in all parts of Bt cotton plants (289i and 289RRi) ie leaves, stems and roots (tap, secondary and fine roots), and not present in the same tissues of conventional cotton plants (Sicot 189). The levels of Bt toxin in 289i and 289RRi leaves ranged from 2,876.6 – 20,287.3 ppb (on plant dry wt. basis). The levels of Bt toxin in the fine roots of these Bt cotton plants ranged from 7,082.3 – 26,009.1 ppb (dry wt. basis). It was noted that the levels of Bt toxin in 289RRi fine roots at weeks 4 and 8 plants were even higher than the levels found in the leaves of these same plants.

The general decrease in Bt toxin levels over time is generally accepted to be due to the ageing of the various plant tissues and gradual breakdown of Bt toxin within these tissues. However, as the plants grew older the levels of Bt toxin in the roots were significantly higher than the leaves as indicated in Figure 1. This increase in Bt toxin below ground can be attributed to the continued growth of new root systems.

These results show that the expression of Bt toxin occurred in every major part of the Bt cotton plants (leaves, stems, and roots) and that root Bt toxin expression was comparable

(or higher in the later stages of cotton plant) to that in cotton leaves. The general assumption, until now, has been that the expression of Bt toxin was confined to the above-ground plant system (leaves and stems) and that the levels found in the leaves are the highest in the total plant. However, the general promoter used for the expression of the inserted *CryIAC* gene is not tissue-specific. Therefore there would be no tissue-specific control over the expression of the *Bt* gene in the cotton plant. Our research is the first to indicate that Bt toxin is found in the roots of the Bt cotton plants in Australia at levels as high and in some instances higher than in the leaves.

To determine if the Bt toxin levels from the controlled environment (glasshouse) cotton plants were indicative of Bt toxin levels in cotton plants grown in the field, field plants were harvested during the 2001–2002 cotton season (weeks 8, 12 and 16) and the Bt toxin levels analysed by ELISA. Results from samples of field-grown cotton plants harvested at week 8 are shown in Table 4. Levels of Bt toxin in 289i and 289RRi leaves ranged from 3,567.7 – 27,395.5 ppb (dry wt basis) and Bt toxin levels in roots ranged from 2,248.1–15,123.4 ppb (dry wt basis). These levels were comparable with the data obtained from glasshouse-grown cotton plants at week 8 and therefore our experiments with glasshouse cotton plants can be taken as indicative of the situation in the cotton fields.

Results of ELISA analyses of the various root types harvested from field-grown cotton plants at 12 and 16 weeks after emergence are shown in Table 5. Of the root types sampled the fine roots exhibited the highest Bt toxin levels, ranging from 412.4 –15,123.4 ppb (dry wt basis) for 289i and 289RRi cotton plants. Even at week 16 we measured significant levels of Bt toxin in all root types ie 288.1 ppb (dry wt basis) for 289i tap roots through to 1,331.0 ppb (dry wt basis) for 289RRi fine roots. These data show a significant amount of Bt toxin in cotton plant roots below the ground late in the growing season.

#### *1.4 Bt toxin presence in Bt cotton root exudates*

Data presented in the previous sections indicate a high level of Bt toxin in the roots of both 289i and 289RRi cotton plants throughout the cotton growing season. Therefore the potential exists for Bt toxin to be released from these roots into the rhizosphere. This ‘exudation’ or ‘root release’ could include leakage of substances from the roots without metabolic energy and/or in the lysates released when cells autolyse (die) or damaged. While we understand that there was no transit peptide signal sequence attached to the *CryIAC* gene when it was engineered into the cotton plant genome, it is uncertain if the absence of targeting to cellular secretion mechanisms would ensure containment of Bt toxin within the roots of the Bt cotton plant.

**Table 4.** Bt toxin levels<sup>1</sup> from various tissues in field-grown cotton plants (week 8)

Variety	Tissue type	Bt toxin levels
<i>Conventional</i>		
Sicot 189	leaf	0
Sicot 189	young leaf	0
Sicot 189	stem	0
Sicot 189	tap root	0
Sicot 189	secondary root	0
Sicot 189	fine root	0
<i>GM cotton</i>		
Sicot 289i	leaf	3,567.7 ± 370.1
Sicot 289i	young leaf	11,401.2 ± 2,104.5
Sicot 289i	stem	887.1 ± 290.0
Sicot 289i	taproot	3,589.6 ± 445.0
Sicot 289i	secondary root	3,327.6 ± 103.7
Sicot 289i	fine root	15,123.4 ± 4,437.4
<i>GM cotton</i>		
Sicot 289RRi	leaf	10,608.7 ± 4,985.9
Sicot 289RRi	young leaf	27,395.5 ± 2,753.3
Sicot 289RRi	Stem	10,610.3 ± 1,278.9
Sicot 289RRi	taproot	3,633.3 ± 175.2
Sicot 289RRi	secondary root	2,248.1 ± 269.3
Sicot 289RRi	fine root	5,397.4 ± 790.0

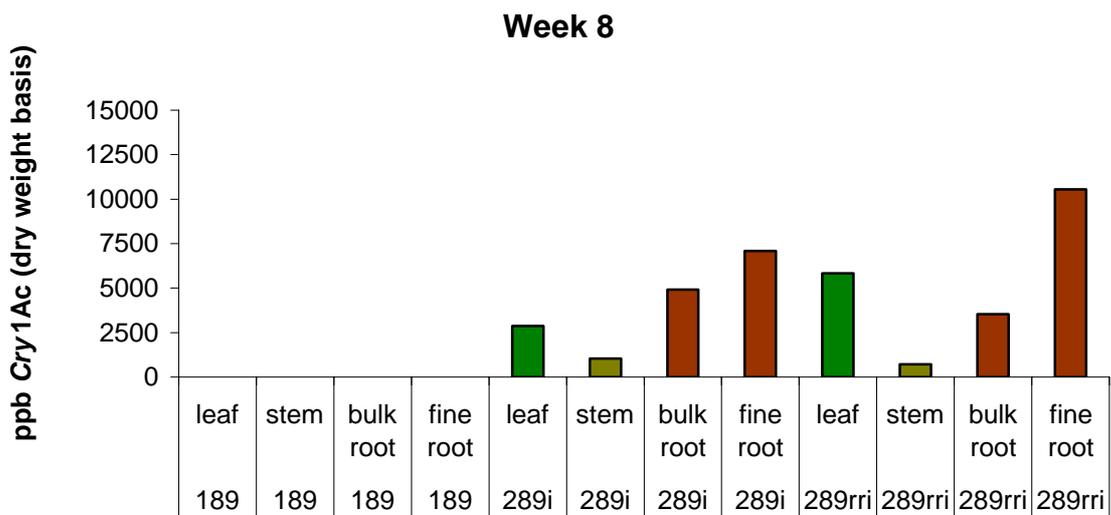
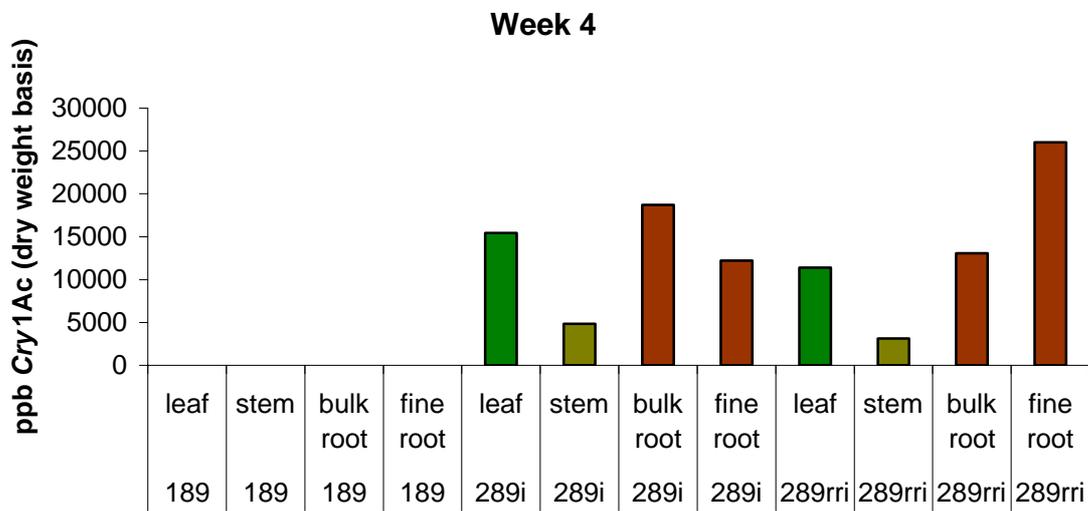
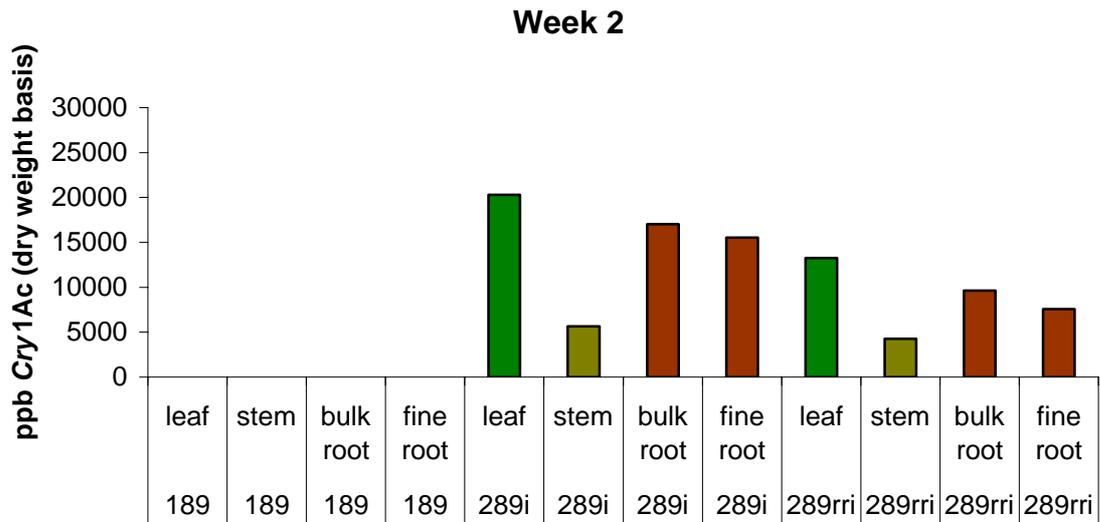
<sup>1</sup> ppb of Cry1Ac (dry weight basis) as determined by ELISA, data is the average of duplicates ± standard error of the mean

**Table 5.** Bt toxin levels<sup>2</sup> in various root types from field-grown cotton plants

Variety	Root type	Week 8 <sup>1</sup>	Week 12	Week 16
<i>Conventional</i>				
Sicot 189	taproot	0	0	0
Sicot 189	secondary root	0	0	0
Sicot 189	fine root	0	0	0
<i>GM cotton</i>				
Sicot 289i	taproot	3,589.6 ± 445.0	1,139.8 ± 305.5	288.1 ± 87.9
Sicot 289i	secondary root	3,327.6 ± 104.0	1,026.0 ± 41.6	244.5 ± 143.3
Sicot 289i	fine root	15,123.4 ± 4,437.0	972.1 ± 41.4	412.4 ± 239.3
<i>GM cotton</i>				
Sicot 289RRi	taproot	3,633.3 ± 175.0	675.4 ± 192.8	540.9 ± 65.6
Sicot 289RRi	secondary root	2,248.1 ± 269.0	715.3 ± 83.6	830.1 ± 16.7
Sicot 289RRi	fine root	5,397.4 ± 790.0	1,004.8 ± 64.8	1,331.0 ± 209.1

<sup>1</sup> Weeks post emergence

<sup>2</sup> ppb of Cry1Ac (dry weight basis) as determined by ELISA, data is the average of duplicates ± standard error of the mean



**Figure 1.** Concentrations of Bt toxin (Cry1Ac) in different parts of non-Bt and Bt cotton varieties grown under glasshouse conditions.

The determination of Bt toxin presence in root exudates from Bt cotton plants was investigated both using the plants grown in sterile solution culture and in soil systems. Initially cotton plants were grown in sterile modified Hoagland's solution to (1) avoid the binding of free Bt toxin by soil particles, (2) reduce the possibilities for breakdown of Bt toxin by microbial action and (3) provide a simple system for collection of root exudates. Cotton plants were also grown in soil and washed roots analysed for Bt toxin release in an attempt to simulate field conditions.

*Initial observations and method development:*

*Solution-grown plants and Dipstick tests* – Early experiments were developed using the Envirologix Cry1Ab/Cry1Ac QuickStix as a rapid method of Bt toxin detection. Surface-sterilised seeds (soaked in 10% bleach for 15 minutes followed by 6 washes in sterile distilled water) were germinated on sterile nylon mesh (~3mm holes) stretched across the top of sterile 120ml containers (10cm diam.) filled with sterile Hoagland's solution (1/10 x strength). Approximately 10 seeds per container were grown for 14 days (post germination) in a 26°C temperature-controlled cabinet (12 hour light/dark cycle). Prior to the collection of root exudates, the growth solution was replaced with fresh Hoagland's solution containing a protease inhibitor cocktail (Sigma, cat. # P2714, 500µl/100ml) to reduce protein degradation. The collection of root exudates was carried out over a 20-hour period at 8°C (to reduce protein degradation).

Detection of Bt toxin in the root exudates of Sicot 189, 289i and 289RRi plants was carried out using the Envirologix Cry1Ac/Cry1Ab QuickStix (dipstick immunological test) instead of the Envirologix ELISA kit to facilitate rapid and simple Bt toxin analysis. Saxena and Stotzky (2000) showed a presence of Bt toxin in root exudates from Bt-corn plant roots using these rapid dipsticks. However, in this study, exudates from 289i and 289RRi cotton roots gave below detectable level results using the Envirologix Cry 1Ac/Cry1Ab QuickStix.

*Solution-grown plants and the Envirologix ELISA test* – Subsequent root exudate samples were analysed with the more sensitive Envirologix ELISA test. However, results indicated that modifications to the exudate collection buffer were required to avoid both false positives and below-detectable readings due to an incompatibility between the exudation buffer (Hoagland's solution) and the ELISA test kit procedure.

*Modifications to solution plant growth* – Modifications were made to both the seedling growth method and the root exudate concentration in an attempt to improve the sensitivity of the ELISA test. Surface-sterilised seeds were germinated on sterile agar and grown for approximately 7 days. In an effort to concentrate the root exudates, the seedlings were transferred to a smaller sterile system using 10ml polypropylene tubes (6 seedlings per tube)

containing beads (30 x 3mm diam.) and approximately 10ml of Hoagland's solution as shown in Photo C. (Appendix 3). The transfer process was done with enough care to prevent the breakage of any lateral roots. The solution-grown plants were kept in a gas permeable, covered container (clear plastic sheet) to maintain sterility, and housed in a temperature-controlled cabinet at 26°C and a 12-hour light/dark cycle. For collection of root exudates, the growth solution was replaced with fresh exudation buffer containing a protease inhibitor cocktail (Sigma, cat. # P2714, 500µl/100ml) to prevent the breakdown of the Bt toxin during the exudation collection. The collection of root exudates was carried out over a 20-hour period at 8°C.

*Modifications in exudate buffers and exudate concentrations* – We replaced the Hoagland's solution exudation buffer with a half strength Envirologix extraction buffer (EB), to maintain the integrity of the ELISA reactions, and added a concentration step prior to measurement. Root exudates were collected from Sicot 189, 289i and 289RRi cotton plants, filtered through 0.2µm filters to remove sloughed cells and fine-root hairs, and concentrated using the Centricon centrifugal filter devices from Millipore (YM-30). These devices concentrated (approx. 100-fold) and retained any protein above 30kDa. The molecular size of the Cry1Ac protein in its truncated form is 73kDa.

*Bt toxin levels in sterile, solution-grown, cell-free, plant root exudates:*

Results in Table 6(a) show the Bt toxin levels in the Envirologix extraction buffer exudates from sterile, 14 day old cotton seedlings. In cell-free, root (unbroken) exudates, Bt toxin was detected at average levels of 218 and 413 ppb/g dry wt for 289i and 289RRi 2 week old seedlings. Levels of Bt toxin in the exudates from 8-week-old seedlings (in a 20-hour assay) were 375 and 267 ppb/g dry wt for 289i and 289RRi, respectively. Bt toxin levels in the roots of these plants were 10,000 and 3,200 ppb (dry wt basis), respectively. No Bt toxin was detected in Sicot 189 root exudates. We found that with a minor root breakage, ie one broken secondary root out of 15 in the 2-week seedlings, there was a significant increase in detectable Bt toxin (6-fold) to levels of 1,323 ppb (dry wt basis). Using this sterile cell-free method, Bt toxin was therefore shown to leak from both unbroken and damaged root systems.

To investigate the effects of the Envirologix extraction buffer on root cells, which could influence measurements, we conducted additional tests with modifications to the exudate collection, concentration and Bt detection protocols. In these tests we utilised the buffer exchange ability of the Centricon centrifugal filter devices. Exudates were initially collected in Hoagland's solution (the nutrient source to plants, but not compatible with the ELISA test kit) and in the process of exudate concentration the buffer was changed to Envirologix extraction buffer (ELISA compatible, but with an unknown effect on roots). As shown in Table 6(b) there was a significant decrease in Bt toxin levels detected in the

exudates from 289i and 289RRi compared to the previous experiment but nonetheless there were clearly measurable levels of Bt toxin present in the sterile, cell-free exudates of the unbroken roots of both 289i and 289RRi and not in the exudates of Sicot 189 roots.

Thus we observed evidence for the exudation of Bt protein from undamaged roots in all these experiments. The actual concentrations of exudates measured depend upon the growth conditions, exudate matrix and the sterility of the system. Even though we used a sterile system (solutions and containers) to set up these experiments we might not have avoided the growth of endophytes carried through the seed which have the potential to degrade the Bt toxin. Our analysis of the growth solution in the experiment with Hoagland's exudates buffer indicated the presence of bacteria suggesting that the degradation of released proteins in the solution was a possibility. We may therefore be underestimating the absolute concentrations of Bt toxin in our different experiments.

We also note, however, that results from the experiments using Enviroligix buffer for exudate extraction, could be overestimates because the Enviroligix buffer contains a detergent. This may cause some cell membrane disruption resulting in artificially increased levels of Bt toxin being released from roots. Nonetheless, experiments using the 1/10 Hoagland's as exudates extraction buffer (Table 6) confirm that release of Bt toxin occurs even from undamaged roots, although at a lower level.

Various types of damage are quite common in naturally-growing roots in soils. As roots try to grow through the different sized pores, cracks and crevices in field soils, and due to the pressures exerted by shrinking and swelling during drying/wetting cycles, damage to cotton roots is likely to happen, and perhaps even more so than in test systems. The data shown in Table 6(a) relating to broken roots showed a 6-fold increase in Bt toxin levels from a single secondary root break. The significance of this observation to the field situation is conjectural. However, in the cotton growing soils of Australia, it is common to find cotton root breakage due to the seasonal swelling and drying of the high clay content soils.

Previous experimental work described by Saxena and Stotzky (2000) detected Cry1Ab toxin in root exudates from Bt corn. They used sterile solution-grown seedlings and seedlings grown in soil, and a 'dipstick' version of the Enviroligix ELISA kit (Quickstix) to detect Bt toxin, ie a qualitative test only. In our work, we were attempting to gain more valuable quantitative data on root exudates in order to assess the levels of Bt toxin that could enter the soil ecosystem from the Bt cotton plant root system.

**Table 6.** Bt toxin levels from exudates of solution-grown cotton plants (sterile conditions).

Variety	Exudation buffer	Bt toxin - No root breakage	Bt toxin - root breakage
<i>(a)</i> <sup>1</sup>			
<i>Conventional cotton</i>			
Sicot 189	½ x EB	0	- <sup>2</sup>
<i>GM cotton</i>			
Sicot 289i	½ x EB	218-375 ppb/g	1323 ppb/g
<i>GM cotton</i>			
Sicot 289RRi	½ x EB	267-413 ppb/g	-
<i>(b)</i> <sup>3</sup>			
<i>Conventional cotton</i>			
Sicot 189	1/10 Hoagland's	0	-
<i>GM cotton</i>			
Sicot 289i	1/10 Hoagland's	+ <sup>4</sup>	-
<i>GM cotton</i>			
Sicot 289RRi	1/10 Hoagland's	+	-

<sup>1</sup> Range of ppb Cry1Ac per g dry root in 20-hour incubation assay. Data are from 5 seedlings (2 and 8 weeks post germination). Bt toxin levels in week 8 seedling roots ranged from 3,200 to 10,000 ppb (289i and 289RRi respectively).

<sup>2</sup> Test not performed

<sup>3</sup> ppb Cry1Ac per g dry root in 20-hour incubation assay, data are the sum of 5 seedlings (2 weeks post germination)

<sup>4</sup> Bt toxin present but accurate quantitative estimations were not done due to the interference of Hoagland's matrix with the ELISA test kit.

#### *Bt toxin in exudates from plants grown in soil:*

*Bt toxin in exudates from washed roots (non-sterile)* – To determine the level of Bt toxin in root exudates from soil-grown cotton plants, Bt and non-Bt cotton varieties were grown in a sandy textured soil from Waikerie, SA. Plants were harvested and roots washed carefully over a 2 ½ hour period to remove soil. The roots (with plant top still attached) were placed in diluted (1/2 x) Enviroligix extraction buffer and incubated at 8°C for 20 hours. Root exudates were concentrated, and where necessary, buffers exchanged using the Centricon as described previously to obtain a concentrated exudate that was analysed using the Enviroligix ELISA kit.

Results in Table 7 show the Bt toxin to be present at high enough levels for quantification, in exudates from both the 289i and 289RRi Bt cotton varieties (but not from Sicot 189 root exudates). The average levels of Bt toxin in 289i root exudates increased from 63 to 4,343 ppb/g (dry weight of roots), from 2 to 14 weeks post germination. The average levels of Bt toxin in 289RRi root exudates increased from 69 to 4,103 ppb/g from 2 to 14 weeks post germination. The significant increase in Bt toxin levels from 8 to 14 weeks could be attributed either to the greater root mass or to the increased proportion of older and damaged roots. The level of Bt toxin detected in 289i (week 2) washed root exudate was significantly lower than that detected in the 289i (week 2) solution-grown/broken root exudate (63 compared to 1332 ppb/g). This could have been due to the non-sterile nature of the washed root system, resulting in Bt toxin degradation despite the addition of a protease inhibitor. Total protein levels in the exudates were analysed using the SIGMA Protein assay kit (P5656) and were found to range from 7.57 to 11.90mg/g dry root. These protein levels are comparable with those reported for sugar beet and corn root exudates (12.0 and 2.1mg/g dry root) in sterile systems (Guckert *et al.*, 1991).

**Table 7.** Bt toxin levels<sup>3</sup> in exudates from washed cotton plant roots (non-sterile conditions)

Variety	Exudation buffer	Week 2 <sup>1</sup>	Week 4	Week 8	Week 14
<i>Conventional cotton</i>					
Sicot 189	½ x EB <sup>2</sup>	0	0	0	0
<i>GM cotton</i>					
Sicot 289i	½ x EB	63±18 <sup>*</sup>	>123±19	219±45	4,343±266
<i>GM cotton</i>					
Sicot 289RRi	½ x EB	69±18	>122±18	370±63	4,103±215

<sup>1</sup> Weeks post germination

<sup>2</sup> ½ x strength of Envirologix Bt toxin ELISA kit extraction buffer (EB)

<sup>3</sup> ppb of Cry1Ac per gram dry weight of root. Data are the average of triplicates ± standard error of the mean. Total protein levels in the exudates ranged from 7.57 to 11.90 mg/g dry root.

<sup>\*</sup> Data presented are underestimates due to the dilution constraints associated with the ELISA test

Our initial efforts with other methods of root washing, eg longer periods of more gentle root washing and using Hoagland's solution as exudation buffer, resulted in the presence of fine organic material attached to the roots which interfered with the detection limits of the ELISA test kit.

Results from all the experiments described above clearly indicate that Bt cotton roots not only contain significant concentrations of Bt toxin but also release Bt toxin through either (probably) passive release and/or cell autolysis. Passive release, whereby substances leak from the root without metabolic energy, and the lysates released when cells autolyse (die) are two ways that substances can be non-specifically transferred from the plant cell to the root exudate (Whipps, 1990).

Also shown was the potential maximum level of Bt toxin to be expected in root exudates with root breakage. The indicated maximum level of Bt toxin in Bt cotton root exudates was approximately 4,300 ppb/g dry root matter (in a 20 hour test assay), although this level may be an overestimate (see above) Bt cotton. The likelihood of root breakage in the cotton fields of Australia is high due to the nature of the clay soils and the constant drying (shrinking) and wetting (swelling) of the soils surrounding the cotton plant roots. The fate of this level of Bt toxin in the soil environment is uncertain. Saxena and Stotzky (2001) suggested that the Bt toxin from the Bt corn root exudates was only briefly available for biodegradation as it was most likely rapidly bound to soil clays, minerals and humic acids allowing it to accumulate in the environment and remain actively insecticidal. Such a build up of Bt toxin in the soil, if it occurred in the field, could potentially influence soil microflora and fauna biodiversity. Whether such a binding and persistence of Bt toxin from Bt cotton plants occurs in soils of Australian cotton fields has yet to be investigated.

### *1.5 Bt toxin in sloughed root epidermal cells and fine-root hairs*

Root hairs and sloughed epidermal cells contribute a significant amount of root material to the rhizosphere of actively growing plants. We therefore measured the levels of Bt toxin in sloughed-off root epidermal cells and broken fine-root hairs of 289i and 289RRi cotton plants.

Samples of this type of root material were collected from root washings prior to using the plant-root assemblage in root exudate experiments, by filtering the exudate solution through a 0.2µm filter and collecting the cells in the filter residue. After removing all fine roots and larger roots, epidermal cells and fine-root hairs, were processed using similar methods for other plant tissues. Results are shown in Table 8. Sloughed epidermal cells and fine-root hairs from 289i plants showed Bt toxin levels of 1317 ppb/g wet weight whereas Sicot 189 cells/fine-root hairs showed no detectable Bt toxin. We assume that the fine-root hairs with their faster growth rates would be contributing to a large portion of this Bt toxin (active growth=active Bt toxin expression). The significance of this Bt toxin source (root turnover) in terms of its potential impacts on soil biota and biological functions is currently unknown.

**Table 8.** Bt toxin levels<sup>1</sup> in sloughed root epidermal cells and fine-root hairs

Variety	Bt toxin
<i>Conventional cotton</i>	
Sicot 189	0
<i>GM cotton</i>	
Sicot 289i	1317

<sup>1</sup> ppb/g dry weight of Cry1Ac (duplicates) as determined by ELISA

Bt toxin levels in the samples of sloughed cells and fine-root hairs collected from the exudate collection chambers (after the 20-hour incubation) were lower than for the fresh samples, perhaps because of microbial degradation in the non-sterile exudate collection solution.

#### *1.6 Bt toxin in rhizosphere soil of cotton plants*

Rhizosphere soil contains root exudates, sloughed off root epidermal cells and broken segments of fine-root hairs. Because Bt toxin was detected in the root exudates extracts, and in sloughed cells and fine-root material of 289i and 289RRi cotton plants, we investigated the potential for the persistence of Bt toxin in the rhizosphere soils of these Bt cotton plants.

#### *Methodology development:*

Rhizosphere soil samples (within 2mm of the root surface) were collected from the roots of Sicot 189 and 289i cotton plants (9 weeks post germination) grown in Avon and Waikerie soils under glasshouse conditions. The various methods evaluated to extract Bt toxin from rhizosphere soil included (1) the bead beating method of Bt toxin extraction with differing rhizosphere soil amounts (0.1g, 0.2g, and 0.5g) in 500µl of Envirologix extraction buffer, using glass or steel beads, different beating times (1–5 minutes), with/without a 70°C heat treatment and with/without a final centrifuge step (16,000g for 10 minutes) to remove soil particles, (2) high speed vortexing of soil/buffer mixture for 2 minutes and (3) shaking (40 minutes at ~300rpm), vortexing (2 minutes) and centrifugation (16,000g for 10 minutes). The first two extraction methods gave inconclusive results (below detection limits and false positives) probably due to the interference from soil organic matter and low levels of recovery. However, the third extraction method gave reliable results, using a sub-sample of 0.5g soil added to 500µl Envirologix extraction buffer (EB) or Palm extraction buffer (Palm *et al.*, 1994). Soil supernatants were collected and processed using the Envirologix ELISA kit.

*Bt toxin detection in rhizosphere soil using an ELISA assay:*

Results in Table 9 show that Bt toxin was present in rhizosphere soil extracts from 289i cotton plants grown in both Avon and Waikerie soils and not present in the equivalent extracts from Sicot 189 cotton plants. Use of both extraction buffers (EB and Palm EB) showed the presence of Bt toxin within the detectable range of the ELISA kit. Results indicated that there was no difference between the two extraction buffers used. Additional modifications to the ELISA test are needed for accurate quantification of Bt in the clay-rich Australian cotton soils. Saxena and Stotzky (2000) have also reported the presence of Bt toxin in rhizosphere soil associated with Bt corn but they did not produce a quantitative value for the Bt toxin, suggesting problems with the methodology associated with accurate quantification of Bt toxin in rhizosphere soils. Palm *et al.* (1994) showed Bt toxin added to soil could be detected by the ELISA method, however modifications were required to both the extraction buffer (Palm EB) and detection buffer to suit each soil type and to increase the sensitivity of the ELISA system.

**Table 9.** Bt toxin detection in rhizosphere soil associated with cotton plant roots

Variety	Soil Type	Extraction buffer	Bt toxin (ELISA)	Bt toxin (Bioassay)
<i>Conventional cotton</i>				
Sicot 189	Avon	EB <sup>1</sup>	0	0
Sicot 189	Avon	Palm EB <sup>2</sup>	0	- <sup>3</sup>
Sicot 189	Waikerie	EB	0	0
Sicot 189	Waikerie	Palm EB	0	-
<i>GM cotton</i>				
Sicot 289i	Avon	EB	+ <sup>4</sup>	+
Sicot 289i	Avon	Palm EB	+	-
Sicot 289i	Waikerie	EB	+	+
Sicot 289i	Waikerie	Palm EB	+	-

<sup>1</sup> Envirologix extraction buffer

<sup>2</sup> Extraction buffer according to Palm *et al.* (1994)

<sup>3</sup> Test not performed

<sup>4</sup> Accurate quantitative estimations of Bt toxin were not calculated due to the high levels of soil inhibition in the ELISA tests reducing the sensitivity of the estimations. Samples were analysed in duplicate

*Bt toxin detection using insect bioassays:*

*Assay development* – A *Helicoverpa armigera* neonate bioassay was set up to detect Bt toxin independent of immunological methods in an attempt to overcome the problems of sample matrix and ELISA incompatibility. *Helicoverpa armigera* caterpillars are susceptible to the Cry1Ac (Bt toxin) protein found in the genetically engineered cotton. The most susceptible stage in the life cycle of this pest is the neonate or newly hatched larvae. Eggs were obtained from a single overnight lay from the Entomology Department, CSIRO Plant Industry, Narrabri (NSW). Freshly hatched neonates were placed in 96 wells (1 per well, 32 wells per sample) each containing approximately 150µl of insect media (Appendix 5) overlaid with 20–50µl of the sample. Wells were sealed, holes pricked for ventilation, and the plates incubated at 25°C for 7 days before calculating percentage neonate death.

*Bt toxin detection in rhizosphere soil using insect bioassays* – Results from insect bioassays of rhizosphere samples (prepared as for ELISA), as shown in Table 9, indicated the presence of detectable levels of Bt toxin in the rhizosphere of 289i plants. Conventional (Sicot 189) cotton plants did not show any detectable levels of Bt toxin in the rhizosphere soil. Further improvements to the sensitivity of the bioassay would be helpful for exact quantification and measurement of bioactivity of persistent Bt toxin in soils. These results concur with the ELISA rhizosphere results and show that active Bt toxin was present in the rhizosphere soil associated with 289i cotton plants.

Even though we observed high levels of Bt toxin in the roots, root exudates, and broken-off root material of 289i and 289RRi plant types, suggesting significant levels of Bt toxin entering the rhizosphere system, we measured low levels in the rhizosphere soil of 289i. An explanation could be that the soil was, in some manner, inhibiting the detection of Bt toxin using this ELISA test kit. Results discussed below support this hypothesis.

*Soil inhibition in the ELISA assays:*

To investigate this potential constraint, equivalent amounts (0.02g) of Bt cotton leaves were processed using the bead beater (as previously described), with and without the addition of soil (0.01g), and subsequent Bt toxin levels measured. Results shown in Table 10 indicated that the presence of soil significantly inhibited the detection of Bt toxin using the Envirologix ELISA kit. The Bt toxin levels declined from 1,755 ppb/g dry weight to undetectable levels with the addition of the soil to the 289i leaves. This suggests that the presence of soil material significantly reduces the levels of Bt toxin detected using the Envirologix ELISA kit. Therefore in the previous rhizosphere soil results, where a ‘below quantitative’ level of Bt

toxin was indicated for 289i rhizosphere soil, there were most likely higher levels of Bt toxin present.

A similar inhibitory effect was also seen using Dipel (MVPII bioinsecticide – a liquid biological insecticide containing crystals of *B. thuringiensis* var. *kurstaki* toxins encapsulated in dead *Pseudomonas fluorescens* cells for increased efficacy) – and the addition of purified clay (montmorillonite) although to a lesser extent. The clay was found to inhibit the Bt toxin levels detected by at least a factor of five (Table 10), from 25,800 ppb/ml down to 5,100 ppb/ml (Dipel). Such constraints and interference of soil components with the ELISA methods have previously been reported for Bt toxin measurements. Palm *et al.* (1994) reported that by modifying the colour development step it was possible to increase the sensitivity of this ELISA test to measure Bt protein in soil and thereby overcome some of these constraints.

**Table 10.** Soil/Clay inhibition of Bt toxin detection in the ELISA test

Cotton leaf variety	Addition of soil/clay	Bt toxin levels
<i>Conventional cotton</i>		
Sicot 189	-	0
Sicot 189	0.01g soil	0
<i>GM cotton</i>		
Sicot 289i	-	1,755 <sup>1</sup> ppb/g
Sicot 289i	0.01g soil	BD <sup>2</sup>
Dipel <sup>3</sup>	-	25,800 ppb/ml <sup>4</sup>
Dipel	0.01g clay	5,100 ppb/ml

<sup>1</sup> Average Cry1Ac ppb/g (dried leaf) of duplicates, as detected by ELISA

<sup>2</sup> Below detectable levels

<sup>3</sup> Dipel – commercial preparation of crystals from *Bacillus thuringiensis* subsp. *kurstaki* encapsulated in dead *Pseudomonas fluorescens* cells

<sup>4</sup> Average ppb/ml Dipel of duplicates, as detected by ELISA

## 1.7 Significance

All the Bt cotton varieties we tested have been released for cultivation in Australia and were positive for the production of Bt toxin in the above ground plant parts. As expected, no Bt toxin was detected in the non-Bt cotton varieties and in soils used in glasshouse studies and field experiments. Overall, the results above indicate that the Bt cotton varieties (V2i, V15i, 289i, and 289RRi) produce Bt toxin in their roots at levels comparable to those in leaves and significant levels of Bt toxin were also found in stems. The presence in soil of Bt toxin from roots suggests that the exposure of soil biota, particularly microorganisms and fauna in the rhizosphere, is at a higher level and for a longer duration than previously supposed. This contrasts with a previous assumption that soil biota are exposed to large concentrations of Bt toxin only after the defoliation of the cotton plant and after harvest.

Our results also show that the Bt toxin levels in roots generally remained high during the first 8 weeks of plant growth after germination in potted plants and up to at least 16 weeks in field plants. Our observations demonstrating significant concentrations of Bt toxin in fine-root hairs and sloughed-off root fragments indicate that expression of the *Bt* genes in the actively growing parts of cotton root system could also be a significant source of Bt toxin in the rhizosphere. In addition, detailed investigations on the environmental fate of the root derived Bt toxin (ie binding to soil components and build up, and movement beyond rhizosphere and root zone) are warranted.

We also found that Bt toxin is released into the rhizosphere and that the levels of Bt toxin released are significantly increased following any damage to the root system, ie cracking and breakage of fine roots etc. We observed root release of Bt toxin both by cotton plants grown *in vitro* (solution culture) and in soil. Thus our observations indicate that Bt cotton releases the Bt toxin into the soil by some mechanism (passive ‘exudation’?) and/or through root turnover, throughout the growing season. We also observed detectable levels of Bt toxin (both by immunological tests and insect bioassays) in the rhizosphere soils of Bt cotton. No detectable Bt toxin was detected in the rhizosphere soils of non-Bt cotton varieties.

Root biomass accounts for approximately 32% of the total above- and below-ground plant biomass 130 days after germination, and stems account for 30% (Grant Roberts, personal communication). Results from various glasshouse and field experiments indicate that roots account for 25–45% of total plant biomass in cotton. It is also known that root turnover accounts for a significant amount of carbon loss (20% or greater of the carbon fixed by photosynthesis and translocated to roots) below ground. Sloughed-off root cells near root tips and older roots are the major food source of root herbivores such as free-living nematodes, protozoa, mites, collembola and macrofauna. Thus populations of these groups of soil fauna

are generally higher (>10-fold) in the rhizosphere compared to bulk soil. This suggests that the rhizosphere-inhabiting soil biota are continuously exposed to Bt toxin produced in the roots of Bt cotton (through root exudation and/or root turnover). Scanning electron micrographs of decomposing stubble (Appendix 10) indicate extensive colonisation of cotton root surfaces by bacteria and fungi.

In Australian soils, the rhizosphere is one of two key zones where the majority of soil biota reside, the other zone being the soil around decomposing crop residues (Detritussphere). Also populations of different groups of microbiota are generally higher (>10-fold) in rhizosphere soils compared to that in bulk soil and rhizosphere biological activity accounts for >60% of overall soil biological activity. Implications of our results on continuous Bt toxin production and release below ground by Bt cotton varieties and the potential effects on rhizosphere biodiversity and biological functions are yet to be fully investigated.

In his review, Stotzky (2000) summarised his research on the persistence and biological activity of insecticidal proteins from *Bacillus thuringiensis* on clays and humic acids. He reports that Bt toxins and DNA from bacteria bind rapidly and tightly to clays, reducing their susceptibility to microbial degradation but retaining their biological activity. The highly reactive smectitic clays, which form the major type of clays in Australian cotton soils, could potentially adsorb the Bt toxin from freshly sloughed-off root cells, root exudates and cell lysates and protect it from microbial degradation. A reliable quantitative measurement of Bt toxin levels in the rhizosphere soil is nevertheless necessary in order to evaluate the persistence of Bt toxin in Australian cotton soils.

## 2. Long term persistence of Bt toxin in decomposing cotton plant residues and soils

### 2.1 Field experimental methodology

#### *Field sites*

The field site with an appropriate previous Bt cotton history was selected for soil sampling. The site was a previous year's experiment on the 'Leitch lease area' adjacent to ACRI (Australian Cotton Research Institute research farm, Narrabri, NSW). The experiments and samples were set up or taken from experimental plots set up by Dr. Greg Constable (CSIRO Plant Industry) to study the 'Management of single and two-gene Bt cotton varieties' (Genetic Manipulation Advisory Committee approval number PR 36X).

In the 'large-scale' replicated trial each plot was 50m wide and 500m long. GPS coordinates for different plots are as follows:

30 11' 31 S    149 35' 42 E,    30 11' 17 S    149 35' 15 E,  
30 10' 46 S    149 35' 51 E,    30 10' 40 S    149 35' 40 E.

We conducted this work in this large-scale replicated trial area, as it has been difficult until now to identify farmer paddocks with full and detailed records of previous Bt cotton use.

The area for the *in situ* residue decomposition experiments (litterbags) was situated in the South-East corner of the ACRI field plots described previously and has GPS coordinates of 30 12.050' S    149 36.395' E. The soil type is self mulching Vertisol. The paddock history is: summer 98/99-fallow; winter 99-fallow; summer 99/2001-cotton (non-GM); winter 2000-wheat; summer 2000/01-fallow; inter 2001-fallow; and summer 2001/02-cotton (non-GM).

#### *Crop residue collection*

Crop residues used in the residue decomposition and Bt toxin persistence experiments were collected from existing field trials. Leaves and stems were collected from genetically modified cotton varieties and their conventional counterparts, eg single gene Bt cotton (Sicot 289i), RoundupReady Bt cotton (Sicot 289RRi) and conventional cotton (Sicot 189). Various Bt cotton plant parts (leaves, stems etc.) were chosen for study, as *Bt* expression is known to be different in different parts of cotton plants.

Sub samples of fresh material (leaves) were snap frozen and stored at -80°C to avoid loss of Bt toxin, whereas stubble samples collected were already dried in the field and were stored at room temperature until use. All leaf material was dried at 40°C for 3 days prior to use in decomposition and Bt toxin persistence experiments. These cotton residue samples

were used in experiments described in sections two (Bt toxin persistence) and three (Bt toxin effects on decomposition).

#### *Cotton residue 'litterbag' experiments (leaf and stubble samples)*

Decomposition studies were set up in the field on 13 September 2001. Litterbags (15cm x 15cm nylon mesh) containing dried cotton stems (10g) or dried cotton leaves (2g) from different cotton varieties were buried at a depth of 2cm in the field on one metre raised beds (Appendix 6, Photos A and B). Cotton leaf litterbags were set up with three treatments (Sicot 189, 289i, 289RRi), using four replicates and five sampling times (0, 2, 4, 6, and 8 weeks), and with two treatments (Sicot 189, and 289i) using four replicates and six sampling times (0, 4, 8, 12, 16 and 20 weeks) (see Appendix 7 for details).

#### *Cotton residue core experiment (leaf samples)*

In addition to the litterbag experiments, we also set up leaf residue experiments using PVC pipe cores to determine the persistence of Bt toxin from cotton leaves in the soil attached to leaves and a few centimeters below. Cotton leaf residues were buried in PVC pipe cores (75mm x 110mm) inserted into soil (100mm depth) in the field. A layer of dried leaves (1g) from the cotton varieties (Sicot 189, 289i, and 289RRi) were placed 15mm from the top of the core and a final 10mm layer of soil placed on top (Appendix 6, Photos C and D). We placed a layer of soil on the top of leaf residue to stop the loss of residue by wind and water erosion and to facilitate a direct contact between leaf residue and soil. These leaf core experiments were set up with the three treatments (Sicot 189, 289i, 289RRi) using four replicates and four sampling times (2, 4, 6, and 8 weeks) (see Appendix 7 for details).

## *2.2 Bt toxin persistence in decomposing cotton leaf residues*

Litterbags containing cotton leaf residues were collected at 0, 2, 4, 6, and 8 weeks post burial. They were transported (cold) to the Adelaide laboratory where leaf sub-samples were taken and stored snap frozen at  $-80^{\circ}\text{C}$  until the Bt toxin analysis using the Envirologix ELISA kit. The methodology previously described for the most efficient Bt toxin recovery was used with a few modifications to take account of sample size (ie 0.05g leaf matter for each analysis). Sub samples were oven-dried ( $60^{\circ}\text{C}$ ) prior to dry weight measurements and dry weights of leaves samples were corrected for ash content as described in section 3.1.

Results in Table 11 show that Bt toxin was detectable in the decomposing leaf residues of both the Bt cotton varieties (289i and 289RRi) but not in the non-Bt cotton leaves. Bt toxin levels in the decomposing leaves from the two-week sampling ranged from 119 ppb/g dry weight (289i) to 247 ppb/g dry weight (289RRi). We found detectable levels of Bt toxin in all the Bt cotton samples through to the eighth week of sampling. We did not find any

detectable Bt toxin in the non-Bt cotton leaves, as expected. Our estimates of Bt toxin levels in the later samplings were only semi-quantitative due to the interference from soil in the ELISA test.

**Table 11.** Bt toxin levels in Bt and conventional cotton leaves from buried litterbags

<b>Cotton variety</b>	<b>Week<sup>1</sup></b>	<b>Week</b>	<b>Week</b>	<b>Week</b>	<b>Week</b>
	<b>0</b>	<b>2</b>	<b>4</b>	<b>6</b>	<b>8</b>
<i>Conventional cotton</i>					
Sicot 189	0	0	0	0	0
<i>GM cotton</i>					
Sicot 289i	1,144 <sup>2</sup>	119	+++ <sup>3</sup>	++	+
<i>GM cotton</i>					
Sicot 289RRi	984	247	+++	++	+

<sup>1</sup> Weeks of field incubation

<sup>2</sup> ppb of Cry1Ac toxin per gram dry leaf (average of triplicates) detected by ELISA

<sup>3</sup> '+' indicates presence of Bt but accurate quantitative estimations of Bt toxin were not performed due to the high levels of soil inhibition in the ELISA tests reducing the sensitivity of the estimations

As indicated before, the presence of soil reduced the level of Bt toxin detected using the ELISA kit and this made accurate quantitative estimations less reliable. Soil inhibition was likely to be a contributing factor in the low detection levels of Bt toxin in the decomposing leaf samples as most samples had a significant amount of soil attached to them that was unable to be removed prior to ELISA analysis. This is supported by the data on ash content given in Appendix 9. This would also suggest that Bt toxin levels shown in Table 11 are most probably underestimates.

The reduction of Bt toxin levels in the decomposing leaves, with time, could be attributed both to the degradation of Bt toxin by soil microbial populations and/or the release and subsequent binding of Bt toxin by soil particles during the decomposition of leaves. Koskella and Stotzky (1997) reported that a number of soil microfloral populations have the ability to degrade Bt toxin, and quickly (within hours). The authors proposed that one of the main reasons for the persistence of Bt toxin in soil is through its binding to soil clays or organic matter making it unavailable for microbial degradation. Our finding of large concentrations of Bt toxin two weeks after field incubation could imply significant soil

binding or slow microbial degradation. An improvement in Bt toxin detection in soil-contaminated samples would provide a better estimate of persistence of Bt toxin in the field, ie quantitative estimations of Bt toxin.

### 2.3 *Bt toxin levels in decomposing cotton stubble residues*

Litterbags containing cotton stubble residues were collected at 0, 4, 8, 12, 16 and 20 weeks post burial. These were transported, sampled and stored as for the leaf litterbags. Bt toxin levels were analysed with the Envirologix ELISA test using the same methodology as for cotton litterbag leaf samples with the exception that only 0.02g of stubble could be used due to pipetting constraints in the ELISA analysis. Sub samples of stubble residues were oven-dried (60°C) prior to dry weight measurements which were corrected for ash content.

Results in Table 12 show that Bt toxin was present in decomposing 289i stubble residues but not in Sicot 189 decomposing stubble residues. Bt toxin level estimations were quantitative, with Bt toxin values of 13 ppb/g during the first four weeks of decomposition. We found detectable levels of Bt toxin in the Bt cotton stubble from later samplings, however the presence of greater amounts of soil attached to the stubble made accurate quantitative estimations difficult. As for the leaf samples, an improvement in Bt toxin detection in soil-contaminated samples would provide a better estimate of persistence of Bt toxin in the field, ie quantitative estimations of Bt toxin in the decomposing cotton stubble. Because the complete decomposition of cotton stubble from one crop season may take three years or more, it is necessary to determine the persistence of Bt toxin from the stubble of Bt cotton varieties.

It was noted that the starting level (week 0) of Bt toxin in 289i stubble residues was significantly higher than the Bt toxin starting levels in the 289i leaf residues. This is because the leaf residues were oven-dried at 40 °C, hence some Bt protein may have degraded. By contrast the stubble residues were naturally dried (at air temperatures).

**Table 12.** Bt toxin levels in *Bt* and conventional cotton stubble from litterbags.

Cotton variety	Week <sup>1</sup>	Week	Week	Week	Week	Week
	0	4	8	12	16	20
<i>Conventional cotton</i>						
Sicot 189	0	0	0	0	0	0
<i>GM cotton</i>						
Sicot 289i	3,465 <sup>2</sup>	13	BD <sup>3</sup>	BD	BD	BD

<sup>1</sup> Weeks of field incubation

<sup>2</sup> ppb of Cry1Ac toxin per gram dry leaf (average of triplicates) detected by ELISA

<sup>3</sup> Below detectable levels in the ELISA test kit. Soil inhibition is the major reason for this low sensitivity

#### 2.4 *Bt toxin persistence in naturally dried, field-grown cotton plant roots*

Naturally dried cotton roots were tested for the presence of Bt toxin twelve months after they were set aside at the end of the cotton season. Sections of secondary root were processed as previously described and Bt toxin levels determined using the ELISA test. Results shown in Table 13 indicate large concentrations of Bt toxin in the dry roots of both 289i and 289RRi (1,199–2,563 ppb/g). These dry roots were stored at room temperature for ~12 months after being pulled from the ground. Bt toxin was not detected in the non-Bt cotton plant roots (Sicot 189). This finding shows the potential for undecomposed Bt cotton roots to provide a significant Bt toxin reservoir in cotton fields after the harvest of Bt cotton.

#### 2.5 *Bt toxin persistence in soils*

Due to the significantly high levels of Bt toxin in cotton plant residues there is a potential for toxin released from degrading plant cells to bind to soil particles and survive microbial degradation. A significant amount of research has been carried out overseas on the binding of Cry toxins to pure clays, soils and humic acids and on persistence (Stotzky, 2000; Palm *et al.*, 1994; and Tapp and Stotzky, 1995). It has been shown that once bound, the Bt toxin appears to be protected from degradation but retains its insecticidal activity, at least over the short term.

Attempts to detect Bt toxin in soils from fields where Bt cotton was grown in previous years (Narrabri, NSW) encountered problems because the current ELISA test is incompatible with soil components such as clay type and organic matter, because it was originally designed for leaf samples. As discussed previously, the presence of soil reduced the sensitivity of the ELISA test and made quantitative estimations difficult. Also, the sensitivity of the current insect bioassay is limited for soil samples. We are attempting to improve the Bt detection methodology for soil samples, eg using the ELISA test, a SDS-PAGE gel system and an insect bioassay. In order to better understand the fate of Bt toxin following its entry into the soil environment, we require a flexible (in terms of type of sample matrix) and quantitative ELISA test for Bt toxin in soils. The aim of our experiments involving Bt cotton leaves in soil cores was to determine Bt toxin persistence after toxin comes into contact with soil. However, due to the problems associated with Bt toxin detection method for soil samples, we have not completed the analyses of soil samples from this experiment.

**Table 13.** Bt toxin levels in naturally dried field-grown cotton plant roots<sup>1</sup>

Variety	Bt toxin levels <sup>2</sup>
<i>Conventional cotton</i>	
Sicot 189	0
<i>GM cotton</i>	
Sicot 289i	1,199 ± 307
<i>GM cotton</i>	
Sicot 289RRi	2,563 ± 187

<sup>1</sup> Levels of Bt toxin in equivalent roots of 16-week-old plants ranged from 2,500 to 8,000 ppb and were higher in fine roots.

<sup>2</sup> ppb/g dry weight of *Cry1Ac* as determined by ELISA. Data is the average of duplicates ± standard error of the mean.

## 2.6 Significance

Different plant parts of Bt cotton (leaves, stubble and roots) have been shown to contain large concentrations of Bt toxin. As these plant residues enter the soil after the harvest for above ground plant parts, decomposition allows the release of Bt toxin into the soil environment. We can assume that Bt toxin from Bt cotton could be degraded by soil microorganisms and/or bound to soil particles (clays, minerals and humic acids).

Our findings showing significant concentrations of Bt toxin (above soil background) in decomposing Bt cotton leaf residues indicate that Bt toxin from dead leaves is not as rapidly degraded by soil microorganisms as might be expected for a protein substance. This suggests that the Bt toxin from the cells of dead leaves may to some extent either not be released for microbial degradation (this is unlikely considering the observed decomposition level) or be bound to soil particles and unavailable for microbial degradation. Unlike stubble residues, dead leaves have lower C:N ratios (ie higher concentrations of nitrogen per unit carbon) and hence are easily degradable. Koskella and Stotzky (1997) proposed that Bt toxins bound to clays potentially resist microbial degradation due to the inaccessibility of amino acid residues and the binding of other substances (ions, humic substances and other organic molecules) onto the clay-toxin complex. In addition, it has been shown that Bt toxins can retain their insecticidal activity whilst bound to clay particles and humic acids (Crecchio and Stotzky, 1998).

A compounding factor with Bt cotton plants is that the Bt toxin gene inserted into the genome has been truncated and encodes an active toxin, not the inactive protoxin form that is produced by the soil bacterium *Bacillus thuringiensis*. Both target and non-target Lepidoptera have been shown to have receptors for this active toxin in their gut (Höfte and Whitely, 1989). If more Bt toxin enters the soil environment than is degraded by microbes, eaten by insect larvae or inactivated by sunlight, the potential exists for the toxin to accumulate if it is bound and protected by soil particles. Accumulation of Bt toxin may constitute a hazard to non-target organisms, impacting the biodiversity and functionality of organisms inhabiting the soil and enhancing the development of insect resistance. Further research on the long-term significance of Bt toxin build up in Australian cotton soils would contribute to a better understanding of the sustainability of Bt cotton varieties and the impacts of Bt cotton agriculture on the environment.

## ***B Assessing the ecological impacts of Bt toxin on one of the key biological processes essential for ecosystem function (cotton residue decomposition)***

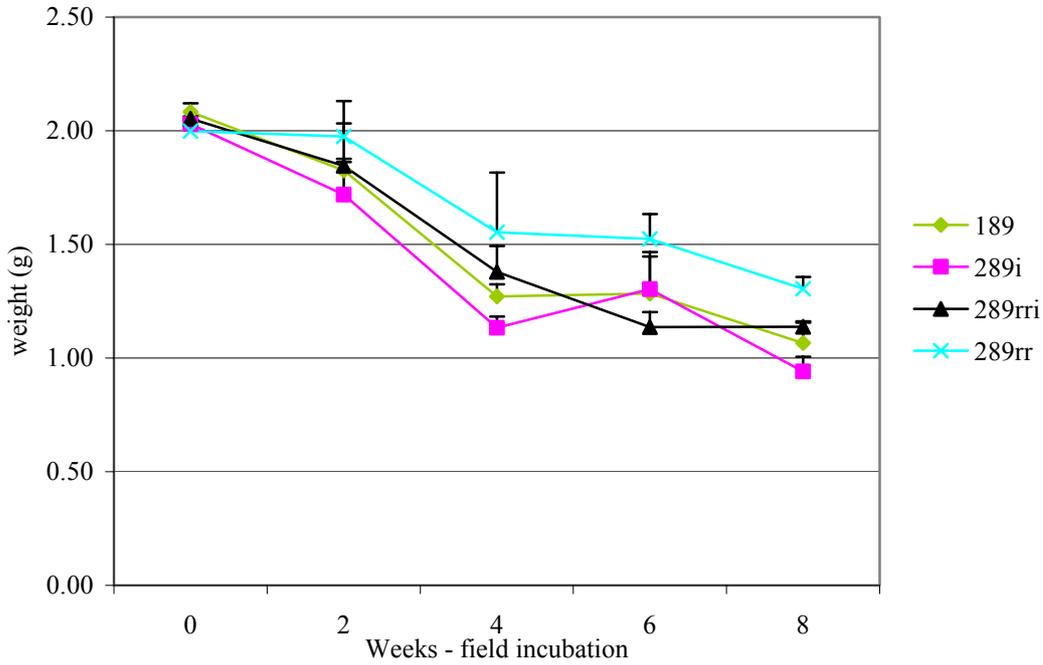
We assessed the impact of Bt toxin on essential biological processes in the soil by investigating decomposition rates, carbon and nitrogen contents, and biological activity associated with decomposing cotton residues.

### **3. Ecological impacts**

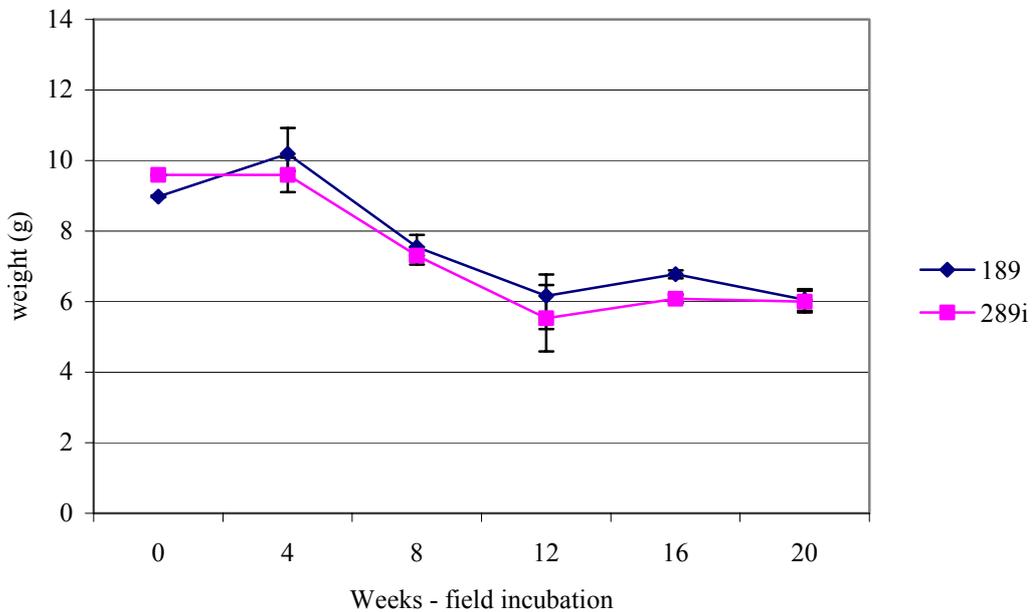
#### *3.1 Decomposition of Bt cotton and non-Bt cotton crop residues*

The decomposition rates associated with Bt and non-Bt cotton residues (leaf and stubble) were determined using the litterbag samples, described previously for the Bt toxin persistence experiments (section 2.1). Cotton varieties Sicot 189, 289i and 289RRi were used in the decomposition experiments with leaf residues, and Sicot 189 and 289i were used in the decomposition experiments with stubble residues. At each sampling, sub-samples of stubble or leaf samples were oven dried (60°C) prior to dry weight measurements. Dried leaf and stubble samples were later ground (<50 µm) and analysed for carbon (C) and nitrogen (N) concentrations as well as ash content. Percentage total C and total N was determined using a CNS 2000 Analyser according to Matejovic (1996) and ash content using a muffle furnace at 600°C for 2 hours according to AOAC Standards for Animal Feed (4.1.10). Results of the crop residue weight loss during field incubation are shown in Figures 2 & 3. All the data for crop residue dry weights and C and N concentrations were corrected for ash content as previously described.

Results for C and N concentrations indicate that non-GM (Sicot 189) leaf material had a higher concentration of nitrogen (3.48%) compared to GM-cotton (Bt, RR and RRi) varieties (2.5–2.8%) resulting in wider C:N ratio for the GM-cotton varieties including Bt cotton. Data on the weight loss shown in Figure 2 indicate that there was only a small difference in the rate of decomposition between leaf residues of Bt and non-Bt cotton varieties during the 8-week incubation experiment. The lowest rate of decomposition of leaves was observed with Roundup tolerant GM-cotton (289RR). This difference in weight loss could be attributed to either the presence of herbicide on the Roundup Ready-cotton and/or the wide C:N ratio of the Roundup Ready-cotton leaf residues. However, results in Figure 4 indicate little difference in terms of the leaf carbon remaining in litterbags for different cotton varieties.



**Figure 2.** Decomposition of cotton leaves in litterbags (expressed as grams dry weight of leaves per bag) buried in a field experiment at Narrabri during 2001.



**Figure 3.** Decomposition of cotton stubble in litterbags (expressed as grams dry weight of stubble per bag) buried in a field experiment at Narrabri during 2001.

Field moist sub-samples of stubble and leaf samples were used to determine the level of microbial activity, substrate-induced respiration and respiratory quotients using a short-term (6 h) laboratory incubation assay (Gupta VVSR, unpublished). We found that the level of microbial populations associated with the non-GM cotton leaf residues was different to that with the Bt cotton and other GM cotton leaf residues, especially during the first four weeks of field incubation (Figure 5). Substrate-induced respiration (SIR), a measurement of the level of microbial populations, was higher for non-Roundup Ready cotton leaf residues compared to all the other Roundup Ready-cotton leaves, after two weeks. RoundupReady cotton leaves exhibited the lowest SIR values (Figure 5).

In addition to the lower SIR values GM-leaf residues also sometimes gave lower respiratory quotient values (RQ, rate of soil respiration in terms of microbial biomass and also known as specific respiration rate) (Table 14). Anderson and Domsch (1990) reported that the RQ of soil organisms could be used to investigate changes in microbial health as a result of soil development, substrate quality, ecosystem development and response to stress. In our laboratory we have found that RQ is a valuable indicator of microbial stress due to pesticide application in soils and is influenced by respirable carbon substrate quality. In addition, it is known that RQ is influenced by the composition of microorganisms and microbial-microfaunal interactions (Frey *et al.*, 2001).

The significant levels of Bt toxin found in Ingard cotton varieties and the differences in C:N ratios of different leaf residues indicate potential Bt toxin effects on both substrate quality and microflora-fauna interactions; in turn, all these could impact on RQ for GM-cotton leaves. By week 8 the RQ values for non-GM cotton leaves were lower compared to that of all the GM-cotton leaves, suggesting significant differences in the microbial successional changes between GM and non-GM cotton leaf residues.

**Table 14.** Respiratory quotient for microorganisms associated with decomposing cotton leaves incubated in litterbags at Narrabri during 2001.

<i>Cotton variety</i>	<i>Weeks Incubation</i>			
	2	4	6	8
Sicot 189	1.70	0.25	0.41	0.65
Sicot 289i	0.86	0.33	0.48	0.91
Sicot 289RRi	0.75	0.26	0.39	0.85
Sicot 289RR	1.22	0.28	0.46	2.11

Note: Respiratory quotient = microbial activity expressed per unit microbial biomass

Results of the weight loss for stubble residues are given in Figure 3. Unlike the leaf residues, the C:N ratio for non-Bt cotton stubble was wider (110.7 for Sicot 189) compared to that for Bt cotton stubble (71.5 for Sicot 289i). We found no dramatic differences in weight loss of stubble between Bt and non-Bt cotton varieties. These results are different to those observed in previous years where we found a 12–16% reduction in the stubble decomposition for Bt cotton compared to the non-Bt cotton stubble in a 12-week experiment.

It is well established that lack of N is one of the reasons for the slower rate of decomposition of stubble compared to the N rich leaf residues. Our results show that rate of decomposition was faster for leaf residues compared to their corresponding stubble samples. Even though the Bt cotton stubble contained higher levels of N (and lower C:N ratios) its rate of decomposition is similar to that of the non-Bt stubble with a wider C:N ratio suggesting differences in biota composition and/or activity levels. Our observations from previous years showed that when the C:N ratios of stubble were similar, the decomposition of Bt cotton stubble was lower than the non-Bt stubble.

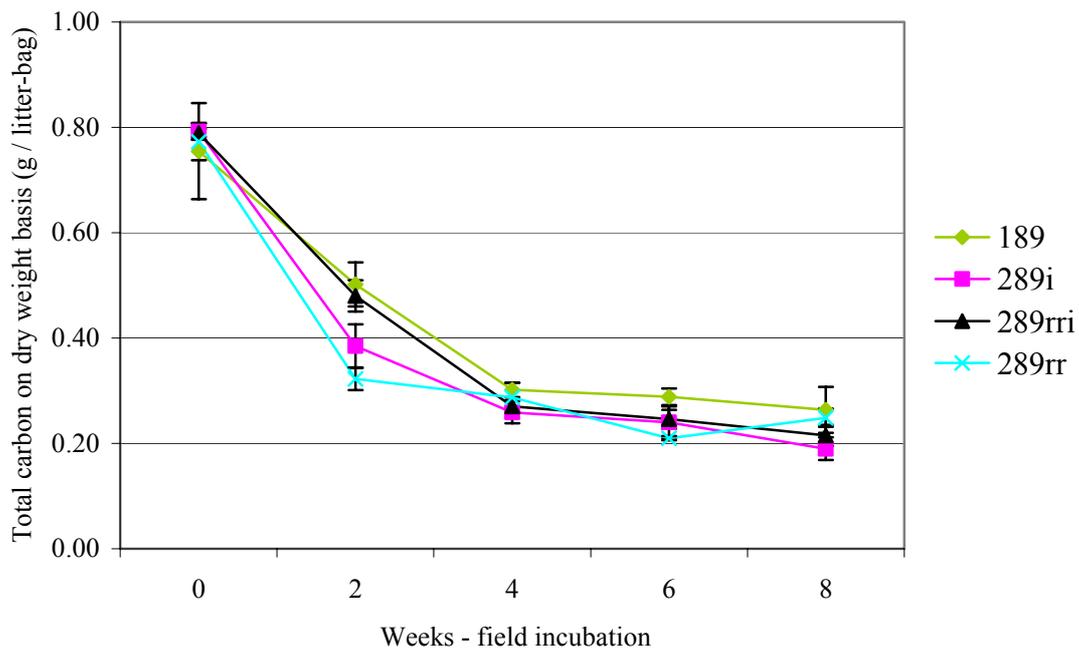
Results shown in Table 15 indicate differences in both the SIR and RQ values between non-Bt cotton (Sicot 189) and Bt cotton (289i) stubble. Seasonal dynamics of different groups of microbiota and biological functions, such as crop residue decomposition, are influenced by resource quality and environmental conditions. Therefore it is necessary to investigate ecological impacts on biota composition and biological functions in more than one season. Results presented in this report are only from a one-year study and clearly experimentation over several years is needed before firm conclusions can be drawn.

**Table 15.** Microbial activity associated with cotton stubble samples in litterbags incubated in a field experiment at Narrabri during 2001.

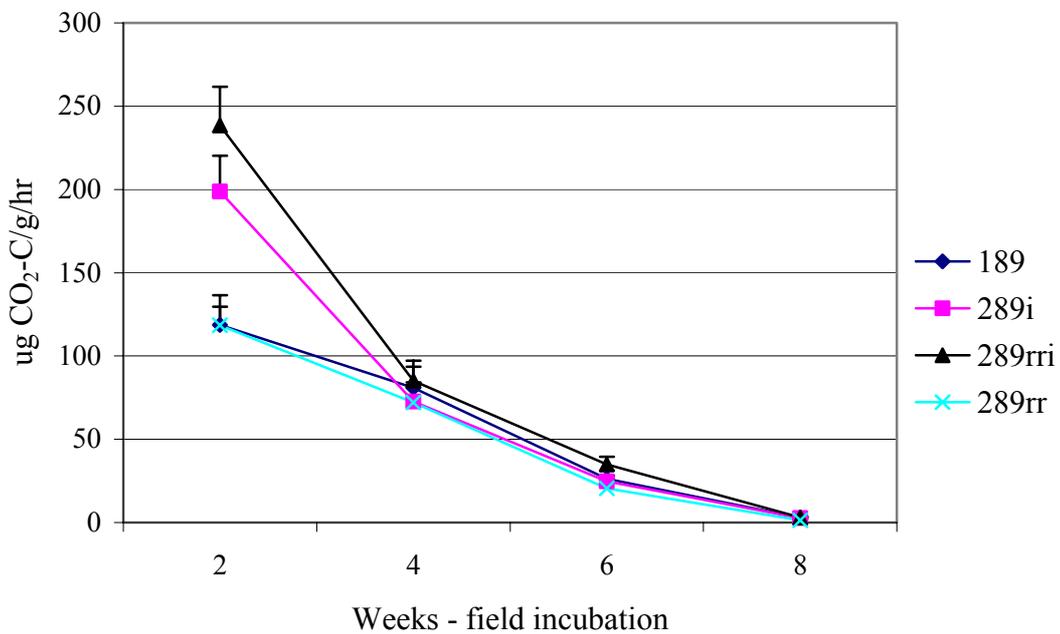
		<i>Cotton variety</i>		
		<i>Weeks incubation</i>		
		4	8	20
<i>Substrate induced Respiration</i> <sup>1</sup>	Sicot 189	20.85 ± 2.37	11.39 ± 2.82	7.60 ± 21.92
	Sicot 289i	18.10 ± 4.97	16.41 ± 4.48	16.90 ± 28.19
<i>Respiration quotient</i> <sup>2</sup>	Sicot 189	2.32	2.80	16.04
	Sicot 289i	3.59	1.97	6.91

<sup>1</sup> measured in units of µg CO<sub>2</sub>-C/g/hr

<sup>2</sup> Respiratory quotient = microbial activity expressed per unit microbial biomass



**Figure 4.** Total carbon levels in litterbags with decomposing leaves buried in a field experiment at Narrabri during 2001



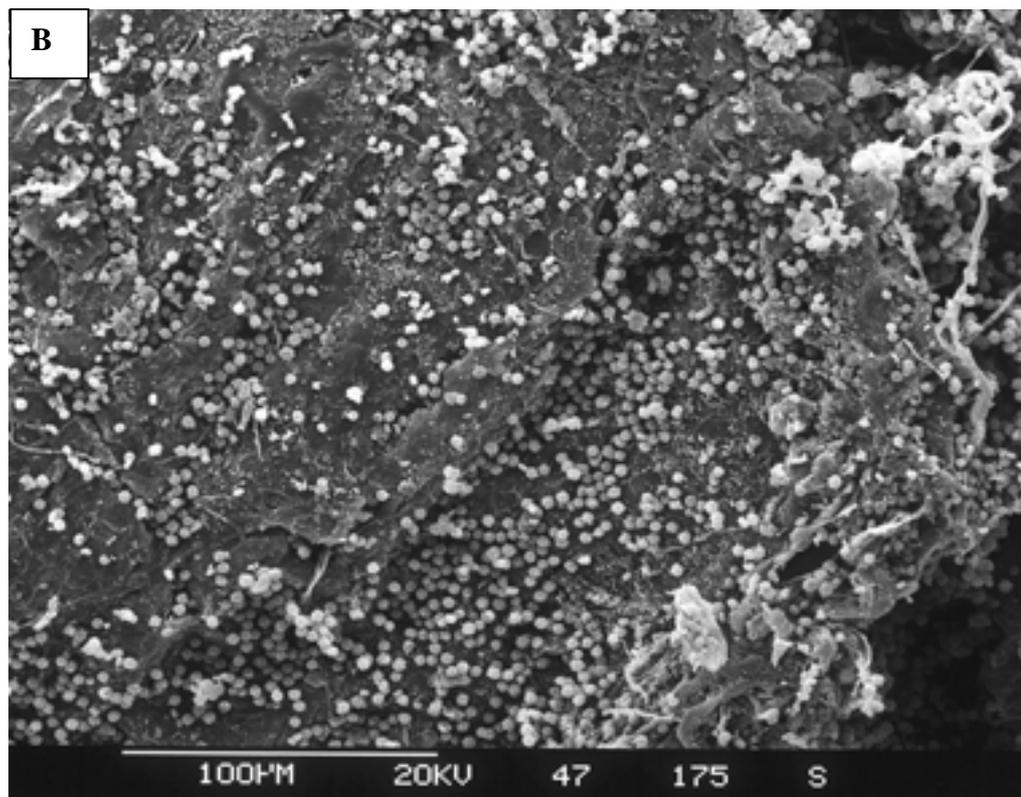
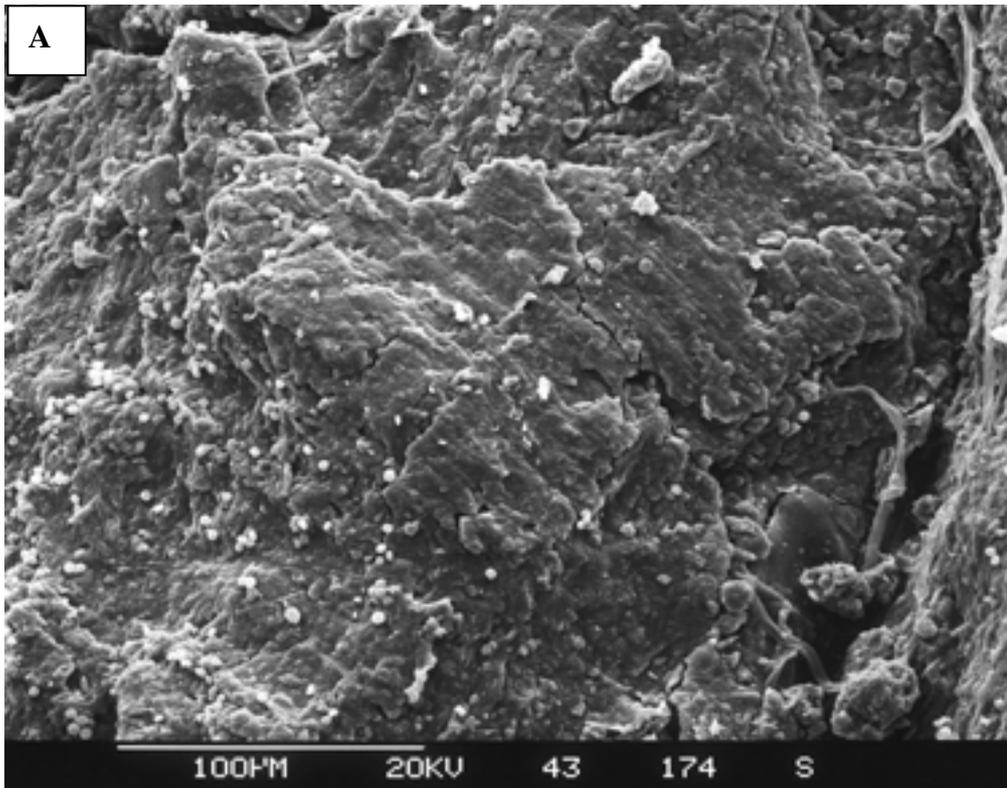
**Figure 5.** Substrate-induced respiration associated with decomposing cotton leaves incubated in litterbags in a field experiment at Narrabri during 2001.

### *3.2 Microbial composition associated with Bt cotton and non-Bt cotton crop residues*

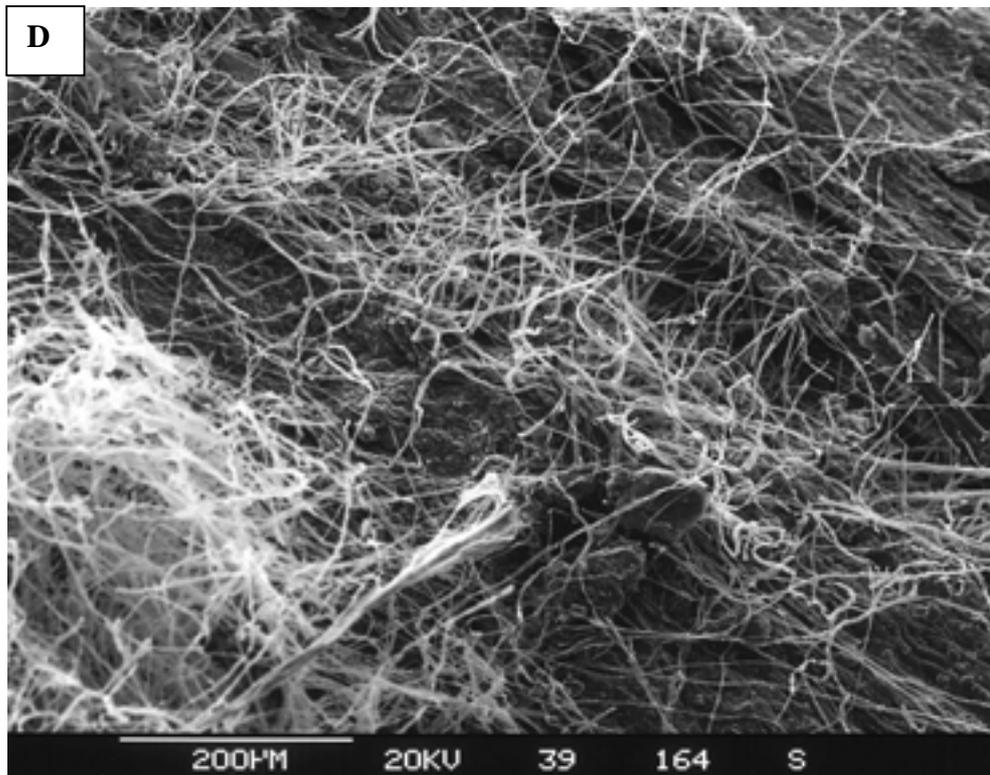
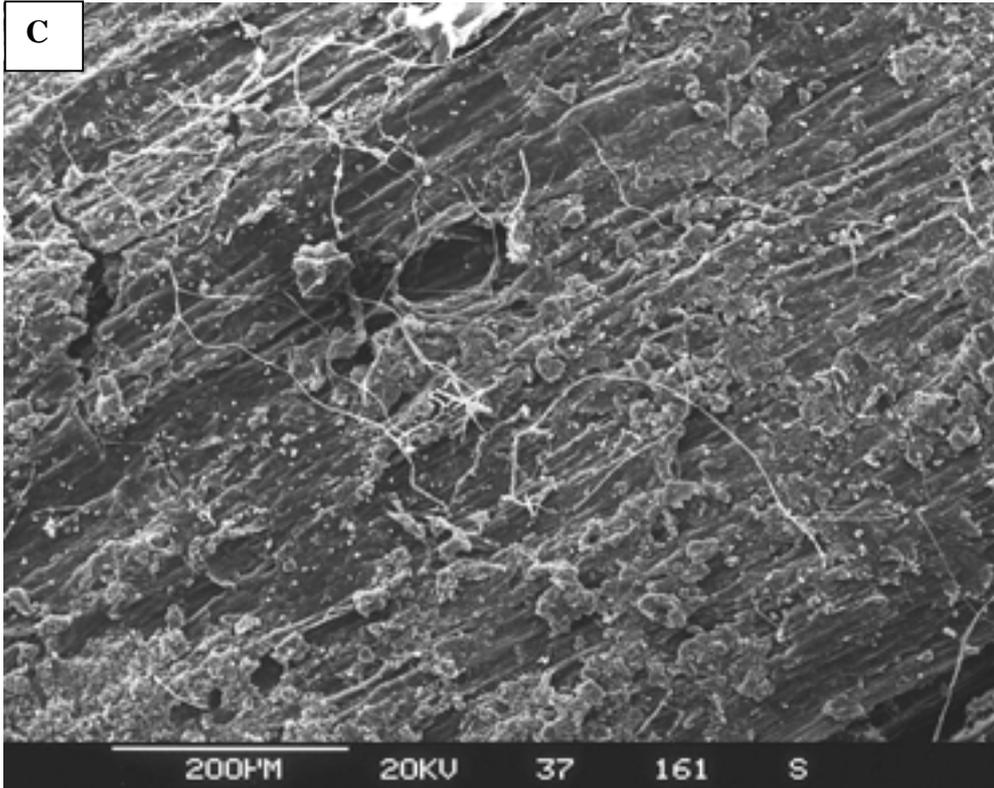
Our initial brightfield microscopic observations of stubble samples indicated that the microflora (eg bacteria, fungi, actinomycetes etc.) associated with the Bt cotton stubble were significantly different to those observed on non-GM cotton. Therefore we conducted detailed scanning electron microscopy (SEM) observations using representative sub-samples of both the Bt and non-Bt cotton stubble from litterbags (eg Photos 1 and 2). Extensive colonisation of Bt cotton residues by fungi (spores and hyphae) was observed compared to the less than prolific fungal colonisation of non-Bt cotton stubble (Photos 1A & B vs. 1C & D). In addition, the types of fungi colonised by the two types of stubble were found to be very different (Photos 2A–C). Our initial measurements (previous years' experimentation) using a selective inhibition-SIR technique showed higher ratios of 'fungi:bacteria' for Bt cotton stubble compared to the non-Bt cotton stubble. Our microscopic observations, both brightfield and SEM, indicated different spore types (colour and type) associated with the Bt and non-Bt cotton stubble (Photos 2 A–C). At present we do not know the species composition of these different microflora and the significance of the difference.

### *3.3 Significance*

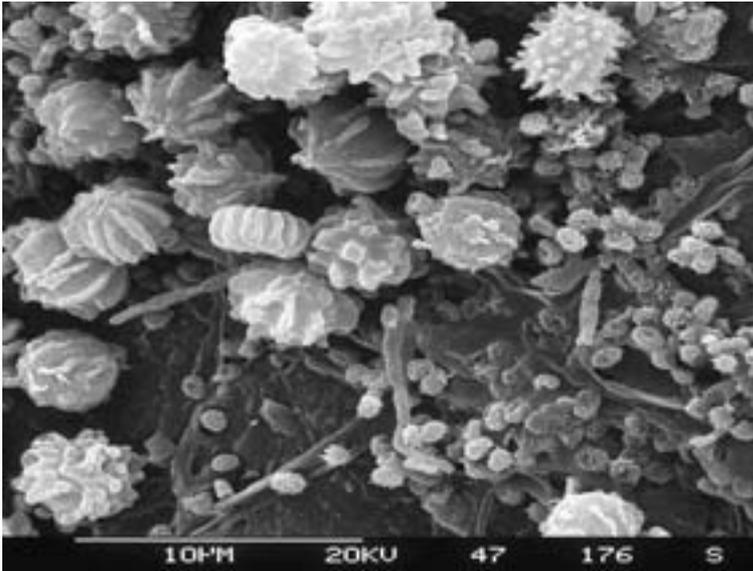
Crop residues are one of the two major sources of carbon for soil biota populations in Australian soils, hence the composition of associated biota has great significance in regulating essential biological functions in the ecosystem. Soil fungi associated with decomposing crop residues can be either non-pathogenic species or species that cause soil-borne plant diseases. Because our observations strongly indicate the proliferation of fungi near decomposing Bt cotton residues, investigation of the details of changes in microbial diversity associated with decomposing Bt cotton residues, in particular over more than one season, is needed to understand potential impacts on the impacts and sustainability of different varieties in cotton farming systems.



**Photos 1A-D:** Scanning electron micrographs of conventional or non-Bt (A and C) and Bt cotton stubble residues (B and D) retrieved from field incubation after 4 weeks (A and B) and 12 weeks (C and B) at Narrabri, NSW (Gupta, McClure and Roberts, unpublished).

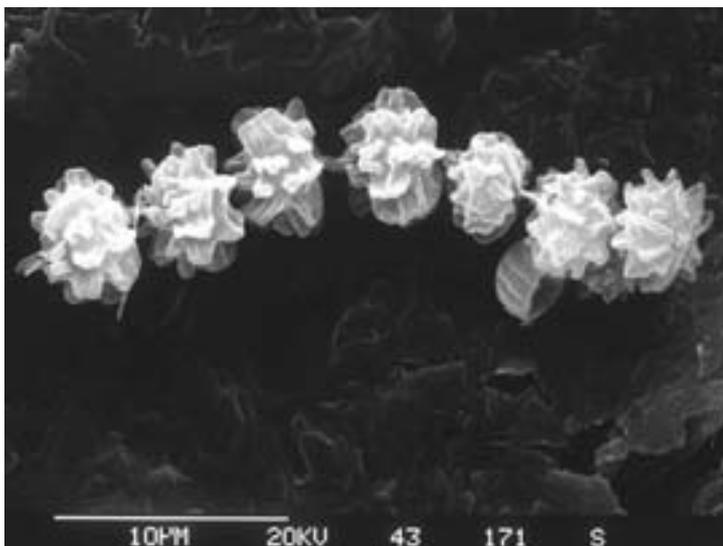
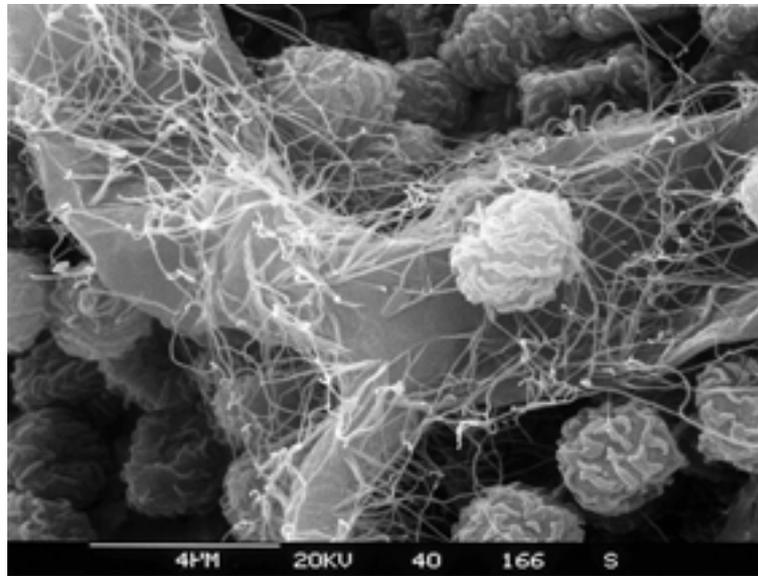


**Photo 1 continued....**



**Photo 2A.** Close-up SEM pictures of Bt cotton stubble showing a diverse range of fungal spores.

**Photo 2B.** Close-up SEM picture of Bt cotton stubble showing fungal and actinomycete hyphae and spores.



**Photo 2C.** Close-up SEM picture of non-Bt cotton stubble showing fungal spores.

#### **4. Conclusions**

1. Bt toxin has been detected in leaves, stems and roots of a number of Bt cotton varieties grown in the field and under glasshouse conditions during the first 16 weeks of the cotton growth season. Bt toxin was also found in root exudates, in sloughed root epidermal cells and fine-root hairs, and in rhizosphere soils.
2. Detectable levels of Bt toxin were found in dried and decomposing leaves and stubble up to or more than 4 weeks after incubation in the field.
3. Significant differences were observed in the composition of biota associated with the crop residues of the Bt cotton and non-Bt cotton varieties.

Estimates of the environmental exposure to Bt toxin from GM crops in Australia have, until this work was done, usually been based on the assumption of only end-of-season exposure to Bt toxin from the whole plant, not of exposure to Bt toxin from the plant throughout the growing season. However, our results suggest further investigations into the environmental exposure and fate of root-released Bt toxin in soil, both during the cotton season and following the harvest of cotton crop, and on the short- and long-term impacts of Bt toxins on soil ecosystems, are warranted, at least to confirm predictions of environmental fate in risk assessments. Technical improvements of methods to quantify Bt toxin levels in soil would be needed for such work.

#### **5. Recommendations for future research**

1. *Improvement of methods to quantify Bt toxin in soil samples*

As indicated above, we used different types of detection techniques (ie ELISA and insect bioassay methods) to determine the presence of, or quantify, Bt toxin in different plant parts, root exudates and soil samples. Even though our ELISA methods were adequate for quantitative estimations of Bt toxin in plant parts and root exudates, they require improvements for a similar sensitivity for soil samples and crop residues with soil because soil appears to interfere with detection.

The literature shows that the *Helicoverpa* bioassay is used regularly as a definitive test for the presence of Bt toxin. We have successfully carried out the insect bioassay using Cry1Ac standards and rhizosphere soil. However, our insect bioassay methods at present are adequate only for semi-quantitative measurements and would

not be suitable for quantitative estimations, especially in soil and stubble samples with lower levels of Bt toxin. Similarly, further modifications are required to the ELISA assay for accurate quantitative estimations of Bt toxin levels in soils with varying Bt cotton history.

Some of the improvements required for the ELISA test system include modifications to the protein extraction and detection buffers suitable for Australian cotton soils in order to overcome the soil inhibition and cross reaction problems. Increasing the sensitivity of both these techniques or the development of additional techniques such as SDS-PAGE gel methods would greatly assist in investigating the long-term persistence and environmental fate of Bt toxin in field situations.

2. *Investigate the long-term persistence of Bt toxin in soils within and outside the cotton root zone*

Because our results indicate more than one avenue for Bt toxin to enter soil both during the growing season and afterwards, it is necessary to investigate the potential for a significant build up of Bt toxin over extended periods (months/years) in Australian cotton soils. In addition, our observations of Bt toxin in decomposing leaf and stubble samples do not clearly indicate that Bt toxin in non-decomposed crop residues would persist in the years following a Bt cotton crop. However, investigations dealing with the fate of Bt toxin in this enriched microsite (ie near roots, in the rhizosphere), and outside the root zone, would assist predictions of the potential ecological impacts of Bt toxin build up.

Australian cotton is mostly grown in clay-rich soils and the clay type in these soils is known to bind proteins under some circumstances, thus making them unavailable for microbial degradation at least over the short term. This could also contribute to the potential for long-term persistence of Bt toxin in these soils.

An empirical approach to investigate the extent of Bt toxin persistence in Australian soils would be to get a better understanding of the nature of potential root release mechanisms, to improve measurements of Bt toxin degradation rates in the field, and to measure Bt toxin levels in soils grown to Bt cotton for varying numbers of seasons in different environments for both Ingard and Bollgard cottons.

3. *Evaluate the non-target effects of Bt cotton on essential functional groups of soil biota and ecosystem functions*

As shown in our SEM photomicrographs and initial measurements of microbial activity, the composition and activity of microbes and other biota associated

with decomposing Bt cotton residues seem to be different to those of non-Bt cotton. Similarly, our work with rhizosphere biota indicates that biota composition and activity associated with Bt cotton plants is significantly different to that of non-Bt cotton plants (Gupta *et al.*, 1998; Gupta *et al.*, 2002). Because crop residues and rhizosphere soil are the two major areas of biota activity in Australian soils, further study of soil biota composition and the ecology of residues of different cotton varieties would help determine if Bt cottons have adverse non-target effects on essential functional groups of soil biota, ecosystem functions, and crop production. The availability of recent molecular and biochemical techniques makes improved determination of the changes in biodiversity in GM-cotton soils possible.

As mentioned previously, the Part 2 phase of this project originally proposed to investigate the environmental impacts of GM-cotton on soil biodiversity and essential ecosystem functions in detail. However this work has not proceeded to date, due to lack of funds.

## **Acknowledgments**

We wish to express our sincere appreciation to our main collaborator Mr. Grant Roberts (Australian Cotton Research Institute, Narrabri) for his efforts with field experimentation and valuable discussions throughout the study. We extend our appreciation to Ms. Clare Fenton-Taylor for her help with technical work in the field, Mr. Colin Tann and Ms. Cheryl Mares (ACRI, Narrabri) for their support with the insect bioassay. We also extend our appreciation to Mr. Bill James (CSIRO Entomology) and Dr. Danny Llewellyn (CSIRO Plant Industry) for their help with the pure toxin and ELISA technique. Dr. Mark Lonsdale (Program Leader of the Component 7, Biodiversity sector, CSIRO) provided invaluable support during both the development and investigation phases of the project.

Funding by Environment Australia (now the Australian Government Department of the Environment and Heritage) for this part of the CSIRO project 'Ecological Impacts of GM Cotton on Soil Biodiversity' is gratefully acknowledged.

## **Publication**

Gupta, V.V.S.R., Roberts, G.N., Neate, S.M., Crisp, P., McClure, S. and Watson, S.K. 2001. Impact of Bt cotton on biological processes in Australian soils. Proceedings of the 4<sup>th</sup> Pacific Rim conference on the biotechnology of *Bt*-Environmental impacts held during October in Canberra, ACT, Australia.

## **Disclaimer**

### **Important Disclaimer**

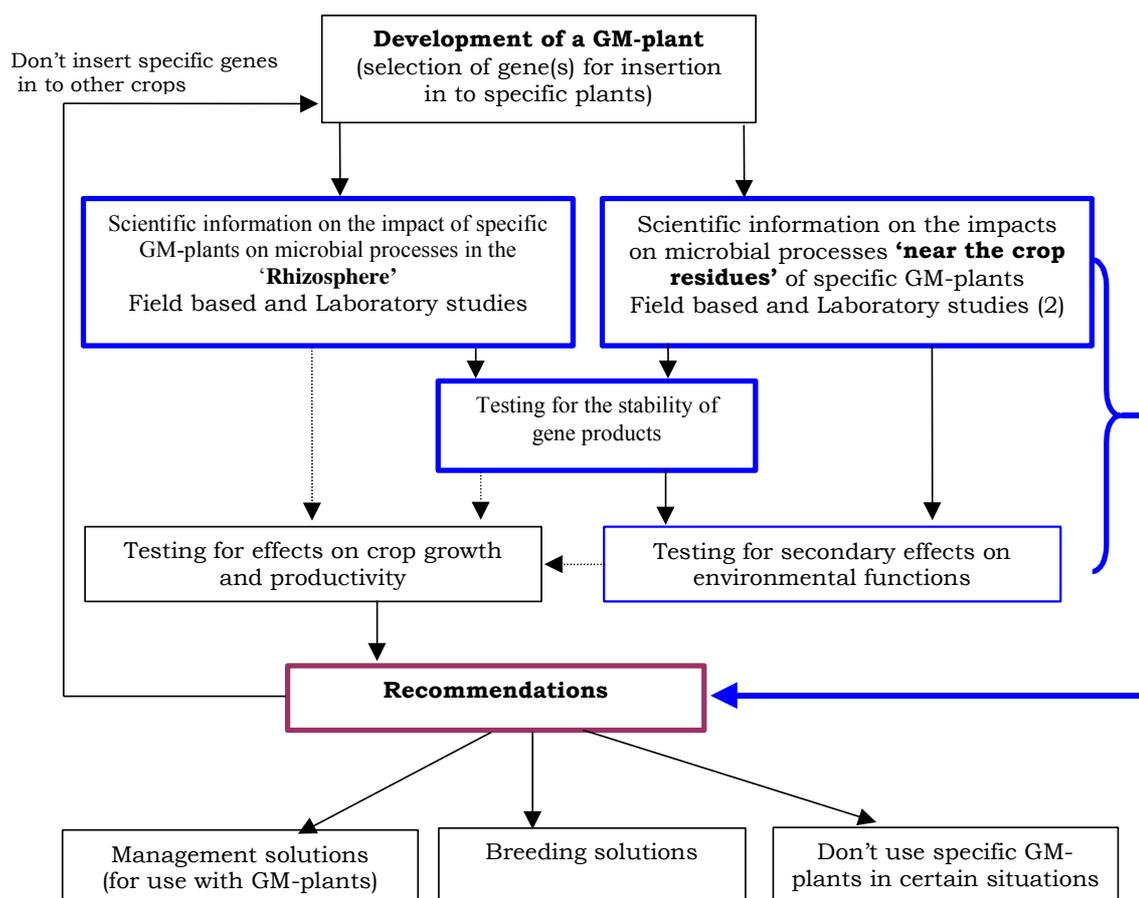
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**Appendix 1.** A flow-chart indicating the role of risk assessment of GM plants for the successful incorporation of GM plants in Australian agriculture with minimal impact on essential ecosystem functions (Gupta, Neate and Roberts, 1999. Research proposal submitted to the Land and Water Australia).



**Appendix 2.** Physical and chemical characteristics of soils used in glasshouse and field experiments (Avon, Waikerie and Narrabri).

<b>Soil characteristics</b>	<b>Avon, SA</b>	<b>Waikerie, SA</b>	<b>Narrabri, NSW</b>
Soil Texture	Sandy loam	Sandy	Clay
Organic C (%)	1.60	0.68	1.13
pH CaCl <sub>2</sub>	8.00	8.00	6.30
Total N (%)	0.15	0.05	0.10
CEC (cmol/Kg)	15.30	-	20.20
% Clay	13.00	6.20	31.00
EC (ds/m)	0.16	0.51	0.08

**Appendix 3.** The different experimental set-up used to grow GM and non-GM cotton plants in controlled environments conditions (glasshouse and growth chamber).



A.



B.



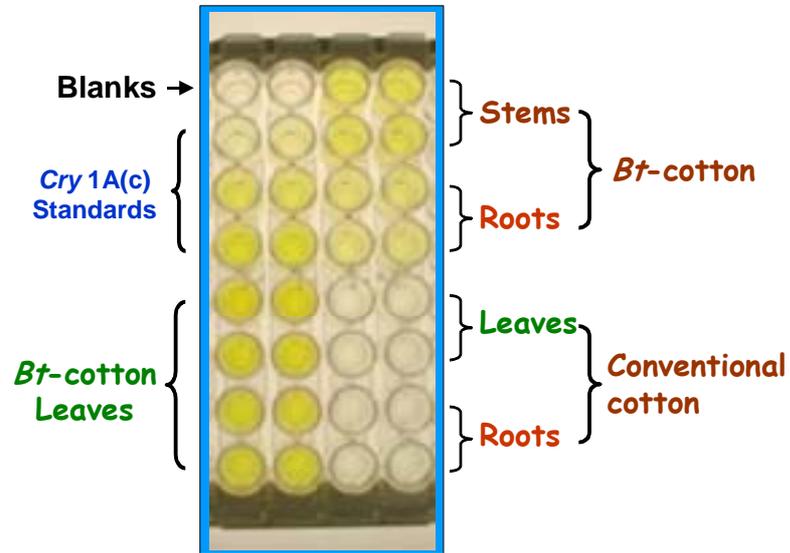
C.



D.

- A. Cotton plants growing in 2.7kg (soil) PVC pots
- B. Cotton plants growing in 1kg (soil) pots
- C. Cotton seedlings growing in sterile Hoagland's solution
- D. Cotton plants harvested prior to root washing

**Appendix 4.** Enviroligix ELISA detection methodology. Bt toxin Cry1Ac levels\* in different parts of Bt cotton plants



\* The Enviroligix ELISA plate shows varying intensity of colour reaction according to the presence of Cry1Ac protein. Higher amounts of Bt toxin are indicated by a higher intensity in colour.

**Appendix 5.** *Heliothis armigera* insect bioassay feeding diet (C. Tann, unpublished – ACRI, Narrabri).

*Ingredients:*

Part A.	Soya flour	65g
	Wheat germ	30g
	Dried yeast	26.5g
	Nipagen	1.65g
	Sorbic acid	0.85g
	Sunflower oil	1 tsp
	Formaldehyde	6.75ml
	Hot water (distilled)	600ml
Part B.	Davis agar	8g
	Water (distilled)	175ml
Part. C.	Ascorbic acid	2.65g

*Method:*

Blend ingredients of Part A well in a food blender for approximately 2 minutes. Microwave Part B for 4 minutes on HIGH and add to Part A. Blend a further 2 minutes. Place in a pre-warmed Pyrex dish in a water bath held at 63°C. When the temperature has dropped to 63°C, add the ascorbic acid and mix well. Pipette into 96-well multiplates and allow to cool. This recipe is enough to do at least 20 plates. Dry the plates for 2 hours in a flow cabinet and store at 4°C (up to 7 days).

**Appendix 6.** Field trial photos of the field-trial set-up at ACRI, Narrabri (NSW) on 13<sup>th</sup> September 2001.



A.



B.



C.



D.

A. Burial of litterbags (one replicate) in 1 metre raised beds in the field at Narrabri

B. Replicates of stubble and leaf litterbags in field trial

C. Leaf core preparation – leaves added to the cores and covered with 10mm of soil

D. Replicates of leaf cores in field trial

## Appendix 7. Litterbag and core field trial set up at Narrabri, NSW.

### Litterbags - leaves

Treatment	Rep 1	Rep 2	Rep 3	Rep 4	
Sicot 189	1	2	3	4	(week 2 sampling)
	5	6	7	8	(week 4 sampling)
	9	10	11	12	(week 6 sampling)
	13	14	15	16	(week 8 sampling)

Treatment	Rep 1	Rep 2	Rep 3	Rep 4	
Sicot 289i	1	2	3	4	(week 2 sampling)
	5	6	7	8	(week 4 sampling)
	9	10	11	12	(week 6 sampling)
	13	14	15	16	(week 8 sampling)

Treatment	Rep 1	Rep 2	Rep 3	Rep 4	
Sicot 289RRi	1	2	3	4	(week 2 sampling)
	5	6	7	8	(week 4 sampling)
	9	10	11	12	(week 6 sampling)
	13	14	15	16	(week 8 sampling)

Treatment	Rep 1	Rep 2	Rep 3	Rep 4	
Sicot 289RR	1	2	3	4	(week 2 sampling)
	5	6	7	8	(week 4 sampling)
	9	10	11	12	(week 6 sampling)
	13	14	15	16	(week 8 sampling)

### Litterbags - stubble

Treatment	Rep 1	Rep 2	Rep 3	Rep 4	
Sicot 189	1	2	3	4	(week 4 sampling)
	5	6	7	8	(week 8 sampling)
	9	10	11	12	(week 12 sampling)
	13	14	15	16	(week 16 sampling)
	17	18	19	20	extra

Treatment	Rep 1	Rep 2	Rep 3	Rep 4	
Sicot 289i	1	2	3	4	(week 4 sampling)
	5	6	7	8	(week 8 sampling)
	9	10	11	12	(week 12 sampling)
	13	14	15	16	(week 16 sampling)
	17	18	19	20	extra

**Cores - leaves**

Treatment

Sicot 189

Rep 1	Rep 2	Rep 3	Rep 4	
1-1	2-1	3-1	4-1	(week 2 sampling)
1-2	2-2	3-2	4-2	(week 4 sampling)
1-3	2-3	3-3	4-3	(week 6 sampling)
1-4	2-4	3-4	4-4	(week 8 sampling)

Treatment

Sicot 289i

Rep 1	Rep 2	Rep 3	Rep 4	
1-5	2-5	3-5	4-5	(week 2 sampling)
1-6	2-6	3-6	4-6	(week 4 sampling)
1-7	2-7	3-7	4-7	(week 6 sampling)
1-8	2-8	3-8	4-8	(week 8 sampling)

Treatment  
Sicot 289RRi

Rep 1	Rep 2	Rep 3	Rep 4	
1-9	2-9	3-9	4-9	(week 2 sampling)
1-10	2-10	3-10	4-10	(week 4 sampling)
1-11	2-11	3-11	4-11	(week 6 sampling)
1-12	2-12	3-12	4-12	(week 8 sampling)

**Appendix 8.** Comparison of dry- and wet-weight Bt toxin levels in field-grown plants.

variety	sample	ppb/g dry	ppb/g wet	ug/g dry	ug/g wet
Sicot 189	leaf	0	0	0	0
Sicot 189	young leaf	0	0	0	0
Sicot 189	stem	0	0	0	0
Sicot 189	taproot	0	0	0	0
Sicot 189	secondary root	0	0	0	0
Sicot 189	fine root	0	0	0	0
Sicot 289i	leaf	3,567.7 ± 370.1	2,130.1 ± 221.0	3.57	2.13
Sicot 289i	young leaf	11,401.2 ± 2,104.5	1,996.8 ± 368.6	11.40	2.00
Sicot 289i	stem	887.1 ± 290.0	157.2 ± 51.4	0.89	0.16
Sicot 289i	Taproot	3,589.6 ± 445.0	567.4 ± 70.3	3.59	0.57
Sicot 289i	secondary root	3,327.6 ± 103.7	689.0 ± 21.5	3.33	0.69
Sicot 289i	fine root	15,123.4 ± 4,437.4	1,672.3 ± 490.7	15.12	1.67
Sicot 289RRi	leaf	10,608.7 ± 4,985.9	4,391.4 ± 799.2	10.61	4.39
Sicot 289RRi	young leaf	27,395.5 ± 2,753.3	1,958.9 ± 508.4	27.40	1.96
Sicot 289RRi	stem	10,610.3 ± 1,278.9	1,882.1 ± 226.9	10.61	1.88
Sicot 289RRi	taproot	3,633.3 ± 175.2	591.0 ± 28.5	3.63	0.59
Sicot 289RRi	secondary root	2,248.1 ± 269.3	398.5 ± 47.7	2.25	0.40
Sicot 289RRi	fine root	5,397.4 ± 790.0	1,411.1 ± 206.5	5.40	1.41

**Appendix 9.** Ash content of leaf samples from litterbag experiments.

<b>Week (field incubation)</b>	<b>Variety GM-cotton</b>	<b>% ash (average 4 reps)</b>
0	Sicot 189	17.82
0	Sicot 289i	17.60
0	Sicot 289rri	19.01
0	Sicot 289rr	18.54
2	Sicot 189	42.07
2	Sicot 289i	53.22
2	Sicot 289rri	51.38
2	Sicot 289rr	60.40
4	Sicot 189	49.89
4	Sicot 289i	50.95
4	Sicot 289rri	62.69
4	Sicot 289rr	59.08
6	Sicot 189	53.91
6	Sicot 289i	54.91
6	Sicot 289rri	53.90
6	Sicot 289rr	69.73
8	Sicot 189	51.59
8	Sicot 289i	63.49
8	Sicot 289rri	60.80
8	Sicot 289rr	60.61

**Appendix 10:** Scanning Electron Microscope photographs of cotton roots.

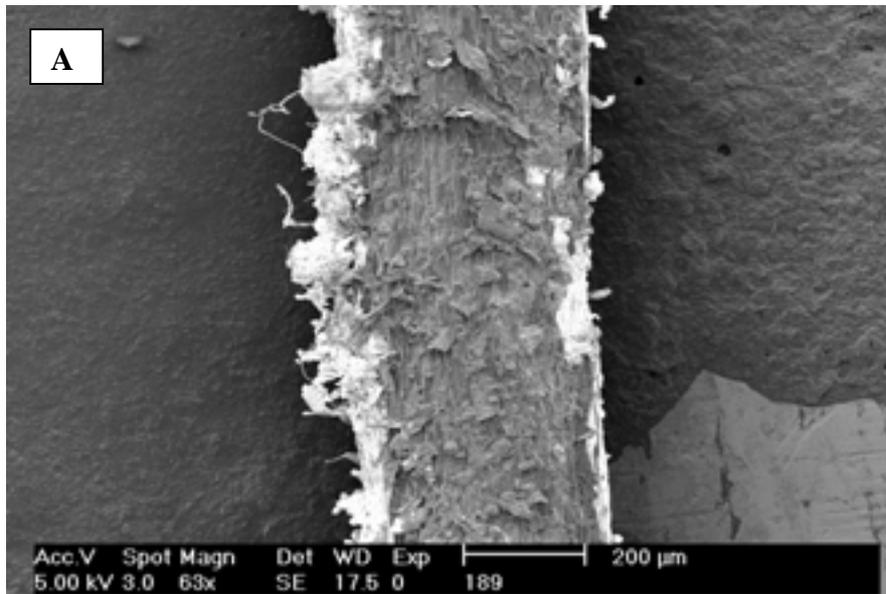


Photo 10A.  
A scanning electron micrograph of cotton root (non-GM variety Sicot 189).

(All SEM photos of roots: Gupta and S. McClure, CSIRO Land and Water, unpublished).

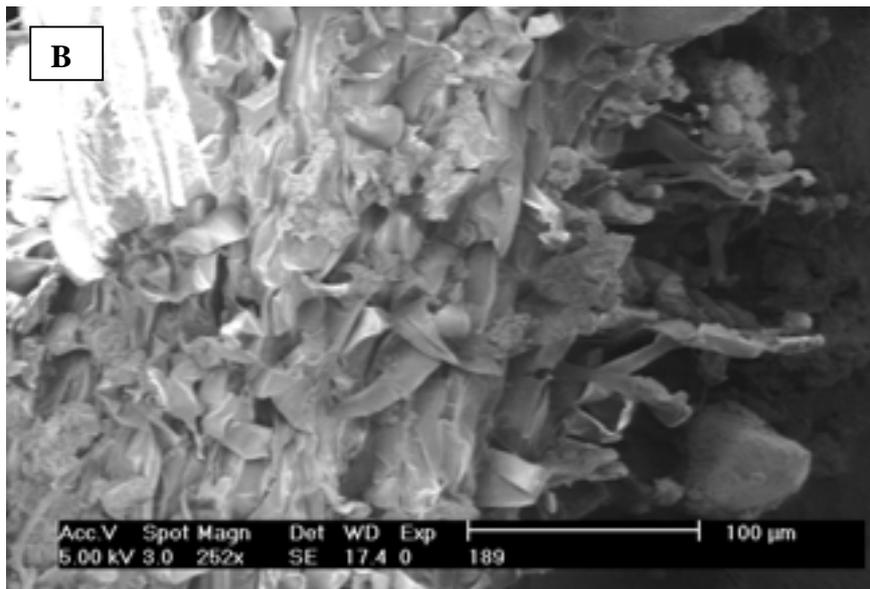


Photo 10B.  
Close-up picture of a cotton root (non-GM cotton variety Sicot 189) showing sloughing of epidermal cells and root hairs.

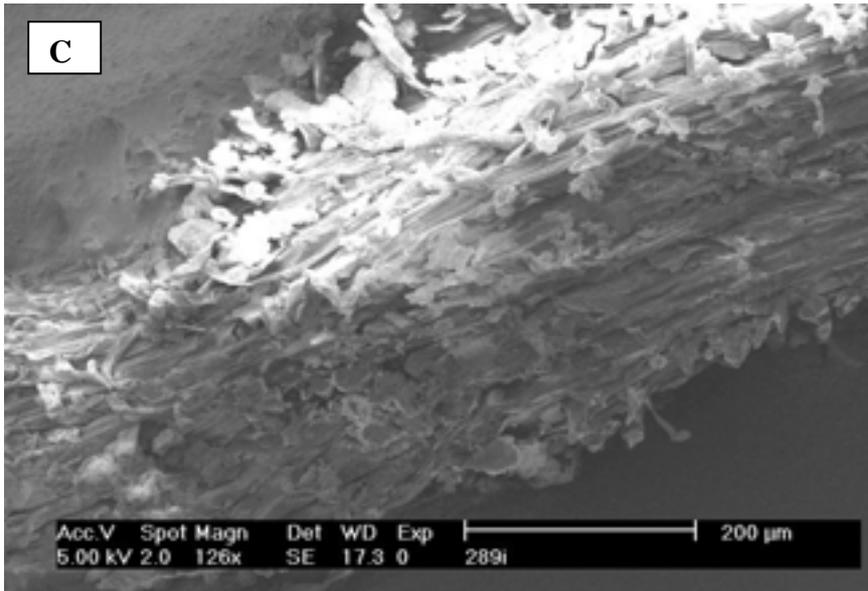


Photo 10C.  
Close-up picture of a cotton root (GM cotton variety Sicot 289i) showing sloughing of epidermal cells.

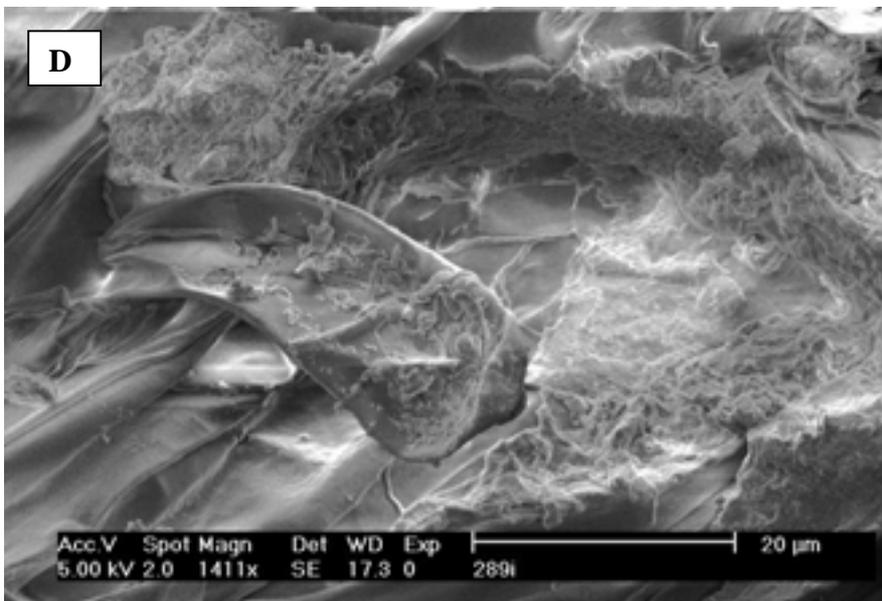


Photo 10D.  
Picture of a fine root hair colonized by bacteria and fungi (variety Sicot 289i).

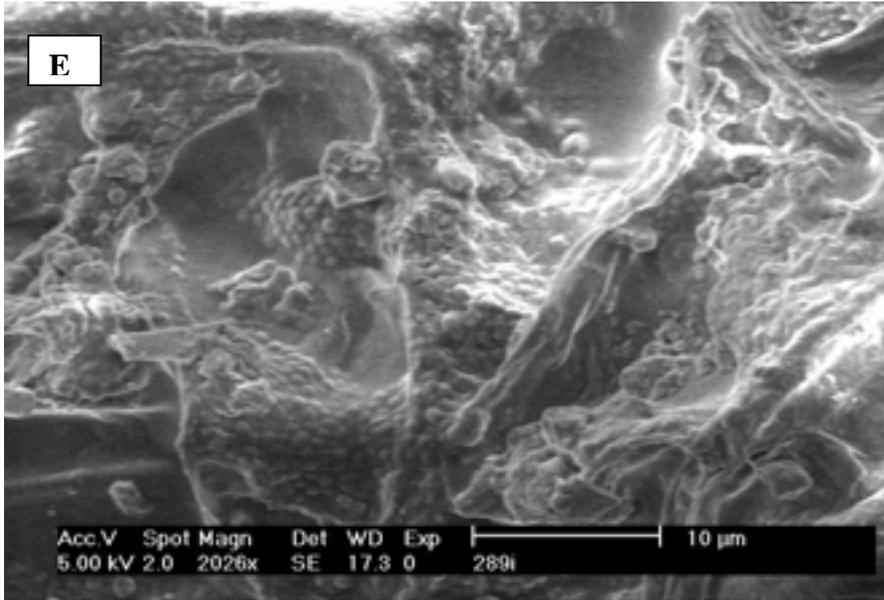


Photo 10E.  
Extensive surface  
colonisation of the  
cotton root by  
bacterial colonies  
(variety Sicot 289i).

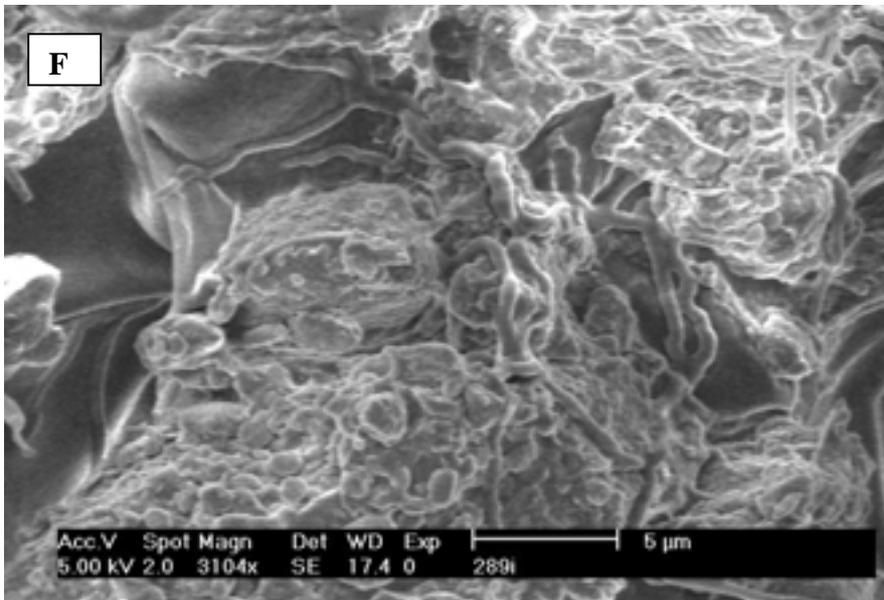


Photo 10F.  
Fungal hyphae  
colonization of the  
cotton root surface  
(variety Sicot 289i)  
and attached soil  
particles  
(rhizosphere  
colonization).

**Appendix 11.** Abstract for the paper presented at the ‘Biotechnology of Bt Environmental Impacts Conference’ held in Canberra during November 2001.

#### IMPACT OF *BT*-COTTON ON BIOLOGICAL PROCESSES IN AUSTRALIAN SOILS

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Transgenic cotton varieties modified to express the *Cry1A(c)* insecticidal toxin effective against lepidopteron insect pests (*Bt*-cotton) are now available for farmer use in Australia. However, little experimental data (especially quantitative) is available on the environmental consequences of the sustained expression and/or presence of *Bt*-toxin in various parts of the plant. Plant residues are the primary source of carbon in Australian soils and the majority of biota populations and biota-mediated processes are concentrated near crop residues and in the rhizosphere of plants. We evaluated the activity and composition of microorganisms and selected groups of soil fauna near decomposing crop residues (in field experiments) and in the rhizosphere of both *Bt*-cotton varieties and their conventional counterparts (in glasshouse).

Analysis of *Bt*-cotton plants using immunological assays revealed the presence of *Cry1A(c)* toxin in the roots and stems in addition to the leaves. Results from litterbag experiments indicated that the decomposition of *Bt*-cotton residues was slower (16%) than that of conventional cotton after 12 weeks of incubation. Microscopy observations showed extensive colonization of *Bt*-cotton residues by fungal hyphae and spores compared to conventional cotton residues, after 4 and 12 weeks of incubation. After 4 weeks of plant growth, total microbial activity (CO<sub>2</sub>-respiration and activity in BIOLOG GN and GP plates) and populations of total bacteria were greater in the rhizosphere of *Bt*-cotton compared to that of conventional cotton. However microbial utilization of <sup>14</sup>C-substrate by rhizosphere microorganisms and the rhizosphere populations of active protozoa and nitrifying microorganisms were lower for *Bt*-cotton compared to conventional cotton. The bacterial communities in the rhizosphere and rhizoplane of *Bt*-cotton compared to conventional cotton showed significant differences in their ability to utilize >35 different carbon substrates. The presence of insecticidal toxin in roots and stems and the significant changes in the populations of some groups of biota and biological functions warrant detailed investigations into the potential environmental consequences from the use of *Bt*-cotton varieties. The potential for persistence of large quantities of *Bt*-toxins in Australian soil and water environments also requires investigation. At present the implications of these results, relative to the potential benefits from reduced pesticide, are not known.

**Appendix 12:** Poster paper for the 12<sup>th</sup> Australian Cotton Conference, Gold Coast, August 2004.

### **Below ground production of *Bt*-protein by Genetically Modified cotton varieties**

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Main collaborator: G.N. Roberts, CSIRO Plant Industry, ACRI, Narrabri, NSW

#### **Abstract**

In this paper we present evidence (in glasshouse and field experiments) of the production of *Bt*-protein in different parts of the cotton root system by *Bt*-cotton varieties. We found that *Bt*-protein levels in the roots were comparable to that observed in leaves and the *Bt*-levels in finer roots were higher than other parts of the root system and plant-age related reductions in the finer roots were small compared to other plant parts. *Bt*-protein expressed in the roots of GM cotton varieties has the potential to be released into the soil through root hair fragments, passive root exudation, sloughed off epidermal cells and cell lysates and available for interaction with soil microorganisms.

#### **Key words**

GM crops, *Bt*-cotton, *Bt*-protein, Microbial functions, Root exudates, Rhizosphere

#### **Introduction**

Genetic modification of plants, microbes and animals to incorporate useful traits is a powerful technology, which is essential for the future development of sustainable agriculture systems from both production and environmental aspects. Plants are being genetically modified to control insect and fungal pathogens (reducing reliance on insecticides and fungicides), to withstand specific herbicide application (better weed management) or environmental conditions (water logging), improve crop quality (nutritional value), bioremediation (phytoremediation) and for biomolecule production (medicinal).

A variety of crops (e.g. cotton, corn, potato, soybean, canola) have been genetically modified to produce *Bt*-proteins such as *Cry* 1Ab, *Cry* 1Ac, *Cry* 2Ab to protect against insect pests. Cotton varieties modified to produce *Cry* 1Ac protein as a protection against the insect pest *Helicoverpa armigera* have been available for farmer use in Australia since 1996. The majority of research on the expression of *Bt*-proteins in genetically modified (GM) cotton varieties have concentrated on the gene expression in above ground plant parts and little experimental information (especially quantitative) is available on the *Bt*-production in the belowground plant parts. Active *Bt*-protein has been reported to be released in *Bt*-corn root exudates (Saxena and Stotzky, 2001) and has been shown to persist in the soil for at least 180 days (Stotzky, 2000). Preliminary evidence from our laboratory indicates differences in rhizosphere microbial populations (measured using carbon substrate utilization profiles and metabolic quotient values) for conventional and Ingard<sup>®</sup> cotton varieties grown under glasshouse conditions (Gupta *et al.*, 1998). No information is available if the *Bt*-proteins could be released by the *Bt*-cotton into soil environments.

Soil biota communities are one of the most diverse groups of earth's biota. They regulate a number of processes that are not only critical for productivity but are also essential for maintenance of ecosystem health (Gupta *et al.*, 1999). Genetically modified plants (GMP)

have the potential to affect rhizosphere microbial dynamics through (1) unintentionally changing rhizosphere chemistry, i.e. quantity and quality of root exudates, (2) through the continuous expression of introduced genes in belowground plant parts or (3) modified response to agrochemical use (Gupta et al., 1999; Gupta et al. 2004). Both the nature and magnitude of GMP effects on soil biota may vary as the influence of each modified gene function on plant composition and rhizosphere chemistry will differ. For GMPs that express the inserted gene only in the above ground plant parts, the soil biota community is exposed to the gene products after crop harvest through crop residues. However when the inserted gene function is also expressed in below ground plant parts, soil biota community is exposed to the gene products throughout the plant growth. In this paper we report results about the production of *Bt*-protein by different GM Cotton varieties, i.e. single gene *Bt*-cotton varieties, Roundup Ready *Bt*-cotton varieties and two gene *Bt*-cotton varieties, in the belowground plant parts.

### Materials and Methods

*Bt*-cotton plants grown in the glasshouse and field conditions provided different plant part materials for above and belowground *Bt*-protein analyses. The different cotton varieties tested include Conventional (Sicot 189) and the GM counterparts; 289i (Ingard<sup>®</sup>), 289RRi (Roundup Ready/Ingard<sup>®</sup>) 189 RR, 289 B (Bollgard II) and 289 BR (Bollgard II BR/Roundup Ready). All varieties were planted in 3.0 kg soil pots (Bollgard varieties in soil that had not been cultivated under GM cotton from Narrabri, NSW and others in a soil from Avon, SA; 4 replicates per variety) and grown in the glasshouse at 26 °C. The same cotton varieties were also planted in fields at Narrabri (NSW) during 2001 (single gene *Bt* varieties) and 2003 (Bollgard varieties). This was to ensure that observations made in controlled (glasshouse) condition experiments were comparable to the field situation. Plants were harvested from the glasshouse experiments at regular intervals and various plant tissue (e.g. leaf, stem, tap root, secondary and fine root) samples taken and snap frozen prior to using the ELISA tests. Similarly, cotton plants harvested from the experimental field at Narrabri at various times after germination, were transported in a cooler to the laboratory where different plant parts were separated and snap-frozen prior to *Bt*-protein analyses.

*Bt*-protein levels in different plant parts were measured using the 'Enviroligix *Cry1Ab/Cry1Ac* ELISA Kit'. This ELISA kit was designed for the quantitative detection of *Bt*-protein in leaf tissue by using specific polyclonal antibodies to the *Cry1Ab* and *Cry1Ac* proteins. These antibodies are enzyme-labelled and results are visualized with a colour development step, the colour level being proportional to the concentration of *Cry1Ab/Cry1Ac* in the sample extract. Prior to this detection process (colour development) the *Bt*-protein was extracted from the plant/soil matrix into a detection buffer. After removing all larger and fine roots, sloughed off epidermal cells and root hairs material were collected from the root washings on a 0.2µm filter prior to use in root exudate experiments. These materials were processed for *Bt* levels using similar methods to other plant tissues.

### Results and Discussion

Results from ELISA analyses demonstrated that measurable concentrations of *Bt*-protein were present in all parts of *Bt*-cotton plants (289i and 289RRi) i.e. leaves, stems and roots (tap, secondary and fine roots), and not present in the same tissues of conventional cotton plants (Sicot 189). The levels of *Bt*-protein in 289RRi fine roots at weeks 4 and 8 were even higher than the levels found in the leaves of these same plants. The general decrease in *Bt*-protein levels over time is generally accepted to be due to the ageing of the various plant tissues and gradual breakdown of *Bt*-protein within these tissues. However, as the plants grew older the levels of *Bt*-protein in the roots were significantly higher than the leaves. These higher levels

of *Bt*-protein below ground can be attributed to the continued growth of new root systems. A decrease in Cry1Ac levels was also observed for both 289B and 289BR glasshouse plants measured at 8, 12 and 16 weeks of age, with virtually no expression detected at 16 weeks. Expression levels had been similar between lateral and tap roots, but were lower for 289B than 289BR.

*Bt*-protein levels in roots of single gene *Bt*-varieties were comparable with the data, obtained from glasshouse-grown cotton plants at week 8. The Bollgard and Bollgard/Round-up Ready field grown varieties indicated higher values than those observed in the glasshouse experiment for roughly the same period of growth. These results suggest that our experiments with glasshouse single gene *Bt*-cotton plants can be taken as indicative of the situation in the cotton fields, but comparisons for the two gene Bollgard varieties would appear to be more complex and requires more detailed study. These results show that the expression of *Bt*-protein occurred in every major part of the *Bt*-cotton plants (leaves, stems, and roots) and that root *Bt*-protein expression was comparable (or higher in the later stages of cotton plant growth) to that in cotton leaves. Of the root types sampled the fine roots exhibited the highest *Bt*-protein levels for 289i and 289RRi cotton plants. Even at week 16 we measured significant levels of *Bt*-protein in all root types for single gene *Bt*-cotton varieties and for two gene *Bt*-cotton in field trials, which demonstrates that a significant amount of *Bt*-protein is present in cotton plant roots below the ground late in the growing season. The general perception, until now, has been that the expression of *Bt*-protein was confined to the plant system (leaves and stems) above ground and that the levels found in the leaves are the highest in the total plant. However, the general promoter used for the expression of the inserted *Cry1Ac* protein gene is not tissue-specific. Therefore there would be no tissue-specific control over the expression of *Bt*-protein in the cotton plant. Our research is the first to indicate that *Bt*-protein is found in the roots of the *Bt*-cotton plants in Australia at levels as high and in some instances higher than in the leaves.

Our analysis of the sloughed epidermal cells and fine-root hairs from 289i plants showed *Bt*-protein levels of 1317 ppb/g wet weight where as Sicot 189 cells/fine-root hairs showed no detectable *Bt*-protein. We assume that the fine-root hairs with their faster growth rates would be contributing to a large portion of this *Bt*-protein (active growth=active *Bt*-protein expression). The significance of this *Bt*-protein reservoir (root turnover) in terms of its potential impacts (both positive and negative) on soil biota and biological functions is currently unknown.

Results from the exudate experiments indicated measurable amounts of *Bt*-protein both in the exudates of solution grown and soil grown plant experiments. As roots try to grow through the different sized pores, cracks and crevices in field soils and due to the pressures exerted by the shrinking and swelling of clay soils during drying/wetting cycles, damage to the cotton roots is more likely to happen resulting in the release of *Bt* from roots.

A variety of soil microorganisms are capable of degrading proteins, including the *Bt*-protein and the degradation of pure proteins is usually faster (hours to days). However it has been shown that the degradation of proteins immobilized by soil clays and organic matter may be slower. Currently we are working to find out the differences, if any, in the rhizosphere microbial communities between conventional and GM-cotton varieties (Knox *et al.*, 2004).

### **Acknowledgements**

Authors wish to express their appreciation to CSIRO Land and Water, Cotton RDC and the Australian Government Department of the Environment and Heritage for the financial support and Ms. Clare Fenton-Taylor for technical assistance.

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