Mechanism and management of insecticide resistance in Australian diamondback moth

Greg Baker
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Project Number: VG08062

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Statement about the purpose of the report
This report details the research and extension undertaken in Project VG08062 on insecticide resistance in diamondback moth in Australian Brassica vegetable crops. Main findings, industry outcomes and recommendations along with suggested areas of future research are discussed.

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2. MEDIA SUMMARY

Diamondback moth (DBM) (*Plutella xylostella* L.) is the main pest of Brassica vegetable crops in Australia, and has international notoriety for rapidly acquiring insecticide tolerance, which then leads to field control failures. DBM resistance to older insecticide classes is widespread in Australia; hence the choices for DBM control are increasingly limited to several newer synthetic pesticides and *Bacillus thuringiensis* products.

In this project we have demonstrated that some field populations of diamondback moth have now developed significant levels of resistance to two important groups of these newer insecticides (IRAC Groups 6 (emamectin benzoate) and 28 (chlorantraniliprole and flubendiamide), and that these resistance levels are already capable of reducing the field control achieved with these Group 6 and 28 insecticides.

We have also demonstrated that high levels of resistance can result in the laboratory from repeated exposure to low concentrations of synthetic (eg. Group 6 insecticide emamectin benzoate) or microbial (eg. *Bacillus thuringiensis* var. *kurstaki*) insecticide. In this selection study we found that in the early stages of tolerance acquisition a novel inducible tolerance mechanism was involved. As the selection process with the synthetic insecticide progressed, a combination of induced metabolic tolerance and genetic mutation(s) were found to contribute to the resistance. Studies are continuing to identify the molecular basis of this resistance, and to determine the full implications of the inducible tolerance process to resistance management.

Further, the DBM lab strain which has acquired high resistance to emamectin benzoate is exhibiting cross-resistance to Group 28 diamides insecticides. Studies are underway to determine if the resistance mechanism(s) in this lab-selected strain is/are the same as those evolving in DBM field populations in response to growers' use of emamectin benzoate. If this is shown to be the case, then cross resistance between Group 6 and 28 insecticides in the field is likely, and the 'two-window' resistance management strategy will need to be revised in the light of such findings.

In summary, in the near future the Australian Brassica vegetable industry is likely to experience declining efficacy with a number of important synthetic insecticides (most notably Group 6 and 28 insecticides). More extensive studies of field strains from all major production areas are required to ascertain the immediacy and magnitude of this threat, and new non-insecticidal technologies for DBM management in Australian Brassica vegetables must be developed to provide sustainable crop production practice.

3. TECHNICAL SUMMARY

3.1. The Problem

Diamondback moth (DBM) (*Plutella xylostella* L.) is the main pest of Australian Brassica vegetable crops. It can rapidly acquire insecticide resistance, which leads to control failures. Insecticides are the only tool presently available to control this pest. DBM resistance to older insecticides is widespread, and choices for DBM control are increasingly limited to several newer synthetic pesticides and *Bacillus thuringiensis* (*Bt*) products.

This project was undertaken to better equip the Australian Brassica industry with knowledge of the current state of resistance levels in DBM field strains, and of the mechanistic processes that lead to the development of resistance, so that through this knowledge better insecticide resistance management (IRM) strategies may be devised.

3.2. The Project science

We have bioassay benchmarked resistance levels in field strains of DBM to emamectin benzoate, indoxacarb, spinosad (now replaced in the Australian market by the closely-related spinetoram), chlorantraniliprole and *Bt* var. *kurstaki* (BtK). We also investigated the role of inducible tolerance mechanisms in the development of resistance, the levels of tolerance DBM can potentially acquire through repeated exposure to low doses of these five toxicants, and whether cross-resistance occurs between any of the newer DBM insecticides (including the IRAC Group-28 insecticides which first became registered in Australia in 2009), and ways to overcome the emergence of high levels of tolerance.

3.3. The Key Research Findings, Extension Highlights and Industry Outcomes

Field surveys

Field strains of DBM have acquired significant tolerance to several of the 'newer' DBM insecticides. In particular, Group 6 and 28 tolerance is at levels which are likely to impair field control with these insecticides in some QLD properties, and similar tolerance levels have been detected in southern Australian properties.

Inducible Tolerance mechanism

In the lab insecticidal tolerance in DBM larvae was induced by repeated exposure to low levels of toxins. The induced insects showed significant tolerance without initially displaying mutational changes in a major resistance gene locus. The novel inducible tolerance was transmitted to offspring by an epigenetic mechanism showing a maternal effect. As selection progressed the epigenetic contribution to the tolerance declined. Fitness costs, associated earlier on with the elevated tolerance, also declined as the selection process continued.

Continuous laboratory selection

Lab selection with low doses of emamectin benzoate and BtK produced major increases in DBM tolerance to each toxicant. The 271 fold emamectin benzoate resistance achieved late in the study

may be due to a recent genetic mutation. By contrast, the laboratory selection with low doses of spinosad and indoxacarb did not significantly increase DBM tolerance.

Cross tolerance relationships

Cross tolerance relationships between the five tested insecticides were established. Significant cross-resistance was conferred to the Group-28 insecticides at an advanced stage in lab selection with emamectin benzoate. If the same mechanism is involved in field-selected DBM, this would indicate a significant cross resistance threat from field use of emamectin benzoate and Group-28 insecticides, and require modification of the National Insecticide Resistance Management (IRM) strategy for southern Australia to place both insecticide groups in the same window.

Industry Outcomes

This project has highlighted the imminent threat to the Australia Brassica vegetable industry posed by the increasing levels of resistance to several of the newer DBM insecticides.

It has identified a novel inducible tolerance mechanism that operates early in the process of selection by a toxicant, and shown that as the selection process progresses a combination of induced metabolic tolerance and genetic mutation(s) contribute to the resistance. Studies are continuing to determine the full implications of the inducible tolerance process to IRM.

The study has identified cross-tolerance relationships for a number of these DBM insecticides, which are important for the design of an effective resistance management rotation strategy.

3.4. Recommendations

1. The project findings, particularly the imminent risk of impaired DBM control with several of the newer DBM insecticides, needs to be communicated to growers, along with advice on IRM practices.

2. If the emamectin benzoate resistance mechanism(s) in the laboratory and field selected strains are the same, modify the DBM '2-window' IRM strategy for southern States (in consultation with CropLife Australia) to take account of the cross-resistance risk for Group 6 and 28 insecticides.

3. Conduct resistance surveys to determine the imminence of the threat to the suite of 'newer' DBM insecticides in southern Australian vegetable crops. This information will inform growers of their current risk , encourage more spray-conserving IPM practice, and provide industry investors with a time frame for the development and phase-in of non-insecticidal management systems.

4. Develop non-insecticidal systems (eg. mating disruption, lure and kill, inundative release of biocontrol agents) for DBM management to provide the industry with the necessary tools for production continuity when the current insecticide arsenal fails, and to position this industry as a market leader in sustainable crop production practice.

4. INTRODUCTION

Resistance in Australian populations of diamondback moth (DBM) to older insecticide classes (eg. synthetic pyrethroids and organophosphates) is widespread and well-documented (Baker and Kovaliski, 1999; Endersby *et al.*, 2004). As a result, the choice of insecticides for the effective control of DBM is increasingly limited to newer synthetic pesticides, such as emamectin benzoate, indoxacarb and spinosad, and Bacillus thuringiensis (Bt) formulations. To help conserve the newer synthetic insecticides a two-window IRM rotation strategy has been actively promoted nationally to the Brassica vegetable industry since 1998-99, but a 2006 national survey revealed that only 35-40% of Brassica vegetable growers are presently following this strategy (Baker *et ai.* 2007). Further, in the same survey it was revealed that 58% of insecticidal sprays currently applied to Australian Brassica crops are either Bt, spinosad, indoxacarb or emamectin benzoate.

Meanwhile, in the Australian canola industry the frequency and severity of losses from DBM outbreaks has markedly increased in the past decade, as warmer, drier weather conditions have favoured the pest. Due to widespread synthetic pyrethroid resistance and resultant spray failures, the use of Bt and off-label use of the newer synthetic DBM pesticides in this industry sector is increasing markedly.

Collectively these developments are placing the effective life of the newer synthetic and bio-pesticides for DBM control under increasing threat from resistance in all Australian Brassica crops. When high-level, widespread DBM resistance last occurred in the early to mid 1990's, the costs incurred by many Brassica vegetable growers due to crop losses, escalating spray costs, additional replant costs and failure to meet supply contracts were dramatic and severe.

In recognition of this threat, and the fact that DBM field resistance to spinosad (Nb. During this project spinosad was replaced in the Australian market by the closely-related spinetoram), indoxacarb and Bt has been documented from a number of countries in Asia and North and Central America (Sarfaz and Keddie, 2005; Sayyed *et ai.*, 2004; Sayyed *et ai.*, 2005; Zhao *et al.* 2006), an insecticide resistance survey was undertaken by Greg Baker in 2006-07 (HAL Project VG04004). This survey revealed an emerging threat by Australian DBM to become tolerant to several of these newer synthetic pesticides. Amongst nine DBM strains collected from commercial vegetable crops in QLD, NSW and SA, nine, four and eight had significantly reduced susceptibility to emamectin benzoate, indoxacarb and spinosad respectively, and in the case of emamectin benzoate four of the nine strains were nearing the point where field performance might be impaired. Since the conclusion of VG04004, the VG08062 project team has further challenged these emamectin benzoate tolerant strains in the laboratory over 2-5 generations with a single dose of emamectin benzoate per generation, and their Resistance Ratio has been substantially increased (eg. from 18 to 140 fold). More importantly, these lab-cultured DBM show inducible tolerance, in other words, in the presence of repeated low doses of the pesticide the insects have developed tolerance to the pesticide in the absence of target site mutations.

In VG08062 we therefore proposed to:

(i) investigate whether the newly-identified tolerance to these newer DBM insecticides is, as suspected, based on inducible tolerance mechanisms;
(ii) determine what levels of tolerance DBM can potentially acquire through continuous exposure to high selection pressure;
(iii) determine whether any cross-tolerance between these three synthetic pesticides and Bt occurs, and finally;
(iv) investigate strategies to overcome the emergence of high levels of tolerance, and devise a theoretically-sound IRM strategy.

In summary, by determining the nature of the resistance mechanism(s) involved in the development of DBM tolerance to these insecticides and applying this understanding to the development of an effective IRM strategy, this project will extend the effective life of DBM insecticides and hence mitigate against the impacts of widespread resistance at a time when the pressure exerted by DBM populations on Australian Brassica production is increasing.

A critical impediment will be the inertia amongst a sector of the industry to embrace proactive IRM strategies. However, given our current understanding of DBM population gene-flows between commercial Brassica properties, in most instances individual growers that do practice these IRM measures will benefit from doing so irrespective of any non-conformance by neighbours.

5. MATERIALS AND METHODS

5.1. Benchmarking insecticide resistance levels of DBM in Brassica vegetable crops.

The DBM cultures:

Waite Susceptible Strain (WSS)

A susceptible laboratory population of *P. xylostella* (Waite Susceptible Strain, WSS) has been maintained on seedling cabbage, *Brassica oleracea* L. variety *capitata* 'Green Coronet' leaves in the laboratory at 25 ±0.5^{0}C and a photoperiod of 14:10 (L:D) h in a separately caged laboratory culture at the Waite Campus, South Australia, without exposure to any insecticides for ≈ 21 years (≈ 240 generations). Cabbage leaves were added periodically, when required for the developing larvae. A thin layer of honey on masking tape and 10% honey solution with 0.1% sorbic acid were provided as food source for the adults. This Waite Susceptible Strain (WSS) was used as the reference strain for comparison with field-collected populations and in genetic crosses.

The Field Strains

In September 2008 we received 10 *P. xylostella* strains (collected by David Carey and his QLD DPI team) from commercial Brassica vegetable crops at Lowood, Lynford, Glenore Grove, Moreton Vale, Lake Clarendon, Gatton East, Gatton West, Granthem, Mt Sylvia I and Mt Sylvia II in SE QLD. In 2009, several *P. xylostella* strains were collected from the same region in SE QLD, and in 2010, two strains were collected from Werombi, NSW. However, throughout the collection period *P. xylostella* field densities were generally low, and the numbers of DBM collected in a number of instances were quite low. We therefore combined several of the field strains according to geographic proximity. In 2011 we received 10 *P. xylostella* strains (collected by David Carey and his QLD DPI team) from commercial Brassica vegetable crops at Lowood, Lynford, Glenore Grove, Moreton Vale, Lake Clarendon, Gatton East, Gatton West, Grantham, Mt Sylvia I and Mt Sylvia II in SE QLD, and also collected a strain from a commercial Brusssels sprouts crop in the Adelaide Hills, SA. In 2012 we received a further six strains from the Lockyer Valley, QLD, one from Werribee, VIC and one from Virginia, SA, all collected from commercial Brassica vegetable crops.

Each of these field strains was reared in a separate cage using the same methods as described for the WSS strain.

The Bioassays:

Bioassays were performed on cabbage leaf discs cut from washed cabbage leaves (90 mm diameter) taken from eight-week-old plants grown in an insect-free glasshouse. The leaf discs were embedded into setting agar in a 90 mm diameter petri-dish with the underside of the leaf facing upwards. Before each large-scale bioassay, a preliminary assay was conducted using a broad range of concentrations of the insecticide to determine the appropriate concentration for

the formal assay. Each bioassay included 10 concentrations at the ratio of either 1:2 or 1:2.5 plus a control (milliQ water), with at least four leaf disks for each concentration. The insecticide solutions were made up in milliQ water to specific concentrations in 100 ml volumetric flasks.

A precise deposit of the test insecticide was administered using a Potter Spray Tower (PST). Ten third instar larvae were placed on each leaf disc, and then each petri dish was sprayed with a 4 ml aliquot of the test solution. Once removed from the PST the dishes were covered with plastic film that was secured with a rubber band (Super^{TM} band). About 100 to 150 fine holes were then punched into the plastic film using a micro needle to allow air exchange. The PST was calibrated before and after each trial allotment, and triple rinsed with AR Acetone and milliQ water between each change in treatment. The treated petri dishes were placed into an incubator at 25±0.5°C (14/10h, L/D photoperiod) with the efficacy of the treatments assessed at defined time period for each toxin.

Full dose-response bioassays using the WSS were conducted for each toxin. Probit analyses using PoloPlus software were performed and discriminating doses (DD) was estimated. The DD was the rounded-upwards value of the dose that killed 99.0% (LC_{99}) of WSS 3rd instar larvae.

The F_1 offspring (3rd instar larvae) from each field collected population were then screened against commercial formulations of emamectin benzoate, spinosad (Nb. During this project spinosad was replaced in the Australian market by the closely-related spinetoram), indoxacarb and BtK toxins at the DD, and in the case of the strains collected in 2008 and 2010 also screened at the 5xDD and 10xDD. The strains collected in 2011 and 2012 were also screened against a formulation of chlorantraniliprole at the DD.

Eighteen, eleven, nine, twelve, one and one of these field strains (or composite strains) were then assessed with full dose response bioassays against emamectin benzoate, spinosad, indoxacarb, chlorantraniliprole, flubendiamide and cyantraniliprole respectively. The results of BtK full dose response bioassays with 11 field strains collected from QLD, NSW and SA in 2007 are also included in this report.

Emamectin benzoate[1], spinosad[2], indoxacarb[3], chlorantraniliprole[3], flubendiamide[4], cyantraniliprole[3] and BtK[5] used for bioassays and laboratory selections were commercial formulations. These were supplied by Syngenta Crop Protection[1], Dow AgroSciences Australia Ltd[2], DuPont Australia Ltd[3], Bayer CropScience[4] and Sumitomo Chemical Australia Pty Ltd[5].

Bioassay Analyses:

Mortality data were analyzed using POLO-PC program (LeOra-software 1997) for probit analysis to estimate the lethal dose concentrations (LCs). Mortality was corrected using Abbott's formula (Abbott 1925) for each probit analysis. Differences in susceptibility were considered significant when the 95% CL of LC_{99} values did not overlap. The resistance ratio (RR) was expressed as the ratio of the LC_{50} and LC_{99} values of the relevant sample to that of the WSS. Developmental penalty and melanisation were analysed using Student *t* test paired analyses (SigmaPlot 10; Systat Software, Inc.).

Field Experiment:

Given the observed level of tolerance to emamectin benzoate and chlorantraniliprole in many of the tested field strains in the laboratory bioassays, a practical question arose as to whether these observed tolerance levels were sufficient to adversely affect the level of control achieved in field crops using these two insecticides at their registered field rates. To answer this question and to determine whether the observed cross-tolerance conferred to chlorantraniliprole by the lab-selected emamectin benzoate strain would affect the level of field control achieved by the registered rate of chlorantraniliprole when applied against the lab-selected emamectin benzoate strain, we devised the following experiment.

Plots in a section of an eight week old Brussels sprouts crop (Cranwell and Sons) at Nairne, SA were sprayed on 8 December 2011 with the registered rate of (i) Proclaim^{TM} (300 g ha^{-1} of product containing 44 g kg^{-1} emamectin benzoate), (ii) Coragen^{TM} (100 ml ha^{-1} of product containing 200 g L^{-1} chlorantraniliprole) and (iii) water control. The treatments were applied at 700L ha^{-1} water volume with the grower's air-assisted boom sprayer, and with 0.01% Agral^{TM} wetting agent and 2.0% TopLine Yellow Fluorescent Dye (the latter to allow selection of leaves with similar, uniform spray coverage. At 2 hours, 3 days and 7 days after application 60 leaves with similar, uniform dye coverage were chosen from each treated plot and transported to the Waite Campus.

In the laboratory a leaf disc was then cut from each leaf and embedded (lower surface upward) in agar in a petri dish as described in the Bioassay methods above. Five 3rd instar DBM larvae from each of three strains ((i) Waite Susceptible Strain, (ii) emamectin benzoate lab-selected strain, generation F_{53}, (iii) composite strain of several 2011 Lockyer Valley, QLD field strains exhibiting emamectin benzoate tolerance) were then enclosed on each petri dish (20 per treatment). The larval mortality was assessed at 72 hours after the larvae were enclosed.

5.2. Investigation of a novel inducible insecticide tolerance mechanism in DBM.

The same culturing, bioassay and Probit analysis methods were used as described in the methods of the Objective I section.

To examine whether the observed fluctuations in insecticidal tolerance are due to metabolic or immune-related induction processes, we measured phenoloxidase activity (melanization) in the hemolymph as an indicator for the larval immune status. Hemolymph was extracted from 20 late 3rd instar larvae by cutting off a foreleg and bleeding each larva directly into 1.5 ml ice–cold phosphate-buffered saline (PBS). The extracted hemolymph was centrifuged for 5 min at 3000g and the cell-free supernatant (plasma) transferred to a cuvette. The absorbance was first measured at 280nm to determine the relative protein concentration, and then the absorbance at 490nm was recorded every minute for 90 min on a Varian DMS 100s spectrophotometer. The rate of the melanization reaction [as the slope of the plot of absorbance against time (arbitrary units)] was used to measure metabolic or immune-related induction in larval insects.

The methods are reported in greater detail in Appendix 2 (2010 Journal of Economic Entomology paper).

5.3. Investigation of acquired insecticidal tolerance through continuous laboratory selection.

A composite of several QLD 2006-07 strains showing reduced susceptibility to emamectin benzoate and BtK toxins was exposed each generation to a sub-lethal concentration of emamectin benzoate (0.022 mg ai L^{-1}, ~1/1000th of registered rate) and BtK formulation (25 µg/ml, ~1/40th of registered rate), and maintained at the same level for five and seven generations respectively (Figure 2). The emamectin benzoate concentration was doubled to 0.044 mg ai L^{-1} at F_6 and to 0.088 mg ai L^{-1} at F_{13}. The concentration of the commercial formulation of BtK (Dipel^{TM}) was doubled to 50µg/ml at F_8 and to 100µg/ml at F_{22}. Emamectin benzoate and BtK tolerant populations are currently at generation 46 (F_{46}) and generation 47 (F_{47}), and are maintained on 0.088 mg ai L^{-1} and 100 mgL^{-1} of emamectin benzoate and BtK toxins respectively (Figure 5).

Similarly, in 2010 we commenced laboratory selection of two DBM strains (each established from a a composite strain collected in 2009 in the Lockyer Valley, QLD and Werombi, NSW) with low concentrations of spinosad (0.06 mg ai L^{-1}, ~1/1000th of registered Success^{TM} rate) and indoxacarb (0.3 mg ai kg^{-1}, ~3/1000th of registered Avatar^{TM} rate).

During the course of the incremental laboratory selections of emamectin benzoate and BtK toxins in field-derived *P. xylostella* populations, we performed the following three routine experiments:

(1) full dose-response bioassays of tolerant strains for every five generation or so,

(2) reciprocal genetic crosses and full dose-response bioassays for reciprocal offspring, and

(3) measurement of genetic and physiological traits, such as developmental penalty. WSS was used to estimate the acquired tolerant level.

The reciprocal genetic crosses (T♀xS♂ and S♀xT♂): These were performed between WS and tolerant strains, and the tolerance levels in their offspring analysed to establish whether the acquisition of tolerance from the continuous incremental selection pressure was caused by inducible immune and metabolic tolerance with an epigenetic transmission, or by resistance alleles that pre-existed in the field populations in low frequencies and which were increased in selected populations.

About 50 pupae each from WS (S♀xS♂), and tolerant strains (T♀xT♂) were sexed. The pupae were kept individually in 45-well plastic trays covered with thin Mylar® film with 10-15 fine holes for air exchange. After adult emergence, for T♀xS♂, 20-25 tolerant females (T♀) were placed with similar number of susceptible males (S♂), and for S♀xT♂, susceptible females (S♀) were placed with tolerant males (T♂) and reared them on toxin-free young cabbages. Full-dose response bioassays of F_1 neonate offspring for each crossing were performed following the methodology mentioned earlier and the tolerance was compared with that of the WS (S♀xS♂) and respective tolerant (T♀xT♂) strains.

5.4. Investigation of cross-tolerance patterns between the synthetic insecticides and BtK.

The same rearing and bioassay methods were used as described in section 5.1 above.

6. RESULTS

6.1 Benchmarking Study.

The project originally proposed to benchmark resistance levels in Brassica vegetable field populations of *P. xylostella* to three synthetic insecticides - emamectin benzoate, spinosad and indoxacarb, and the microbial insecticide *Bacillus thuringiensis* subsp. *kurstaki* (BtK). However during the course of the project a new group of synthetic insecticides, the Group-28 diamides, were introduced into Australia for the control of caterpillar pests in Brassica vegetable crops. Hence the benchmarking study was expanded to include these new Group-28 chemistries, namely chlorantraniliprole, flubendiamide and cyantraniliprole.

Discriminating Dose Bioassays:

The discriminating dose results are presented in Table 1(a-d). We consider that less than 95% mortality at the DD indicates a significant decline in susceptibility to the test toxicant has occurred. As the DD mortality response declines, this indicates that the loss of susceptibility is moving from incipience along a gradient towards potential field failure.

Emamectin benzoate

Our results demonstrate that for 28 of the 29 strains that were bioassayed with the DD of emamectin benzoate the mortality response was less than 95%, and for 12 of these 29 strains the mortality response was less than 75% (Table 1a). These bioassays recorded significant declines in emamectin benzoate susceptibility in DBM collected from all four States surveyed (QLD, NSW, VIC and SA), but the greatest shifts were observed in strains from QLD and VIC. The lowest mortality response to the emamectin DD (38.1%) was recorded with a 2011 QLD strain. However there was no overall decline in susceptibility to emamectin benzoate apparent during the years of this study; for example, for the QLD strains collected in 2008, 2009, 2011 and 2012 the mean % mortalities recorded at the DD were 70.7%, 41.5%, 74.3% and 74.9% respectively.

For the nine strains tested in 2008-10 with the 5xDD and 10xDD of emamectin, significant survivorship occurred in five and two strains respectively. Clearly such survivorship at these higher doses is concerning.

Spinosad

For 17 of the 29 strains that were bioassayed with the DD of spinosad the mortality response was less than 95%, and for nine of these 29 strains the mortality response was less than 75% (Table 1b). Significant shifts in spinosad susceptibility were observed in DBM strains collected from all four States, with the lowest mortality response recorded being 39.0% for a 2009 combined QLD strain. Strangely all of the 2011 strains that were tested (ten from QLD and one from SA) had 100% mortality at the DD, which is unexpected given the results from the other years (2006 as reported in VG07030, and 2008, 2009, 2010 and 2012 in this study). We consider that this is more likely to be an experimental artefact rather than a real measure of the susceptibility of these strains, but are unaware of any specific methodological mistake having occurred. Discounting the 2011 data-set, there was a small decline in susceptibility to spinosad apparent during the

years of this study; for example, for the QLD strains collected in 2008, 2009 and 2012 the mean % mortalities recorded at the DD were 76.9%, 39.0% and 70.5% respectively.

As previously mentioned in this report, during the life of this project spinetoram replaced spinosad as the active ingredient in the Dow Agrosciences Success™ foliar spray formulation for use in Australian horticulture. Spinetoram and spinosad are very closely related chemical structures, and presumed to exhibit cross resistance.

Indoxacarb

For 12 of the 27 strains that were bioassayed with the DD of indoxacarb the mortality response was less than 95%, and for five of these 27 strains the mortality response was less than 75% (Table 1c). Significant shifts in indoxacarb susceptibility were observed in DBM strains collected from all four States, with the lowest mortality response recorded being 61.9% for a 2010 NSW strain. As reported for the 2011 spinosad results, six of the eight strains that were tested with indoxacarb in 2012 (five from QLD and one from SA) had 100% mortality at the DD, which is unexpected given the results from the other years (2006 as reported in VG07030, and 2008, 2009, 2010 and 2011 in this study). Once again we are unaware of any specific methodological mistake having occurred. Discounting the 2012 data-set, there was no apparent decline in susceptibility to indoxacarb during the years of this study; for example, for the QLD strains collected in 2008, 2009 and 2011, the mean % mortalities recorded at the DD were 90.9%, 77.5% and 88.8% respectively.

BtK

For only one of the 21 strains that were bioassayed with the DD of BtK was the mortality response less than 95% (Strain QLD 2008 (f), 93.5% mortality) (Table 1c). These results indicate that, in contrast to the tested synthetic insecticides, there has been no apparent change in susceptibility of the surveyed DBM field strains to the biopesticide BtK.

Chlorantraniliprole

The new Group-28 insecticides were not included in the original project specification, but following reports of the early onset of resistance in DBM populations in various South-East Asian countries to the recently-registered Group-28 insecticides, we decided to expand our screening program in 2011 to include chlorantraniliprole, the active in two or the three Group-28 products registered in Australian Brassica vegetable crops.

For 13 of the 19 strains (from QLD, VIC and SA) that were bioassayed with the DD of chlorantraniliprole the mortality response was less than 95%, and for five of these 19 strains the mortality response was less than 75% (Table 1d). Significant shifts in chlorantraniliprole susceptibility were observed in DBM strains collected from QLD and VIC, but not from the two SA properties. The lowest mortality response recorded was 65.0% for a 2012 VIC strain. The observed level of decline in chlorantraniliprole susceptibility in these DBM strains only four years after the first registration of Group-28 chemistry in Australia is cause for great concern for the longer-term field efficacy of this new, valuable, IPM-compatible insecticide group.

Table 1a. The percentage mortality of F_1 3rd instar *P. xylostella* larvae from field-collected populations during 2008-2012 tested against a commercial formulation of emamectin benzoate at the DD, 5 x DD and 10 x DD. The DD is the rounded-upwards value for the LC_{99} of the WSS strain.

Chemicals	Strain	% larval mortality		
		DD	5 x DD	10 x DD
Emamectin benzoate	QLD 2008 (a)	92.5	100	100
	QLD 2008 (b)	70.0	90.2	100
	QLD 2008 (c)	88.1	100	100
	QLD 2008 (d)	84.3	89.5	100
	QLD 2008 (e)	72.3	92.5	100
	QLD 2008 (f)	95.0	100	100
	QLD 2008 (g)	82.4	100	100
	Combined QLD strain 2009	41.5	90.9	90.2
	NSW 2010 (a)	85.0	87.5	92.5
	NSW 2010 (b)	87.5	100	100
	SA 2011	85.4	-	-
	QLD 2011 (h)	75.0	-	-
	QLD 2011 (d2)	66.4	-	-
	QLD 2011 (d1)	80.5	-	-
	QLD 2011 (c)	38.1	-	-
	QLD 2011 (b1)	72.5	-	-
	QLD 2011 (b2)	62.2	-	-
	QLD 2011 (e)	81.9	-	-
	QLD 2011 (f)	80.0	-	-
	QLD 2011 (i)	94.9	-	-
	QLD 2011 (j)	91.7	-	-
	QLD 2012 (d1)	89.1	-	-
	QLD 2012 (d2)	72.5	-	-
	QLD 2012 (k)	70.3	-	-
	QLD 2012 (b2)	65.3	-	-
	QLD 2012 (c)	67	-	-
	QLD 2012 (b1)	85	-	-
	SA 2012	82.9	-	-
	Vic 2012	50.0	-	-

Table 1b. The percentage mortality of F_1 3rd instar *P. xylostella* larvae from field-collected populations during 2008-2012 tested against a commercial formulation of spinosad at the DD, 5 x DD and 10 x DD. The DD is the rounded-upwards value for the LC_{99} of the WSS strain.

Chemicals	Strain	% larval mortality		
		DD	5 x DD	10 x DD
Spinosad	QLD 2008 (a)	72.5	95.0	100
	QLD 2008 (b)	57.5	97.5	100
	QLD 2008 (c)	90.5	100	100
	QLD 2008 (d)	85.0	100	100
	QLD 2008 (e)	60.0	100	100
	QLD 2008 (f)	85.0	100	100
	QLD 2008 (g)	87.5	100	100
	Combined QLD strain 2009	39.0	100	100
	NSW 2010 (a)	57.5	97.5	100
	NSW 2010 (b)	95.0	100	100
	SA 2011	100	-	-
	QLD 2011 (h)	100	-	-
	QLD 2011 (d2)	100	-	-
	QLD 2011 (d1)	100	-	-
	QLD 2011 (c)	100	-	-
	QLD 2011 (b1)	100	-	-
	QLD 2011 (b2)	100	-	-
	QLD 2011 (e)	100	-	-
	QLD 2011 (f)	100	-	-
	QLD 2011 (i)	100	-	-
	QLD 2011 (j)	100	-	-
	QLD 2012 (d1)	90	-	-
	QLD 2012 (d2)	57.9	-	-
	QLD 2012 (k)	46.2	-	-
	QLD 2012 (b2)	72.5	-	-
	QLD 2012 (c)	73.8	-	-
	QLD 2012 (b1)	82.5	-	-
	SA 2012	83.3	-	-
	Vic 2012	77.5	-	-
	QLD 2011 (e)	100	-	-
	QLD 2011 (bf)	100	-	-
	QLD 2011 (i)	100	-	-
	QLD 2011 (j)	100	-	-

Table 1c. The percentage mortality of F_1 3rd instar *P. xylostella* larvae from field-collected populations during 2008-2012 tested against commercial formulations of indoxacarb and BtK toxins (Dipel®) at the DD, 5 x DD and 10 x DD of the respective toxicant. For each toxicant, the DD is the rounded-upwards value for the LC_{99} of the WSS strain.

Chemicals	Strain	% larval mortality		
		DD	5 x DD	10 x DD
Indoxacarb	QLD 2008 (a)	95.0	100	100
	QLD 2008 (b)	76.3	100	100
	QLD 2008 (c)	94.8	100	100
	QLD 2008 (d)	100	100	100
	QLD 2008 (e)	87.5	100	100
	QLD 2008 (f)	87.5	100	100
	QLD 2008 (g)	95.1	100	100
	Combined QLD strain 2009	77.5	93.0	100
	NSW 2010 (a)	72.5	100	100
	NSW 2010 (b)	61.9	85.0	100
	SA 2011	82.9	-	-
	QLD 2011 (h)	100	-	-
	QLD 2011 (d2)	72.2	-	-
	QLD 2011 (d1)	69.4	-	-
	QLD 2011 (c)	86.1	-	-
	QLD 2011 (b1)	100	-	-
	QLD 2011 (b2)	100	-	-
	QLD 2011 (e)	96.7	-	-
	QLD 2011 (f)	72.2	-	-
	QLD 2011 (i)	96.7	-	-
	QLD 2011 (j)	95.1	-	-
	QLD 2012 (d1)	100	-	-
	QLD 2012 (d2)	100	-	-
	QLD 2012 (k)	100	-	-
	QLD 2012 (b2)	100	-	-
	QLD 2012 (c)	95.1	-	-
	QLD 2012 (b1)	100	-	-
	SA 2012	100	-	-
	Vic 2012	92.5	-	-
BtK	QLD 2008 (a)	100	100	100
	QLD 2008 (b)	100	100	100
	QLD 2008 (c)	97.5	100	100
	QLD 2008 (d)	97.6	100	100
	QLD 2008 (e)	95.3	100	100
	QLD 2008 (f)	95.0	100	100
	QLD 2008 (g)	93.5	100	100
	Combined QLD strain 2009	97.6	100	100
	NSW 2010 (a)	97.5	100	100
	NSW 2010 (b)	100	100	100
	SA 2011	100	-	-
	QLD 2011 (h)	100	-	-
	QLD 2011 (d2)	100	-	-
	QLD 2011 (d1)	100	-	-
	QLD 2011 (c)	100	-	-
	QLD 2011 (b1)	100	-	-
	QLD 2011 (b2)	100	-	-
	QLD 2011 (e)	100	-	-
	QLD 2011 (bf)	100	-	-
	QLD 2011 (i)	100	-	-
	QLD 2011 (j)	100	-	-

Table 1d. The percentage mortality of F_1 3rd instar *P. xylostella* larvae from field-collected populations during 2008-2012 tested against a commercial formulation of chlorantraniliprole at the DD, 5 x DD and 10 x DD. The DD is the rounded-upwards value for the LC_{99} of the WSS strain.

Chemicals	Strain	% larval mortality		
		DD	5 x DD	10 x DD
Chlorantraniliprole	SA 2011	100	-	-
	QLD 2011 (h)	92.3	-	-
	QLD 2011 (d2)	71.1	-	-
	QLD 2011 (d1)	86.8	-	-
	QLD 2011 (c)	73.7	-	-
	QLD 2011 (b1)	60.5	-	-
	QLD 2011 (b2)	97.4	-	-
	QLD 2011 (e)	89.5	-	-
	QLD 2011 (f)	89.5	-	-
	QLD 2011 (i)	97.5	-	-
	QLD 2011 (j)	100	-	-
	QLD 2012 (d1)	87.9	-	-
	QLD 2012 (d2)	95	-	-
	QLD 2012 (k)	75	-	-
	QLD 2012 (b2)	92.5	-	-
	QLD 2012 (c)	73.2	-	-
	QLD 2012 (b1)	94.9	-	-
	SA 2012	100	-	-
	Vic 2012	65.0	-	-

Full Dose Bioassays:

The results of the full dose bioassays are presented in Table 2.

For the 18 strains bioassayed with emamectin benzoate the resistance ratio (RR) ranged between 3.4 and 28.6 at the LC_{50} value, and between 5.7 and 222.9 at the LC_{99} value. For the six QLD and one VIC strain(s), where the RR at the LC_{99} was 36.3 or greater, we consider that the field efficacy of DBM control with emamectin could soon be (if not already) adversely affected by this loss of insecticidal susceptibility.

For the 11 field strains bioassayed with spinosad the resistance ratio (RR) ranged between 0.5 and 4.3 at the LC_{50} value, and between 2.1 and 13.2 at the LC_{99} value. Albeit there has been a low to moderate shift in susceptibility to spinosad in several of these tested strains, most notably with the VIC 2012 strain, we do not consider that the field efficacy of DBM control with spinosad is in any imminent risk at these surveyed field sites.

For the nine field strains bioassayed with indoxacarb the resistance ratio (RR) ranged between 1.2 and 12.0 at the LC_{50} value, and between 2.2 and 18.4 at the LC_{99} value. Albeit there has only been a low to moderate shift in susceptibility to indoxacarb in the tested strains, there is a possibility that for the two strains which had RR values of 18.4 (NSW 2010 (b)) and 13.9 (QLD 2011 (c)) that the field efficacy of the registered product (Avatar®) could be affected. This is because Avatar is registered at a relatively low field rate of indoxacarb.

For the 12 strains bioassayed with chlorantraniliprole the resistance ratio (RR) ranged between 3.4 and 55.2 at the LC_{50} value, and between 7.3 and 844.7 at the LC_{99} value. Given that chlorantraniliprole was first registered in Australian Brassica vegetables only in 2009, the levels of tolerance evident in all 12 of these strains tested in 2011 and 2012 is most unexpected and concerning. Also, the tolerance to chlorantraniliprole was not only evident in the Queensland strains, but was also found in the SA and VIC strains tested in 2012.

For the one field strain bioassayed with flubendiamide and cyantraniliprole a low to moderate shift in tolerance was recorded (RR of 15.1 and 5.5 respectively at the LC_{99} value).

For the 11 QLD, NSW and SA strains collected in 2007 that were bioassayed with BtK the RR ranged between 1.4 and 3.7 at the LC_{50} value, and between 1.2 and 3.7 at the LC_{99} value. RR values of this small magnitude are well within the population and experimental variability range, and hence do not indicate any shift in susceptibility to BtK in these tested field strains.

Field Experiment:

The results are presented in Figure 1. The mortality of the Waite Susceptible strain (WS) was 98.2% and 98.9% when exposed on Day 0 to the Proclaim™ and Coragen™ treated foliage respectively, indicating that the coverage achieved by the grower's spray operation had been highly effective. However, despite the effective spray coverage, the mortality of the QLD strain on Day 0 was only 37.8% on the Proclaim™–treated foliage and 78.0% on the Coragen™-treated foliage. At Day 3 the mortality of the Waite Susceptible strain (WS) was 37.6% and 87.4% exposed to the Proclaim™ and Coragen™-treated foliage respectively, and the corresponding mortalities for the QLD strain were 3.3% and 66.7%. At Day 7 the mortality of the Waite Susceptible strain (WS) was 16.3% and 85.9% exposed to the Proclaim™ and Coragen™ treated foliage respectively, and the corresponding mortalities for the QLD strain were 0.0% and 30.8%. These results provide a compelling indicator of the actual effect on field control of the resistance now emerging in field strains of DBM in QLD (and other States) to both emamectin benzoate and the Group-28 chemistry.

The laboratory strain (EBS), which had been selected over 53 generations with low concentrations of the Proclaim™ formulation of emamectin benzoate, exhibited almost complete tolerance to the Proclaim™–treated foliage. Specifically, on Days 0, 3 and 7 the larval mortality was 0.6%, 3.0% and 0.0% respectively. Surprisingly, there was a significant reduction in the effect of the Coragen™ treatment on the EBS strain, which had never been exposed to chlorantraniliprole nor any other Group-28 insecticide. Specifically, on Days 0, 3 and 7 the larval mortality of the EBS strain on the Coragen™-treated foliage was 55.4%, 30.5% and 1.0% respectively, which compared with 98.9%, 87.4% and 85.9% mortality respectively for the susceptible WS strain. This result provides clear evidence of cross-resistance being conferred in the EBS strain to chlorantraniliprole. This is an important finding, but the full significance of this finding to industry depends on (i) whether this is a generalized effect with all Group-28 insecticides (which it appears to be, see Objective IV section), and (ii) the similarity of the resistance mechanism involved in the lab-selected EBS strain compared to the mechanism involved in the field strains that are exhibiting emamectin benzoate tolerance. The mechanism(s) involved in these two emamectin benzoate tolerant strains is/are still being investigated; however, if the(se) mechanism(s) is/are identified, and prove to be similar in both strains, the 2-

window IRM strategy for southern Australia will need to be modified to take account of the cross-resistance risk for emamectin benzoate and the Group-28 insecticides.

Table 2. Full dose response bioassay of G_2 3rd instar *P. xylostella* larvae from field collected populations during 2008-2012 tested against commercial formulations of emamectin benzoate, spinosad, indoxacarb, chlorantraniliprole, flubendiamide, cyantraniliprole and BtK.

Insect strains	LC_{50} mg aiL^{-1}	CL	RR	LC_{99} mg aiL^{-1}	CL	RR	Slope+/-S.E	N
Emamectin Benzoate								
WS	0.0272	0.0213-0.0344	1	0.207	0.1285-0.5109	1	2.745+/-0.303	266
QLD 2008 (b)	0.1496	0.11-0.22	5.5	11.44	4.708-42.856	55.2	1.24+/-0.121	361
QLD 2008 (c)	0.1276	0.0836-0.1760	4.7	4.286	2.6664-8.756	20.7	2.08+/-0.238	362
QLD 2008 (d)	0.2508	0.1848-0.3256	9.2	3.824	2.2748-8.448	18.5	1.96+/-0.202	363
Combined QLD strain 2009	0.176	0.1364-2244	6.5	4.488	2.64-9.372	21.7	1.657+/-0.161	320
NSW 2010 (a)	0.2024	0.1496-0.2684	7.4	2.055	1.2232-4.7652	9.9	2.311+/-0.218	360
NSW 2010 (b)	0.09152	0.0717-0.1135	3.4	1.184	0.7568-2.3276	5.7	2.094+/-0.235	287
QLD 2011 b(b1+b2)	0.1302	0.0752-0.1989	4.8	1.879	0.8976-8.8352	9.1	2.007+/-0.311	152
QLD 2011 (c)	0.7788	0.4664-1.3024	28.6	35.552	11.924-324.016	171.6	1.401+/-0.209	145
QLD 2011 d(d1+d2)	0.2068	0.0880-0.3828	7.6	7.524	2.552-100.584	36.3	1.495+/-0.214	151
QLD 2011 (e+f)	0.2068	50.336-145.200	7.6	4.924	2.1604-26.0392	23.8	1.693+/-0.275	150
QLD 2011 (i+j)	0.2068	0.0924-0.3696	7.6	14.212	4.356-208.736	68.6	1.264+/-0.194	150

Table 2 (cont'). Full dose response bioassay of G_2 3rd instar *P. xylostella* larvae from field collected populations during 2008-2012 tested against commercial formulations of emamectin benzoate, spinosad, indoxacarb, chlorantraniliprole, flubendiamide, cyantraniliprole and BtK.

Insect strains	LC_{50} mg aiL^{-1}	CL	RR	LC_{99} mg aiL^{-1}	CL	RR	Slope+/-S.E	N
Emamectin Benzoate								
QLD 2012 (b1)	0.1408	0.0440-0.2816	5.2	17.292	4.136-776.16	83.4	1.111+/-0.161	202
QLD 2012 (b2)	0.1804	0.088-0.3036	6.6	4.343	1.791-287.41	21	1.677+/-0.223	200
QLD 2012 (c)	0.3067	0.2407-0.3912	11.3	3.863	2.372-7.867	18.6	2.115+/-0.200	280
QLD 2012 (d2)	0.0792	0.0528-0.1144	2.9	1.861	0.9724-5.8916	8.9	1.711+/-0.263	201
QLD 2012 (k)	1.012	0.2904-2.0284	3.7	46.2	9.724-1881.13	222.9	0.875+/-0.150	201
SA 2012	0.0924	0.0704-0.1232	3.4	1.804	0.968-4.62	8.7	1.792+/-0.175	283
Vic 2012	0.2684	0.1786-0.4180	9.9	13.816	5.236-71.896	66.7	1.359+/-0.152	241
Spinosad								
WS	0.290	0.245-0.346	1	1.922	1.327-3.326	1	2.941+/-0.323	240
QLD 2008 (a)	0.78	0.612-0.984	2.7	19.2	11.28-40.56	10	1.670+/-0.157	359
QLD 2008 (b)	0.972	0.792-1.188	3.4	11.088	7.332-19.776	5.8	2.200+/-0.204	360
Combined QLD strain 2009	0.324	0.252-0.408	1.1	5.4	3.384-10.632	2.8	1.913+/-0.198	320

Table 2 (cont'). Full dose response bioassay of G_2 3rd instar *P. xylostella* larvae from field collected populations during 2008-2012 tested against commercial formulations of emamectin benzoate, spinosad, indoxacarb, chlorantraniliprole, flubendiamide, cyantraniliprole and BtK.

Insect strains	LC_{50} mg aiL^{-1}	CL	RR	LC_{99} mg aiL^{-1}	CL	RR	Slope+/-S.E	N
Spinosad								
Combined QLD strain 2009	0.324	0.252-0.408	1.1	5.4	3.384-10.632	2.8	1.913+/-0.198	320
NSW 2010 (a)	0.816	0.660-0.996	2.8	10.116	6.684-18.012	5.3	2.123+/-0.194	360
QLD 2012 (b1)	0.276	0.132-0.444	1.7	5.532	3.276-13.116	2.9	2.240+/-0.304	200
QLD 2012 (b2)	0.696	0.528-0.888	2.4	6.684	3.996-15.516	3.5	2.359+/-0.315	203
QLD 2012 (c)	1.344	1.0944-1.644	4.6	13.200	8.784-23.520	6.9	2.346+/-0.230	279
QLD 2012 (d2)	0.132	0.060-0.216	0.5	3.972	1.896-17.988	2.1	1.575+/-0.300	205
QLD 2012 (k)	1.200	0.912-1.560	4.1	14.520	8.64-32.88	7.6	2.147+/-0.260	201
SA 2012	0.396	0.300-0.528	1.4	4.776	2.712-12.036	2.5	2.151+/-0.216	280
Vic 2012	1.236	0.888-1.776	4.3	25.32	12.48-79.56	13.2	1.777+/-0.183	242
Indoxacarb								
WS	3.99	3.12	1	31.99	20.925-75.42	1	2.623+/-0.316	261

Table 2 (cont'). Full dose response bioassay of G_2 3rd instar *P. xylostella* larvae from field collected populations during 2008-2012 tested against commercial formulations of emamectin benzoate, spinosad, indoxacarb, chlorantraniliprole, flubendiamide, cyantraniliprole and BtK.

Insect strains	LC_{50} mg aiL^{-1}	CL	RR	LC_{99} mg aiL^{-1}	CL	RR	Slope+/-S.E	N
Indoxacarb								
Combined QLD strain 2009	12.9	9.3-16.5	3.2	197.1	118.8-438.3	6.2	1.964+/-0.247	280
NSW 2010 (a)	9.18	7.26-11.13	2.3	71.1	48.0-133.5	2.2	2.614+/-0.333	286
NSW 2010 (b)	48.0	29.7-63.0	12.0	588.0	357.0-1524	18.4	2.136+/-0.367	204
QLD 2011 (c)	13.8	9.0-20.1	3.5	444.3	207.3-1630.2	13.9	1.546+/-0.222	150
QLD 2011 d(d1+d2)	11.7	8.7-15.3	2.9	72	45.9-168.6	2.3	2.965+/-0.504	151
QLD 2011 (e+f)	5.4	2.16-9.9	1.4	374.1	141.3-2994.6	11.7	1.274+/-0.252	148
QLD 2011 (i+j)	3	0.9-5.7	0.8	129.6	51.0-1433.1	4.1	1.426+/-0.347	150
QLD 2012 (c)	6.60	4.20-9.60	1.7	235.8	109.5-877.5	7.4	1.507+/-0.162	281
Vic 2012	5.13	3.45-7.11	1.2	106.2	56.52-302.91	3.3	1.767+/-0.217	240
Chlorantraniliprole								
WS	0.058	0.046-0.07	1	0.778	0.508-1.404	1	2.051+/-0.186	358
QLD 2011 b(b1+b2)	0.7	0.18-1.38	12.1	6.72	2.66-509.56	8.64	2.366+/-0.422	154
QLD 2011 (c)	3.2	1.58-6.66	55.2	657.2	119.4-43052	844.7	1.006+/-0.191	150

Table 2 (cont'). Full dose response bioassay of G_2 3rd instar *P. xylostella* larvae from field collected populations during 2008-2012 tested against commercial formulations of emamectin benzoate, spinosad, indoxacarb, chlorantraniliprole, flubendiamide, cyantraniliprole and BtK.

Insect strains	LC_{50} mg aiL^{-1}	CL	RR	LC_{99} mg aiL^{-1}	CL	RR	Slope+/-S.E	N
Chlorantraniliprole								
QLD 2011 d(d1+d2)	1.22	0.76-1.84	21	31.4	14.0-136.4	40.36	1.649+/-0.231	149
QLD 2011 (e+f)	0.83	0.46-1.32	14.3	72.66	27.14-434.38	93.39	1.198+/-0.189	150
QLD 2011 (i+j)	2.24	0.84-5.22	38.6	126.8	30.2-7366.8	163.0	0.328+/-0.216	150
QLD 2012 (b1)	0.46	0.22-0.74	7.4	12.58	5.58-6.74	13.6	1.620+/-0.256	206
QLD 2012 (b2)	0.44	0.26-0.62	7.1	7.88	4.18-25.30	8.5	1.859+/-0.307	200
QLD 2012 (c)	0.854	0.68-1.68	13.8	12.74	8.00-24.72	13.8	1.983+/-0.193	280
QLD 2012 (d2)	0.210	0.091-0.340	3.4	6.80	3.20-32.38	7.3	1.542+/-0.299	204
QLD 2012 (k)	1.46	0.90-2.16	23.5	74.0	31.4-333.0	80.3	1.363+/-0.204	199
SA 2012	0.28	0.22-0.36	4.8	11.0	5.6-30.8	14.1	1.454+/-0.153	281
Vic 2012	1.046	0.588-1.772	18.0	53.9	17.44-587.4	69.3	1.359+/-0.194	242

Table 2 (cont'). Full dose response bioassay of G_2 3rd instar *P. xylostella* larvae from field collected populations during 2008-2012 tested against commercial formulations of emamectin benzoate, spinosad, indoxacarb, chlorantraniliprole, flubendiamide, cyantraniliprole and BtK.

Insect strains	LC_{50} mg aiL^{-1}	CL	RR	LC_{99} mg aiL^{-1}	CL	RR	Slope+/-S.E	N
Flubendiamide								
WS	0.259	0.197-0.322	1	2.03	1.334-3.960	1	2.593+/-0.310	361
QLD 2011 Combined (G_5)	0.941	0.605-1.292	3.6	30.624	16.608-83.664	15.1	1.537+/-0.205	362
Cyantraniliprole								
WS	0.048	0.039-0.059	1	0.518	0.339-0.948	1	2.247+/-0.219	280
QLD 2011 Combined (G_8)	0.28	0.23-0.34	5.8	2.85	1.88-5.20	5.5	2.308+/-0.230	282
BtK (Nb: the unit for these BtK vales are µg ai L^{-1})								
QLD 2007 Crowley Vale (a)	110.1	86.6-142.2	3.70	2032	1135-4863	3.70	1.837+/-0.198	320
QLD 2007 Crowley Vale (b)	58.4	38.0-84.4	1.99	1139	534-4896	2.10	1.803+/-0.310	320
QLD 2007 Lowood (a)	48.1	36.6-62.7	1.64	1446	788-3519	2.66	1.573+/-0.162	320
QLD 2007 Lowood (b)	51.9	36.9-69.1	1.77	660	385-1608	1.22	2.106+/-0.262	280
QLD 2007 Lowood (c)	42.5	27.5-59.7	1.45	1323	674-3097	2.44	1.559+/-0.217	320
QLD 2007 Gatton (a)	41.7	32.2-53.3	1.42	886	518-1930	1.63	1.753+/-0.178	320
QLD 2007 Gatton (b)	102.2	81.7-128.1	3.50	724	463-1485	1.33	2.736+/-0.354	280

Table 2 (cont'). Full dose response bioassay of G_2 3rd instar *P. xylostella* larvae from field collected populations during 2008-2012 tested against commercial formulations of emamectin benzoate, spinosad, indoxacarb, chlorantraniliprole, flubendiamide, cyantraniliprole and BtK.

Insect strains	LC_{50} mg aiL^{-1}	CL	RR	LC_{99} mg aiL^{-1}	CL	RR	Slope+/-S.E	N
BtK (Nb: the unit for these BtK vales are µg ai L^{-1})								
QLD 2007 Dalby	79.8	61.6-100.4	2. 72	746	472-1561	1.37	2.396+/-0.310	320
QLD 2007 Upper Tenthill	77.5	60.1-100.9	2.64	1951	1058-4830	3.59	1.661+/-0.175	320
NSW 2007 Werombi	58.4	37.9-84.4	1.99	1139	534-4896	2.10	1.803+/-0.310	320
SA 2007 Nairne	80.2	59.9-108.6	2.73	1837	938-5299	3.38	1.711+/-0.208	280

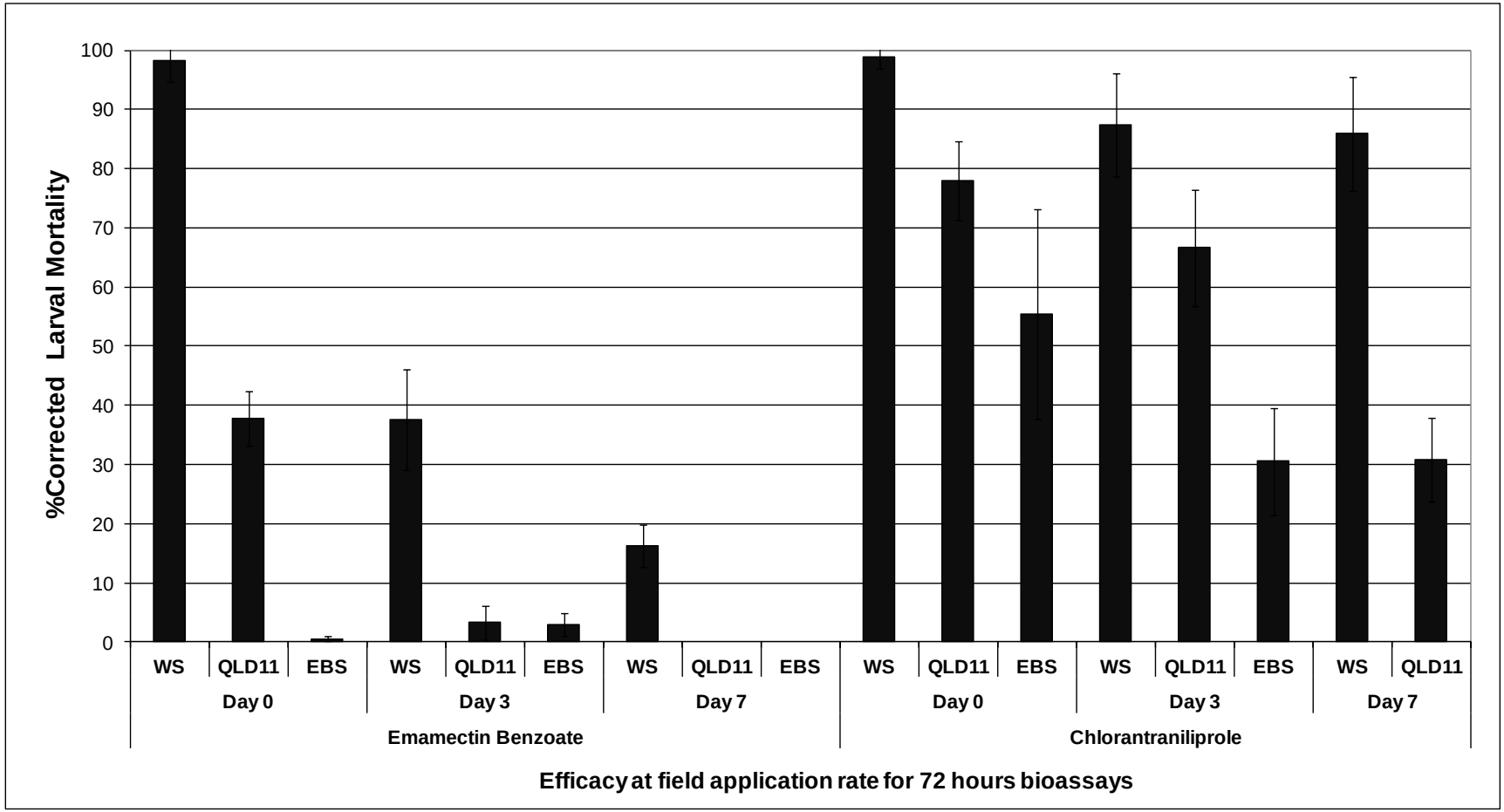


Figure 1. The mean percentage mortality (Abbott's corrected for control mortality) of 3rd instar DBM larvae of three strains (WS – Waite Susceptible; QLD11 – composite Lockyer Valley 2011 field strain; EBS – emamectin benzoate selected laboratory strain at F_{53}) exposed to Brussels sprouts foliage at 0, 3, and 7 days after field-spraying with the label rates of either Proclaim™ (44 g kg^{-1} emamectin benzoate) or Coragen™ (200 g L^{-1} chlorantraniliprole). The bars indicate the standard errors of the mean values.

6.2. Novel inducible insecticide tolerance mechanism.

This study is reported in greater detail in Appendix 2 (2010 Journal of Economic Entomology paper).

The field-derived populations were compared with the WSS in terms of their relative tolerance to emamectin benzoate, spinosad, indoxacarb, and BtK toxins, and respective ability to be induced by exposure to a sub-lethal concentration of the test insecticide. We observed that repeated exposures to insecticide selection pressure in the field caused elevated levels of tolerance, which decreased in field-collected populations after maintaining insects with pesticide-free diet in the laboratory. This suggests that the observed tolerance in the field is transient.

For example, when one *P. xylostella* population with persistently high levels of tolerance against emamectin benzoate during 2006-2007 was cultured in the laboratory in the absence of the insecticide, within three generations the tolerance ratio was reduced more than half with further reduction in subsequent generations (Figure 2). Conversely, when the same insects were exposed to a sub-lethal concentration (0.022 mg ai L^{-1}) of the insecticide in generation F_8, the resistance ratio increased within one generation to the levels found in the field, and this increased further in subsequent generations under incremental selection to reach tolerance ratios that are several-fold of the initial field populations (Figure 2). This suggests that repeated exposure to insecticides in the field and under laboratory conditions increased the level of tolerance, which in turn decreases within a few generations under non-selective conditions.

Again, 2006-2007 field-collected populations with relatively high level of tolerance to BtK formulation were reared in the laboratory without any selection (on toxin-free cabbage seedlings) the acquired tolerance levels declined over subsequent generations (unpubl. data). Moreover, when these populations (a composite of less susceptible 2006-2007 field populations of Gatton II & Lowood II, QLD and Theresa Park & Werombi, NSW) were exposed to a sub-lethal concentration (25µg/ml) of BtK toxins (LC_{20}, 12µg/ml; LC_{50}, 28µg/ml; LC_{90}, 102µg/ml and LC_{99}, 291µg/ml for WSS) in cabbage seedlings, the tolerance levels increased accordingly within a few generations (Figure 1b). This indicated that *P. xylostella* populations acquired tolerance within a few generations under selection pressure, but returned to susceptible levels in the absence of toxins. Similar processes appear to occur in the field. For example, populations collected at Crowley Vale and Gatton II in early spring had tolerance values close to the WSS for BtK toxins, whereas populations collected from the same sites had significant shift in tolerance when collected late spring (Figure 3).

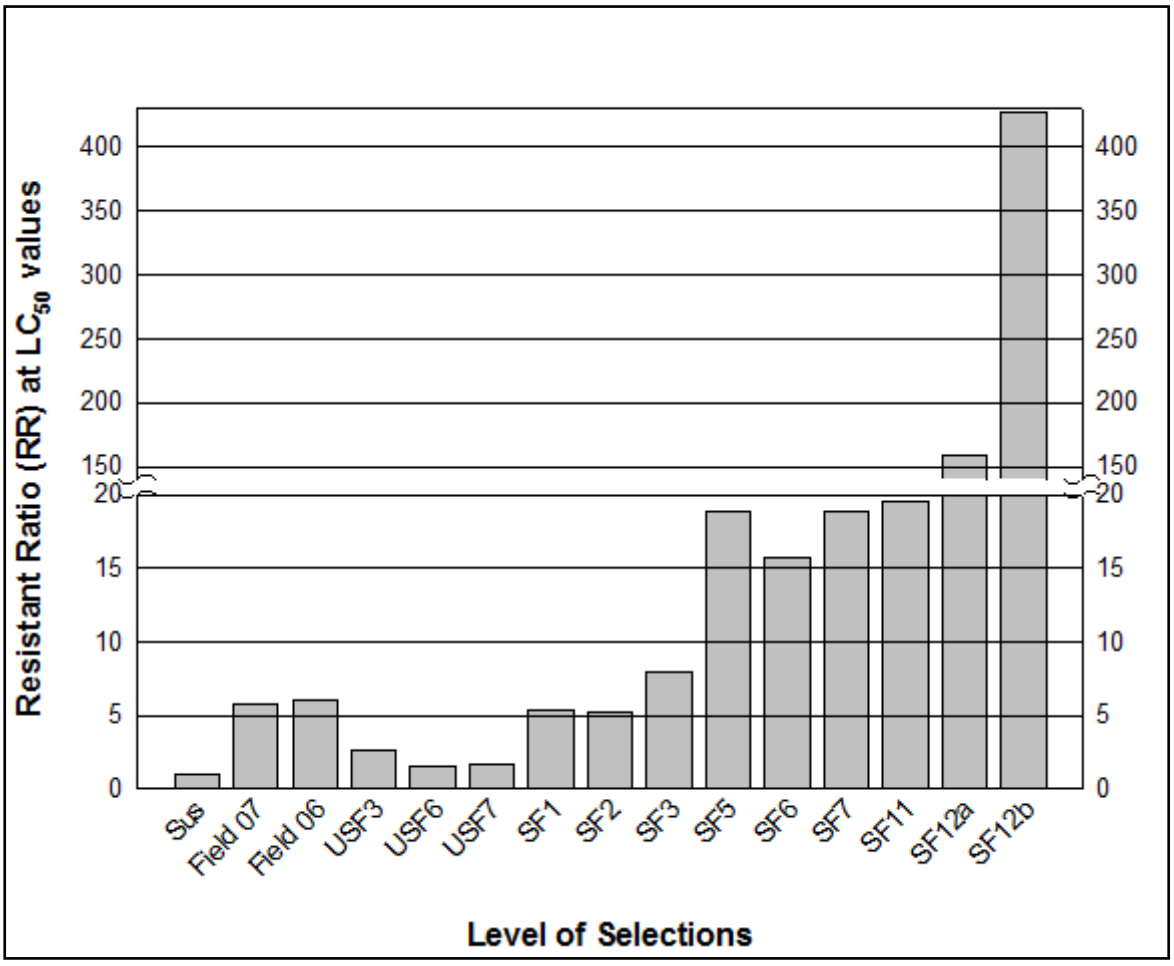


Figure 2. Tolerance to emamectin benzoate in a field derived *P. xylostella* population (Field06 and Field07) when culturing in the laboratory in the presence and absence of the insecticide. The field populations were cultured in the laboratory for seven generations in the absence of emamectin benzoate and bioassays were performed after three (USF_3), six (USF_6) and seven (USF_7) generations, when tolerance levels were almost at the level of the WSS used as a control. All subsequent generations were exposed to a sub-lethal concentration of emamectin benzoate (0.022 mg aiL^{-1}) and tolerance levels measured at generation one (SF_1), two (SF_2), three (SF_3) and five (SF_5). The emamectin benzoate concentration was increased to double (0.044 mg aiL^{-1}) at 6^{th} generation and maintained until 12^{th} generation. Full dose response bioassays were performed at generation six (SF_6), seven (SF_7), eleven (SF_{11}) and twelve (SF_{12}). Resistant ratios (RRs) showed here based on LC_{50} values.

This suggests that transient induction of tolerance is the result of repeated exposure to toxins in the field and under laboratory conditions, which in turn decreases within a few generations under non-selective conditions. The conclusion is that the observed tolerance in field collected populations is less likely due to genetic resistance (which produces individuals resistant to high toxin concentrations), but may be the result of induction of immune and metabolic responses, resulting in low-level resistance (which we refer to here as inducible tolerance).

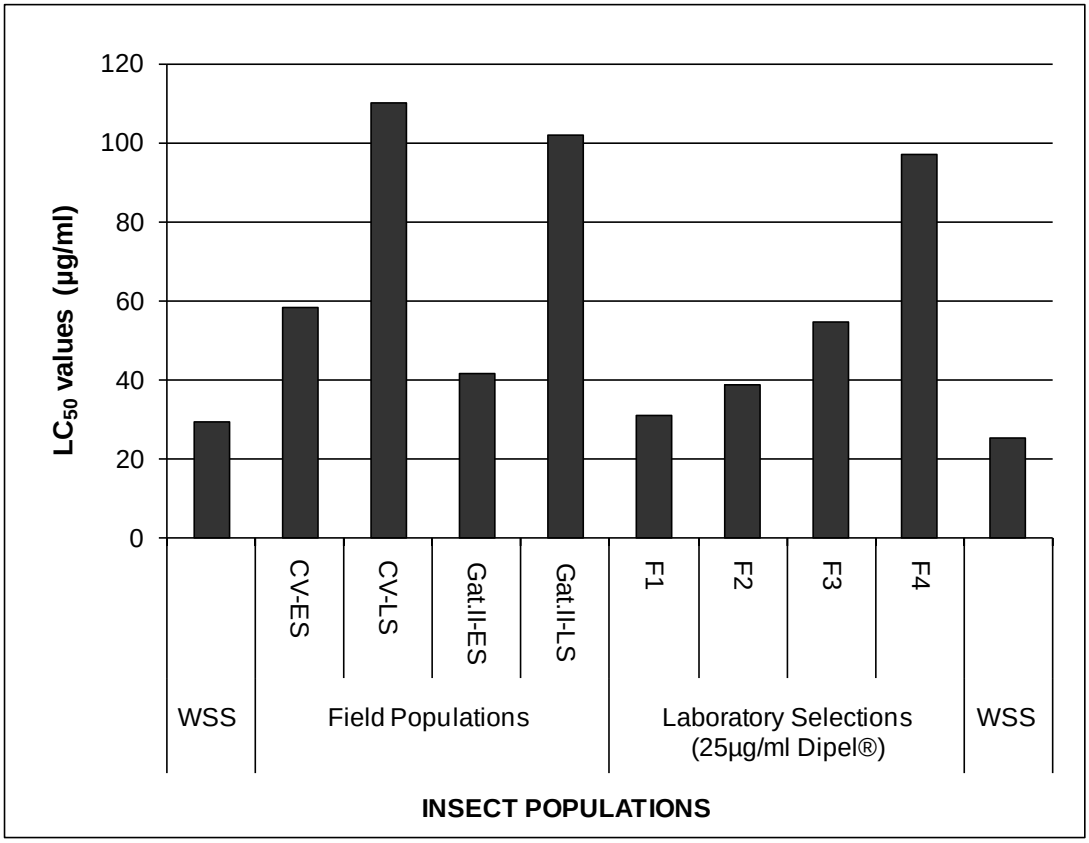


Figure 3. Elevated tolerance in field populations of *P. xylostella* to a commercial formulation of BtK-toxins (Dipel®), and following four rounds of laboratory selection (F_1-F_4 generation) to neonate offspring (a composite of Gatton II & Lowood II, QLD and Theresa Park & Werombi, NSW 2006 field populations) with a low concentration (25 µg/ml). Field populations shown are Crowley Vale (CV) and Gatton II (Gat.II), QLD collected early spring (ES) and late spring (LS) during 2007. WSS was used to compare the shift in susceptibility under field and laboratory selections.

Inducible tolerance in the flour moth *Ephestia kuehniella* has been shown to be associated with the relative amounts and activities of immune and metabolic components at the gut lining (Rahman et al, 2004, 2007; Ma et al, 2005). As suggested by these researchers, we assume that the phenoloxidase mediated immune response discriminates between susceptible and tolerant and/or resistant insects.

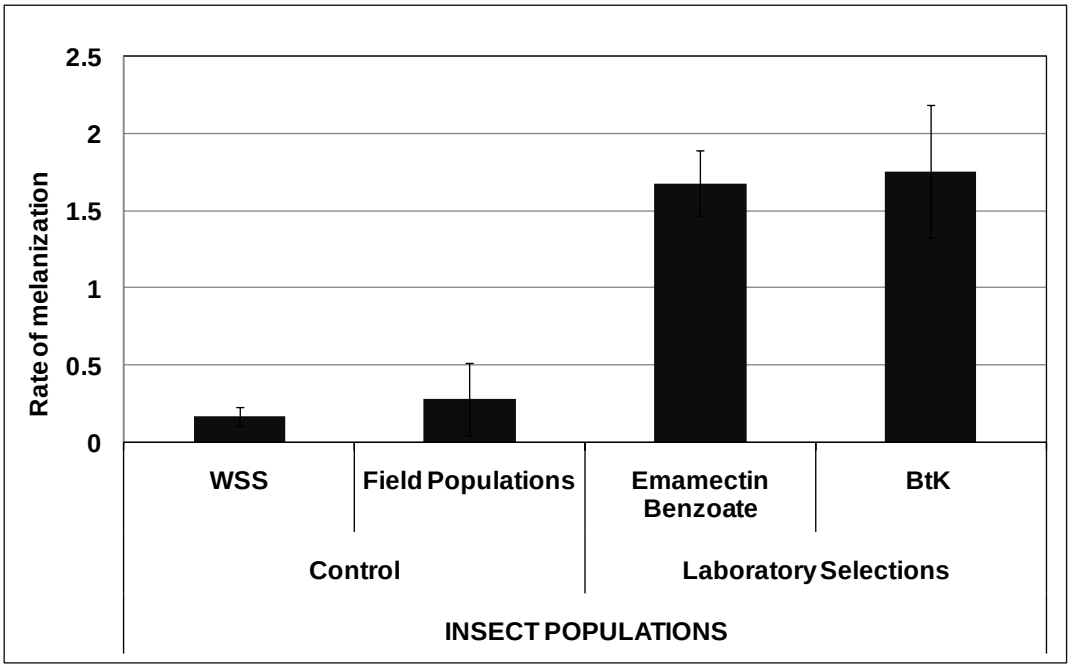


Figure 4. Induction of the humoral immune response in 4th instar larvae of the field derived laboratory populations of *P. xylostella* selected with a low concentration of emamectin benzoate (0.022 mg ai L^{-1}) and BtK toxins (25 μg/ml), as indicated by the rate of the melanization reaction (as the slope of the plot of absorbance against time in arbitrary units) of cell-free haemolymph in larvae from WSS, field and laboratory selected populations.

Field-collected insect survivors were analysed for immune- or metabolic up-regulation. However, direct measurements from field-collected larvae were not possible, since collected insects have to be kept for at least one generation to monitor for any parasitism. By that time any transient induction process may have dissipated. We therefore decided to use our WSS and tolerant laboratory cultures to study immune and/or metabolic activities. Laboratory assessment did not reveal any signs of differential immune and/or metabolic activities in field derived laboratory insects compared to WSS but a significant increase in melanization was observed in laboratory selected insects (Figure 4). Given that the differential immune and/or metabolic activities are part of a transient regulatory process, we suspect that the effects generated in the field could have faded away by the time it was examined. We therefore conclude that if tolerance in the field was due to differential regulation of immune and/or metabolic activities, it was indeed transient and in the absence of the toxin and elicitors, the tolerance would disappear within a few generations.

6.3. Acquired insecticidal tolerance through continuous laboratory selection.

1. The emamectin benzoate, BtK, spinosad and indoxacarb laboratory selections:

Full dose response bioassays demonstrated that laboratory selections with sub-lethal doses of emamectin benzoate and BtK toxins produced a major increase in *P. xylostella* tolerance (Figures 1 & 5, Tables 3 & 4).

By early 2013 the tolerance of the laboratory strains selected with low doses of emamectin benzoate and BtK respectively had reached have reached 271 fold emamectin resistance (LC_{50}) at the F_{55} generation of selection and 45 fold BtK resistance (LC_{50}) at F_{63}. We suspect that the 271 fold emamectin resistance may be due to a recent genetic mutation, and are initiating a study to identify this mechanism (see section below). Starting with the F_{45} generation of the BtK low-dose selected strain we have commenced a 'high-dose' BtK (2.0 g Dipel^{TM} product L^{-1}) selection program to potentially select a genetic mutation(s).

By contrast, the tolerance of the spinosad-selected and indoxacarb-selected strains had not significantly increased by early 2013 (7 fold spinosad resistance (LC_{50}) at F_{25} and 1.4 fold indoxacarb resistance (LC_{50}) at F_{16}). As a result we have now discontinued the indoxacarb selection; and with the spinosad-selected strain we are in the process of replacing spinosad with spinetoram (reflecting the recent change in active constituent in Dow AgroSciences' spinosyn products) and increasing the selecting concentration to determine whether a higher dose selection strategy may elicit a greater tolerance response.

To determine whether low-level selection with Group 28 diamides can result in the development of high-level tolerance, we have commenced a similar low-rate selection study using chlorantraniliprole.

These results suggest that these newer DBM insecticides may differ in their risk profile to selection for resistance, e.g., chemistries such as indoxacarb and spinosad (and presumably spinetoram) are likely to be at lesser risk than chemistries such as emamectin benzoate and the Group 28 diamides. This information should be factored into industry management plans.

2. The reciprocal genetic crossing experiments:

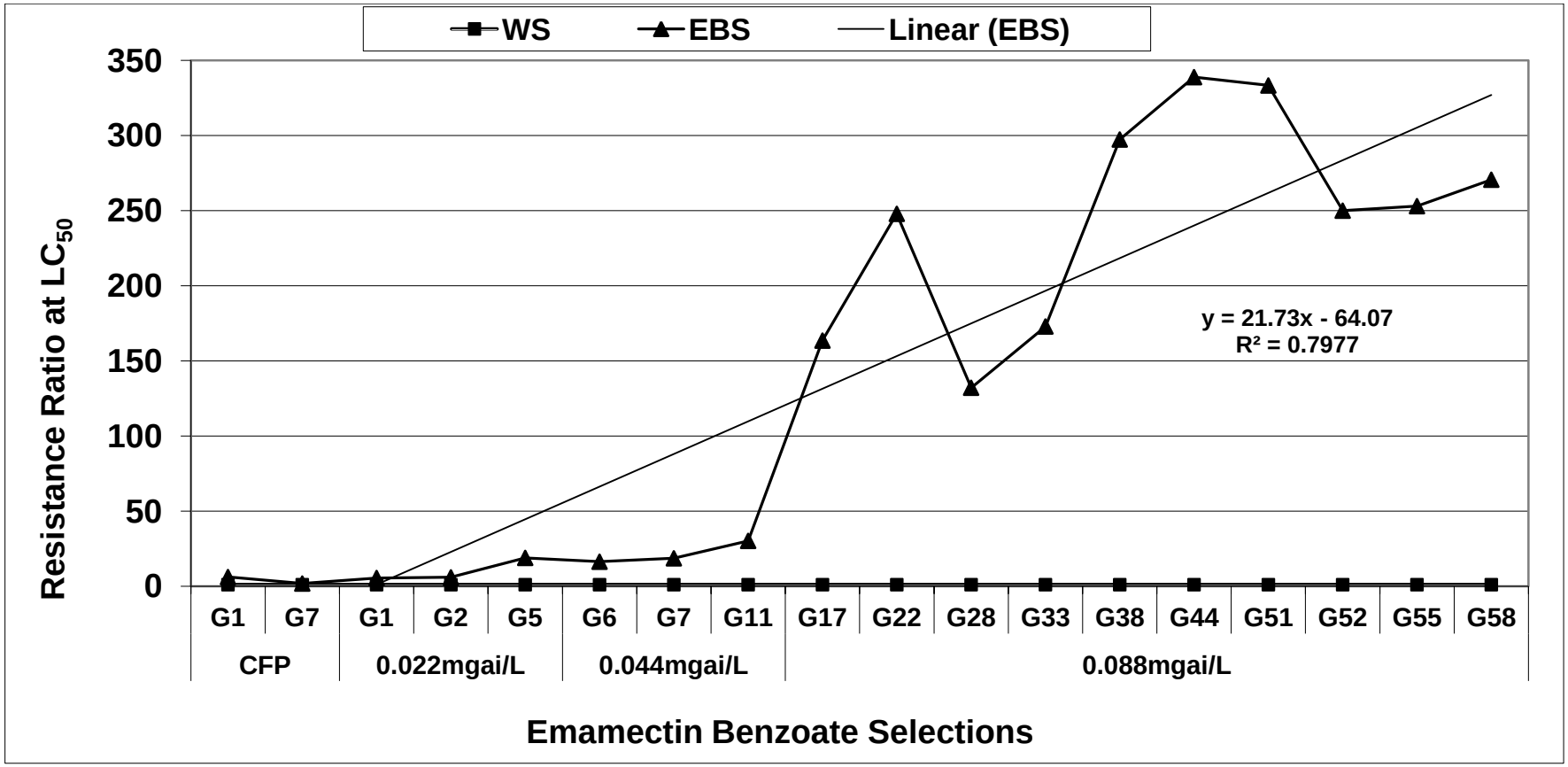
Given that the crossing of tolerant and susceptible individuals will produce different outcomes depending on the mode of transmission of the phenotypic trait, we performed reciprocal genetic crosses allowing virgin females from the WS and tolerant populations mated with males from their reciprocal counterparts, and analyzed tolerance levels in the offspring. This allowed us to investigate whether the acquisition of tolerance under continuous incremental selection pressure is caused by resistance alleles that pre-existed in the field populations in low frequencies and which were increased in selected populations, or alternatively by inducible immune and metabolic responses that can be transmitted to offspring by epigenetic inheritance mechanisms (caused by gene and protein regulatory mechanisms).

The full-dose response bioassays of reciprocal genetic crosses of emamectin benzoate (Table 3a) and BtK tolerant (Table 4a) strains suggest that in both strains the tolerance was primarily

transmitted to offspring by epigenetic inheritance mechanisms. After establishing epigenetic transmission by reciprocal genetic crosses, for example in generation 5 for emamectin benzoate, the insects were kept under continuous selection pressure until generation 12, when another reciprocal genetic cross was performed and offspring examined for tolerance. In generation 12 tolerance level has increased to 158-fold (Table 3b). While the tolerance levels in F_1 offspring of the two reciprocal crosses (RR 9.2 in SXT, RR 15.4 in TXS; Table 3b) were somewhat higher than in generation 5 (RR 2.3 in SXT, RR 5.45 in TXS; Table 3a), the relative contribution has decreased compared to the levels in the tolerant parent (Figure 6a & 6b). While the dominant contribution in generation 5 was about one third of the tolerance levels of the tolerant parent (Table 3a), it amounts to about one tenth of the tolerant parent in generation 12 (Table 3b). Likewise, the maternal effect (difference in RRs between SXT and TXS crosses) is 3.15 in generation 5 and 6.2 in generation 12, about half of the RRs of the TXS offspring. The full-dose response bioassays of reciprocal genetic crosses of BtK tolerant strain at generation 23 (Table 4a & Figure 7a) and generation 45 (Table 4b & Figure 7b) indicated similar mechanisms. This suggests that while epigenetic contributions are significant to incremental increases in tolerance in the earlier generations after exposure to low level of insecticide, other resistance mechanisms that are transmitted genetically predominate in subsequent generations under incremental selections to the insecticide.

Figure 5. Tolerance to commercial formulations of a) emamectin benzoate and b) BtK toxins (Dipel®) in field-derived laboratory populations of *P. xylostella* under incremental selections. WSS was used as control.

a) Emamectin benzoate tolerant population



b) BtK toxins tolerant population

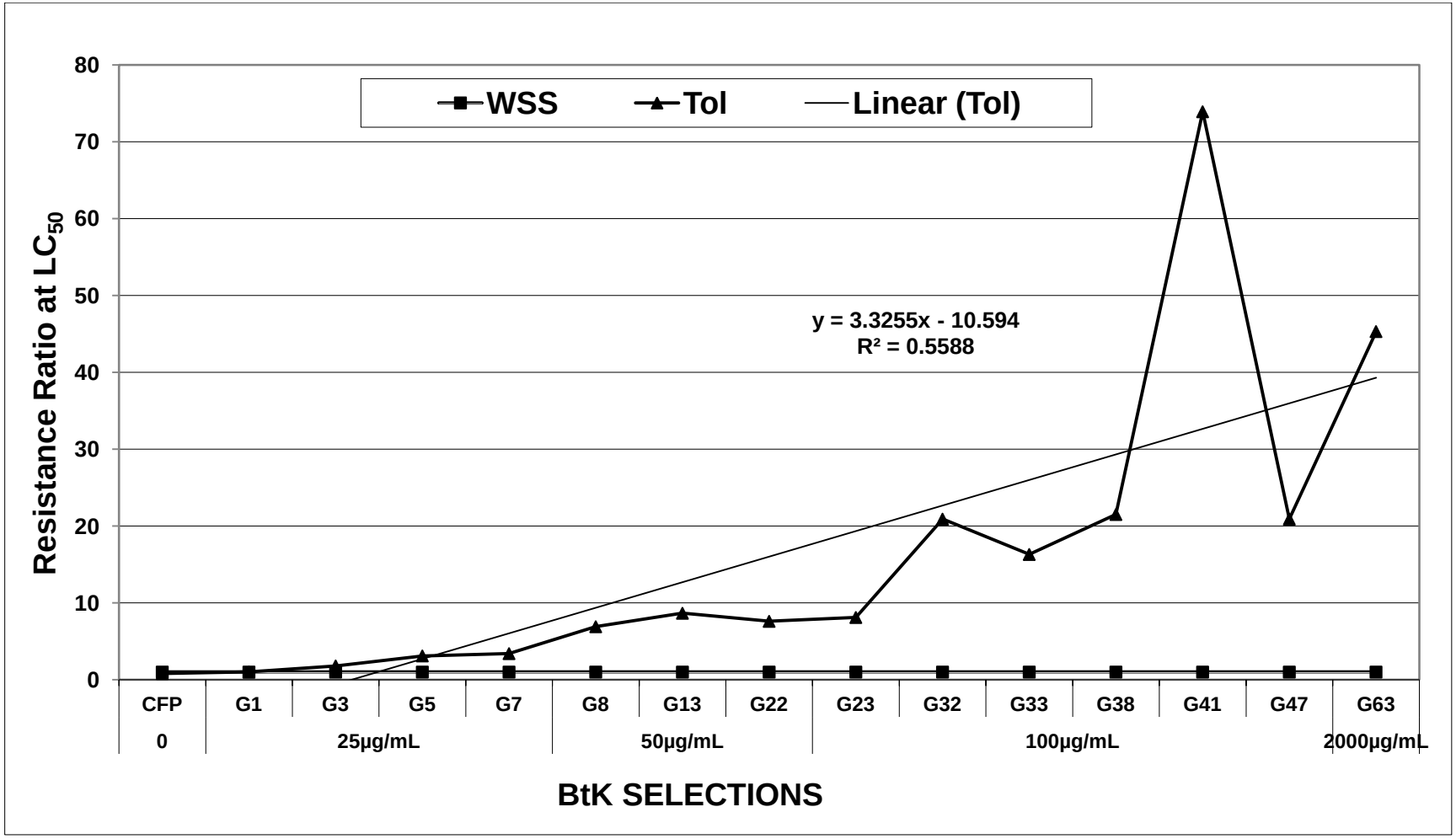


Table 3. Full dose response bioassays of emamectin benzoate-susceptible (WSS: as S♀xS♂), tolerant (T♀xT♂), and their reciprocal genetic crosses (as S♀xT♂ and T♀xS♂). The bioassays were performed using 3rd instar *P. xylostella* larvae from F_1 offspring. a) generation five and b) generation twelve in the course of incremental laboratory selection with emamectin benzoate.

a. generation five (F_5)

Crosses ♀ x ♂	**LC_{50} mg ai L^{-1}**	**95% C.L.**	**RR†**
S×S	0.04268	0.03608-0.0484	1
T×T	0.80388	0.632-1.04	18.83
S×T	0.09548	0.0594-0.137	2.3
T×S	0.23276	0.165-0.321	5.45

b. generation twelve (F_{12})

Crosses ♀ x ♂	**LC_{50} mg ai L^{-1}**	**95% C.L.**	**RR†**
S×S	0.0286	0.02376-0.03476	1
T×T	4.532	3.52-5.72	158.5
S×T	0.264	0.211-0.326	9.2
T×S	0.44	0.339-0.572	15.4

†Resistance ratio (the ratio of the LC_{50} value of the test strain to the LC_{50} value of the Waite Susceptible strain (SxS)).

Table 4. Full dose response bioassays of BtK toxins-susceptible (WSS: as S♀xS♂), tolerant (T♀xT♂), and their reciprocal genetic crosses (as S♀xT♂ and T♀xS♂). The bioassays were performed using 3rd *P. xylostella* instar larvae from F_1 offspring. a) generation 23 and b) generation 45 in the course of incremental laboratory selection with BtK toxins.

a) generation twenty three (F_{23})

DBM Strain ♀ x ♂	LC_{50} (µg/ml)	95% C.L.	RR	LC_{90} (µg/ml)	95% C.L.	RR	LC_{99} (µg/ml)	95% C.L.	RR†
S×S	44.8	36.8-54.4	1	149.19	114.9-211.9	1	398	269.5-692	1
T×T	391.8	254.7-802.4	8.8	4945.5	1870-33098	33.2	39076	8957-728588	98.2
S×T	135.2	82.9-255.5	3	2140	776.7-23748	14.3	20345	3892-1182509	51.1
T×S	166.9	100.6-357.4	3.7	3479.8	1075-65233	23.3	41401	6161-5479344	104

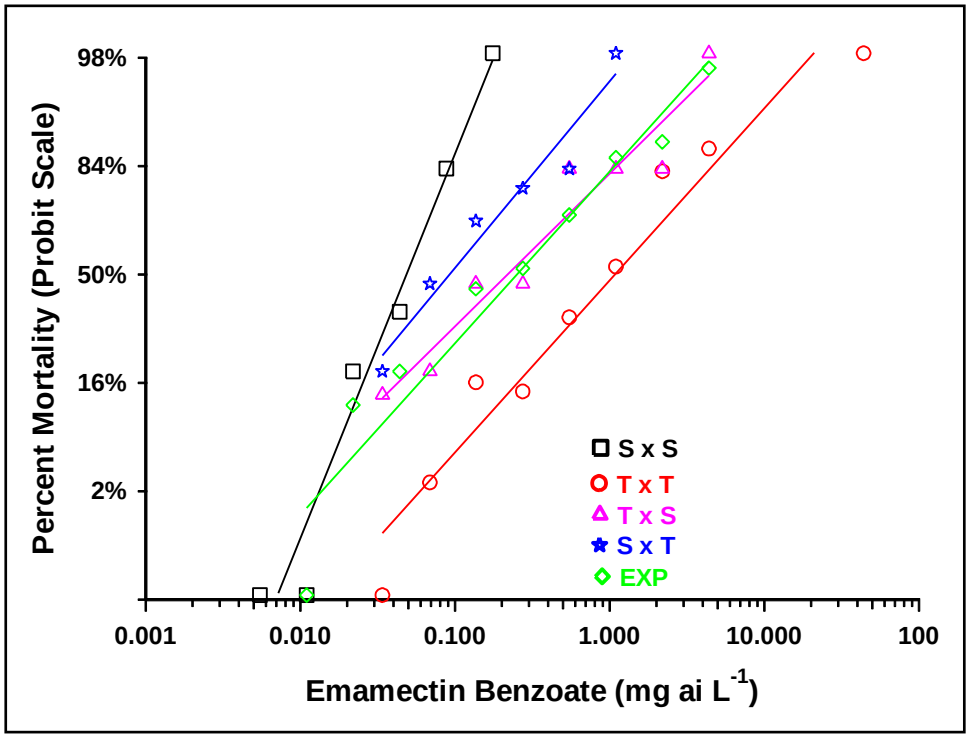
b) generation forty five (F_{45})

DBM Strain ♀ x ♂	LC_{50} (µg/ml)	95% C.L.	RR	LC_{50} (µg/ml)	95% C.L.	RR	LC_{99} (µg/ml)	95% C.L.	RR†
S×S	26.8	20.6-34.4	1	98.14	70.5-159.5	1	283.1	171.5-622.4	1
T×T	1978.5	1199-4022	73.9	20798	8439-111405	212	141566	37159-1862143	500
S×T	186.8	139-249.4	7	904.7	621.3-1527	9.2	3273.5	1869-7534	11.6
T×S	220.3	169.5-286	8.2	1224.9	857.7-1971.7	12.5	4960.2	2898-10562	17.5

†Resistance ratio (the ratio of the LC_{50} value of the test strain to the LC_{50} value of the Waite Susceptible strain (SxS)).

Figure 6. (a) Log dose versus percentage of mortality (probit scale) of the susceptible strain (S × S, □) and field strain exposed to emamectin benzoate for five generations (T × T, o). The corresponding genetic crosses (T♀xS♂, Δ) and (S♀xT♂, ∗) are shown together with the values predicted for a single gene transmission (EXP,◊). (b) Log dose versus percentage of mortality (probit scale) of the susceptible strain and field strain exposed to emamectin benzoate for 12 generations. Reciprocal genetic crosses and abbreviations are as in (a).

a. Generation five (F_5)



b. Generation twelve (F_{12})

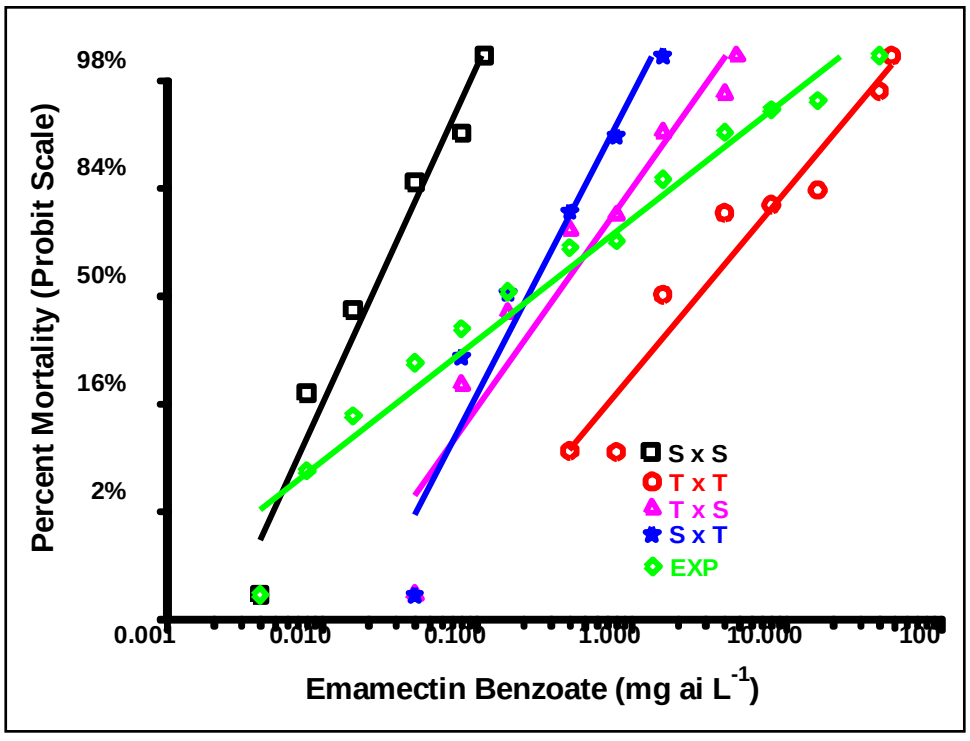
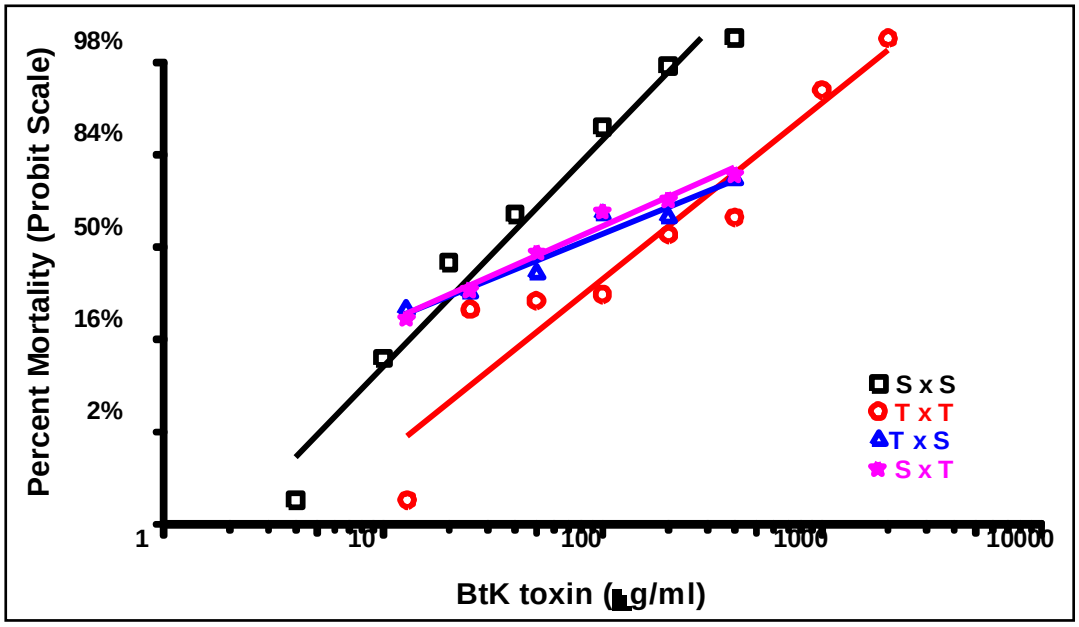
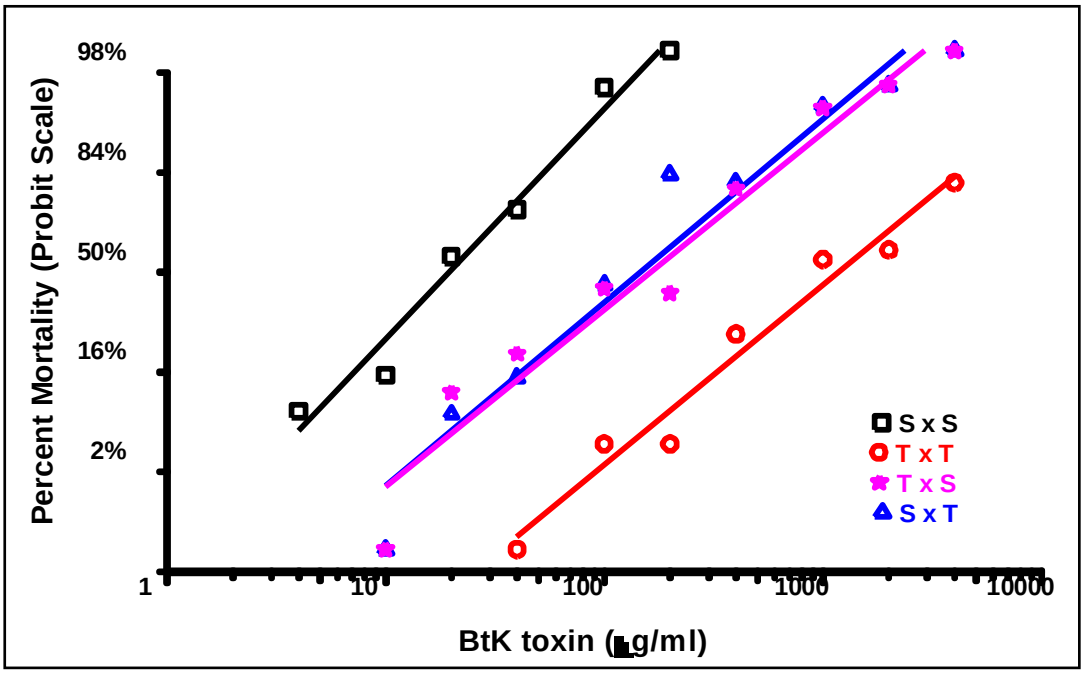


Figure 7. (a) Log dose versus percentage of mortality (probit scale) of the susceptible strain (S × S, □) and field strain exposed to BtK toxins for 23 (F_{23}) generations together with their reciprocal genetic crosses (T♀xS♂, Δ) and (S♀xT♂, ✱). (b) Log dose versus percentage of mortality (probit scale) of the susceptible strain and field strain exposed to BtK toxins for 45 (F_{45}) generations. Reciprocal genetic crosses and abbreviations are as in (a).

a. Generation 23 (F_{23})



b. Generation 45 (F_{45})



3. Developmental Penalties:

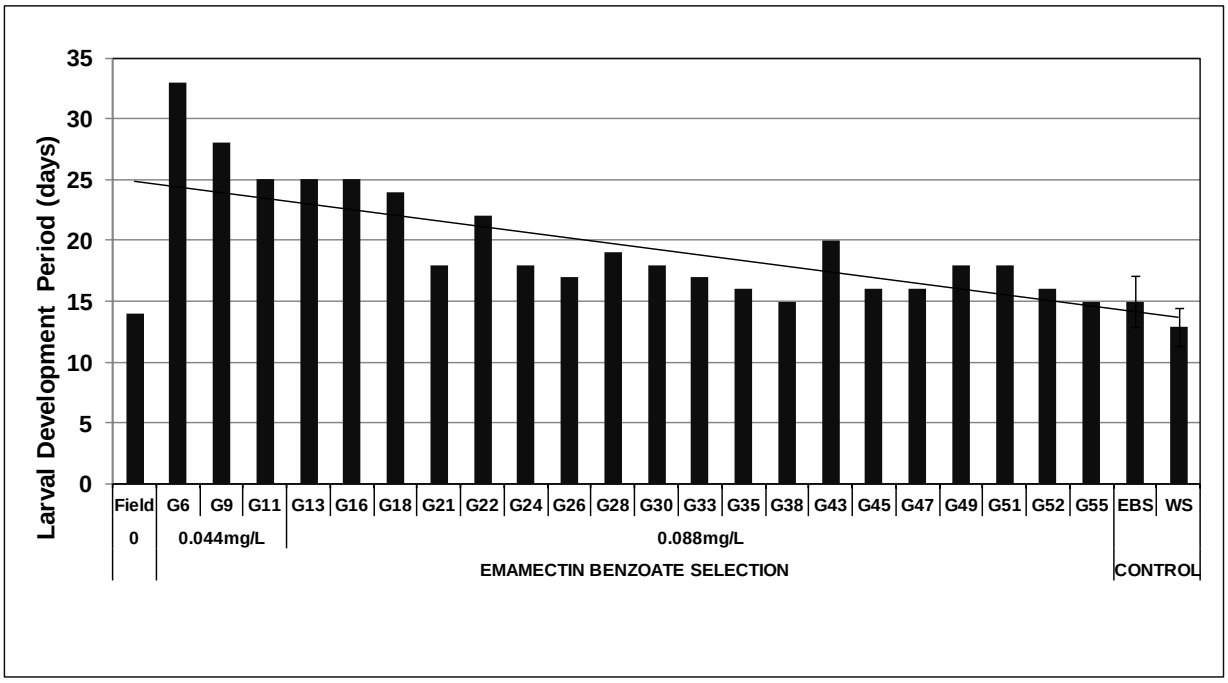
The laboratory selections with low doses of emamectin benzoate and BtK toxin seem to have imposed a significant developmental penalty, as evidenced by a delay in larval development. Here we consider that the term developmental penalty is different from the term "fitness cost," which is generally used in the context of mutational alterations in resistance genes (Gahan et al., 2005) causing pleiotropic effects due to absence of resistance gene products or changes in the function of mutant gene products. However, once the populations were kept at constant toxin levels, the developmental penalty slowly diminished over subsequent generations (Figure 8).

This could indicate possible genotypic selection of allelic combinations of multi-gene functions that reduce developmental penalty. This may in long term provide tolerant populations with the adaptive potential to acquire resistance mechanisms that are genetically transmitted and involve target site mutations in important resistance genes. Since it is unlikely that this reduction in developmental penalties is due to genetic adaptation, it is important to understand how immune and metabolic activities in induced larvae influence the developmental penalties as well as tolerance to the toxin.

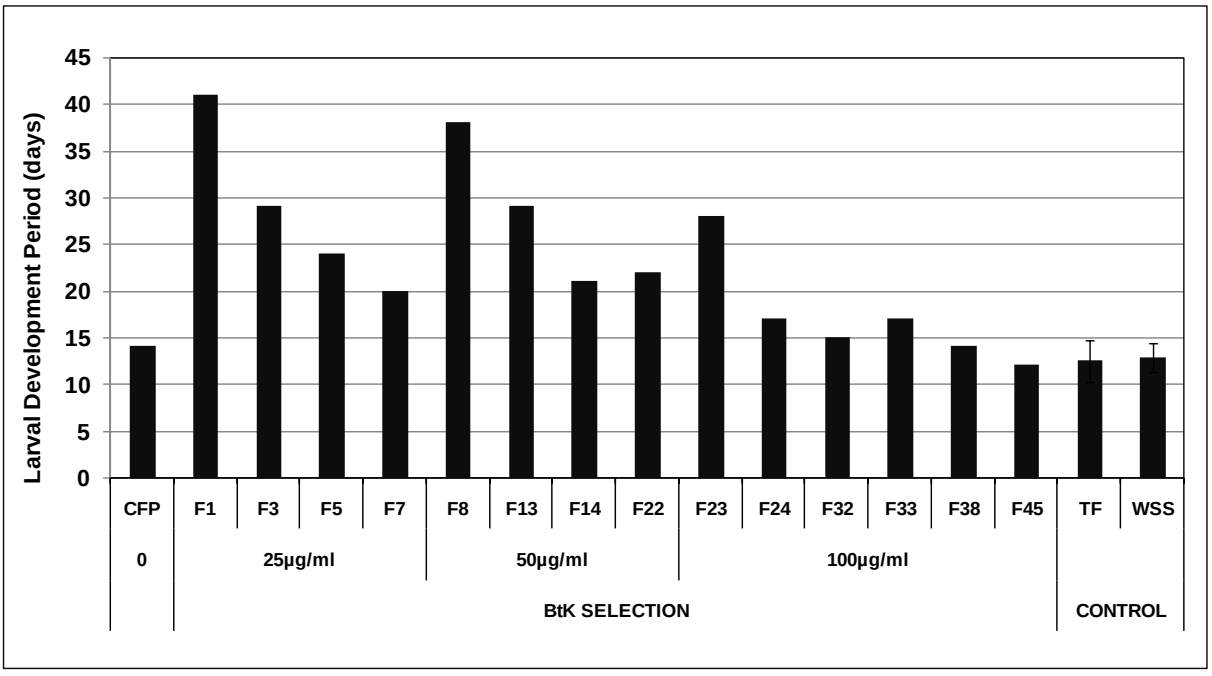
In summary, our results suggest that incremental increases in tolerance to emamectin benzoate and BtK toxins in field-derived DBM are achievable under laboratory selection conditions to levels where there is potential to cause insecticidal control failures. The tolerant phenotype is caused by a novel inducible tolerance that is transmitted to offspring by an epigenetic mechanism showing a maternal effect. While the epigenetic contribution to incremental increases in tolerance is prominent under low to medium selection pressure, other resistance mechanisms that are transmitted genetically may predominate over time with incremental increase in toxin exposure. Further, the elevated tolerance carries high fitness costs in the form of developmental delays, but toxin-exposed insects can reduce fitness costs in subsequent generations. It is plausible that insects initially respond to toxin exposure by an elevated immune and metabolic status which is broad initially and becomes more specific through genotype selection after subsequent generations. Hence the initial broad tolerance may be lost together with the reduction of fitness penalty. Whether this inducible immune and metabolic tolerance mechanism is a threat to resistance management in the field will depend upon the extent of cross-resistance to other frequently-used insecticides, and the size of the developmental penalties associated with the tolerance.

Figure 8. Developmental penalties (extended larval development from egg-hatch neonate to pupa) in a) emamectin benzoate and b) BtK toxins selected field derived laboratory populations of *P. xylostella*. Combined field population (CFP) was initially selected with a low concentration of emamectin benzoate (0.022 mg ai L^{-1}, ~$1/1000^{th}$ of registered rate, data not shown) and BtK toxins (25µg/ml) and maintained for several generations. The emamectin benzoate concentration was doubled to 0.044 mg ai L^{-1} at F_6 and to 0.088 mg ai L^{-1} at F_{13}. The BtK toxins concentration was doubled to 50µg/ml at F_8 and to 100µg/ml at F_{22}. WSS and tolerant insects reared on toxin free condition (TF) were used as control.

a) Emamectin benzoate tolerant strain



b) BtK toxins tolerant strain



6.4. Cross-tolerance patterns between the synthetic insecticides and BtK.

Cross tolerance patterns between emamectin benzoate, spinosad, indoxacarb and BtK:

A significant cross tolerance was observed when emamectin benzoate tolerant insects were tested with spinosad routinely throughout the selection period, but the level of the cross tolerance was reduced as the selection progressed (Table 5). No to low cross tolerance was observed when emamectin benzoate tolerant insects were tested with indoxacarb and Btk toxins (Table 5). Moderate to high level of cross tolerance was observed when BtK tolerant insects were tested with emamectin benzoate, spinosad and indoxacarb in the early generation during the course of laboratory selection, but most of the cross tolerance was lost as the selections progressed (Table 5). Spinosad tolerant insects showed low to moderate cross tolerance to emamectin benzoate and indoxacarb, but no cross tolerance with BtK toxins.

Table 5. Cross tolerance between emamectin benzoate, spinosad, indoxacarb and BtK toxins in a field derived *P. xylostella* population when selected with a sub-lethal concentration of each toxin with incremental increased in toxin concentration under laboratory condition. Full dose response bioassays were performed using 3rd instar larvae reared on toxin free cabbage seedling from egg-hatch. WSS was used as control.

INSECT STRAINS/ GENERATION	LEVEL OF CROSS TOLERANCE (WSS AS CONTROL)					
	LC_{50} (RR)	LC_{99} (RR)	LC_{50} (RR)	LC_{99} (RR)	LC_{50} (RR)	LC_{99} (RR)
Emamectin Benzoate	Spinosad		Indoxacarb		Btk Toxins	
F_1	6	22	1.7	1.3	1.64	2.7
F_5	11.46	10.85	-	-	-	-
F_6	13.42	41.97	-	-	-	-
F_{15}	6.3	14.3	1.8	2.2	2.3	2.1
F_{22}	4.6	18.1	1.7	1.7	1.4	0.4
F_{33}	9.1	10.6	3.6	4.3	0.8	0.7
F_{45}	4.8	10.4	2.5	5.4	1.1	0.6
Btk Toxins	Emamectin Benzoate		Spinosad		Indoxacarb	
F_1	3	-	-	-	10.5	44.4
F_6	1.8	0.3	-	-	3.1	24.5
F_{10}	1.4	23.3	3.4	6.4	1.2	1.1
F_{27}	2.2	7	1.9	3.7	1.2	1.8
F_{35}	0.98	1.9	1.8	3.2	0.7	1.1
Spinosad	Emamectin Benzoate		Indoxacarb		Btk Toxins	
F_1	4.1	22.4	3.6	8.4	1.1	1
F_5	4.7	10.4	2.6	3	1.7	1.5

Our observations suggest that elevated tolerance carries high fitness costs in the form of developmental delays, but toxin-exposed insects can reduce fitness costs in subsequent generations. It is plausible that insects imposed on fitness penalty show cross-resistance to other damaging pathogens and possibly pesticides by an elevated immune and metabolic status which is broad initially and becomes more specific through genotype selection after subsequent

generations. This cross tolerance may be lost together with the reduction of fitness penalty. Finally, whether this inducible immune and metabolic tolerance mechanism is a threat to resistance management in the field will depend upon the extent of cross-resistance to other frequently-used insecticides, and the size of the developmental penalties associated with the tolerance.

Cross tolerance patterns between emamectin benzoate and the Group-28 diamides (chlorantraniliprole, flubendiamide and cyantraniliprole):

The cross tolerance patterns exhibited by the emamectin benzoate selected (EBS) laboratory strain when bioassayed with the three Group-28 insecticides between generations F_{52-55} are presented in Table 6. At the time the RR value at the LC_{50} for emamectin benzoate was 253, and the corresponding RR values for chlorantraniliprole, flubendiamide and cyantraniliprole were 98.5, 35.8 and 47.5. These results clearly demonstrate significant levels of cross resistance being conferred to the Group-28 chemistries by the EBS strain, as a result of the tolerance acquired through lab selection by repeated exposure to low doses of emamectin benzoate.

As mentioned in the Objective III section, we have commenced a low dose selection program with chlorantraniliprole. If significant Group 28 tolerance develops in this chlorantraniliprole-selected strain, we will determine whether cross-tolerance is conferred in the other direction from Group 28 insecticides to emamectin. At the time of reporting we had bioassay results after only three generations of selection (G_3).

Given the observed cross-tolerance conferred by our emamectin-resistant strain to Group 28 diamides, we sent samples of this strain in 2012 to Dr Ralf Nauen**,** Bayer CropScience, Research Pest Control, Germany, for genetic comparison with diamide-resistant DBM from Asia. This showed that the mutation responsible for the Asian diamide resistance (membrane-spanning domain of the ryanodine receptor) is not involved in our Australian emamectin-selected resistance.

These results demonstrating Group-28 cross-resistance in our emamectin benzoate selected strain corroborate the results of the field experiment reported in section 6.1, and have important implications for the management of DBM resistance to these important Group 6 and 28 insecticides. At the present we are uncertain of the molecular/genetic basis of the high-level resistance now being expressed in the low-dose lab-selected strain, and whether it is the same mechanism as that responsible for the emerging resistance to emamectin benzoate in field populations of DBM in Australian Brassica vegetables. This knowledge is now essential to determining the full significance of this cross resistance to industry, and to devising an IRM response if the resistance mechanisms in the lab and field populations are related.

In collaboration with Dr Simon Baxter, Ramsey Research Fellow, Adelaide University, we are now endeavouring, with Grains Research and Development Corporation funding, to characterise the molecular mechanism of this emamectin resistance. Firstly, we plan to perform analysis of transcriptomes from three time points (initial selection, mid-selection and late selection) to find the gene responsible for the regulation of the initial tolerance. We will also perform reciprocal backcrosses for genetic characterization of the resistance. Using this material, we will then

perform genotype-phenotype based analysis to determine whether mutation(s) in the GABA receptor, the primary target of emamectin, is the cause of resistance. If this is not the case, we will perform RADseq on multiple backcross families to pinpoint the resistance locus, with the aid of the DBM genome, recently published by Baxter and colleagues in Nature Genetics.

We have recently demonstrated that piperonyl butoxide (PBO), a known insecticidal synergist, does significantly synergize (and thereby diminish) the effect of the emamectin benzoate resistance in both the lab-selected (EBS) and tolerant QLD 2011 field strains (Table 7). For example, when bioassayed with emamectin benzoate alone the RR for the EBS strain at the LC50 was 165 fold, but this was reduced to a RR of only 16.3 when bioassayed with emamectin benzoate + 0.01% PBO. Similarly, when bioassayed with emamectin benzoate alone the RR for the QLD 2011 field strain at the LC50 was 17.3 fold, and this was reduced to a RR of only 3.0 when bioassayed with emamectin benzoate + 0.01% PBO. PBO also provided synergism of chlorantraniliprole in the EBS, QLD 2011 and chlorantraniliprole-selected (CS) strains (Table 8). Given that PBO is an inhibitor of cytochrome P450 enzymes, which are important detoxification agents for various insecticides, these PBO effects suggest that P540 detoxification may be involved in the observed emamectin benzoate and Group-28 tolerance mechanism.

Table 6. Resistance and cross resistance to commercial formulations of emamectin benzoate, chlorantraniliprole and flubendiamide in field-derived emamectin benzoate selected (EBS) laboratory *P. xylostella* population (F_{52-55}, selected with 0.022-0.088mgaiL^{-1}). WS population was used as reference strain.

Insect strains	LC_{50} mg ai L^{-1}	95%CL	RR	LC_{90} mg ai L^{-1}	95%CL	RR	Slope+/-S.E	N
Resistance to emamectin benzoate								
WS	0.017	0.011-0.025	1	0.059	0.039-0.118	1	2.387+/-0.259	243
EBS	4.29	3.31-5.49	253	23.5	16.896-36.30	398	2.073+/-0.217	360
Cross-resistance to chlorantraniliprole								
WS	0.066	0.054-0.08	1	0.212	0.166-.298	1	2.503+/-0.246	320
EBS	6.5	3.89-10.58	98.5	94.12	45.36-329.9	443.9	1.104+/-0.118	315
Cross-resistance to flubendiamide								
WS	0.3696	0.2688-0.4848	1	1.1472	0.8352-1.8432	1	2.602+/-0.323	279
EBS	13.22	7.96-21.18	35.8	195.89	96.18-654.67	170.8	1.095+/-0.118	315
Cross-resistance to cyantraniliprole								
WS	0.04	0.033-0.049	1	0.134	0.101-0.196	1	2.445+/-0.247	327
EBS	1.898	1.237-2.744	47.5	8.21	5.32-16.18	61.27	2.015+/-0.235	317

In summary, we have identified that significant cross-tolerance between emamectin benzoate and spinosad (and to a lesser extent indoxacarb) occurred early in the lab-selection process, but the cross-tolerance declined as the selections continued. In contrast, significant cross-resistance was conferred to the Group-28 insecticides at an advanced stage in the laboratory selection with emamectin benzoate. If the same mechanism is responsible for the emerging resistance to emamectin benzoate in field populations of DBM as in the lab-selected EBS strain, these results would indicate a significant cross resistance threat in field population of DBM in Australia, and would require modification of the National 'Two-Window' IRM strategy for southern Australia to place both emamectin benzoate and the Group-28 products in the same window.

Table 7. Synergistic effect of 0.01% piperonyl butoxide (PBO) on the tolerance of emamectin benzoate to Waite susceptible (WS), emamectin benzoate selected (EBS) laboratory and field (QLD11) strains of *P. xylostella* larvae. (0.01% PBO alone resulted in larval mortalities of 3.85%, 1.57% and 7.3% in the WS, EBS and QLD11 strains respectively after control mortality correction, n=40.)

Insect strain	Treatment	LC_{50}	95%CL	RR	LC_{90}	95%CL	RR	Slope+/-S.E	N
WS	Emamectin benzoate	0.024	0.018-0.031	1	0.057	0.042-0.091	1	3.434+/-0.412	240
	Emamectin benzoate +PBO	0.008	0.007-0.009	0.33	0.016	0.014-0.022	0.28	4.189+/-0.638	241
EBS	Emamectin benzoate	3.96	2.86-5.324	165	16.324	11.35-27.324	286	2.084+/-0.214	353
	Emamectin benzoate +PBO	0.392	0.128-0.748	16.3	11	6.556-24.684	193	0.883+/-0.114	317
QLD 2011_G_6	Emamectin benzoate	0.416	0.313-0.541	17.33	2.301	1.632-3.648	40.37	1.725+/-0.170	362
	Emamectin benzoate +PBO	0.072	0.055-0.092	3	0.366	0.265-0.572	6.42	1.821+/-0.205	362

Table 8. Synergistic effect of 0.01% piperonyl butoxide (PBO) on the tolerance of chlorantraniliprole to Waite susceptible (WS), emamectin benzoate selected (EBS) laboratory, field (QLD11) and chlorantraniliprole selected (CS) strains of *P. xylostella* larvae. (0.01% PBO alone resulted in larval mortalities of 13.6%, 0%, 2.6% and 0% for WS, EBS, QLD11 and CS strains respectively after control mortality correction, n=40.)

Insect strain	Treatment	LC_{50}	95%CL	RR	LC_{90}	95%CL	RR	Slope+/-S.E	N
WS	Chlorantraniliprole	0.052	0.042-0.066	1	0.206	0.15-0.33	1	2.171+/-0.239	282
	chlorantraniliprole +PBO	0.034	0.026-0.044	0.65	0.088	0.078-0.238	0.43	3.180+/-0.438	284
EBS_G_{58}	Chlorantraniliprole	11.26	8.54-14.90	217	54.82	37.68-92.54	266	1.865+/-0.168	321
	chlorantraniliprole +PBO	4.24	3.34-5.40	81.5	14.96	10.86-23.70	72.6	2.340+/-0.229	321
QLD 2011_G_{10}	Chlorantraniliprole	0.396	0.296-0.498	7.6	1.196	0.912-1.834	5.8	2.674+/-0.410	321
	chlorantraniliprole +PBO	0.098	0.064-0.126	1.9	0.290	0.224-0.460	1.4	2.736+/-0.541	318
CS_G_3	Chlorantraniliprole	1.548	1.17-2.096	29.8	6.78	4.5-12.38	32.9	2.000+/-0.193	317
	chlorantraniliprole +PBO	0.234	0.168-0.326	4.5	0.79	0.528-1.522	3.8	2.426+/-0.236	280
	emamectin benzoate	0.275	0.208-0.366	11.5	0.924	0.647-1.562	16.2	2.442+/-0.232	317

7. DISCUSSION

The benchmarking study was undertaken to provide a measure of the tolerance levels that have developed in DBM populations on commercial Brassica vegetable properties in Australia to the newer synthetic insecticides and the microbial insecticide *Bacillus thuringiensis* var *kurstaki* (BtK). Given that historically in Australia DBM insecticidal resistance has been first observed in the Lockyer Valley, QLD (see Appendix 1), we focused the study on field collections from this QLD production region, but also included several properties in NSW, VIC and SA .

The study revealed that significant declines in susceptibility to a number of the main 'newer' insecticides registered for DBM control have occurred, and in some instances the resultant tolerance/resistance is at levels that will significantly impair field control with the affected insecticides.

For emamectin benzoate (IRAC Group 6, registered as Proclaim^{TM}), we consider that for an increasing percentage of properties nationally (the incidence of properties at risk in each State still needs to be determined) the field efficacy of DBM control will soon be (if not already) adversely affected by the increasing resistance levels.

For indoxacarb (IRAC Group 22, registered as Avatar^{TM}), although there has only been a low to moderate shift in susceptibility in the field strains tested in this study, there is a possibility that strains exhibiting the upper range of observed decline in susceptibility could experience reduced field control, because Avatar^{TM} is registered at a relatively low field rate of indoxacarb.

For spinosad (IRAC Group 5, registered as Success^{TM}; nb. during this project the closely-related spinetoram replaced spinosad as the active in this product), we do not consider that the observed tolerance in the field strain tested in this study is in any imminent risk of significant impairment of field control.

For the biopesticide BtK, we found no evidence of any apparent change in susceptibility of the surveyed DBM field strains.

For the IRAC Group-28 insecticides (registered as Coragen^{TM}, Belt^{TM}, Durivo^{TM}), which were added to the benchmarking study in 2011, the magnitude and geographic spread of the tolerance evident in the field strains screened in this study are unexpected and concerning, particularly given that this insecticide group was only first registered in Australian Brassica vegetables in 2009.

The field experiment conducted in SA in December 2011, in which the registered rates of Proclaim^{TM} and Coragen^{TM} were sprayed onto a Brussels sprouts crop and the mortalities that resulted from enclosing larvae of the susceptible and QLD (2011) field strains on samples of the sprayed foliage were measured, demonstrated a significant reduction in the efficacy of these Group 6 and 28 chemistries against the QLD (2011) field strain, thereby reinforcing the benchmarking results for these chemistries.

In summary, the development of Group 6 and 28 tolerance / resistance is at levels which is likely to be reducing the efficacy of DBM control in some properties in the Lockyer Valley, QLD, and similar tolerance has been detected in the very limited survey of southern Australian properties.

A more comprehensive study to determine the extent of insecticidal tolerance/resistance across the main production regions of southern Australia is required.

To examine potential cross-resistance patterns between the various insecticides studied in this project, we conducted a range of bioassays with the various lab-selected strains. Moderate cross-tolerance was observed when emamectin benzoate tolerant insects were tested with spinosad throughout the laboratory selection period, but the level of the cross tolerance was reduced as the selection progressed. Nil to low cross tolerance was observed when emamectin benzoate tolerant insects were tested with indoxacarb and BtK toxins. Moderate to high levels of cross tolerance was observed when BtK tolerant insects were tested with emamectin benzoate, spinosad and indoxacarb in the early generations during the course of laboratory selection, but most of the cross tolerance was lost as the selections progressed. Spinosad tolerant insects showed low to moderate cross tolerance to emamectin benzoate and indoxacarb, but no cross tolerance with BtK toxins.

Cross-resistance to Group-28 insecticides was evident in the emamectin benzoate selected laboratory strain (F_{52-55}). This has important implications for the management of DBM resistance to these important Group 6 and 28 insecticides.

In summary, we identified that significant cross-tolerance between emamectin benzoate and spinosad (and to a lesser extent indoxacarb) occurred early in the lab-selection process, but the cross-tolerance declined as the selections continued. In contrast, significant cross-resistance was conferred to the Group-28 insecticides at an advanced stage in the laboratory selection with emamectin benzoate. If the same mechanism is responsible for the emerging resistance to emamectin benzoate in field populations of DBM as in the lab-selected EBS strain, these results would indicate a significant cross resistance threat in field population of DBM in Australia, and would require modification of the National 'Two-Window' IRM strategy for southern Australia to place both emamectin benzoate and the Group-28 products in the same window.

We had observed in the field surveys of emamectin benzoate tolerance levels that insecticide exposure in the field caused elevated levels of tolerance, which decreased in field-collected populations after maintaining insects with pesticide-free diet in the laboratory.

A key question for resistance management is whether insect populations in the field are responding to increased selection pressures by acquiring inducible tolerance to the selective agent. If so, another question is whether such potentially multigenic tolerance traits, either alone or in combination with other recessive or low level resistance alleles, can cause problems in resistance management. Firstly, the insect has to respond to the selective agent or associated elicitors with the induction of its natural defense and metabolic activities. The second requirement is that the elevated immune status is transmitted to offspring. If these two preconditions exist in an insect species, it should be possible to increase tolerance levels by an incremental increase of the elicitor or agent in subsequent generations. This was demonstrated by Rahman *et al*. (2004) with a laboratory selected population of the stored foodstuffs pest *Ephestia kuehniella*. For the observed increase of tolerance levels by increments, an uninterrupted line of females with an elevated immune status is essential. This requirement is often not met with most leaf-eating field insects, but an exception is DBM. It may be more than a coincidence that

diamondback moth is one of the few insect species that have evolved resistance in the field to endotoxins from *B. thuringiensis* and to most synthetic pesticides. We therefore wanted to investigate the existence of inducible tolerance mechanisms in diamondback moth under laboratory and field conditions.

To answer the above questions, we experimentally explored the genetic and epigenetic potential of inducible tolerance in *P. xylostella* in a continuous selection program exposing each successive generation to a low dose of a specific toxicant (emamectin benzoate, spinosad, indoxacarb, and commercial BtK toxins). For emamectin benzoate and BtK the selection program has continued for approximately 60 generations. For the emamectin benzoate selected strain tolerance was shown to increase, but in back-crossed individuals no significant resistance alleles were identified. Hence the observed increase in emamectin benzoate tolerance was probably not based on preexisting recessive resistance mechanisms in the population. Instead, the genetic analysis after five and 12 generations of selection with a low dose of emamectin benzoate was consistent with a transient up-regulation of the immune and metabolic status in tolerant insects, which can be transmitted to offspring by a maternal effect.

The observation that initially most of the tolerance is transmitted to offspring by a maternal effect is an indication that the increase in tolerance under selection pressure is not due to preexisting resistance alleles in the population, but rather based on transient regulatory gene activities. The induction may involve a broad range of gene activities from immune-gene products to metabolic gene products. The epigenetic transmission could in principle be based on several mechanisms, the most likely being that increased levels of immune and metabolic components in the circulation of induced females results in their inclusion into the oocyte, triggering induction processes during embryogenesis. This mode of transmission is independent of the genotype of males and only requires a continuous line of immune-induced females, resulting in neonates that are already on an elevated immune and metabolic status.

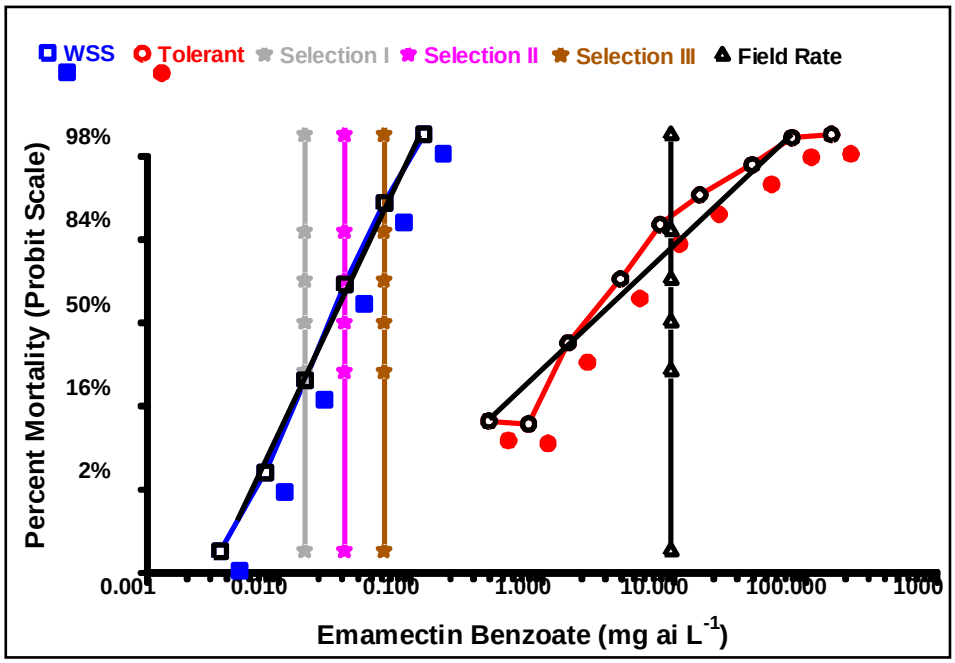
Although the relative contribution of inducible tolerance mechanisms is relatively high in the first few generations, the significant increase in tolerance observed between generation 5 and 12 is based on mechanisms that are genetically transmitted. If we accept the notion that tolerance is based on inducible regulatory mechanisms, it is possible that some of the regulatory pathways are genetically fixed. Alternatively, genotype selection of multiple allelic combinations that contribute to tolerance may have been achieved over the prolonged exposure to the pesticide. Finally, rare mutations may have emerged in target sites of metabolic or other genes that would have contributed to the observed increase in tolerance at earlier generations, and this needs to be investigated.

To summarize, tolerance in DBM larvae can be induced by exposure to low level of toxins and creates sub-populations of insects that show significant levels of tolerance that may lead to mutational changes in a major resistance gene locus in the long run. Our results suggest that incremental increases in tolerance to emamectin benzoate and BtK toxins in field-derived DBM are achievable under laboratory selection conditions to levels where there is potential to cause insecticidal control failures (Figure 9). We have discovered a new mechanism that generates tolerance under continuous selection, whereby the tolerant phenotype is caused by a novel induction process that is transmitted to offspring by an epigenetic mechanism showing a

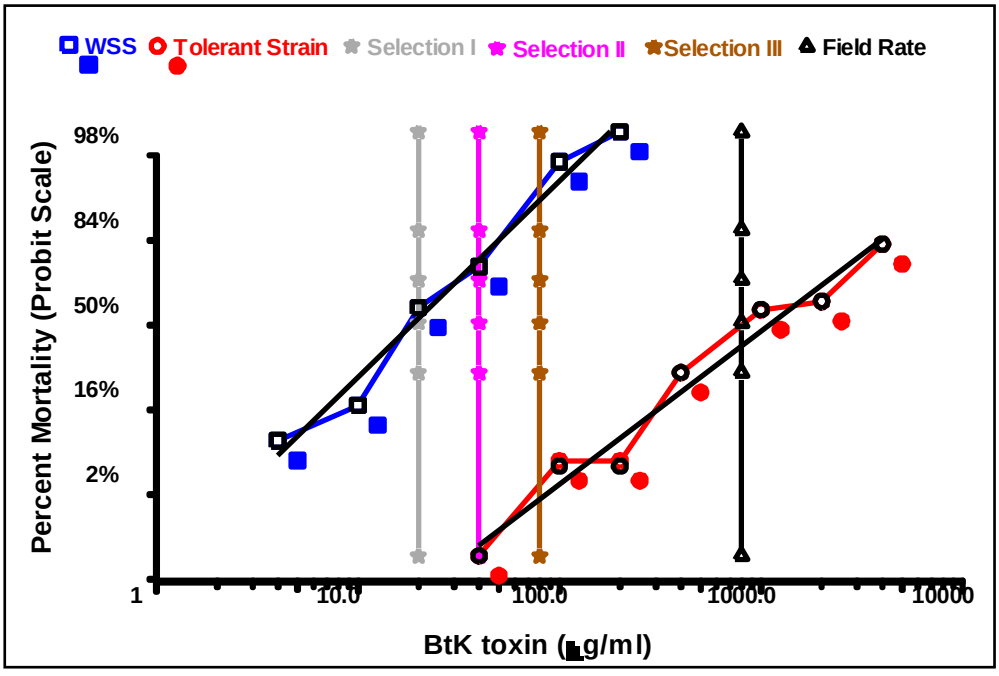
maternal effect. While the epigenetic contribution to incremental increases in tolerance is prominent under low to medium selection pressure, other resistance mechanisms that are transmitted genetically may predominate over time with incremental increase in toxin exposure.

Figure 9. A significant increased in tolerance and/or resistance to commercial formulations of a) emamectin benzoate (at F_{44}) and b) BtK toxins (at F_{45}) in field-derived laboratory populations of *P. xylostella* achieved under incremental selections started with low concentrations of each toxicant. Laboratory tolerant populations survived at field application rate for both emamectin benzoate and BtK toxins. WSS was used as control.

a) Emamectin benzoate tolerant strain



b) BtK toxins tolerant strain



The magnitude of the threat that this new tolerance mechanism represents to resistance management in the field is unclear and will be dependent on possible cross-tolerance to other pesticides and the size of the fitness penalties associated with the tolerance. Further studies are underway to investigate these aspects of the observed tolerance to synthetic pesticide. However so far we have observed that cross-tolerance can occur, as observed with the conferring of Group-28 tolerance by lab selection with emamectin benzoate (Table 6 and Figure 1), and in turn the fitness penalties evident early in the continuous selection process decline over time (Figure 8).

Several experiments are now underway to determine the molecular basis of the observed resistance, and the relatedness of the resistance mechanism(s) in the lab-selected and field-selected emamectin resistant strains. Once this is known, we will be able to determine whether Group 6 and Group 28 cross-tolerance needs to be managed in the field, and re-arrange the 'two-window' IRM rotation strategy accordingly.

How to address the other major research finding of this study, namely the epigenetic induction of tolerance through a maternal inheritance mechanism, is less clear. We had thought that the inducible tolerance mechanism may have been restricted to Bt toxins, but have found that it can occurs with synthetic insecticides as well (at least emamectin benzoate). This finding suggests that the induction process can occur from repeated exposure to doses (even very low doses) of a wide range of insecticides. Interruption of this selection process in the field may be able to be achieved by a complete break from spraying for a period of some months by the deployment of an alternative management strategy, for example, mating disruption (MD) using pheromones or lure-and-kill using a DBM version of a product such as Magnet^{TM} (used for attracting and killing cotton bollworm moths).

(At the start of the project we had considered that a refuge strategy, in which unsprayed refuge crops were planted in close proximity to the sprayed crops, might be used as an IRM tactic to help counter epigenetic resistance. However we now consider that although this would potentially dilute the proportion of the DBM population in the sprayed crop that possessed the induced epigenetic resistance, we do not believe it would interrupt the maternal transmission amongst the induced proportion of the population.)

In respect of MD, the pheromone application technologies have advanced considerably in the past decade or so (eg. ISCA Technologies market SPLAT^{TM}, an extrudable and spray-on pheromone with about a 10-week diffusion life, ideally suited to the life-span of the main Brassica vegetable crops), and at the same time the scale of Brassica vegetable production on many farm enterprises has substantially increased. These changes mean that MD may now be a cost-effective alternative to an insecticide spray program. In respect of lure-and-kill, Dr Peter Gregg (University of New England) and Ag Biotech Australia Pty. Ltd. are testing a DBM moth attractant in a Magnet-like formulation for deployment in canola crops (GRDC Project UNE00016). If this proved successful in canola, it would very likely be cost-effective for deployment in Brassica vegetables.

It is essential that several new alternative technologies be developed to manage DBM in Australian Brassica vegetables, because the development pipe-line of new insecticides is diminishing, and the findings of this project indicate that the effective life of a number of the existing chemistries is quite finite, and existing resistance management tactics will not alone suffice to adequately delay resistance development.

However the molecular mechanism findings of this project will allow rapid molecular diagnostic assays to be developed in the future which growers can use to assess the resistance status of the DBM population on their property at the beginning of the DBM season. Such assays, allied with knowledge on cross-resistance patterns (knowledge which this project has generated), will allow growers of the future to design property-specific insecticide programs which combine the use of the most effective products for targeting the particular DBM population on their property in a rotation pattern best suited to control their DBM population with minimized risk of cross-resistance.

8. TECHNOLOGY TRANSFER

The project findings and their implications of this project have been communicated to industry in a round of workshops held in Queensland (Gatton), New South Wales (Bathurst, Richmond), Victoria (Werribee, Cranbourne), Tasmania (Devenport), South Australia (Lenswood) and Western Australia (Perth, Manjimup) in August-September 2010.

The updated findings and their implications were presented by Mr David Carey (QLD DAFF) at a Brassica grower meeting in Gatton, Queensland in April 2012, and by Mr Greg Baker (SARDI) at an AUSVEG grower meeting in Virginia, South Australia in March 2013.

The findings have also been communicated at teleconferences in 2011, 2012 and 2013 to the AIRAC committee of CropLife Australia (which includes the three VC supporting insecticide companies) and the Diamide Working Group (technical representatives of the companies that are marketing Group-28 diamide chemistries in Australia).

The findings were also communicated to an international audience at the Sixth International Diamondback Moth Workshop held in Thailand in March 2011 (see Appendix 2), and through the publication of a peer-reviewed scientific paper in the Journal of Economic Entomology in 2010 (see Appendix 1).

9. RECOMMENDATIONS

1. The project findings, particularly the imminent risk of an increasing incidence of impaired DBM control with several of the newer DBM insecticides, needs to be broadly communicated to Australian Brassica vegetable growers. This communication should include reinforcement of advice on insecticide resistance management practices.

2. If the emamectin benzoate resistance mechanism(s) in the laboratory and field selected strains are the same, then in consultation with CropLife Australia modify the DBM '2-window' IRM strategy for southern Australia to take account of the cross-resistance risk for Group 6 and 28 insecticides.

3. As a priority undertake resistance screening surveys to determine the imminence of the threat to the suite of 'newer' DBM insecticides in southern Australian Brassica vegetable production regions. This information will inform growers of their current risk exposure, help encourage more spray-conserving IPM practice, and provide industry investors with an estimated time frame for the development and phase-in of non-insecticidal alternative management systems.

4. Research and develop non-insecticidal systems (eg. mating disruption, lure and kill, inundative release of biocontrol agents) for DBM management by the Australian Brassica vegetable industry to provide the industry with the necessary tools for production continuity when the current insecticide arsenal fails, and to position this industry as a market leader in sustainable crop production practice for the benefit of Australian consumers and environment.

5. Determine the nature of the molecular mechanism responsible for the observed epigenetic and genetic resistance in DBM, and the causes(s) of cross-tolerance between the different synthetic and biological insecticides. This knowledge would allow the design and testing of new IRM strategies and rapid resistance-screening tools.

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Finally, I wish to acknowledge the scientific legacy of Professor Otto Schmidt (ex Adelaide University), whose research and theoretical concepts on inducible tolerance helped shape the project's research.

11. LIST OF TABLES

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APPENDIX 1:

Crucifer Vegetable Insecticide Resistance Management Strategies and Issues in Australia

Paper presented at the Sixth International Diamondback Moth Workshop, Thailand, March 2011.

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ABSTRACT
The diamondback moth (DBM) (*Plutella xylostella*) is internationally notorious for evolving resistance to insecticides. Historically DBM resistance problems have been severe in Australian crucifer vegetable production, particularly in Queensland. This paper outlines the insecticide resistance management (IRM) program that is currently employed in Australia to maximize the number of effective applications that can be made of the available DBM insecticides, and thereby to maximize effective pest control and long term sustainability and profitability of crucifer vegetable production.

A central component of this IRM program is a national "two-window" insecticide rotation strategy. This rotation strategy includes the six different mode-of-action insecticide groups registered in Australia since 1998. The chosen calendar periods provide similar market share for the insecticides in each window, and take account of the dynamics of the pest complex in each State. A national resistance screening program documents changes in susceptibility to these newer compounds, and this information assists in property and regional level resistance management.

Other Australian crucifer IRM strategies, which are based upon well-established pest management principles, include natural enemy conservation, threshold-based crop monitoring and well-timed and well-calibrated spray application.

Recent results of the resistance screening program indicate that moderate shifts in tolerance to emamectin benzoate, indoxacarb and spinosad have occurred in some production areas. However use of these products has declined following the 2009-10 registration of three Group-28 diamide products. The high level of diamide usage by crucifer growers nationally, and the particular resistance risk presented by one of these diamide products formulated for seedling drench application, now presents a major ongoing challenge for DBM resistance management in Australia.

Keywords: Insecticide resistance management (IRM), diamondback moth (DBM), crucifer vegetables, window strategy.

INTRODUCTION

The diamondback moth (DBM) (*Plutella xylostella*) is internationally notorious for evolving resistance to insecticides, particularly in intensively sprayed vegetable production regions. In Australia, routine spraying for DBM and other pests in crucifer vegetable crops has led to the development of resistance to organophosphate and synthetic pyrethroid insecticides, and to tolerance to some of the newer insecticides registered since the late 1990's.

This paper provides an outline of crucifer vegetable crop production in Australia, the documented history of insecticide resistance in Australian populations of DBM, and the

insecticide resistance management (IRM) program that is currently employed in the Australian vegetable industry to maximize the number of effective applications that can be made of the available DBM insecticides, and thereby to maximize the long term profitability of crucifer vegetable production.

THE AUSTRALIAN CRUCIFER VEGETABLE INDUSTRY

The approximately 13,000 ha (AUSVEG 2009) of crucifer vegetable production in Australia is largely concentrated around the main urban population centers in each of the five mainland States and in northern Tasmania (Figure 1).

Figure 1: The location (indicated by the red circles) of the six main crucifer vegetable production regions of Australia. The five southern regions are climatically temperate, and the Queensland region is subtropical.

Typically a crucifer-producing farm enterprise plants between 20 to 200+ ha of crucifer vegetables per annum. In the climatically temperate southern States DBM is the key pest, and typically has six to nine generations per annum. The great majority of the insecticide sprays, which may range from three to eight or more applications per *Brassica oleracea* crop, are applied for DBM control. In the subtropical production region of southern Queensland (Lockyer Valley) DBM has approximately 12 generations per annum, and is one of a complex of six lepidopteran (the other pest species are *Crocidolomia pavonana*, *Helicoverpa armigera*, *H. punctigera*, *Hellula hydralis* and *Spodoptera litura*) and two sucking pests (*Bemisia tabaci* biotype B and *Thrips tabaci*) that commonly require control. The number of insecticide sprays applied to Lockyer Valley crucifer crops generally ranges between 8 to 12 per crop (D. Carey, pers. comm.).

Insecticide sprays are generally applied using standard hydraulic nozzles mounted on approximately 10 m width boom arms. These boom spray units are tractor-mounted, and some are fitted with ducted air-assistance. Spray volumes range from 500 (transplant) to 1000 (maturity) L ha^{-1}. Some growers are starting to use fan-assisted, multiple spray heads and lesser spray volumes with excellent success (G. Furness, pers comm.).

HISTORY OF INSECTICIDE RESISTANCE IN POPULATIONS OF DBM IN AUSTRALIAN VEGETABLE CROPS

From the end of WWII to the late 1970's DDT and a range of cyclodiene, organophosphate and carbamate insecticides were used for pest control in Australian crucifer vegetable crops (Baker 1994; Endersby and Ridland 1994; Heisswolf and Hargreaves 1994). During this period there were no documented studies of insecticidal tolerance or resistance in Australian populations of DBM.

Following the registration of permethrin for the control of DBM and several other lepidopteran pests in Australian crucifer vegetable crops in 1978, a further three synthetic pyrethoids (es-fenvalerate, deltamethrin and alpha-cypermethrin) were registered in the early 1980's. Synthetic pyrethroid resistance was first identified in DBM in populations in the Lockyer Valley of Queensland in the mid 1980's (Wilcox 1986; Altmann 1988), approximately seven years after their initial registration. Widespread spray failures and crop losses occurred in the Lockyer Valley during 1985 to 1987, which led to the development and implementation of an insecticide resistance management (IRM) strategy in 1988 (Deuter 1989). The strategy was based on the

rotation of four insecticide groups - synthetic pyrethroids, carbamates, organophosphates and cyclodienes) (Deuter 1989) - and was subsequently broadened to include complementary IPM practices such as summer crop production break, improved spray application, pest scouting and the use of *Bacillus thuringiensis* (*Bt*) products) (Heisswolf 1992).

In southern Australia reports of DBM control failures first emerged in 1990 in South Australia, and were confirmed to be related to resistance to synthetic pyrethroid and organophosphate insecticides (Baker 1994; Baker and Kovaliski 1999). Further reports of DBM control failures followed in Victoria and New South Wales in 1993-94 and in Western Australia and Tasmania in 1995. Endersby and Ridland (1997) confirmed moderate to high level resistance to permethrin in strains of DBM collected from crucifer vegetable crops in each of Victoria, New South Wales, Western Australia and Tasmania. Despite the documented resistance, the use of organophosphate and synthetic pyrethroid insecticides for the control of DBM in southern Australian crucifer vegetable crops continued. The reason for this was that between 1978 and 1997-98 no new class of insecticide was registered in Australian crucifer vegetables, and the only available insecticidal alternatives were *Bt* products. Hence control was achieved by the more frequent use of the available synthetic insecticides supplemented with the use of the *Bt* products. The broad resistance to the classes of registered synthetic insecticides limited the prospects for effective resistance management.

THE DEVELOPMENT OF A NATIONAL IRM STRATEGY FOR DBM IN AUSTRALIAN CRUCIFER VEGETABLES

A new opportunity to introduce a more effective platform of IRM strategies against DBM in Australian crucifer vegetables emerged in 1996-97. For the first time a national DBM IPM program principally involving public sector research and extension workers in each State was established. A second key stimulus was the impending registration in quick succession of four highly effective insecticides - fipronil, chlorfenapyr, emamectin benzoate and spinosad - in new chemical groups that showed no apparent cross-resistance to one another.

This presented a unique opportunity for a resistance management strategy to be developed based upon the rotation of these new insecticides.

Thus in August 1996 state and university researchers and pesticide company representatives began formal discussions on the development of an Australia-wide IRM rotation strategy. Over the following year they negotiated the framework and detail of the rotation strategy.

From a purely resistance management perspective, the best strategy would have been to have four windows, one for each of the new products. In theory, this would increase the number of effective applications that could be obtained from each insecticide compared to an *ad hoc* mosaic approach (Roush 1989; Immaraju et al. 1990; Roush 1993). However, given the staggered times over which the products were being registered, difficulties in dividing the year into even quarters in terms of number of spray applications and market share, and excessive complications to the message that could be delivered to pesticide resellers and vegetable growers, a simpler two window strategy was devised.

From considerations of pesticide use patterns and pest population pressure, the year was divided into two relatively equal periods. However, in recognition of the regional/State differences in the crucifer pest complex and timing of the peak periods of DBM pressure and consequent pesticide use patterns, three different regional versions of the strategy were devised - 1. Queensland; 2. Western Australia and 3. South-eastern Australia (South Australia, Victoria, Tasmania and New South Wales) - each with differing calendar dates for the two window periods.

With respect to the older synthetic insecticides, a review of the literature on resistance mechanisms and Australian data on cross-resistance in DBM suggested that sufficient levels of cross resistance between carbamates, organophosphates and synthetic pyrethroids would likely limit any potential windowing benefit. Further, it was anticipated that the use of these older chemistries was likely to sharply decline in favour of the new products. For these reasons these older chemistries were not windowed.

Figure 2: Version I (1998-99) of the Australian "two-window" DBM IRM rotation strategy for the south-eastern Australian geographic region.

Because of their selectivity, leaving *Bt* products available year round was considered preferable to restricting them to one window. Hence the use of *Bt* in the early phase of crop development (eg. 6-10 leaves), when some damage can be tolerated and spray coverage is generally good, is promoted year round to help colonizing natural enemies to establish and to reduce the selection pressure on other insecticides.

Once the "two-window" DBM IRM strategy was agreed upon in later 1997, it was actively promoted in an IRM awareness campaign run in 1998. Glossy colour A4 flyers of the window strategy were circulated to all crucifer growers, crop scouts, consultants, chemical resellers and chemical company field personnel.

The first version (Figure 2) of the strategy windowed fipronil (Regent®) and chlorfenapyr (Secure®), which were the first of the new products to obtain Australian registration. In 1999-2000 spinosad (Success™) and emamectin benzoate (Proclaim®) were registered and added to the strategy (version II), and in 2001-02 the third version was released with the registration of indoxacarb (Avatar®). In 2009-10 the strategy was again updated (version IV, Figure 3) to

include the newly registered Group-28 chemistries flubendiamide (Belt®) and chlorantraniliprole (Coragen®). All three of the regional versions of the strategy can be viewed at the web address: http://www.sardi.sa.gov.au/pestsdiseases/horticulture/horticultural_pests/diamondback_moth/insecticide_resistance_management

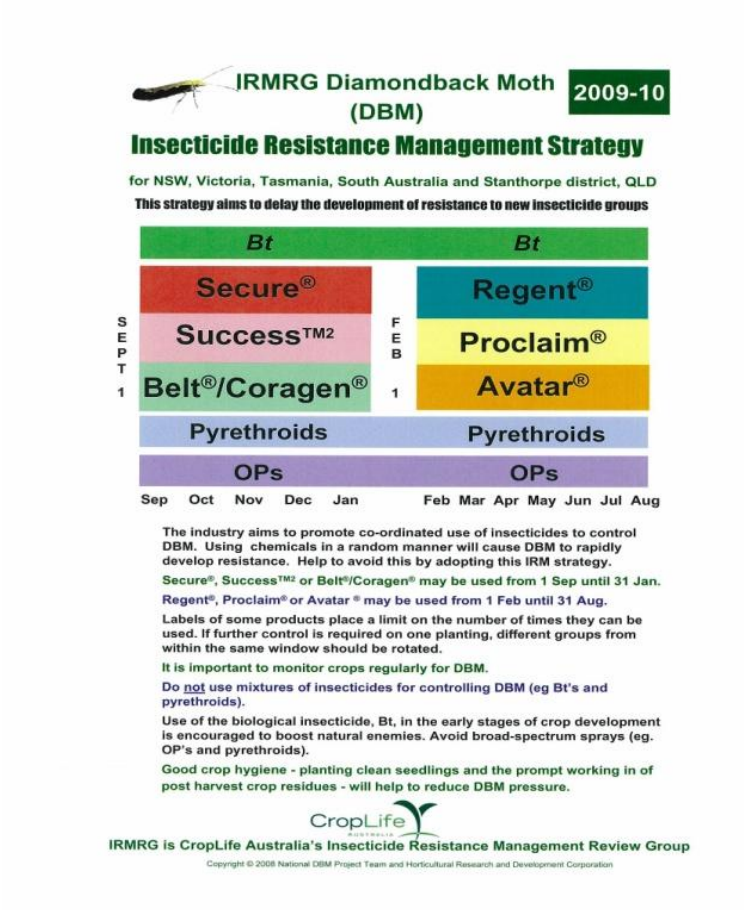

Figure 3: Version IV (2009-10) of the Australian "two-window" DBM IRM rotation strategy for the south-eastern Australian geographic region.

To assist in educating growers and other industry personnel about the different insecticide classes, the opposing face of the "two-window" IRM strategy flyer contains the IRAC chemical groups & product names information.

Grower compliance with the "two-window" IRM strategy is only moderate. In a 2006 national survey of crucifer vegetable growers, 60% of respondents knew of the "two-window" DBM IRM strategy, but only 39% said that they practiced the strategy (Baker and Maratos 2007). A common reason given by growers for not using the strategy was that DBM was not considered a major pest on their property. This is concerning because if growers are still spraying frequently, albeit against low density DBM infestations, selection for resistance will continue to occur.

This level of non-compliance with the "two-window strategy" means that a mosaic of treatment patterns is often occurring in neighbouring crops. Clearly if gene flows are occurring between these crops, this will result in simultaneous selection with several pesticides at the same time. However, if neighbouring crucifer properties are at least one to two kilometres apart and growing crucifer crops all year round, as is often the case in southern Australia, the gene flow between neighbouring properties may be quite low. For example, in release-recapture studies using laboratory-reared and fluorescent-marked DBM adults, Mo and co-workers (2003) estimated that

99.9% of the released moths in commercial cauliflower and broccoli crops in South Australia were expected to remain within 113 to 300 m of the release point. Hence growers that do adhere to the "two-window" strategy may still benefit from doing so, irrespective of their neighbours' non-compliance.

In an endeavour to increase the industry awareness of the threat of DBM resistance and of good IRM practices, including the national "two-window" strategy, a DVD titled "The Reality of Resistance: Insecticide resistance development and management in Brassica vegetables" was produced in 2007 and distributed to all chemical resellers in vegetable production areas nationally.

OTHER KEY IRM STRATEGIES

In addition to the "two-window" rotation strategy, a number of other practices, which are based upon well-established pest management principles, are actively promoted for the resistance management benefits they provide. These practices aim to preserve susceptible individuals by reducing spray frequency, and include natural enemy conservation, threshold-based crop monitoring, crop breaks and well-timed and well-calibrated spray application.

Natural enemy conservation:

Growers are encouraged to choose selective chemistries to minimize the disruptive effect of the spray program on the natural enemy complex. To assist growers a glossy colour "Impact of Insecticides on Natural Enemies Found in Brassica Vegetables" chart has been produced, hard copies distributed nationally, and also made available at the web address: http://www.sardi.sa.gov.au/pestsdiseases/research_projects2/research_projects/diamondback_moth/impact_of_insecticides_chart.

Spray decision making:

The national DBM IPM program has actively promoted the uptake of spray decision making based on crop scouting and the use of economic thresholds. The use of crop scout consultants, particularly those that encourage growers to implement an IPM strategy (such as IPM Technologies Pty. Ltd.), has been promoted. This program has also developed an electronic sampling plan, which assesses the need to spray based on the number of plants infested with DBM larvae, the crop type, market destination, the stage of crop development and parasitism levels (Hamilton et al. 2004). A sub-component of the sampling plan, a DBM rate of development model which requires daily max/min temperatures, assists with optimizing spray timing. Accessible at the web address: http://new.dpi.vic.gov.au/agriculture/pests-diseases-and-weeds/pest-insects/ag0512-diamondback-moth/sampling-plan, this plan interfaces with other tools developed by the national DBM program. For example, if the plan recommends a spray, then there is a direct link to the "two-window" IRM strategy so that only those chemicals available at that time of the year are recommended. Next, of the chemicals recommended, there is a link to the Beneficial Insect Toxicity Chart. To help conserve beneficial insects, the grower can then choose insecticides that will be less disruptive to the types of beneficials that were present during crop monitoring.

Crop break and hygiene:

A crop break is promoted in Queensland from November to January inclusive, as this summer period is sub-optimal for the production of quality crucifers, particularly cauliflowers and cabbages. Good crop hygiene is promoted nationally, particularly the planting of clean seedlings and the prompt working in of post harvest crop residues. These strategies are designed to reduce DBM population densities on farm and hence to reduce spraying frequency.

RESISTANCE SCREENING PROGRAM

A national DBM resistance screening program was established in 1999. The program involves testing of field populations of DBM from the major crucifer production regions with a variety of newer (post 1997 registration) and long-established insecticides to detect substantial change in susceptibility and to confirm resistance in the event of field control failure. Tests conducted between 2000-03 using the leaf dip method (Tabashnik and Cushing 1987) revealed moderate levels of resistance to synthetic pyrethroids (resistance ratios, RR, of field populations compared with susceptible Waite laboratory population up to 18 fold), no evidence of tolerance to either chlorfenapyr (RR of 0.42-2.04), emamectin benzoate (RR of 0.72-4.43), spinosad (RR of 0.68-3.40) or indoxacarb (RR of 0.83-2.48), and, with the exception of one Queensland field population (RR of 11.03), no evidence of fipronil tolerance (RR of 0.99-2.37) (Endersby et al. 2004).

A further round of bioassays were conducted between 2006-10 with the three most frequently used of the newer DBM insecticides, emamectin benzoate, indoxacarb and spinosad, and *Bt* subsp. *kurstaki*. These were tested against fourteen, six, eight and two field strains respectively. The test insecticide doses were applied by Potter tower to third instar DBM larvae on 90 mm diameter cabbage leaf discs.

In 18 of the 30 bioassays nil or very low insecticide tolerance (RR<5) was recorded (Table 1). In the case of emamectin benzoate, seven of the fourteen bioassays indicated low to moderate tolerance (5<RR<15), with the highest RR being 12.4 for a 2007 Queensland strain. With indoxacarb, four of the six bioassays indicated low to moderate tolerance, with a maximum RR of 13.3 recorded for a 2010 New South Wales strain. With spinosad, only one of the eight strains had a RR exceeding 5.0 (a 2007 Queensland strain with RR of 6.0), and with the two Bt subsp. kurstaki bioassays no tolerance was evident. Over the four years that the bioassays were conducted there was little indication of an increase in tolerance to the tested products.

The small sample size of this 2006-10 survey does not preclude that higher tolerance levels to these insecticides may occur in some populations of DBM in Australian crucifer vegetable crops. However thus far there have been no reports of control failures with these products nationally. Further, the tested strains were chosen from high-risk districts with a history of DBM resistance to older chemistries, so the non-detection of higher levels of resistance in these strains is an encouraging result.

Table 1: LC_{50} estimates for 14 DBM field strains collected from commercial crucifer vegetable crops in Queensland (QLD), New South Wales (NSW) and South Australia (SA), 2006-10.

FIELD STRAIN	LC_{50} †	95% CI	RR
EMAMECTIN BENZOATE:			
QLD†† 2006	0.260	0.167-0.392	6.1
QLD 2007A	0.083	0.047-0.126	1.9
QLD 2007B	0.139	0.110-0.173	3.2
QLD 2007C	0.216	0.163-0.286	5.1
QLD 2007D	0.069	0.057-0.083	1.6
QLD 2007E	0.528	0.427-0.660	12.4
QLD 2007F	0.229	0.123-0.383	5.4
QLD 2007G	0.251	0.202-0.308	5.8
QLD 2008A	0.150	0.110-0.220	5.4
QLD 2008B	0.128	0.084-0.176	4.6
QLD 2008C	0.251	0.185-0.326	9.0
QLD 2009A	0.176	0.136-0.224	4.1
NSW 2010A	0.202	0.150-0.268	4.7
NSW 2010B	0.092	0.072-0.114	2.1
INDOXACARB:			
QLD 2007A	30.9	24.9-38.4	8.5
QLD 2007C	21.3	16.2-27.3	5.9
QLD 2007H	21.15	16.08-26.97	5.9
QLD 2009A	12.9	9.3-16.5	3.6
NSW 2010A	9.18	7.26-11.13	2.6
NSW 2010B	48.0	29.7-63.0	13.3
SPINOSAD:			
SA 2006	1.032	0.828-1.272	3.4
QLD 2007A	1.404	1.152-1.716	4.7
QLD 2007G	1.80	1.32-2.40	6.0
QLD 2007H	1.26	1.02-1.55	4.2
QLD 2008A	0.972	0.792-1.188	3.1
QLD 2008D	0.780	0.612-0.984	2.5
QLD 2009A	0.324	0.252-0.408	1.1
NSW 2010A	0.816	0.660-0.996	2.7
BACILLUS THURINGIENSIS SUBSP. *KURSTAKI*:			
QLD 2009A	90780	68839-111080	0.7
NSW 2010A	143770	106790-182779	1.1

† LC_{50} VALUES EXPRESSED AS MG A.I. L^{-1} FOR THE 3 SYNTHETICS, AND INTERNATIONAL UNITS OF POTENCY L^{-1} FOR THE BTK.
††QLD=LOCKYER VALLEY, QLD; NSW=SYDNEY BASIN, NSW; SA=ADELAIDE HILLS, SA. FOR A GIVEN YEAR, THE SAME LETTER INDICATES A STRAIN FROM THE SAME PROPERTY.

MANAGEMENT OF THE NEW GROUP-28 DIAMIDES

In 2009 the first of the Group-28 diamide insecticides were registered in Australian crucifer vegetables as foliar spray formulations for the control of a suite of lepidopteran pests, including DBM. The chemicals were flubendiamide (registered as Belt® 480SC and Belt® 240 WG containing 480 g ai L^{-1} and 240 g ai kg^{-1} respectively) and chlorantraniliprole (registered as Coragen® containing 200 g ai L^{-1}). They combine high insecticidal activity and selectivity on target lepidopterans, thereby providing excellent field control with minimal toxicity to beneficials and other non-target organisms, including humans.

These new Group-28 foliar spray products were placed in the first window of the Australian "two-window" DBM IRM strategy in 2009. This was publicized with the national mail-out of an updated flyer in early 2010 (Figure 3).

However looking forward there are several specific challenges to the effective management of the resistance risk to the Group-28 chemistry. Firstly, in the two years since their initial registration the uptake of Group-28 products by Australian vegetable growers has been rapid, and their resultant market share is already significant. Secondly, in 2010 a seedling drench formulation (Durivo®) containing a mixture of chlorantraniliprole (100 g ai L^{-1}) and thiamethoxam (200 g ai L^{-1}) was registered for use in this crucifer vegetable market for lepidopteran and sucking pest control. The convenience and insurance value of a prophylactic seedling drench treatment is likely to encourage many growers to treat successive plantings of crucifer seedlings. This seedling drench treatment is more persistent than a foliar application. Both these factors combine to increase the resistance selection risk.

In regard to the persistence concern associated with Durivo®, an experiment was conducted recently in an Adelaide Hills Brussels sprouts crop. Seedlings sourced from three different nurseries were treated with the registered rate of Durivo® (3 g chlorantraniliprole per 1000 seedlings). At a range of intervals after transplanting, third instar DBM larvae were placed on leaf discs form the youngest fully expanded leaf from each of the nursery sources. The Abbott's corrected mortality of these larvae remained at nearly 100% for 30 DAT, and then progressively declined from approximately 90% at 35 DAT to 20% at 57 DAT (Figure 4). Unfortunately a comparative data-set for the foliar formulation of chlorantraniliprole is not presently available. The relatively long period of partial control observed in this South Australian study is a concern, and the total exposure period is problematic for achieving compliance with two of the key global guidelines of the IRAC Diamide Working Group (IRAC 2010). These guidelines advise to "avoid exposure of consecutive insect pest generations" to Group 28 insecticides, and that the "total exposure period of all Group28-active windows applied through the crop cycle (from seedling to harvest) should not exceed 50% of the crop cycle". In all crucifer production regions of Australia it would be unavoidable to expose consecutive DBM generations to chlorantraniliprole in any Durivo®-treated crop; and in leafy crucifer vegetable, broccoli, cabbage and cauliflower crops treated with Durivo® the total exposure period of chlorantraniliprole will likely exceed 50% of the crop cycle.

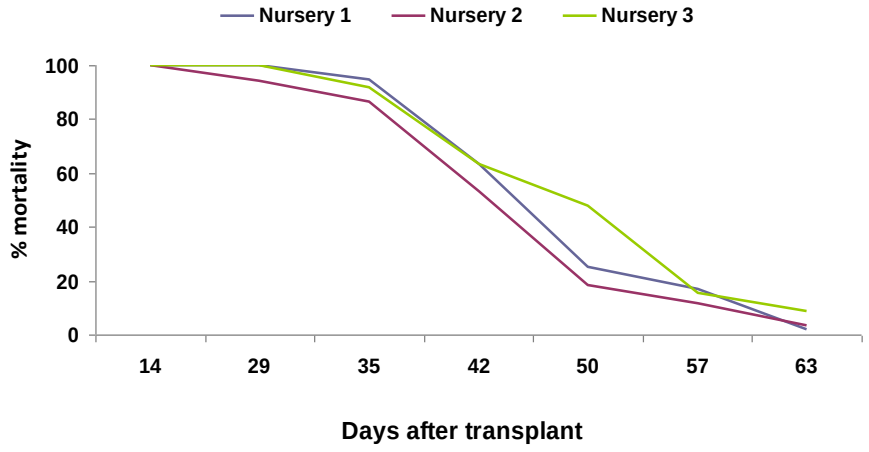

Figure 4: The Abbott's corrected percentage mortality of third instar DBM larvae after 72 hours exposure on leaf discs cut from Durivo®-treated Brussels sprouts plants at 14, 29, 35, 42, 50, 57 and 63 days after transplant, Adelaide Hills, South Australia. The transplants were drench-treated with Durivo® (30 ml product per 1000 seedlings) and planted on 6 December 2010.

A future challenge also looms with the anticipated registration of more Group 28 products, and the possibility that some of these future registrants may not wish to 'window' their products.

To provide stewardship of the Group-28 chemistry in Australia, and to help address the aforementioned challenges, an Australian Diamide Working Group has been formed with chemical company, reseller and state entomologist representatives. An initial recommendation of the Working Group to be promoted to industry is that a grower that has used Durivo® should, to help counter the risk of selection for Group 28 resistant heterozygotes, automatically rotate to another mode of action insecticide from 30-35 days after transplant and refrain from any further use of Group 28 insecticides on the crop.

CONCLUSION

Significant improvements in Australian crucifer pest management and, specifically, in the management of insecticide resistance in diamondback moth populations in this cropping system, have been achieved in the past fifteen years. The challenge to further increase grower adoption of these practices and to effectively incorporate new chemistries, particularly seedling drench formulations, into these IRM strategies is ongoing and increasing.

Acknowledgements

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APPENDIX 2:

Induction and Transmission of Tolerance to the Synthetic Pesticide Emamectin Benzoate in Field and Laboratory Populations of Diamondback Moth

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Abstract

Field surveys of pest insect pest populations in agroecosystems reveal low but significant levels of tolerance to synthetic and biological pesticides but fail to uncover resistance alleles in test crosses. To study the potential of inducible mechanisms to generate tolerance to synthetic pesticides, we performed baseline susceptibility studies in field and laboratory populations of diamondback moth, *Plutella xylostella* (L.). to commercial formulations of emamectin benzoate. Pesticide exposure in the field caused elevated levels of tolerance, which decreased in field-collected populations after maintaining insects with pesticide-free diet in the laboratory. Because no significant resistance alleles were identified in back-crossed individuals, the observed increase in tolerance was probably not based on preexisting recessive resistance mechanisms in the population. Instead, the genetic analysis after five and 12 generations is compatible with a transient up-regulation of an immune and metabolic status in tolerant insects that can be transmitted to offspring by a maternal effect. Although the epigenetic effects contributed to incremental increases in tolerance in the first five generations, other resistance mechanisms that are transmitted genetically predominate after 12 generations of increased exposure to the pesticide.

Introduction

Improvements in synthetic and biological pesticides and their application in the field impose extreme selection pressures on target and nontarget insect populations (Roush and Tabashnik 1990). For example, the selection pressure imposed on insect populations in transgenic crops expressing *Bacillus thuringiensis* (Bt) toxin proteins effectively eliminates most susceptible insects, but those individuals that survive pose a threat for widespread resistance (Shelton et al. 2002). Resistance management strategies for all insecticidal control tactics, such as the so-called high-dose strategy for Bt (Bates et al. 2005), are based on understanding the evolution and genetics of resistance, which has tended to focus on single major genes (Roush and McKenzie

1987) that confer resistance via decreased sensitivity of the target sites or increased metabolism, binding, and excretion of the pesticides (Roush and Tabashnik 1990).

However, in the older insecticide resistance management literature, there was considerable discussion of "tolerance" or "vigor tolerance," which seems generally vague in explanation but was not considered to be "real" resistance nor thought to be due to major genes specific to insecticides. Gordon (1961), for example, concluded that resistant strains could be more tolerant to stresses from lack of water or food, and that nutritional factors could influence resistance.

Furthermore, even in more recent studies, researchers often comment upon (but generally do not publish) low levels of decreased susceptibility to pesticides that seem to wax and wane irregularly and are difficult to repeat (Ahmad 1999). These effects seem to be separate from target site mutations, providing protection to low to medium pesticide doses (Rahman et al. 2004). These tolerance mechanisms are difficult to study and given that they provide only a small proportion of the resistance levels usually achieved by target site mutations, were mostly ignored until recently. However, observations in some insect populations suggest that insects exposed to extreme selection pressures have the potential under laboratory conditions to increase the levels of tolerance significantly (Rahman et al. 2007).

A key question for resistance management is whether insect populations in the field are responding to increased selection pressures by acquiring inducible tolerance to the selective agent. If so, another question is whether such potentially multigenic tolerance traits (Rahman et al. 2004), either alone or in combination (Gonzalez-Cabrera et al. 2001) with other recessive or low level resistance alleles, can cause problems in resistance management. This would be most likely for pests that are somewhat naturally tolerant to the insecticides being used. A key example might be *Helicoverpa* species (bollworms), which are less sensitive to Bt cotton, *Gossypium hirsutum* L., than other targeted Lepidoptera, such as pink bollworms and tobacco budworms.

Firstly, the insect has to respond to the selective agent or associated elicitors with the induction of its natural defense (Freitak et al. 2007) and metabolic activities (Li et al. 2007). There are several ways to measure immune induction. One way is by using a biochemical approach to measure the level and activity of key enzymes in insect immune defense, such as prophenoloxidase (Kanost et al. 2004), by extracting hemolymph and showing melanization in cell-free plasma before and after challenging the insect with the immune elicitor. The other ways are phenotypic approaches, by performing bioassays before and after immune induction. For example, the larval immune system can be induced by feeding a sublethal dose of a Bt toxin formulation to the flour moth *Ephestia kuehniella* Zeller, and tolerance levels measured by challenging the same larvae a few days later with a lethal dose of the formulation. The survival rate correlated with the elevated immune status in surviving larvae, which suggested that the immune status of insects may have caused the increased tolerance to the biopesticide (Rahman et al. 2004).

The second requirement is that the elevated immune status is transmitted to offspring. Transmission of immune phenotypes by epigenetic effects have been discovered in crustaceans (Huang and Song 1999) and insects, including aphids (Little et al. 2003) and bees (Moret and Schmid-Hempel 2001), but transmission of biopesticide tolerance to subsequent generations of insects was only recently demonstrated (Rahman et al. 2004). In some insects from the field, more than one resistance mechanism has been identified (Gonzalez-Cabrera et al. 2001) involving a recessive (high-dose) resistance trait (Bird and Akhurst 2004) in conjunction with dominant (low-dose) tolerance mechanisms (Ma et al. 2005). So far, transmission of tolerance seems to occur predominantly by a maternal effect (Rahman et al. 2004), which could be due to epigenetic modifications of maternal genes, or incorporation of hemolymph immune components

into oocytes in induced females and subsequent immune induction in the embryos. The fact that lipid particles (Jung and Yun 2007), lectins (Amanai et al. 1994), hexamerins (Kang et al. 1995), and phenoloxidase (Schmidt et al. 2005) are stored in the ooplasm could make them candidates for embryonic elicitors, but more research is required to uncover the molecular basis of the maternal effect transmission of the elevated immune status.

Finally, if the two preconditions mentioned above exist in an insect species, it should be possible to increase tolerance levels by an incremental increase of the elicitor or agent in subsequent generations. This was demonstrated in *E. kuehniella* laboratory populations. Starting with the semilethal dose of 500 ppm of the Bt formulation, the amount of toxin in the food was doubled every few generations to 32,000 ppm over a period of 3 yr (Rahman et al. 2007). For the observed increase of tolerance levels by increments, an uninterrupted line of females with an elevated immune status was essential. This requirement rules out insect species showing high levels of inbreeding depression, where genetic out-crossing with susceptible (female) insects is expected to disrupt the maternal effect transmission. Insects that can be cultured from single breeding pairs are usually found in storage pests, whereas most leaf-eating field insects, such as *Helicoverpa* and *Pieris* species (Roush 1986), show inbreeding depression. One of the exceptions in field insects is the diamondback moth, *Plutella xylostella* (L.). which can be propagated from a single pair for many generations (M.M.R., unpublished data). It may be more than a coincidence that diamondback moth is one of the few insect species that have evolved resistance in the field to endotoxins from *B. thuringiensis* (Shelton et al. 2002) and to most synthetic pesticides (Talekar and Shelton 1993). We therefore wanted to investigate the existence of inducible tolerance mechanisms in diamondback moth under laboratory and field conditions.

Given that synthetic pesticides have different modes of action and different resistance pathways (Li et al. 2007) compared with those of biopesticides, such as Bt-toxin (Tabashnik et al. 2000), the emergence of resistance to synthetic pesticides and its management in the field have been approached differently from biopesticides in conceptual and practical terms.

Here we report elevated levels of tolerance to a chemical pesticide (emamectin benzoate) in diamondback moth field populations that have been under continuous selection pressure. The tolerance is correlated with an elevated immune and metabolic status and is transmitted to offspring by an epigenetic process showing a maternal effect.

Materials and Methods

Insects. A susceptible laboratory population of P. *xylostella* (Waite susceptible strain) has been maintained on seedling cabbage, *Brassica oleracea* L. variety *capitata* 'Green Coronet' leaves in the laboratory at 25 ± 0.5°C and a photoperiod of 14:10 (L:D) h in a separately caged laboratory culture at the Waite Campus, South Australia, without exposure to any insecticides for ≈18 yr (≈205 generations). To increase larval numbers on a large scale for bioassays, neonate larvae were reared on seedling canola, *Brassica napus* L. 'Monty', stands grown on vermiculite in 500-ml Plaspak plastic pots. Cabbage leaves were added periodically, when required for the developing larvae. A thin layer of honey on masking tape and 10% honey solution with 0.1% sorbic acid were provided as food source for the adults. This Waite susceptible strain (WSS) was used as the reference strain for comparison with field-collected populations and in genetic crosses.

Fourteen populations of *P. xylostella* were collected in 2006 and 20 populations in 2007 from crucifer vegetable fields in different regions of Queensland, New South Wales, and South

Australia, Australia. These sites were selected because they are important crucifer production areas with a history of intensive use of emamectin benzoate for management of *P. xylostella*. Approximately 150–200 *P. xylostella* larvae and pupae were collected at each location and transported to the Waite Campus (SARDI Entomology), South Australia, where bioassays were performed. These were then reared on cabbage leaf substrate using the same methodology mentioned above. Because high levels of parasitism existed in most of the field-collected populations, parasitoid-free cultures from each field strain were first established from *P. xylostella* adults emerging from the field-collected population, which were reared on dense canola seedlings as described above.

Insecticide and Bioassays. Emamectin benzoate (Syngenta 2004) was supplied by Syngenta Crop Protection (Greensborough, NC). Bioassays were performed on insecticide-coated leaf discs. Cabbage leaf discs were cut from washed cabbage leaves (90 mm in diameter) taken from 8-wk-old plants grown in an insect-free glasshouse. The leaf discs were embedded into setting agar in a 90-mm-diameter petri dish with the underside of the leaf facing upward. Before each large-scale bioassay, a preliminary assay was conducted using a broad range of concentrations of the insecticide to determine the appropriate concentration for the formal assay. Each bioassay included 10 concentrations at the ratio of either 1:2 or 1:2.5 plus a control (Milli-Q water, Millipore, Billerica, MA), with at least four leaf disks for each concentration. The insecticide solutions were made up in Milli-Q water to specific concentrations in 100-ml volumetric flasks.

A precise deposit of the test insecticide was administered using a Potter Spray Tower (PST). Ten third instar larvae were placed on each leaf disc, and then each petri dish was sprayed with a 4-ml aliquot of the test solution. Once removed from the PST the dishes were covered with plastic film that was secured with a rubber band (Super band). ≈100–150 fine holes were then punched into the plastic film by using a microneedle to allow air exchange. The PST was calibrated before and after each trial allotment, and triple rinsed with AR Acetone and Milli-Q water between each change in treatment. The treated petri dishes were placed into an incubator at 25 ± 0.5°C and a photoperiod of 14:10 (L:D) h, with the efficacy of the treatments assessed at 72 h.

Full dose-response emamectin benzoate bioassays using the WSS were conducted. Probit analyses using PoloPlus software were performed and a discriminating dose (DD) was estimated. The DD was the rounded upward value of the dose that killed 99.0% (LC_{99}) of WSS larvae, which was 0.22 mg (AI) $liter^{-1}$ emamectin benzoate. Third-instar larvae from field collected populations of *P. xylostella* were then screened against the DD and 10× DD of emamectin benzoate in different generations between F_0 to F_{12}. To enable seasonal comparison at five of the field collection sites, diamondback moth populations were collected in the early and late spring during 2007. Populations that survived 10× DD were considered to have tolerance greater than what would be considered to be within the natural variability range for an unselected population. Therefore, a further full dose line assessment of the strain was carried out and compared with the susceptible reference strain.

Table 1. Susceptibility of third instar larvae from field-collected diamondback moth populations during 2006 tested against commercial formulations of emamectin benzoate at the DD and 10× DD

Pop	G[a]	% mortality	
		DD	10× DD
Lowood I, QLD	F_0	87.5	97.5
Lowood II, QLD	F_1	95	100
Gatton I, QLD	F_0	77.5	100
Gatton II, QLD	F_8	95	100
Glenore Grove, QLD	F_8	97.5	100
Tenthill I, QLD	F_3	90	95
Mt Tarampa, QLD	F_0	90	100
Theresa park, NSW	F_7	100	100
Werombi, NSW	F_2	95	100
Virginia, SA	F_3	87.5	100

[a] Generation at test in the laboratory without selection.

Field-collected insects were exposed to 0.022 mg (AI) liter^{-1} emamectin benzoate (SF1) and maintained for five generations when reciprocal genetic crosses were performed (SF_5). Subsequently, the pesticide concentration was increased to 0.044 mg (AI) liter^{-1} and maintained until generation 12, when reciprocal genetic crosses were performed again (SF_{12}).

Statistical Analysis. The POLO program was used for probit analysis of dose-response data (LeOra Software 1997). Mortality was corrected using Abbott's formula (Abbott 1925) for each probit analysis. Differences in susceptibility were considered significant when the 95% CL of LC_{99} values did not overlap. Resistance ratios (RRs) were calculated based on LC_{50} and LC_{99} values, by dividing the LC value of a field population by the corresponding LC value of the WSS.

Results

Tolerance to Emamectin Benzoate in Diamondback Moth Field Populations. Comparison of mortality in field populations and a standard susceptible P. *xylostella* population (WSS) in 2006 and 2007 (Tables 1 and 2) revealed a clear trend toward higher survival rates in the subsequent year of field collection after continued field exposure through routine use. Fulldose line bioassays indicated that insects from various geographic locations surviving the 10× DD showed low but significant levels of "tolerance" to the insecticide (Table 3).

One population with persistently high levels of tolerance in both years (Field 06 and Field 07) was cultured in the laboratory in the absence of the insecticide (Fig. 1a and b). Within three generations the tolerance ratio was reduced more than half (Fig, 1, unselected F_3, USF_3), with further reduction in subsequent generations (Fig. 1, USF_6 and USF_7). Conversely, when insects were exposed to a sublethal concentration 0.022 mg liter^{-1} of the insecticide in generation F_8, the resistance ratio increased within one generation to the levels found in the field (Fig. 1, selected F_1, SF_1) and increased further in subsequent generations under incremental selection (Fig. 1, SF_2– SF_{12}) to reach tolerance ratios that are several-fold of the initial field populations (Fig. 1, SF_7–SF_{12}). This suggests that repeated exposure to insecticides in the field and under laboratory conditions increased the level of tolerance, which in turn decreases within a few generations under nonselective conditions.

Table 2. Susceptibility of third instar larvae from field-collected diamondback moth populations during 2007 tested against commercial formulation of emamectin benzoate at the DD and 10× DD

Pop[a]	G[b]	% mortality	
		DD	10× DD
[E]Lowood I, QLD	F_0	85	95
[L]Lowood I, QLD	F_0	27.5	87.5
Lowood II, QLD	F_0	82.5	85
Glenore Grove, QLD	F_1	97.5	97.5
[E]Gatton I, QLD	F_0	82.5	100
[L]Gatton I, QLD	F_0	65	87.5
[E]Gatton II, QLD	F_1	75	100
[L]Gatton II, QLD	F_0	82.5	87.5
[E]Tenthill I, QLD	F_0	82.5	100
[L]Tenthill I, QLD	F_0	64	95
Werombi, NSW	F_0	85	100
[E]Nairne, SA	F_0	90	100
[L]Nairne, SA	F_0	75	97.5

[a] [E], collected early spring; [L], collected late spring.
[b] Generation at test in the laboratory without selection.

Tolerance Is Transmitted by a Maternal Effect. To establish whether the acquisition of tolerance under selection pressure is caused by resistance alleles that preexisted in the field populations in low frequencies and which were increased in selected populations, we performed reciprocal crosses and analyzed tolerance levels in F_1 and F_2. Reciprocal crosses of laboratory selected lines with the WSS showed a strong maternal effect in F_1 larvae (Table 4). Offspring from susceptible females and tolerant males (S × T) had tolerance ratios closer to the susceptible strain, whereas offspring from tolerant females and susceptible males (T × S) were significantly greater (nonoverlap of 95% CL) and closer to the tolerant strain.

Table 3. Full dose-response bioassays of field populations that survived at 10 × DD emamectin benzoate

Strain[a]	LC_{50} mg (AI) liter^{-1}	95% CL	R.F	LC_{90} mg (AI) liter^{-1}	95%CL	R.F	Slope ± SE
WSS	0.04268	0.03608–0.0484	1	0.238	0.145–0.326	1	3.46 ± 0.405
Field strains							
Lowood I, 06	0.2596	0.1672–0.392	6.1	5.808	2.244–19.36	24.4	1.84 ± 0.169
[E]Lowood I, 07	0.1386	0.1104–0.173	3.2	2.675	1.672–5.126	11.3	1.810 ± 0.162
[L]Lowood I, 07	0.2508	0.2024–0.308	5.8	3.74	2.376–7.04	15.7	1.98 ± 0.184
Lowood II, 07	0.2156	0.1628–0.286	5.1	3.344	1.936–7.612	14.1	1.96 ± 0.18
Glenore Grove, 07	0.08316	0.04664–0.126	1.9	3.916	1.804–15.444	16.5	1.39 ± 0.153
[E]Tenthill L07	0.528	0.4268–0.660	12.4	9.24	5.72–18.48	38.9	1.87 ± 0.289
[L]Tenthill L07	0.2288	0.1232–0.383	5.4	4.167	1.768–26.04	17.5	1.84 ± 0.171

[a] [E], collected early spring; [L], collected late spring.

Because the females are the heterogametic sex in Lepidoptera, this effect is in the opposite direction of what could be attributed to sex linkage. If sex linkage were the case, the crosses with resistant males would be expected to contribute two resistance alleles to the offspring, with the females being hemizygotes and very resistant, while the male offspring would be heterozygotes. In the crosses using susceptible males, only the male offspring would carry resistance. The fact that tolerance was transmitted by the females and not the males suggests that a large fraction of

the tolerance levels observed in laboratory-selected lines was not genetically controlled, but transmitted by an epigenetic effect.

Tolerance Is Correlated with an Elevated Immune Status. Field-collected lines that were kept in the laboratory under selection pressure (exposure to DD of the pesticide) and in the absence of the pesticide, were compared for their respective immune status, by measuring the rate of melanization in larval cell-free hemolymph (plasma). Emamectin benzoate-selected insects (PS) showed >10× higher rates of melanization than unselected (US) insects (Fig. 2). This suggests that pesticide-exposed insects have an induced immune status in the humoral part (plasma) of the immune system.

Genetic Transmission Versus Epigenetic Transmission of Tolerance. After establishing epigenetic transmission by reciprocal genetic crosses in generation 5, the insects were kept under continuous selection pressure until generation 12, when another reciprocal genetic cross was performed and offspring examined for tolerance to Bt toxin. In generation 12, the tolerance level has increased to 158-fold (Table 4, bottom). Although the tolerance levels in F_1 offspring of the two reciprocal crosses (RR 9.2 in S × T, RR 15.4 in T × S) were somewhat higher than in generation five (RR 2.3 in S × T, RR 5.45 in T × S), the relative contribution has decreased compared with the levels in the tolerant parent (Fig. 3a and b). Although the dominant contribution in generation 5 was approximately one third of the tolerance levels of the tolerant parent (Table 4, top), it amounts to about one tenth of the tolerant parent in generation 12 (Table 4, bottom). Likewise, the maternal effect (difference in RRs between S × T and T × S crosses) is 2.15 in generation 5 and 6.2 in generation 12, approximately half of the RRs of the T × S offspring. This suggests that although epigenetic contributions are significant in the first generations after exposure to the pesticide, most of the increase in tolerance observed between generation 5 and 12 was based on mechanisms that are genetically transmitted.

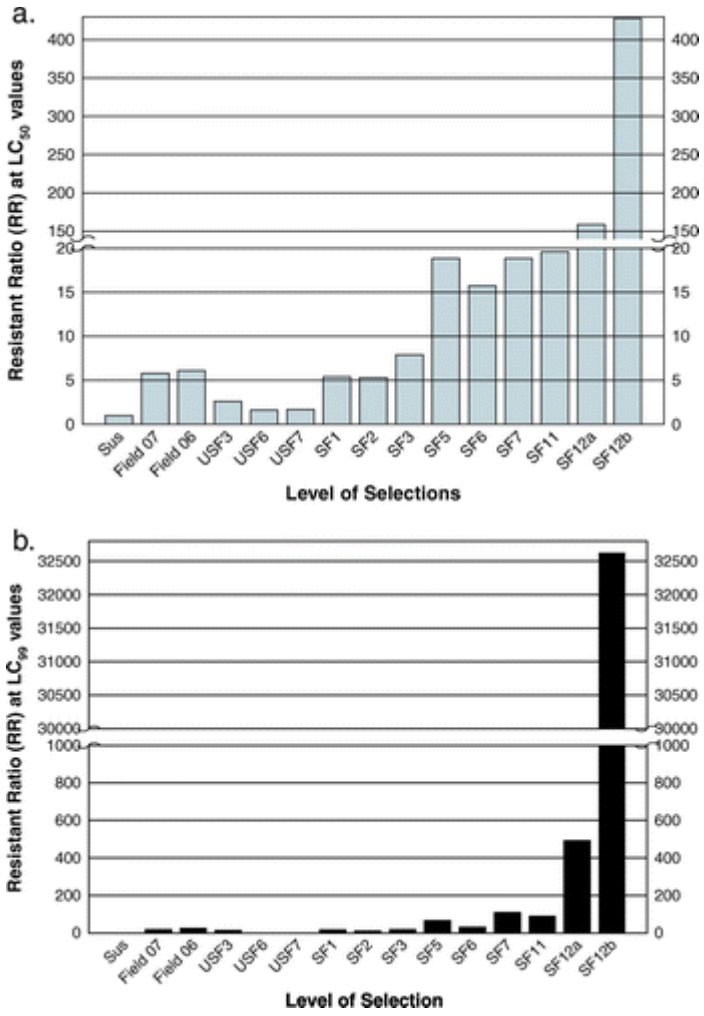

Fig. 1. Tolerance to emamectin benzoate in a diamondback moth field population and after culturing in the laboratory in the presence and absence of the insecticide. Tolerance levels are presented for a field population collected in 2006 (Field 06) and 2007 (Field 07) with the Waite susceptible strain (Sus) used as a control. The population Field 06 was cultured in the laboratory for seven generations in the absence of emamectin benzoate and bioassays were performed after three (USF3), six (USF6), and seven (USF7) generations, when tolerance levels were almost at the level of the control. All subsequent generations were exposed to emamectin benzoate (0.022 mg [AI] liter^{-1}) and tolerance levels measured after one (SFl), two (SF2), three (SF3), five (SF5), six (SF6), seven (SF7), eleven (SF11) and twelve (SF12) generations. For the SF11 and subsequent generations, the concentration of emamectin benzoate was doubled to 0.044 mg (AI) liter^{-1}. (a) RRs based on LC_{60} values; (b) RRs based on LC_{99} values.

Discussion

Field monitoring of baseline susceptibility and geographic variation in insect pests to biopesticides revealed several fold differences in resistance to standard susceptible strains, compatible with the existence of incomplete recessive resistance alleles (Ahmad 1999). However, attempts to uncover any preexisting resistance alleles by appropriate genetic crosses are more often than not unsuccessful with variable resistance levels in field-collected insects (Ahmad 1999). Similar observations have been made with regards to field-derived tolerance to synthetic pesticides, which did not uncover recessive resistance alleles in test crosses. The

absence of resistance alleles in these phenotypes could be explained by complex multigenic origins of the observed field resistance.

Table 4. Reciprocal crosses between emamectin benzoate-susceptible (WSS: as S × S) and tolerant adults (T × T), and the analysis of tolerance levels in F_5 offspring (top) and generation F_{12} in the course of selection (bottom)

	LC_{50} mg (AI) liter^{-1}	95% CL	RR
Cross (F_5)			
S × S	0.04268	0.03608–0.0484	1
T × T	0.80388	0.632–1.04	18.83
S × T	0.09548	0.0594–0.137	2.3
T × S	0.23276	0.165–0.321	5.45
Cross (F_{12})			
S × S	0.0286	0.02376–0.03476	1
T × T	4.532	3.52–5.72	158.5
S × T	0.264	0.211–0.326	9.2
T × S	0.44	0.339–0.572	15.4

Here, we show that field and laboratory populations of *P. xylostella* that have been exposed to the synthetic pesticide emamectin benzoate acquire tolerance to the pesticide by inducible mechanisms that are transient and in the absence of selection pressure disappear from the population within one to three generations. The elevated immune status and tolerance to the pesticide is transmitted to offspring in part by epigenetic transmission processes. Although this transient and inducible tolerance to emamectin benzoate and its transmission to offspring by a maternal effect has similar phenotypic properties to a recently described tolerance mechanism to Bt toxin (Rahman et al. 2004), the two mechanisms may differ considerably at the molecular level.

First, the induction process by synthetic pesticides is not clear. Given that emamectin benzoate is administered with the food it may act as a gut-derived elicitor to stimulate the immune system in the hemolymph, similar to the induction by allelochemicals (Li et al. 2007). However, although it is possible to speculate on nonlethal damage of the gut lining by sublethal doses of the Bt toxin that allow gut-derived elicitors, such as enterobacteria (Broderick et al. 2006) and other components of Bt formulations, to contact and induce the hemolymph-derived immune system (Rahman et al. 2004), it is difficult to envision synthetic pesticides as elicitors of the immune system. Nevertheless, metabolic enzymes involved in pesticide resistance, such as glutathione transferases, are induced by plant-derived allelochemicals (Li et al. 2002b), which are sometimes similar in structure to synthetic pesticides. But although allelochemical-producing plants may have coevolved with insects and produced recognition proteins, synthetic pesticides are unlikely to be recognized by specific binding proteins. The question is therefore, how are synthetic pesticides recognized by the insect immune system in the absence of specific recognition proteins?

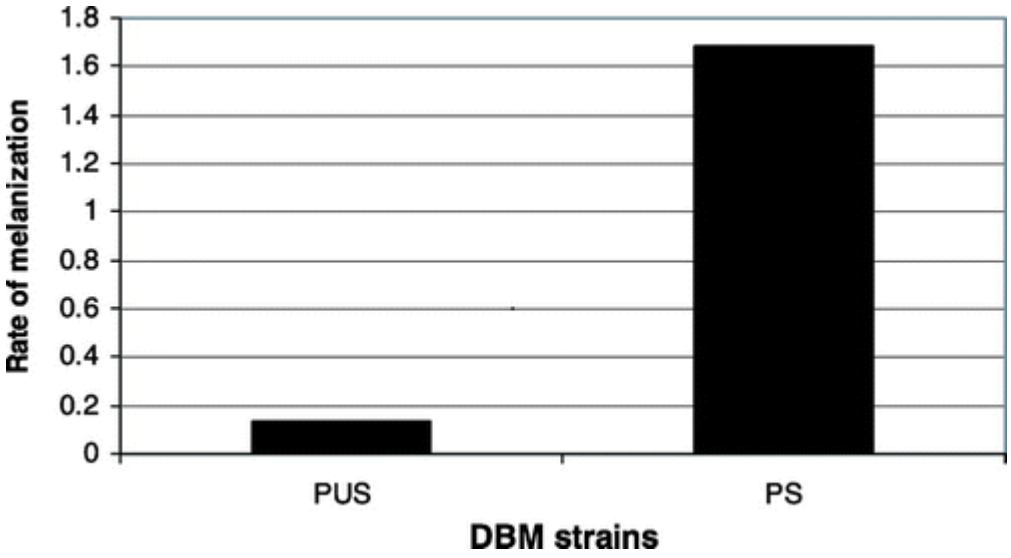

Fig. 2. Induction of the humoral immune response of susceptible diamondback moth larvae exposed to sublethal concentrations (0.5 µg/ml) of emamectin benzoate. The graph shows the rate of the melanization reaction (as the slope of the plot of absorbance against time in arbitrary units) of cell-free hemolymph in larvae from an unselected (PUS) and a selected (PS) population.

Whatever the recognition mechanism, some components, such as glutathione transferases, are found in immune-related coagulation reactions (Li et al. 2002a) as well as in pesticide detoxification (Huang et al. 1998), and may possibly be regulated by similar mechanisms. Even if synthetic pesticides are able to induce immune and metabolic activities by unknown mechanisms, how this translates into inactivation and sequestration of synthetic pesticides remains to be uncovered by further experiments.

The observation that initially most of the tolerance is transmitted to offspring by a maternal effect is an indication that the increase in tolerance under selection pressure is not due to preexisting resistance alleles in the population, but rather based on transient regulatory gene activities. The induction may involve a broad range of gene activities from immune-gene products, such as prophenoloxidase to metabolic gene products, such as P_{450} monooxygenases, esterases, and glutathione transferases (Li et al. 2007). The epigenetic transmission could in principle be based on several mechanisms (Jablonka 2004), the most likely being that increased levels of immune and metabolic components in the circulation of induced females results in their inclusion into the oocyte, triggering induction processes during embryogenesis. This mode of transmission is independent of the genotype of males and only requires a continuous line of immune-induced females, resulting in neonates that are already on an elevated immune and metabolic status.

Although the relative contribution of inducible tolerance mechanisms is relatively high in the first few generations, the significant increase in tolerance observed between generation 5 and 12 is based on mechanisms that are genetically transmitted. If we accept the notion that tolerance is based on inducible regulatory mechanisms, it is possible that some of the regulatory pathways are genetically fixed. Alternatively, genotype selection of multiple allelic combinations that contribute to tolerance may have been achieved over the prolonged exposure to the pesticide. Finally, rare mutations may have emerged in target sites of metabolic or other genes that would have contributed to the observed increase in resistance.

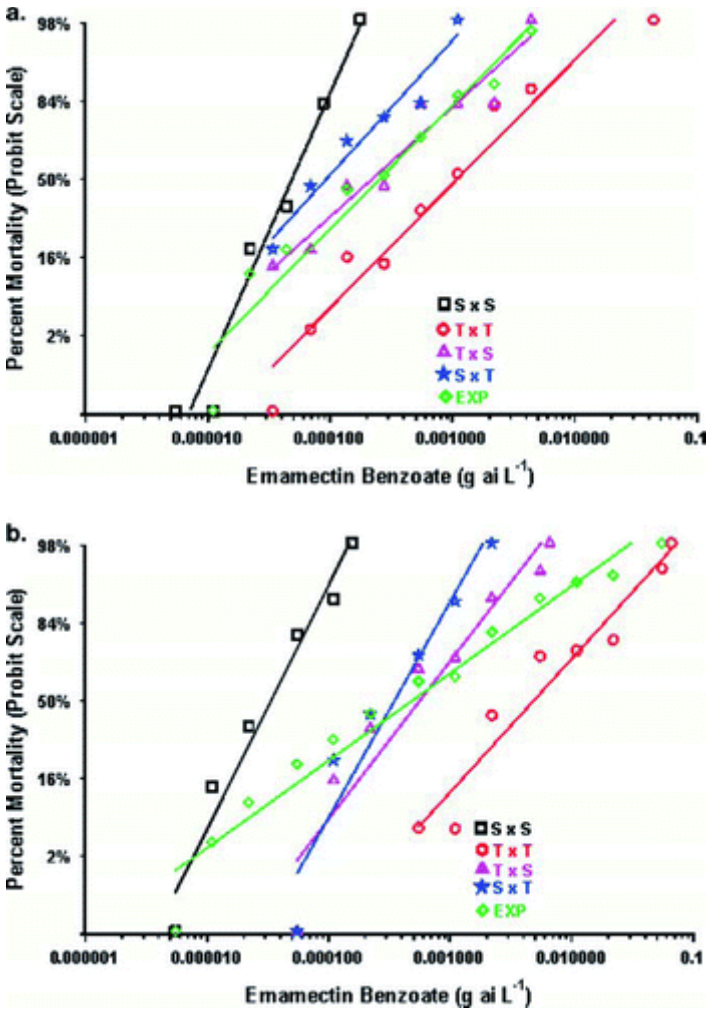

Fig. 3. (a) Log dose versus percentage of mortality (probit scale) of the susceptible strain (S × S, □) and field strain exposed to emamectin benzoate for five generations (T × T, ○). The corresponding genetic crosses ($T_{female} \times S_{male}$, △) and ($S_{female} \times T_{male}$. ☆) are shown (see Fig. 1) together with the values predicted for a single gene transmission (EXP, ◊). (b) Log dose versus percentage of mortality (probit scale) of the susceptible strain and field strain exposed to emamectin benzoate for 12 generations. Reciprocal genetic crosses and abbreviations are as in a, (Online figure in color.)

In summary, we have uncovered a new mechanism that can generate tolerance to the synthetic pesticide emamectin benzoate. Although this mechanism provides tolerance to pesticide concentrations of low to medium toxicity, it has the potential to increase tolerance levels by increments if the selection pressure is maintained over subsequent generations. Prolonged exposure to the pesticide may provide tolerant insect populations with the adaptive potential to acquire resistance mechanisms that are transmitted by genetic means and could involve target site mutations in crucial resistance genes. Whether this new tolerance mechanism is a threat to resistance management in the field is not known and will be dependent on possible cross-tolerance to other pesticides and the size of the fitness penalties associated with the tolerance. Further studies are under way to investigate these aspects of the observed tolerance to synthetic pesticide.

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