Part 1 - Summary Details

CRDC Project Number: **DAN 172**

Project Title: Biochemical mechanisms of resistance to Bacillus *thuringiensis* endotoxins in *Helicoverpa armigera*

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CRDC Program: On-Farm

Part 2 – Contact Details

Administrator: Mr Graham Denny
Organisation: New South Wales Department of Primary Industries
Postal Address: LMB 21 Orange
Ph: (02) 6391 3554 **Fax:** (02) 6391 3327 **E-mail:** graham.denny@dpi.nsw.gov.au

Principal Researcher: Dr Robin Gunning
Organisation: New South Wales Department of Primary Industries
Postal Address: Tamworth Agricultural Institute, 4 Marsden Park Road, Calala NSW 2340
Ph: (02) 6763 1128 **Fax:** (02) 6763 1222 **E-mail:** robin.gunning@dpi.nsw.gov.au

Supervisor: Dr Bob Martin
Organisation: New South Wales Department of Primary Industries
Postal Address: Tamworth Agricultural Institute, 4 Marsden Park Road, Calala NSW 2340
Ph: (02) 6763 1258 **Fax:** (02) 6763 1222 **E-mail:** bob.martin@dpi.nsw.gov.au

Researcher 2: Dr Graham Moores
Organisation: Rothamsted Research
Postal Address: Harpenden, Herts., AL52JQ, UK.
Ph: **Fax:** **E-mail:** graham.moores@bbsrc.ac.uk

Signature of Research Provider Representative: _____

Part 3 – Final Report Guide (due 31 October 2008)

Background

The use of conventional insecticides to control Helicoverpa on cotton in Australia has led to insecticide resistance and environmental problems. As an alternative strategy for pest management, we have introduced *Bacillus thurlingiensis* (*Bt*) protein toxins both as applied insecticides and in transgenic cotton. Bt toxins are ingested by the insect as pro-toxins and activated by mid-gut proteases. The activated toxins disrupt the mid-gut membrane wall and lead to *Helicoverpa* death. In Australia, two *Bt* delta-endotoxins are used against Helicoverpa. Australian transgenic cottons express Cry 1Ac (in Ingard®)) and Cry2Ab (in combination with Cry1Ac in Bollgard®). Continuous toxin expression in transgenic cotton, presents an enduring threat of resistance in *Helicoverpa armigera* and *H. punctigera*.

In 2001, an *H. armigera* strain ("silver strain") was formed by Dr Ho Dang, from field survivors of the Cry1Ac resistance monitoring programme, from New South Wales and Queensland cotton areas. Dr Dang's work suggested that the strain was Cry 1Ac resistant, however, due to inadequate Cry 1Ac bioassay methods used by both Dr Dang and CSIRO researchers, the resistance status of the silver strain was never properly resolved. Preliminary resistance mechanism studies by Robin Gunning, however, suggested that the silver strain was able to metabolise Cry 1Ac via esterase iso-enzymes.

Objectives

- To revise Cry 1Ac bioassay protocols to resolve the resistance status of Ho Dang's silver strain
- To determine whether "silver strain" *H. armigera* esterase to binds to other Bt toxins
- Study mechanisms of esterase/Bt toxin binding. Metabolism or sequestration or protease action.
- Determine whether esterase inhibitors will prevent *cry*1Ac binding to esterase in *H. armigera*.
- Examine esterase-binding ability of other Bt *cry* crystal proteins, with particular emphasis genes may be incorporated into transgenic crops in Australia.
- Determine the amino acid sequences of *cry* binding esterases and model sites of *cry* protein attachment.

Methods

3.1 Insect strains

Australian *Helicoverpa armigera* strains used in this work were: A Cry1Ac susceptible, laboratory strain, the silver strain, silver selected strain and reciprocal backcross of the susceptible and silver selected strains. The silver strain originated from Cry1Ac resistance screen survivors from NSW and Queensland cotton (Emerald, Q; Darling Downs, Q; St George, Q; Macintrye Valley, Q and NSW, Namoi Valley, NSW, Macquarie Valley, NSW and Hillston, NSW).

Five, Cry1Ac susceptible *H. armigera* field strains, collected from conventional cotton and maize crops in 2002, which been exposed to a wide variety of conventional insecticides, were

also used for comparison of esterase activity in this study. (Strain 1, Warren NSW, Strain 2, Narromine, NSW; Strain 3, Gunnedah; Strain 4, Griffith, NSW; and Strain 5, Willow Tree, NSW).

3.2 Diet Incorporation Bioassay
Feeding bioassays on third instar larvae were used, where Cry1Ac was incorporated into an artificial diet. Formulated Cry1Ac (MVP®) was serially diluted with distilled water containing 0.1% Triton X-100 and pipetted onto the diet surface. Larvae were confined on the Bt treated diet at 25 ^{o}C for 4 days before being transferred to fresh, non- BT diet.

Mortality was assessed 14 days after Cry1Ac dosage. Dosage mortality data were analysed by probit analysis (3). We bioassayed the silver strain and a lab susceptible strain but since the silver strain" had been maintained for some time, to better gauge the magnitude of resistance, the silver strain was selected once, with MVP® at the LC_{50} concentration and the F_1 progeny silver sel. bioassayed. The silver sel. strain was also reciprocally back-crossed to the susceptible strain (silver sel. females x susceptible males; silver sel. males x susceptible females) and progeny were bioassayed with MVP.

3.3 Feeding Bioassays On Cotton Leaves
First instar larvae from the susceptible and silver selected strains were placed on cotton leaf discs of non-*Bt* cotton (Sicot 189), Ingard (Sicot 189i) and Bollgard II (Sicot 289b) Ingard and Bollgard II cottons were expressing Cry1Ac toxin at a rate of 1.2 – 1.4 ppm). The larvae were sealed into vented, air tight, polystyrene 5 cm diameter petri dishes (5 larvae per petri dish). Larvae were allowed to feed on the cotton leaf discs at 25^{o}C. Each experiment was replicated 8 times. Additional leaf material was provided if required and mortality was assessed at 10 days. Surviving larvae were retained on artificial diet to pupation, and then to eclosion, to verify normal development, fertility, and fecundity into next generation.

3.4 Electrophoresis
Polacrylamide gel electrophoresis preparation methods were similar to those used by Devonshire and Moores (2). Larvae, (3-4 mg) were homogenised in microtitre plates, with 200 µl of 1.6% Triton X-100 in distilled water containing 10% sucrose and a few grains of bromocresol purple. Aliquots (10µl), were loaded directly onto a polyacrylamide gel. Gels were run at 250V and maximum current until the solvent front had run off the gel. The gels were stained for esterase activity in a solution of 0.1mM 1-naphthyl acetate 0.2% Fast Blue RR salt in phosphate buffer (pH 6.0) for 30 mins in darkness at 25^{o}C and fixed in 5% acetic acid.

3.5 Total Esterase Determinations
Small, (3 – 4mg) larvae, were used for total esterase determinations. Fifty larvae from each strain were homogenised in 2.5 ml of 0.02M phosphate buffer (pH 7.0) containing 0.05% Triton X-100. Replicated aliquots (10µl), were transferred to a clean microplate containing 240 µl of 0.2M phosphate buffer (pH 6.0) with 0.6% Fast Blue RR salt and 1.86% 1-naphthyl acetate. Kinetic assays were immediately performed on a microplate reader utilising kinetic software, taking absorbance readings (450nm) automatically at 14 second intervals. The kinetic velocity was calculated by the online computer as the slope of the fitted regression line using an absorbance limit of 2000mOD.

3.6 In Vivo Esterase Determinations

The *in vivo* esterase determination method was as follows. First instar, susceptible and silver sel.. larvae, were fed on a Cry1Ac treated diet (0.0012 mg Cry1Ac/0.5g diet) or Cry1Ac expressing, Ingard cotton leaves at 25$_{o}$C. At time intervals from 30 minutes to 70 hours later, samples of larvae were taken and stored at –15$_{o}$C until the conclusion of the experiments. Control larvae were fed non- *Bt* cotton or diet. Larval tissue, (20 mg) from each sampling time, was assayed for esterase activity.

3.7 Inhibition of esterase by Cry1Ac toxin

Cry1Ac pro-toxin (crystal and spores) and activated Cry1Ac (crystal) were dissolved in 50 mM carbonate buffer (pH 9.5). Small (3-4 mg.) larvae were used for these experiments. Fifty larvae from each strain were homogenised in 1.0 ml of 0.02M phosphate buffer (pH 7.0) containing 0.05% Triton X-100. Aliquots (50 µl), were pipetted into Eppendorf tubes. Cry1Ac was added to the aliquots and volume was adjusted to 150 µl with carbonate buffer. Tubes were incubated for 60 min at 25$_{o}$ C. Esterase activity was determined on replicated, 10 µl aliquots. Final concentration of Cry1Ac pro-toxin and activated toxin ranged from 2.0 x 10^{-8} – 2,0 x 10^{-5} µg/assay and 5.0 10^{-8} – 5.0 x 10^{-6} µg/assay respectively.

3.8 Surface Plasmon Resonance

Surface plasmon resonance (SPR) techniques using a BIAcore® was used to study *H. armigera* esterase / Cry1Ac interactions. Esterase isoenzymes from the resistant silver sel. strain were purified by anion exchange chromatography. Approximately 1800 response units (RU) of activated Cry1Ac toxin in 10mM sodium acetate (pH 4.0) was bound to a CM5 carboxymethyl surface using EDC-NHS (1-ethyl-3-{3-dimethylaminopropyl}-carbodiimide and N-hydroxy-succinimide) chemistry. Purified *H. armigera* esterases from the Cry1Ac silver sel.ected strain were then passed across this surface at concentrations ranging 1.74µM – 27.8µM in 10 mM disodium tetraborate, 1 M NaCl, pH 8.5 (to approximate mid-gut conditions). The Cry1Ac surface was regenerated between each injection with two 10 second injections of glycine, (pH 1.5).

3.9 Spinosad Bioassays

Technical grade spinosad was dissolved in acetone and serially diluted. Spinosad biosaays were done by topical application onto the dorsal thorax of 30-40 mg. larvae. Dosage mortality data were analysed by Probit Analysis.

Results

4.1 Resistance to Cry1Ac in "silver strain" H. armigera

The *Bt* resistance monitoring programme showed a some survival of Cry1Ac in cotton populations of *H. armigera*. This apparent resistance has now been studied in some detail (DAN 172C) by Robin Gunning (NSWDPI) and Graham Moores (Rothamsted research, UK). Resistance was confirmed in the *H. armigera* strain ("silver strain") formed from survivors the resistance -monitoring programme (Table 1). Through a single selection of the silver strain, the resistance factor was estimated at 150 and 275 fold at the LC$_{50}$ and LC$_{99.9}$ levels respectively. The resistance was genetically inherited as a semi-domminant, non sexlinked trait (Table 1).

Table 1 *Response of susceptible, cry1Ac selected and non-selcted "silver strain"and "silver strain x susceptible F1 cross, populations of H. armigera to cry1Ac toxin.*

Strain	Slope	X^2	LC_{50} (95% fiducial limits) mg CRY 1Ac / 0.5 g diet.	Resistance Factor*	$LC_{99.9}$ (95% fiducial limits) mg CRY 1Ac / 0.5 g diet.	Resistance Factor*
Susceptible	3.1	2.0	0.000125 (0.00010 – 0.00015)	1	0.0012 (0.00069 – 0.0023	1
"Silver strain" 2001/2	1.9	5.9	0.0018 (0.001-0.003	14	0.063 (0.0075 – 0.57)	50
"Silver sel."	2.5	0.7	0.019 (0.010 – 0.0.24)	150	0.33 (0.15 – 0.69)	275
Silver sel. + 10µg PBO	2.5	2.9	0.0009 (0.00075 – 0.0011)	7.2	0.015 (0.0075 – 0.029)	12.5
Silver sel (m) x Sus (f) F1	2.6	1.4	0.0049 (0.0041 – 0.0061)	39	0.08 (0.036 – 0.17)	67
Silver sel (f) x Sus (m) F1	2.8	0.7	0.0046 (0.003 – 0.006)	37	0.061 (0.022 – 0.16)	51

4.2 Leaf Feeding Bioassays

Feeding bioassays showed that 70% of the "silver selected strain" could survive on Ingard cotton and 5% survived on Bollgard cotton(Figure 2.)

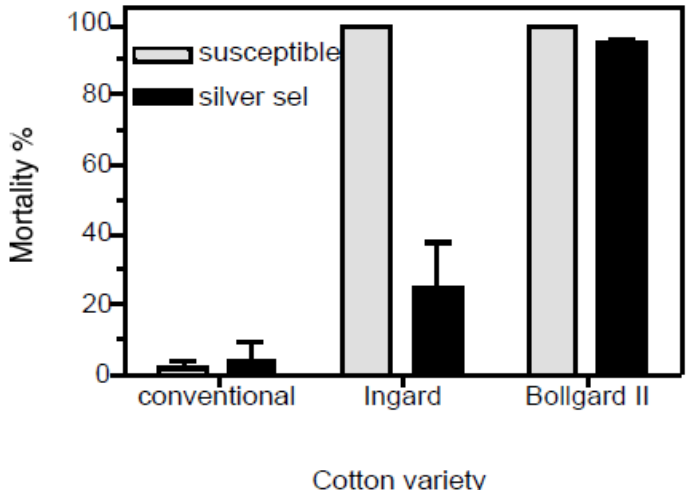

Figure 1 *Survival of Cry1Ac susceptible and silver selected strains of H. armigera larvae, on leaves of conventional non-Bt (Sicot 189) Ingard (Sicot 189i) and Bollgard II (Sicot 289b) cotton varieties. (Errors bars represent 95% confidence intervals).*

4.3 Resistance in field strains of H. armigera

Given that the "silver strain" had now been held in the laboratory (albeit unselected), for some time, field strains of *H. armigera* from cotton, were bioassayed with Cry1Ac to determine whether resistance observed in Dr Dang's resistance monitoring, was still present (Table 2). All four strains tested, (Narrabri 2003, Narrabri 2004, St George 2004 and Goondiwindi 2004, showed significant resistance at the $LC_{99.9}$ level (28,7,13,18 fold respectively). These data are indicative of heterogeneous populations containing a low, but significant frequency of Cry1 Ac resistant individuals. Proof of Cry1Ac resistance in the field strains was provided by selecting each once (LC_{50} of susceptible strain) with Cry1Ac. Results showed that one selection, increased the resistance factors and slope values of the dose response curves. Resistance factors were 38,20, 184 and 30 fold respectively at the LC_{50} and 208.28.,4916 and 496 respectively at $LC_{99.9}$ levels . The data prove resistance in the field strains and demonstrate ease of resistance selection.

Table 2. *Response of susceptible and Cry1Ac selected(LC_{50} of the susceptible strain) and nonselected cotton populations of H. armigera to Cry1Ac toxin.*

Strain	Slope	X^2	LC_{50} (95% fiducial limits) mg CRY 1Ac / 0.5 g diet.	Resistance Factor*	$LC_{99.9}$ (95% fiducial limits) mg CRY 1Ac / 0.5 g diet.	Resistance Factor*
Susceptible	3.1	2.0	0.000125 (0.00010 – 0.00015)	1	0.0012 (0.00069 – 0.0023	1
Narrabri field strain, 2003	1.4	32	0.00018 (0.00009 – 0.0003)	1.4	0.034 (0.003 – 0.43)	28
Narrabri field strain 2003selected	1.8	7.1	0.0048 (0.0035-0.0066)	38	0.25 (0.095-0.62)	208
Narrabri field strain, 2004	1.8	6.2	0.00013 (0.000096 – 0.00095)	1	0.0084 (0.0026 – 0.018)	7
Narrabri field strain, 2004 sel.	2.7	5.6	0.0024 (0.0017 – 0.0033)	20	0.034 (0.012 – 0.081)	28
St George field strain, 2004	1.7	11.9	0.00049 (0.000083 – 0.0015)	3.9	0.015 (0.0033 – 0.075)	13
St George field strain, 2004 sel.	1.4	4.7	0.023 (0.014 – 0.038)	184	5.9 (0.90 – 38.1)	4916
Goondiwindifield strain, 2004	1.4	12.1	0.00015 (0.000063 – 0.0035)	1.2	0.021 (0.0025 – 0.26)	18
Goondiwindifield strain, 2004 sel.	1.7	2.7	0.0060 (0.0038 – 0.099)	50	0.59 (0.12 – 2.94)	492

Resistance in the field strain was further demonstrated by leaf feeding bioassays with Ingard and conventional cotton. Data showed that there was survival in both the field strains and selected field strains on Ingard cotton (Figure 2). These data indicate the field significance of the Cry1Ac resistance.

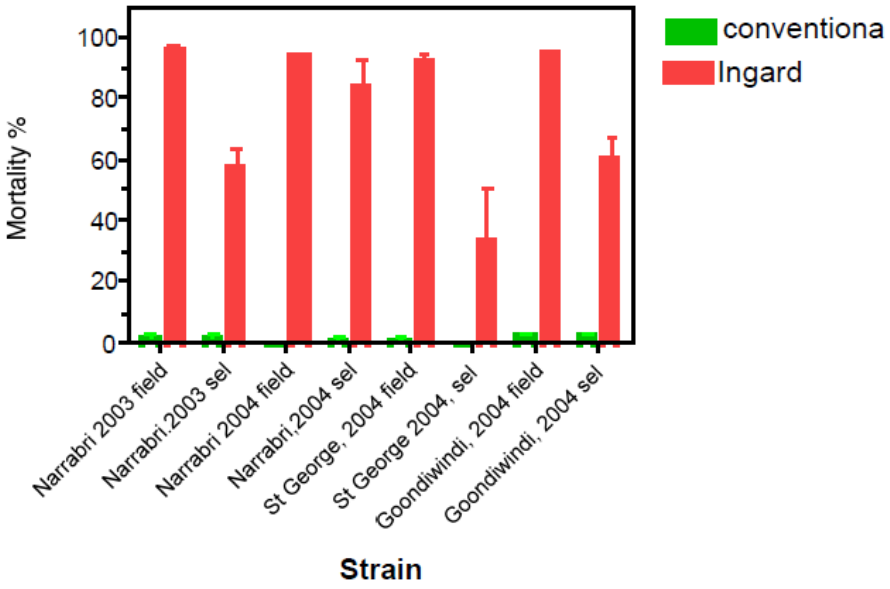

Figure 2 *Survival of Cry1Ac susceptible, field and field selected strains of H. armigera larvae, on leaves of conventional non-Bt (Sicot 189) Ingard (Sicot 189I). (Errors bars represent 95% confidence intervals).*

4.4 Resistance Mechanism Studies

A class of serine hydrolases, called non-specific esterases, which are found in the insect gut, have been implicated as an insecticide-resistance mechanism in numerous insect pests due to their ability to hydrolyse insecticidal esters and their ability to sequester xenobiotics. In Australia, *H. armigera* resistance to insecticide groups as diverse as pyrethroids, spinosad and chlorfenapyr have been linked these enzymes In CRDC project DAN 172C, we demonstrated an esterase resistance mechanism "crossing over' from conventional chemistry and conferring resistance to Cry1Ac in *H. armigera*.

The Cry1Ac resistance mechanism was identified as an over production of esterase isoenzymes (Figs. 3 and 4), inherited as a semi-domminant trait, along with the resistance (Figure 3). Overproduced esterase binds to Cry1Ac (Figure 5). Sequestration) of Cry1Ac is the most probable form of detoxification. Esterase binding to Cry 1Ac was also inherited as a semi-domminant trait .

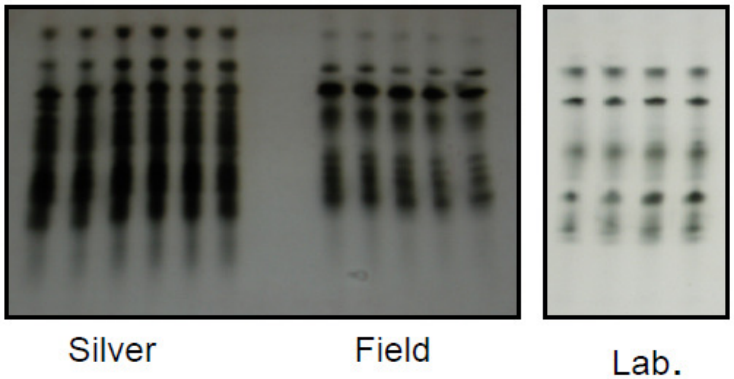

Figure 3 *Polyacrylamide gels showing esterase activity in lab susceptible, field susceptible and silver sel. strains H. armigera larvae. (Each track contains the equivalent of 0.05 of a 3-4 mg larva).*

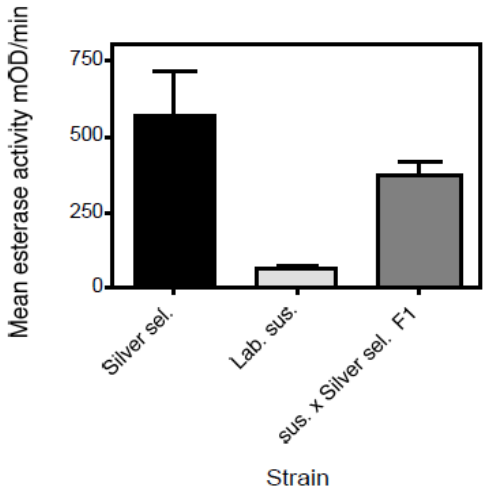

Figure 4. *Total esterase activity in 3 – 4 mg larvae of strains of H. armigera silver sel. strain, Cry1Ac susceptible lab strain and F1 backcrosses. Error bars represent 95 % confidence intervals.*

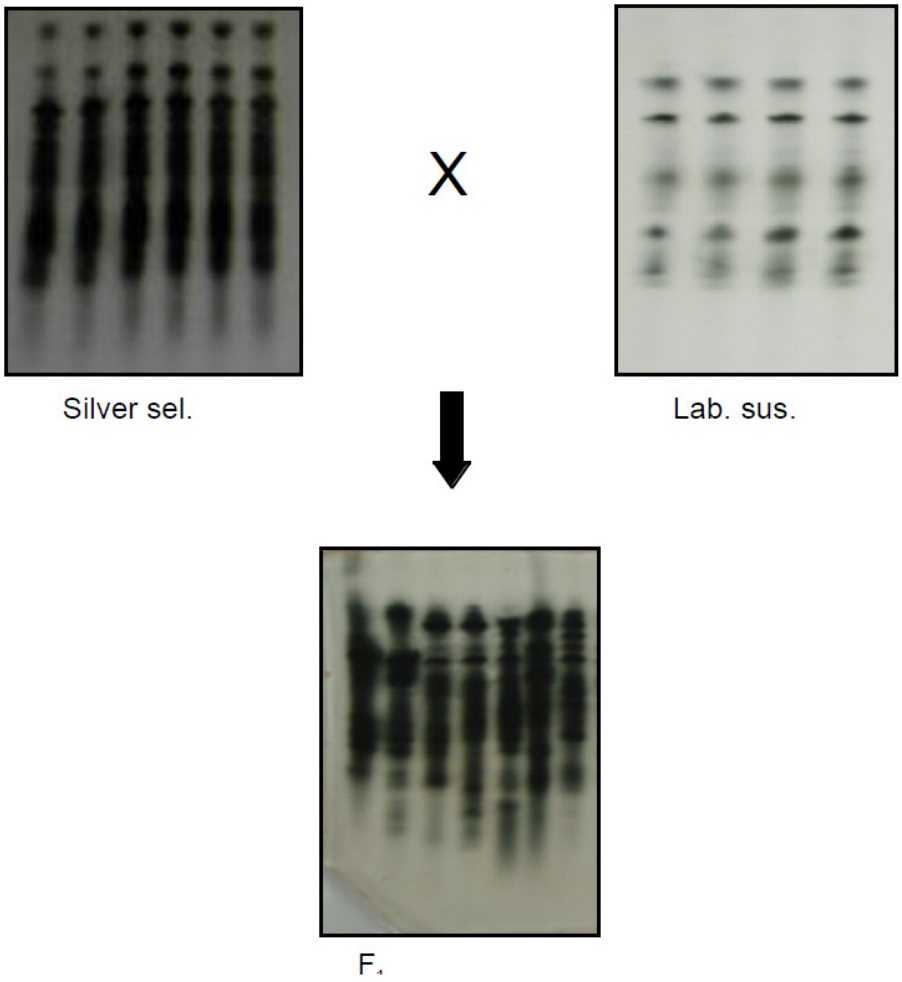

Figure *5 Polyacrylamide gels showing esterase activity in Lab susceptible, silver sel. strain and F1 cross, H. armigera larvae. (Each track contains the equivalent of 0.05 of a 3-4 mg larva).*

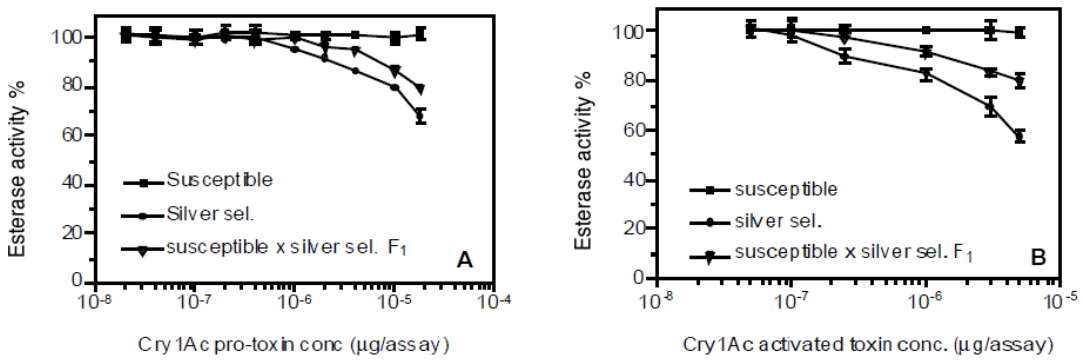

Figure 6. *In vitro esterase inhibition by Cry1Ac pro-toxin (A) and activated toxin (B) in Cry1Ac susceptible, resistant silver sel. and F1 back-cross strains of H. armigera. Error bars represent 95 % confidence intervals*

Cry1Ac binding to resistant esterase was confirmed directly, using real time bimolecular interaction analysis (Biacore™) and this demonstrated a strong affinity of esterase, for Cry1Ac (Fig. 7).

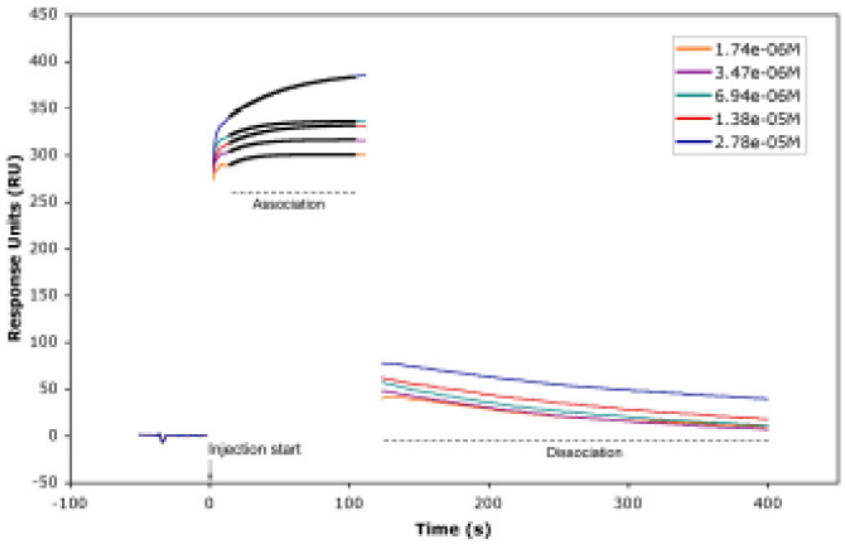

Figure 7*. Sensogram plot of association and dissociation curves of purified esterase, from Cry1Ac resistant, silver sel. H. armigera to activated Cry1Ac toxin.*

4.5 Demonstration of Resistance Mechanism In Live, Small Larvae

Whilst the *in vitro* data clearly showed that silver sel. strain esterase bound to Cry1Ac toxin and susceptible esterase did not, the resistance significance for *Bt* cotton requires the ability of esterases in neonate larvae to sequester Cry1Ac toxin *in vivo*. In the field, *H. armigera* are exposed as first instar larvae to Cry1Ac toxin, and this resistance mechanism would have little ecological significance for resistance selection unless it was expressed in live first instar larvae. Esterase inhibition by Cry1Ac was studied *in vivo*, in first instar silver sel. Strain larvae, using either formulated Cry1Ac in the diet or by feeding leaves of Cry1Ac expressing, Ingard cotton plants. Larvae were sampled at regular time intervals for esterase analysis. *In vivo* inhibition data are shown in Fig. 8. Esterase inhibition, compared to non-Cry1Ac diet controls, was detected 2 hours after feeding on formulated Cry1Ac diet and continued to increase for 16 hours (Fig 8A). Esterase activity remained significantly inhibited whilst the

actively feeding larvae had access to Cry1Ac treated diet. Susceptible strain larvae did not survive the course of the experiment. With Ingard cotton, (Fig. 8B), esterase inhibition was detected after 4 -5 hours of active feeding, esterase activity continued to decline for the duration of the experiment. Esterase activity remained inhibited whilst the feeding larvae had access to Ingard cotton. Susceptible strain larvae did not survive the course of experiment on Ingard cotton.

The *In vivo* data (Fig. 8) demonstrate field significance of the esterase resistance mechanisms. An esterase sequestration, resistance mechanism, potentially, has great ecological significance because of the capacity for selection by transgenic cotton crops. Findings that resistant larvae apparently sequester and detoxify Cry1Ac toxin, whilst feeding on transgenic cotton, are highly significant because they also provide a direct, field mechanism for the observed resistance to Cry1Ac in *H. armigera*.

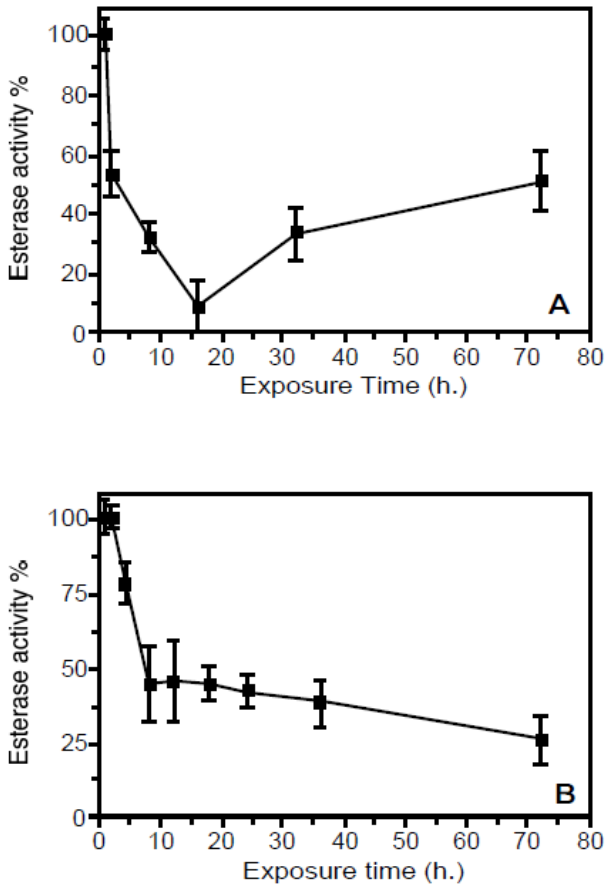

***Figure 8**. In vivo esterase inhibition (with respect to non-Cry1Ac fed controls) in silver sel. Strain larvae, fed with formulated Cry1Ac (0.0012 mg Cry1Ac / 0.5 g diet.), (A), or on actively expressing Cry1Ac Ingard® cotton leaves (B). Error bars represent 95 % confidence*

4.6 Cry1Ac Synergism by Piperonyl butoxide

The insecticide synergist piperonyl butoxide (PBO) is an effective inhibitor of esterases in *H. armigera*. Experiments using PBO, topically applied to third instar larvae (fed a Cry1Ac diet) and first instar larvae fed PBO dipped Ingard and Bollgard leaves, showed that PBO was an effective synergist of Cry1Ac resistant *H. armigera*,(Table 1, Figures 9 and 10). These data further demonstrate the importance of esterase sequestration as a resistance

mechanism in Cry 1Ac resistant *H. armigera.* The ability of PBO to "cross over" from synergising conventional insecticides, to synergism of transgenic cotton against resistant pests, represents a considerable breakthrough in the battle to effectively manage transgenic crops and to retain their efficacy against resistant insects.

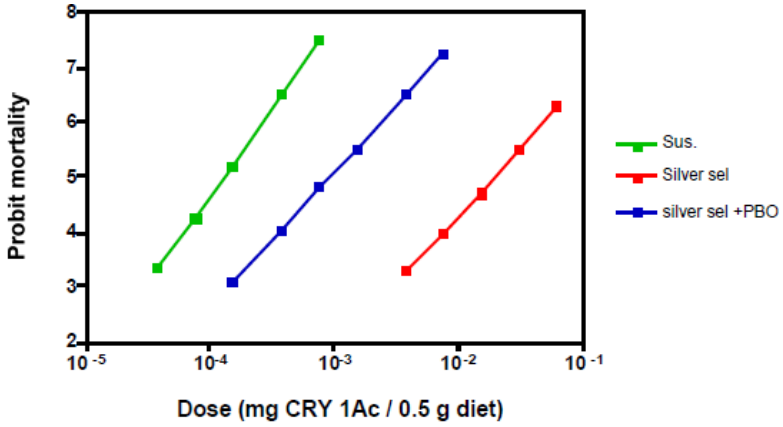

***Figure** 9 Dose response curves (diet incorporation bioassay), showing the synergistic effects of topically applied PBO (10µg) on Cry1Ac toxicity against resistant "silver sel" H. armigera.*

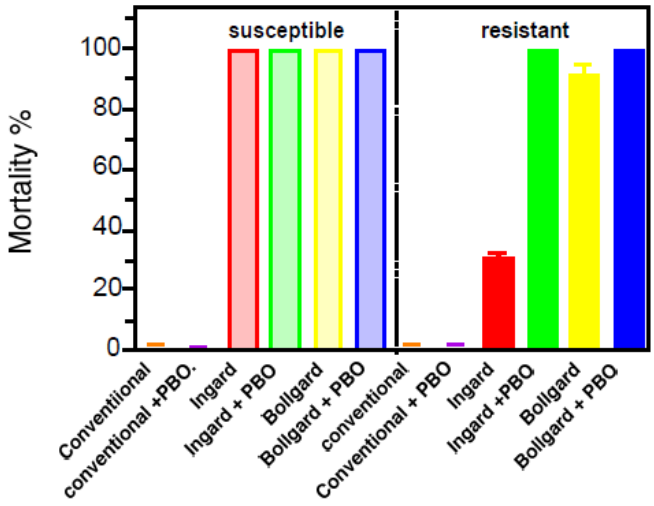

***Figure 9.** First instar leaf feeding bioassays using conventional, Ingard and Bollgard cottons, showing the synergistic effects of PBO against Cry1Ac resistant H. armigera. Error bars represent 95% confidence intervals.*

4.7 Cross- Resistance To Spinosad

In Australia, *H. armigera* resistance to insecticide groups as diverse as pyrethroids, spinosad and chlorfenapyr have been linked to sequestration by esterase iso-enzymes. This project has shown cross-resistance between Cry1Ac and spinosad *H. armigera* (Figure.10). Spinosad large, biological molecule, is used as a foliar spray against *H. armigera*. Spinosad has a history of resistance development in Australia, coincident with Cry1Ac resistance detected by Dr Ho Dang's Cry 1Ac resistance monitoring (Figure 11).

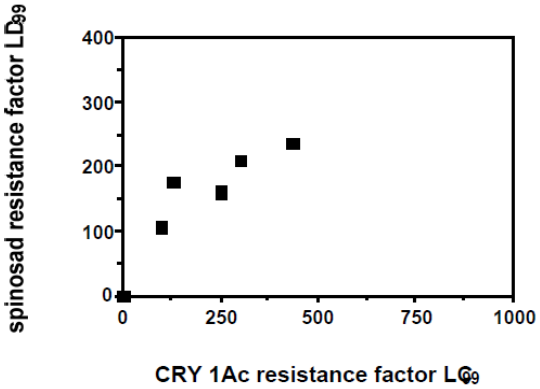

Figure 10. *Relationship between Cry1Ac and Spinosad resistance in Cry 1Ac resistant strains of third instar H. armigera*

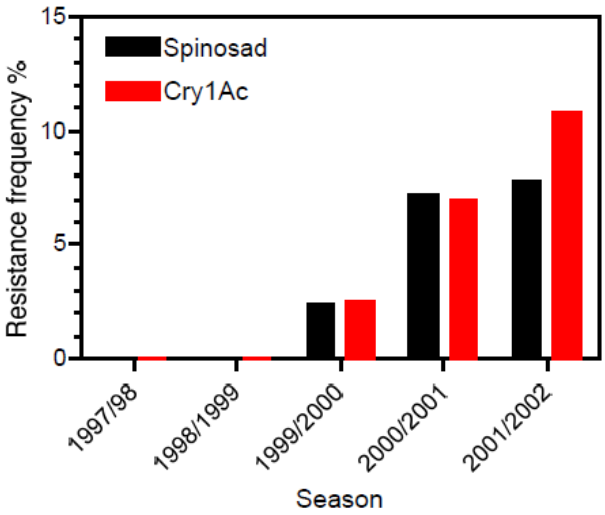

Figure 11 *Frequency of Spinosad and Cry1Ac (MVP®) resistance in cotton populations H. armigera larvae 1999 – 2002.*

4.8 Spinosad Resistance Mechanism

The cross-resistance between Cy1Ac and spinosad in *H. armigera*, is the result of a common resistance mechanism, sequestration by esterase. Esterase from Cry1Ac resistant *H. armigera* had a great affinity for binding to spinosad (Fig.12). Like Cry1Ac resistance, spinosad resistance in the silver sel. strain is suppressed by piperonyl butoxide (Fig 13).

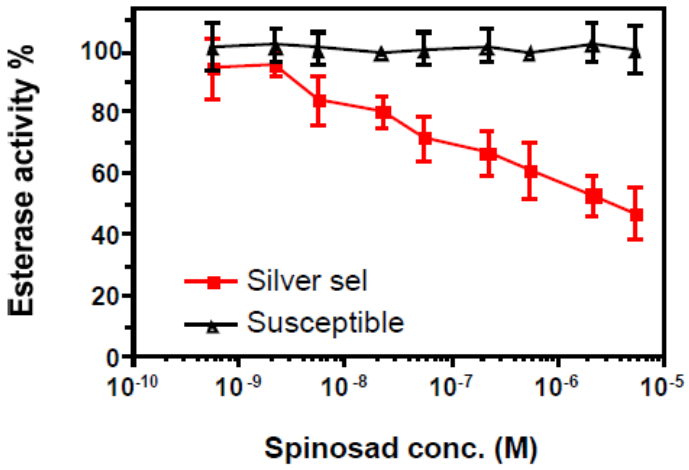

Figure 12. *Binding of spinosad to esterase from "silver selected" Cry1Ac resistant H. armigera*

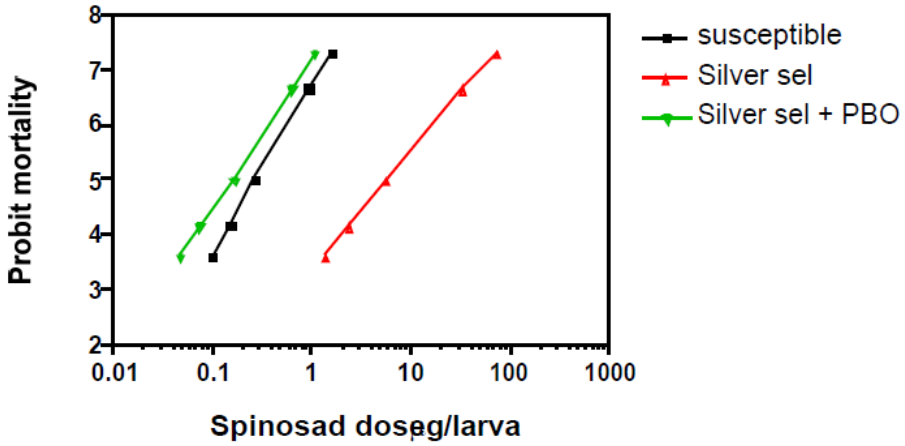

Figure 13. *Synergism of spinosad resistance by piperonyl butoxide in silver sel strain, Cry 1Ac resistant larvae (30 – 40 mg).*

4.9 Protease Action Of Esterase

There are previous reports of 'potentiation' of *Bt* by use of serine protease inhibitors and these could have been as a result of inhibition by esterases, since esterases often 'double' as serine proteases. Biamolecualr analysis data (Figure 12), with silver selected strain esterase, showed that protease inhibitor (Ala-Ala-Ala p-nitroanilide) could prevent the binding of esterase to Cry 1Ac, which may support the hypothesis that esterase is also acting as a protease against *Bt* toxins.

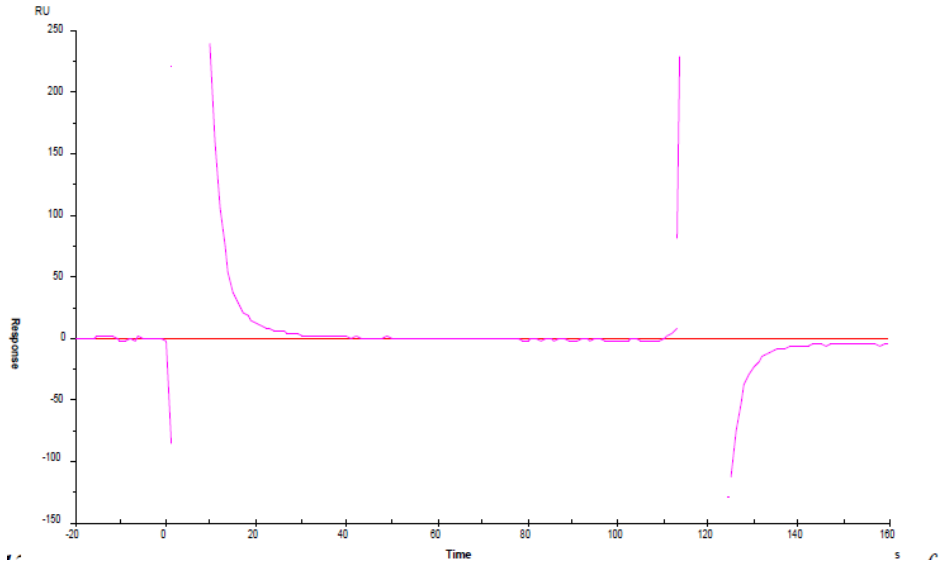

Figure 12. *Sensogram plot of association and dissociation curves of purified esterase, from Cry1Ac resistant silver sel. H. armigera to activated Cry1Ac toxin in the prewsence of protease inhibitor.*

Outcomes

- All objectives of this project were achieved. Given that some Cry1Ac resistant *H. armigera* are still present in cotton populations of *H. armigera*, there is some survival on Bollgard cotton and that resistance is linked to spinosad resistance, we consider that there is a considerable threat from the esterase resistance mechanism, to the future of Bollgard cotton in Australia. The threat may be exacerbated, by increasing registrations of spinosad for *H. armigera* control on other crops
- Our findings, that there has been a "crossing over" of resistance mechanisms between conventional chemistry and a toxin produced by Bollgard II cotton have serious implications for pest control and resistance management for insecticides and transgenics.
- The ability of PBO to "cross over" from synergising conventional insecticides to synergism of transgenic cotton against resistant pests represents a considerable breakthrough in the battle to effectively manage transgenic crops and to retain their efficacy against resistant insects.
- Studies of esterase binding to Cry 1Ac have used ground-breaking surface Plasmon resonance real-time bimolecular analysis techniques.

The research project addressed all three CRDC outputs (economic, social and environment), by providing the means to prolonging the life of Bollgard cotton. Resistance to transgenic cotton is one of the greatest threats to the sustainability of the cotton industry because insect pest control is a major cost factor. Effective resistance management in Bollgard will reduce the need for supplementary insecticide use, protecting natural resources, and the community.

Conclusion

Whilst in all insect species, there is some naturally occurring variation in susceptibility to insecticides, mere vigour tolerance can be discounted in the silver strain because of the ease of selection for higher levels of resistance. Had the observed resistance level (14-fold at the

LC_{50}) been the result of mere variation in susceptibility within susceptible strains, one mild selection with Cry1Ac would not have resulted in any resistance. The increase in resistance observed in the silver sel. strain, indicated that genetic resistance was already present in the silver strain and this was proven by the back-cross data. Conclusions are therefore, that silver strain H. armigera, bred from field survivors of Cry1Ac resistance monitoring, were clearly resistant to Cry1Ac toxin. Transgenic cotton feeding bioassay data, confirmed Cry1Ac resistance to Ingard cotton and further emphasises the potential field significance of the resistance.

Findings of esterase binding to Cry1Ac are very significant, because sequestration is recognised as a potential *Bt* toxin, resistance mechanism. Given the greatly increased esterase titre in the silver sel. strain, esterase could have the ability to bind to and thus detoxify considerable quantities of Cry1Ac. F_1 backcross data proved that extra esterase and ability of that esterase to bind to Cry1Ac was undoubtedly linked to resistance. Although no appreciable hydrolysis of the protein by resistant esterase may take place, the very large molar amounts of esterase in resistant *H. armigera* could be sufficient to sequester quantities the toxin, thus rendering it harmless before reaching the target-site. Suppression of resistance by an esterase inhibitor, PBO, supports the esterase resistance mechanism hypothesis.

In vivo data (Fig. 8) showed that esterase, in live first instar silver sel. strain *H. armigera*, binds to Cry1Ac and thus could be , of significance in the field. Esterase sequestration, resistance mechanism potentially, has great ecological significance because of the capacity for selection by transgenic cotton crops. Findings that resistant larvae apparently sequester and detoxify Cry1Ac toxin, whilst feeding on transgenic cotton, are highly significant because they also provide a direct, field mechanism for the observed resistance to Cry1Ac in silver strain *H. armigera*.

Previous mechanisms reported to confer resistance to *Bt* toxins are based on modifications to the receptor binding site , or alterations to the proteases that cleave the pro-toxin processing it into a smaller active toxin . The potential for *H. armigera* esterase to bind to and detoxify *Bt t*oxins, is of great concern, because esterase-based resistance mechanisms in insects are not uncommon. The vast over production of esterase in the silver sel. strain is not unique to Cry1Ac resistance, and is also causing cross-resistance to spinosad. However, given that Australian *H. armigera* have developed esterase based resistance mechanisms in the past, it is still unclear whether this esterase-mediated Cry1Ac resistance mechanism, is in any way connected with widespread use of other conventional insecticides. Cross-resistance studies and further experiments with other *Bt* resistant species, with widespread resistance to both chemical and *Bt* insecticides, may help clarify the situation.

Confirmation of Cry1Ac resistance in a strain of *H. armigera* derived from survivors of a field Cry1Ac resistance monitoring programme in Australia and findings of inherited increased esterase activity, which genetically segregates with the resistance and can sequester Cry1Ac, are important to the future of *Bt* crops. Of further concern, is the semi-domminant status of the resistance mechanism, which will make *H. armigera* resistance management on Bollgard II cotton more difficult. Survival on transgenic cottons further emphasises the field significance of resistance to Cry1Ac. Cry1Ac resistance will place additional selection pressure on the Cry2Ab toxin component of Bollgard II cotton. Given that *H. armigera* is a cosmopolitan pest of cotton and other crops, the finding of an esterase-mediated resistance

mechanism may pose a considerable threat to the future efficacy of *Bt* transgenic crops, world-wide.

The discovery that resistance to Cry1Ac can be overcome by an insecticide synergist, PBO, may have considerable implications for future control by *Bt*. The mechanism of PBO synergism of Cry1Ac appears to be a simple inhibition of the esterase iso-enzymes that are either sequestering and/or metabolising by other means, the Cry1Ac toxin. Whilst excellent Cry1Ac synergism with PBO (formulated as an emulsifiable concentrate) was achieved on transgenic cotton in the laboratory, in field conditions the lack of PBO photo-stability may present a limitation to prolonged synergistic effects. This could be overcome by using a microencapsulation of PBO to provide formulations that give continuous release of this synergist.

Extension Opportunities

Technological Advances

- The ability of PBO to "crossover" from synergising conventional insecticides to synergism of transgenic cotton against resistant *H. armigera* represents a considerable breakthrough in the battle to effectively manage transgenic crops and to retain their efficacy against resistant insects. However, there are no intellectual property implications.
- Studies of esterase binding to Cry 1Ac have used ground-breaking surface Plasmon resonance real-time bimolecular analysis techniques.

Dissemination Of Project Outcomes

Clearly, the area of biochemical resistance to *Bt* toxins in *H. armigera* is of great relevance to the future of transgenic cotton in Australia. An understanding of Cry toxin structure, with relationship to esterase binding is required so that future toxins may be selected to avoid this resistance mechanism. It is hoped that the information from this project will be eventually incorporated into the resistance management plan for transgenic cotton. There is, however, a certain reluctance in some sectors of the cotton industry, to accept the concept of *Bt* resistance at all and this is presenting a very considerable barrier to dissemination of project outcomes.

The project outcomes are progressively being published in scientific journals and presented at scientific conferences.

> Gunning, R. V. (2004) – Role of esterases in resistance to biological insecticides in *Helicoverpa armigera*. *International Congress of Entomology*, Brisbane, August, 2004.

Publications

Gunning, R. V., Dang, H. T., Kemp, F. C., Nicholson, I. C. and Moores, G. D. - New Resistance mechanism in *Helicoverpa armigera* threatens transgenic crops expressing *Bacillus thuringiensis* Cry1Ac toxin. *Applied and Environmental Microbiology*. **71**, 2558 – 2563.

A second publication, detailing the PBO synergism work will shortly be submitted to *Nature Biotechnology*. A publication on the spinosad cross-resistance work will follow thereafter

Impact on the Australian Cotton Industry

The results and conclusions of this research threaten the future of Bollgard cotton in Australia unless steps are taken to manage this resistance. Given the seriousness of the threat, the benefits of the project greatly out-weigh, the small costs involved.

Part 4 – Final Report Executive Summary

In Australia, the cotton bollworm, *Helicoverpa armigera,* has a long history of resistance to conventional insecticides, Transgenic cotton (expressing *Bt* toxin Cry1Ac) has been grown for *H. armigera* control since 1996.

This project demonstrated that the strain of *H. armigera*, which came from from the survivors of Dr Ho Dang's resistance monitoring programme, are resistant to Cry1Ac toxin (275 fold). Some 70% of resistant *H. armigera* were able to survive on Cry1Ac transgenic cotton (Ingard®), a small but significant proportion (5%) also survived on Bollgard II cotton. The resistance is inherited as a non sex-linked semi-domminant trait. Resistance was associated with elevated esterase iso-enzyme levels, which were inherited with resistance. Studies of esterase binding to Cry 1Ac by conventional enzymatic techniques and ground-breaking surface plasmon resonance real-time bimolecular analysis techniques showed that resistant strain esterase could bind to Cry1Ac pro-toxin and activated toxin. Studies with live, first instar larvae, showed that Cry1Ac resistant larvae, fed on Cy1Ac cotton or Cry1Ac treated artificial diet, had lower esterase activity than non-Cry1Ac fed larvae, thus giving direct mechanism for the selection of this esterase based resistance mechanism on transgenic cotton.

Cross-resistance studies in the Cry 1Ac resistant strain, showed that Cry 1Ac resistance was linked to spinosad resistance. Spinosad resistance in *H. armigera* is also due to esterase sequestration and thus both Cry 1Ac and spinosad have a common resistance mechanism in *H. armigera*. Both Cry 1Ac and spinosad esterase mediated resistances were suppressed by the insecticide synergist piperonyl butoxide.

Confirmation of Cry1Ac resistance in a strain of *H. armigera* derived from survivors of a field Cry1Ac resistance monitoring programme in Australia and findings of an esterase mediated resistance mechanism that can sequester Cry1Ac, are important to the future of *Bt* crops. Of further concern, is the semi-domminant status of the resistance mechanism, which will make *H. armigera* resistance management on Bollgard II cotton more difficult. Survival on transgenic cotton, further emphasises the field significance of resistance to Cry1Ac. Cry1Ac resistance will place additional selection pressure on the Cry2Ab toxin component of Bollgard II cotton. Given that *H. armigera* is a cosmopolitan pest of cotton and other crops, the finding of an esterase-mediated resistance mechanism may pose a considerable threat to the future efficacy of *Bt* transgenic crops, world-wide. The ability, however, of PBO to "crossover" from synergising conventional insecticides to synergism of transgenic cotton against resistant pests represents a considerable breakthrough in the battle to effectively manage transgenic crops and to retain their efficacy against resistant insects.