If you are participating in the presentations this year, please provide a written report and a copy of your final report presentation by 31 October. If not, please provide a written report by 30 September.

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: **DAN197**

Project Title: Sustainable Chemical Control of Aphids, Twospotted Mite and Mirids in Cotton

Project Commencement Date: 1 July 2008 **Project Completion Date:** 30 June 2011

CRDC Program: 2. Farming Systems

Part 2 – Contact Details

Administrator: Mr Graham Denney, Manager External Funding
Organisation: NSW DPI
Postal Address: LMB 21 Orange 2800
Ph: 02 6391 3219 **Fax:** 02 6391 3327 **E-mail:** graham.denney@industry.nsw.gov.au

Principal Researcher: Dr Grant Herron, Senior Research Scientist
Organisation: NSW DPI
Postal Address: PMB 4008 Narellan2567
Ph: 02 4640 6471 **Fax:** 02 4640 6300 **E-mail:** grant.herron@industry.nsw.gov.au

Supervisor: Dr Robert Mensah, Acting RL Plant Entomology
Organisation: NSW DPI
Postal Address: Locked Bag 1000, Narrabri NSW 2390
Ph: 02 6799 1525 **Fax:** 02 67991503 **E-mail:** robert. mensah@industry.nsw.gov.au

Supervisor: Dr Lewis Wilson, Program Leader
Organisation: CSIRO
Postal Address: Locked Mail Bag 59, Narrabri NSW 2390
Ph: 02 67991550 **Fax:** 02 67931186 **E-mail:** lewis.wilson@csiro.au

Signature of Research Provider Representative: _____

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

(The points below are to be used as a guideline when completing your final report.)

Background

1. Outline the background to the project.

With the introduction of transgenic cotton in Australia to control *Helicoverpa* spp., a reduction in chemical insecticide usage has occurred. Subsequently, there has been an increase in the populations of sucking insect pests such as green mirids and green vegetable bugs. Control of these emerging pests with broad-spectrum insecticides depletes beneficial insect populations and often leads to outbreaks of secondary pests, such as mites, aphids and silver leaf whitefly, and inevitably selects for insecticide resistant strains. Dealing with the likelihood of resistance requires on-going monitoring for resistance to key insecticides if future control problems are to be averted.

In Australia, cotton aphid reproduces almost exclusively asexually, essentially they clone themselves. This method of reproduction allows the very rapid fixing of genotypic changes into a population as there is no inclusion of alleles from male cotton aphid. This is particularly evident with insecticide resistance genes and the rapid appearance of resistant strains seen shortly after insecticide usage. Cotton aphid is resistant to a range of insecticides in many crops and countries. Some ten years ago high-level resistance to organophosphates (omethoate and dimethoate) and some carbamates (pirimicarb) developed in cotton aphid strains causing control failures (Herron *et al*. 2001) but in recent seasons the efficacy of both products has been recovered. More recently *Prima Facia* neonicotinoid resistance was detected in two cotton aphid strains during the 2007-2008 season and during the following 2008-2009 season neonicotinoid control failures were reported for the first time and resistance increased in both level and abundance (Herron and Wilson 2011). Similarly, TSM is notorious world-wide for developing miticide resistance and this has occurred many times in Australia. TSM insecticide resistance continues to evolve in cotton and most recently caused chlorfenapyr (Intrepid®) resistance (Herron *et al*. 2004).

Green mirid in particular are proving to be a serious emerging pest in Bollgard II® crops. This is primarily due to the reduction in insecticides used against *Helicoverpa* spp., which also formerly suppressed mirid populations. There has been an increase in spray formulations specifically targeting mirids. Currently there is a high reliance on dimethoate, clothianidin and fipronil for mirid control and there is a risk that resistance will occur and potentially serious crop losses. Overseas data indicate that similar sucking bug pests, such as, *Lygus lineolaris* in the south eastern USA can quickly develop resistance to organophosphates and pyrethroids (Scott and Sondgrass 2000). However, Australian resistance researchers currently do not possess the capability to detect resistance in green mirids.

Continued insecticide resistance monitoring is essential for effective ongoing resistance management of these sucking pests of cotton. For this reason the major thrust of DAN197 is the use of resistance monitoring technologies, both conventional bioassay and molecular genetic, to monitor resistance in TSM and cotton aphid against key pesticides used for their control. Additionally, there is a need to establish and verify a practical bioassay methodology that can be used with green mirids as a first step

to establishing robust baseline data that is essential for resistance monitoring. Finally new methodologies, both for conventional bioassay and molecular genetic tests, are required for new unique mode of action chemicals and to help speed up testing to reduce the delays inherent with bioassay based research.

References

Herron, G.A., Powis, K. Rophail, J. (2001) Insecticide resistance in *Aphis gossypii* Glover (Hemiptera: Aphididae), a serious threat to Australian cotton. *Australian Journal of Entomology* **40** (1): 85-89.

Herron, GA and Wilson LJ. (2011) Neonicotinoid resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) from Australian cotton. *Australian Journal of Entomology* **50**: 93-98.

Herron, G.A., Rophail, J. and Wilson, L. (2004) Chlorfenapyr resistance in two-spotted spider mite (Acari: Tetranychidae) from Australian cotton. *Experimental & Applied Acarology* **34**: 315-321.

Scott, W.P., and Snodgrass, G.L. (2000) A review of chemical control of the tarnished plant bug in cotton. *Southwest Entomologist* **23**: 67-81.

Objectives

2. List the project objectives and the extent to which these have been achieved.

1. *Monitor resistance in two-spotted mite and cotton aphid against key pesticides*.

Two-spotted mite and cotton aphid were collected by Drs Herron and Wilson during their annual end of season collecting trips and by growers and consultants. These were returned to the EMAI insectary for subsequent culturing and resistance testing against key pesticides. The objective for aphids was fully met with all strains being successfully cultured and tested for resistance using both molecular genetic and bioassay techniques. The objective for two-spotted mite was not fully met as no mites were tested for resistance during season 2009-2010 although collections were made by Drs Herron and Wilson for that purpose. Unfortunately in that season mite strains died out for no apparent reason after collection and numbers never increased to levels necessary for testing. This last 2010-2011 season two-spotted mite were again slow to increase and careful checking found contaminant Tarsonemid mites. Unfortunately, species specific identification was not possible and taxonomists were not certain if the Tarsonemid mite was an obligate plant feeder. The identifying taxonomist noted that within the family Tarsonemidae there are some species that have been recorded as predating on insect eggs and perhaps that may also include mite eggs. Although speculative, we can confirm that once the Tarsonemid mites were removed from the cultures the two-spotted spider mite populations recovered and were tested for resistance.

2. *Develop and or refine methods to handle and breed mirids.*

The objective was not met for two reasons. Firstly, a field collected strain of mirids was being maintained during 2007/2008 but the culture was left to die out so resources could concentrate on aphid resistance bioassay due to the emerging neonicotinoid resistance

problem at that time. Mirid culturing is extremely time consuming and technically difficult requiring a huge resource input. However, with the explosion of neonicotinoid resistance in cotton aphid extra chemicals had to be tested to evaluate the risk of cross resistance within the neonicotinoid group, such as clothianidin and imidacloprid. This required development of full log dose probit analysis for each insecticide. For that reason the establishment and bioassay of mirids was delayed until the 2008/2009 aphid resistance testing was complete. In addition, a new chemical called spirotetramat was made available that required generating baseline data using 2009/2010 aphid strains which we have maintained for this purpose. This has generated an enormous amount of unplanned, but essential testing. Finally, in the 2009/2010 season a more sustainable solution was decided upon where mirids were to be collected locally, tested immediately, and data used to generated and establish a baseline response. That removed the need to maintain a perennial strain saving some 3 days FTE of staffing per week trying to keep mirids ready for testing.

3. *Develop and/or refine methods to test mirids for resistance*

The objective was met during season 2009/2010 when green mirids were collected from EMAI grown lucerne and a bioassay methodology was tested against them. Mirids were returned to the laboratory and sprayed with the insecticides fipronil, clothianidin or dimethoate with the aid of a Potter spray tower. Post spray mirids were maintained on bean pods set in agar within a Petri dish. Data generated was assessed at a 24, 48 and 72 h with holding period (WHP) with 48 h being chosen for subsequent assays because mortality had stabilised and control mortality at the 48 h was usually acceptable.

4. *Extension*

The objective was fully met over the entire duration of the study. Dr Herron gave an invited presentation on aphid resistance and management at the 14th Australian cotton conference. Additionally, Dr Herron participated in the resistance extension road show in the years when it was undertaken by the CRDC and actively participated in the annual TIMS Technical Review meetings that then progressed into timely resistance management strategy modifications via the Cotton Pest Management Guide. Finally Dr Herron ensured that growers and consultants spoken to before the annual collections for resistance testing received a personal emailed report of that seasons testing.

Methods

3. Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.

Chemicals tested

Aphids were treated with endosulfan (Thiodan®), acetamiprid (Intruder®), clothianidin (Shield®), diafenthiuron (Pegusus®), imidacloprid (Confidor®) and thiamethoxam (Actara®) but not all chemicals were tested all seasons. All were proprietary commercial

insecticide formulations except diafenthiuron (Pegasus®) for which the UV activated carbodiimide derivative of diafenthiuron, CGA-140408, was tested instead. This was necessary because diafenthiuron is activated by exposure to UV light, which would not normally occur in the laboratory. Note that clothianidin (Shield®) and thiamethoxam (Actara® or the seed dressing Cruiser®) are from the same neonicotinoid chemical group. Some aphid strains were further tested against spirotetramat (Movento®) and sulfoxaflor (Transform™) to establish baseline data for resistance monitoring.

TSM were treated against abamectin (Agrimec®), bifenthrin (Talstar®), propargite (Comite®), and diafenthiuron (Pegasus® as CGA140408).

Mirids were tested against fipronil (Regent®), clothianidin (Shield®) or dimethoate (eg Dimethoate 400 or Saboteur®)

Aphid collection and culturing

Aphids were collected by researchers, CRC Regional Extension Officers, consultants and growers from commercial cotton fields or cotton plants in the vicinity of commercial crops. They were sent to the bioassay laboratory at Camden (Elizabeth McArthur Agricultural Institute, EMAI) and each field strain was cultured separately on pesticide-free cotton at 25 ± 4 ^{o}C under natural light. Strain integrity was assured by maintaining populations in purpose built insect proof cages. If the field sample contained sufficient aphids a small subpopulation of each field strain was collected for use in the molecular assays immediately. Any subpopulations taken immediately after collection from the original field submissions are indicative of farm level resistance.

Resistance detection

Via Bioassay. Adult apterous aphids (except spirotetramat that were 0-24 h old nymphs) were tested by placing them in a 35 mm Petri dish on an excised cotton plant leaf disc fixed in agar (Herron *et al*. 2001). Briefly, batches of ten adult (except spirotetramat that used nymphs) female aphids per leaf disc were then sprayed with a discriminating dose of insecticide with the aid of a Potter spray tower. All tests were replicated (unless otherwise marked) and included a water-only sprayed control. After spraying, clear plastic film was used to cover the Petri dishes, which were then maintained at 25 ± 0.1 ^{o}C in 16:8 L:D for 24 h (except spirotetramat 72h) after which mortality was assessed. Some strains were further tested at multiple insecticide concentrations to allow probit regression and resistance factor calculation. Strains subjected to probit analysis had LC_{50} values determined that were used to calculate LC_{50} level resistance factors plus their 95% confidence interval using methods outlined in Robertson and Preisler (1992). Against spirotetramat (Movento®) and sulfoxaflor (Transform™) probit analysis was used to establish a baseline response for future resistance monitoring.

Via Molecular Assay. Pirimicarb (Pirimor®) and organophosphate resistances were detected via an established DNA based method (M^{c}Loon and Herron 2009). Briefly, DNA

is isolated from 20 individual aphids from each of the different field strains and individual aphid DNA extractions were subject to PCR amplification of the *Ace*I gene (covering the mutation responsible for resistance) using PCR followed by restriction enzyme digests with the enzymes; *Ssp*I (carbamate resistance) and *Pdi*I (organophosphate resistance). Note that the *Ssp*I enzyme detects resistance to pirimicarb, which would normally also give cross resistance to dimethoate and omethoate, while the *Pdi*I enzyme detects another resistance mechanism to organophosphates (profenofos and chlorpyrifos) based on a second mutation within the *Ace*I gene. Agarose gel electrophoresis was performed to visualise the result of the enzyme digests. Gel concentrations were 2%, run for 90 minutes at 94V and saved as digital images using the Gel Dock System (Bio Rad).

Two-spotted mite

Strains of TSM are collected from a range of cotton fields in NSW and put into culture as above. The bioassay procedure required young adult female mites to be transferred from culture to French bean leaf discs (Herron *et al*. 2004). Briefly, mites and leaf discs were then sprayed with a discriminating dose of insecticide with the aid of a Potter spray tower as above. Each test was replicated (unless otherwise indicated) and included a water only sprayed control that did not exceed 15%. After spraying, mites on leaf discs were maintained at 28 ± 0.1 ^{o}C in constant light for 48 h after which mortality is assessed.

Mirids

Adult green mirid was tested via bioassay using methods modified for aphids given above. A piece of bean pod was set in agar rather than a cotton leaf disc and mortality assessed at 24, 48 and 72 h post treatment.

References

Herron, G.A., Powis, K. Rophail, J. (2001) Insecticide resistance in *Aphis gossypii* Glover (Hemiptera: Aphididae), a serious threat to Australian cotton. *Australian Journal of Entomology* **40** (1): 85-89.

Herron, G.A., Rophail, J. and Wilson, L. (2004) Chlorfenapyr resistance in two-spotted spider mite (Acari: Tetranychidae) from Australian cotton. *Experimental & Applied Acarology* **34**: 315-321.

McLoon MO and Herron GA (2009) PCR detection of Pirimicarb resistance in Australian field isolates of *Aphis gossypii* Glover (Aphididae: Hemiptera). *Australian Journal of Entomology* **48**: 65-72.

Robertson JL and Preisler HK (1992) *Pesticide Bioassays With Arthropods*. CRC press, Boca Raton.

Results

4. Detail and discuss the results for each objective including the statistical analysis of results.

Aphids

For the first time since the 1999-2000 control failures pirimicarb (Pirimor®) and general OP resistance was not detected in any of the 24 cotton aphid strains collected during 2008-2009 (Table 1). Interestingly, endosulfan resistance was detected in a single strain, something that has also not occurred for several seasons. Unexpectedly, there were multiple survivors in multiple strains detected at the discriminating dose against the neonicotinoid insecticides acetamiprid (Intruder®), thiamethoxam (Cruiser®), clothianidin (Shield®) and imidacloprid (Confidor® or Gaucho®). This suggests a significant increase in the level and abundance of neonicotinoid resistance compared to the previous year. As individual strains were often resistant to more than one neonicotinoid insecticide it implies cross resistance between those insecticides tested. Unfortunately one of the neonicotinoid strains tested (E Wer) was associated with a neonicotinoid field control failure. Surprisingly, low level suspect diafenthiuron (Pegasus®) resistance was also detected in some strains tested during 2008-2009. Encouragingly there does not seem to be any obvious cross resistance relationship to the neonicotinoid resistance nor any control failures reported against diafenthiuron (Pegusas®).

In 2009-10, for a second season in a row neither pirimicarb (Pirimor®) nor general OP resistance was detected in the 14 cotton aphid strains collected (Table 2). Endosulfan resistance was again detected at a very low level in two strains. There were again multiple survivors in multiple strains detected at the discriminating dose against the neonicotinoid insecticides with 78% of the strains tested showing some neonicotinoid resistance. Thiamethoxam (Cruiser®) and clothianidin (Shield®) resistance was 69 and 15 fold respectively. Again low level suspect diafenthiuron (Pegasus®) resistance was detected in two strains with the maximum level of 4.0 fold.

For a third season in a row pirimicarb (Pirimor®) and general OP resistance was not detected in the cotton aphid strains collected during 2010-2011 (Table 3). There was again a single diafenthiuron (Pegasus®) survivor but the result seems indicative of vigour tolerance because any response is low level and does not change with selection (Figure 1). There were again multiple survivors in multiple strains detected at the discriminating dose against the two neonicotinoid insecticides tested. Alarmingly, 96% of the strains tested showed some neonicotinoid resistant aphids. Baseline data were generated for spirotetramat (Movento®) (Figure 2) and sulfoxaflor (Transform™) (Figure 3). Sulfoxaflor (Transform™) proved a good fit to the probit model with current bioassay data indicating a discriminating dose of 0.01 g sulfoxaflor / L (approximate $LC_{99.9}$ upper 95% CI fiducial limit for the most tolerant strain Alch)(Figure 3). In contrast, spirotetramat (Movento®) data poorly fitted the probit model with doses required to achieve 100% control of individual strains varying more than an order of magnitude. For that reason it is not possible to calculate a reliable spirotetramat (Movento®) discriminating dose.

Two-spotted mite (TSM)

For season 2008-2009 TSM were collected from NSW only (Table 4). Abamectin (Agrimec®) and diafenthiuron (Pegasus®) resistance were not detected. Propargite (Comite®) resistance was detected in one strain only and very low level chlorfenapyr (Intrepid®) resistance detected in four of the six strains tested. Bifenthrin (Talstar®) resistance was evident in all strains tested with some strains showing a very high proportion of resistant individuals (Table 4). Although collected in 2009-2010,TSM failed to establish into culture and could not be tested.

Seven strains of TSM were collected for 2010-2011 but one was lost to predatory mites and another two succumbed to Australia Post handling (Table 5). Four strains were tested for resistance and no propargite (Comite®) or diafenthiuron (Pegasus®) resistance was detected. Abamectin resistance was detected in three of the four strains tested at frequencies less than 10 percent and so likely one abamectin application away from a spray failure. Talstar® resistance was detected in all strains tested with some strains having a high proportion of resistant individuals that peaked at 60% in strain Nan Back F1.

Green Mirid.

Dose response data were obtained for fipronil, clothianidin and dimethoate with the aid of a Potter spray tower (Table 6). Data generated was assessed at a 24, 48 and 72 h after treatment with a 48 h (see Table 6, Regent® replicates) with-holding period (WHP) being the best compromise between excessive control mortality and bioassays reaching a stable end point mortlaity (see Table 6, dimethoate replicates). Any future bioassays with mirids will therefore use a 48 h WHP.

Discussion

Despite the overall reduction in sprays associated with Bollgard II®, resistance causing control failure against cotton aphid remains an issue. During the last season of the previous cotton study, DAN184, a Gwydir strain of cotton aphid was shown to have acetamiprid (Intruder®), thiamethoxam (Cruiser®) and thiacloprid (Calypso®) survivors. These discriminating dose survivors indicated a *Prima Facie* detection of resistance. For this current study DAN197, the 2008-2009 season data saw neonicotinoid resistance increase in both level and abundance. For the first time neonicotinoid resistance causing control failure was seen in a single strain known as E Wer. Interestingly, the failure occurred despite a relatively low resistance factor of 3.1 fold suggesting the strain was very heterogeneous for resistance and so resistance levels could significantly increase with more selection. For strain E Wer, clothianidin (Shield®) was noted as not working at all and resistant individuals were detected. Another strain known as Fair was collected after an acetamiprid (Intruder®) application and poor product performance was noted. Again acetamiprid (Intruder®) resistant individuals were detected in the Fair strain. Worryingly, aphids that survived a field rate of clothianidin (Shield®) were also likely cross resistant to other neonicotinoid products. The

cross resistance detected is consistent with the study of Wang *et al*. (2007) that demonstrated a relationship between imidacloprid (Gaucho®) and acetamiprid (Intruder®) resistance in cotton aphid and that of Alyokhin *et al*. 2007 that linked imidacloprid (Gaucho®) resistance to thiamethoxam (Cruiser®) in Colorado potato beetle. Consequently Australian populations of cotton aphid must also be considered a single cross-resistance group as suggested by Nauen and Denholm (2005). Additionally some strains showed a low level of diafenthiuron (Pegusus®) tolerance but it did not appear related to the neonicotinoid resistance.

During that 2008-2009 season neonicotinoid resistance was detected in 82% of the stains tested with thiamethoxam (Cruiser®) and clothianidin (Shield®) resistance peaking at 18 and 10 fold respectively. Interestingly, the proportion of strains showing neonicotinoid resistance in 2009-2010 remained approximately static at 78% but maximum levels have risen. In the 2009-2010 season thiamethoxam (Cruiser®) and clothianidin (Shield®) resistance peaked at 69 fold and 15 fold resistance respectively. Such resistance must make the neonicotinoids unreliable for cotton aphid control and growers may well be better off using alternative foliar products for aphid control if neonicotinoid seed dressings have been used

Interestingly, though the proportion of strains showing neonicotinoid resistance in 2009-2010 remained approximately static at 78% but for season 2010-2011 the frequency of strains showing resistance increased to an extreme 96% of strains tested. Such resistance must make the neonicotinoids unreliable for cotton aphid control and preliminary glasshouse trial data generated at EMAI (DAN 1201 and 1203) suggests neonicotinoid seed treatments may not control all neonicotinoid resistant aphids. Growers may well be better off using alternative foliar products for aphid control if neonicotinoid seed dressings have been used. It is noteworthy that IPM friendly pirimicarb (Pirimor®) is currently resistance free so it would be a better first choice for an aphid specific foliar spray. Other alternatives are also available including, diafenthiuron (Pegusus®), spirotetramat (Movento®), pymetrozine (Chess®) an OP (e.g. chlorpyrifos) or spray oil. If foliar neonicotinoids are to be used against aphids I recommend the following to help manage resistance:

1. Try to limit in-season use of foliar neonicotinoids for aphid control if they have been used as a seed treatment.
2. If foliar sprays are required for aphid control and neonicotinoid seed treatments have been used it is most important that the first foliar spray is not from the neonicotinoid chemical group. It is essential to alternate.

I now consider the low frequency of diafenthiuron (Pegasus®) discriminating dose survivors is due to vigour tolerance rather than resistance. In 2008-2009 13% of strains tested did have discriminating dose survivors that suggested resistance. These initial resistance detections during 2008-2009 were low level with a maximum response level of 3.6 fold. The following 2009-2010 season found a similar 14% of strains showing discriminating dose survivors and again response levels remained static, peaking at 4.0 fold. Testing of aphids during 2010-2011

found a single aphid surviving a discriminating dose and again the strain would not pressure and levels did not exceed those already detected. Such a result is indicative of high level vigour tolerance (a super tolerant susceptible) rather than resistance. For this reason the diafenthiuron (Pegusus®) discriminating dose will be doubled for season 2011-2012 to 0.003%.

The false positive seen against diafenthiuron (Pegusus®) and cotton aphid highlights the need for robust and reliable baseline data. Baseline data generated to date for spirotetramat (Movento®) against cotton aphid has failed to produce consistent baseline data that can be used to discriminate between vigour tolerance and resistance. The bioassay methodology used for spirotetramat (Movento®) against cotton aphid was adapted from Elbert et al. (2008) except a Potter spray tower was used to apply product rather than a leaf dip. Elbert et al. (2008) proposed a spirotetramat (Movento®) cotton aphid discriminating dose of 0.03 g / L (30 ppm), yet baseline generated here shows the susceptible strains had survivors up to 0.25 g / L implying a discriminating dose nearer to 1 g / L (1000 ppm). Further, we tested a cotton aphid strain from horticulture with spirotetramat (Movento®) with reported control issues but baseline data is so variable we couldn't confirm or refute resistance. Variation in response between strains in this study is not the few fold suggested by Elbert et al. (2008) but more than an order of magnitude. Clearly something is wrong, either resistance or methodology issues. Resistance seems unlikely as our susceptible strain SB gave the most tolerant response (Figure 2). That then implies a problem with method adaption from Elbert et al. (2008) methodology being used here in Australia against cotton aphid. Methodology likely requires further modification that will be done in the forthcoming 2011-2012 cotton season (project DAN 1203) and will likely require a change to the bioassay withholding period.

Resistance testing of TSM continues to be problematic with some strains collected not surviving long enough to be tested for resistance. The loss of two strains from Hillston during season 2010-2011 is unfortunate but a direct consequence of a lack of overnight couriers from the area. The loss of a TSM strain to predators is regrettable but much improved on the last 2009-2010 season where all strains were lost before testing without any obvious cause. A possible predator, that was very hard to see, was found in season 2010-2011 and confirmed as a Tarsonemid mite by taxonomists. Unfortunately, a specific identification was not possible and taxonomists were not certain if the Tarsonemid mite was a plant feeder. The identifying taxonomist noted within the family Tarsonemidae that some species have been recorded as predating on insect eggs and perhaps they may also eat mite eggs. We can confirm that once the Tarsonemid mites were removed from the cultures they recovered and could be tested for resistance.

Subsequent resistance testing of TSM during 2010-2011 produced unexpected positive results against abamectin. Abamectin resistance monitoring against TSM has rarely detected positive resistance results and from 2002-2003 abamectin resistance was not detected in TSM until season 2007-2008 when it was detected in a single strain. The following season all TSM strains were lost and now for 2010-2011 three out of the four TSM strains tested show some

abamectin resistance. Clearly something has changed with the way abamectin is being used against TSM in Australian cotton – and there was in some regions a high reliance of abamectin against mites due to high efficiacy and low price – with many field sprayed once, quite a few 2-3 times and some even more. If abamectin use against TSM continues at this high level than it will select resistant individuals and future control issues seem likely.

References

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Wang, KI., Guo, QL., Xia, XM., Wang, HY. and Liu, TX. (2007) Resistance of *Aphis gossypii* (Homopterous: Aphididae) to selected insecticides on cotton from five cotton production regions in Shandong, China. *Journal of Pesticide Science* **32**: 372-378.

Outcomes

5. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

- Cotton aphid and two-spotted mite were collected from Australian cotton growing regions.

- Two-spotted mite was susceptible against propargite (Comite®) and diafenthiuron (Pegasus®) but bifenthrin (Talstar®) and abamectin (eg Wizard®) resistance was detected. The detection of abamectin (eg Wizard®) resistance in 3 of the 4 strains during 2010-2011 was highly unusual and may relate to high product use.

- For the third consecutive season DAN197 ran molecular testing that did not detect pirimicarb (Pirimor®) or organophosphate resistance in field collected cotton aphid. These chemicals can now be used by cotton growers with confidence.

- Neonicotinoid resistance in cotton aphid increased in both level and abundance as the study progressed. In 2010-2011, some 96% of the strains tested showed some level of neonicotinoid resistance (ie Actara®-Cruiser® or Shield®). Such ubiquitous resistance makes the neonicotinoids unreliable for cotton aphid control and growers may well be better off using alternative foliar products for their aphid control if a neonicotinoid seed dressing has been

used. Alternatives products available include pirimicarb (Pirimor ®), diafenthiuron (Pegusus®), spirotetramat (Movento®), an OP or spray oil.

- Diafenthiuron (Pegasus®) cotton aphid survivor were found in small numbers though-out the study but no control issues were reported with any suspect diafenthiuron (Pegusus®) resistant strains. Subsequent diafenthiuron (Pegasus®) pressuring could not change any strain's response to diafenthiuron (Pegasus®) implying vigour tolerance rather than resistance. For the 2011-2012 season, the diafenthiuron (Pegasus®) discriminating dose will be adjusted to control vigour tolerant cotton aphid.

- Green mirid were tested against fipronil (Regent®), clothianidin (Shield®) and dimethoate (Rogor®) at 24, 48 and 72 h and results indicate that a 48 h withholding period would be suitable for future bioassays.

6. Please describe any:-
   a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);

   - Nil

   b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and

      - Detection of *kdr* Pyrethroid Resistance in the Cotton Aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) using an PCR-RFLP Assay

   c) required changes to the Intellectual Property register.

      - Nil

Conclusion

7. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

Cotton aphid is an important pest of cotton due to its ability to reduce yield through feeding damage. Until the introduction of *Bt*-cotton to Australia in the mid 1990s cotton aphids were considered late season secondary pests because they were suppressed by insecticides used against other pests. However, from 1998-1999 season aphids have been more troublesome initially with control failures against pirimicarb (Pirimor®) and omethoate (Folimat®) making sticky cotton a real possibility and subsequently nearly a decade later more failures with neonicotinoids. Chemical

control failures necessitated a complete re-think and modification of the aphid IRMS that is still developing and evolving to this day. The strategy is underpinned by resistance monitoring and mitigation methods based on chemical alternation and non-sequential use. This is augmented by a series of adjunct methods of aphid control that help put the resistance gene(s) at a selective disadvantage. Although the newly emerged neonicotinoid resistance in cotton aphid is a serous concern the Australian cotton industry is now much better placed to cope with resistance than back in 1998-1999. With the help of good resistance management pirimicarb (Pirimor®) and omethoate (Folimat®) now work and new chemistry called spirotetramat (Movento®) is available and from 2010-2012 will be further augmented by the reintroduction of pymetrozine (Chess®).

Unfortunately, for season 2010-2011 the frequency of strains showing neonicotinoid resistance increased to an extreme 96% of strains tested. Such resistance must make the neonicotinoids unreliable for cotton aphid control and preliminary glasshouse trial data generated at EMAI (DAN1201 and 1203) suggests neonicotinoid seed treatments also may not control all neonicotinoid resistant aphids. Growers may well be better off using alternative foliar products for aphid control if neonicotinoid seed dressings have been used. It is noteworthy that IPM friendly pirimicarb (Pirimor®) is currently resistance free so it would be a better first choice for an aphid specific foliar spray. Other alternatives are also available including, diafenthiuron (Pegusus®), spirotetramat (Movento®), pymetrozine (Chess®) an OP or a spray oil. If foliar neonicotinoids are to be used against aphids then try to limit in-season use of foliar neonicotinoids for aphid control if they have been used as a seed treatment. If foliar sprays are required for aphid control and neonicotinoid seed treatments have been used it is most important that the first foliar spray is not from the neonicotinoid chemical group. Finally, cotton gowers should consider the implications of coincident aphids if spraying neonicotinoids against other pests such as mirids. Aphids will develop resistance even if the sprays are not targeted directly against them.

Table 1. Pirimicarb and Organophosphate (OP) susceptibility using molecular diagnosis plus bioassay determination of endosulfan (Thiodan®), acetamiprid (Intruder®), clothianidin (Shield®), diafenthiuron (Pegasus® (CGA140408)), imidacloprid (Confidor®) and thiamethoxam (Cruiser®) via percent mortality at the discriminating dose (ie percent susceptible) and resistance factor (RF_{50})(95% confidence interval in brackets) for various strains of cotton aphid collected during season 2008-2009

Strain	Region	OP (*Pdi*I)	Pirimor® (*Ssp*I)	Endo 0.035%	Intruder® 0.0002%	Actara® 0.002%	Pegasus® 0.0015%	Shield® 0.005%	Confidor® 0.002%
The Ovr 08	Narrabri	Susc	Susc	100%	100%	100	NR	100% RF_{50}0.1 (0.05-0.2)	NR
Ros R2	Dalby	Susc	Susc	100%	100%	80%	100%	93% RF_{50}1.7 (0.5-2.4)	92%
Brook	St George	Susc	Susc	100%	100%	100%*	100%	100% RF_{50}0.8 (0.4-1.5)	92%
War F1	Namoi Valley	Susc	Susc	100%	100%	100%	100%	100% RF_{50}0.4 (0.3-0.6)	100%
W Lag V	Namoi Valley	Susc	Susc	100%	76%	86%	100%	95% RF_{50}4.8 (2.9-7.8)	89%
Elra	Darling Downs	Susc	Susc	100%	82%	44% RF_{50}18 (7.4-44.5)	100%	99% RF_{50}3.0 (1.9-4.5)	92%
Bin	Moree	Susc	Susc	100%	77%	57%	100%	89%* RF_{50}2.0 (0.9-4.0)	96%
The My	Namoi Valley	Susc	Susc	100%	100%	100%	100%	91% RF_{50}2.9 (1.3-6.2)	100%
Kat Vol	St George	Susc	Susc	100%	74% RF_{50}6.4 (3.8-10.6)	70%	95% RF_{50}2.6 (1.6-4.2)	80% RF_{50}6.2 (3.0-13.0)	89% RF_{50}3.9 (2.2-6.7)
St R	Dalby	Susc	Susc	100%	49% RF_{50}3.1 (1.7-5.6)	42% RF_{50}14.0 (6.5-30.3)	100%	98% RF_{50}1.4 (0.5-4.0)	94%
Kat S	St George	Susc	Susc	100%	76%* RF_{50}1.2 (0.7-2.1)	73%	100%	70% RF_{50}5.9 (2.9-11.7)	82% RF_{50}8.1 (4.6-14.2)

Strain	Region	OP (*Pdi*I)	Pirimor® (*Ssp*I)	Endo 0.035%	Intruder® 0.0002%	Actara® 0.002%	Pegasus® 0.0015%	Shield® 0.005%	Confidor® 0.002%
The Ovr 09	Dalby	Susc	Susc	100%	100%	98%	100%	100% RF_{50}1.6 (0.9-3.0)	98%
Cav	Dalby	Susc	Susc	100%	100%	72%	100%*	90% RF_{50}2.8 (1.0-8.0)	88% RF_{50}5.1 (2.7-9.7)
Ros R9	Dalby	Susc	Susc	100%	100%	66%	100%	86% RF_{50}3.8 (2.4-6.2)	84% RF_{50}9.1 (6.3-13.1)
Tull	Namoi Valley	Susc	Susc	100%*	92%	71%	100%	98% RF_{50}3.2 (1.9-5.5)	94%
Wam	Darling Downs	Susc	Susc	100%	98%	100%	100%	91%	99%
War Vol	Namoi Valley	Susc	Susc	100%	100%	100%	100%	100%	100%*
Bal	St George	Susc	Susc	100%	92%	100%	100%*	100%* RF_{50}1.6 (0.6-4.0)	100%
Nar	Darling Downs	Susc	Susc	100%	91%	80%	95% RF_{50}3.6 (2.1-6.2)	50% RF_{50}10 (1.8-56)	100%
Went F6	Namoi	Susc	Susc	99% RF_{50}1.8 (1.3-2.6)	97%	100%	100%	100%*	99%
W Lag F5	Namoi Valley	Susc	Susc	100%	98%	98%	100%	95% RF_{50}2.1 (1.3-3.5)	100%
Yar	Namoi Valley	Susc	Susc	100%	99%	100%	100%	98% RF_{50}4.1 (2.1-8.2)	95%
Fair	Dalby	Susc	Susc	100%	87%	100%	100%	100%*	100%
E.Wer	Dalby	Susc	Susc	100%	94%	36% RF_{50}3.1 (0.5-20)	99% RF_{50}2.7 (1.7-4.2)	80% RF_{50}1.9 (1.0-4.0)	97%

* not replicated

Table 2. Pirimicarb and Organophosphate (OP) susceptibility using molecular diagnosis plus bioassay determination of endosulfan (Thiodan®), clothianidin (Shield®), diafenthiuron (CGA140408) (Pegasus®), and thiamethoxam (Actara or Cruiser®) resistance via percent mortality at the discriminating dose (ie percent susceptible) and resistance factor (RF_{50})(95% confidence interval in brackets) for selected strains of cotton aphid collected during season 2009-2010

Strain	**Region**	**OP (*Pdi*I)**	**Pirimor® (*Ssp*I)**	**Endo 0.035%**	**Actara® 0.002%**	**Pegasus® 0.0015%**	**Shield® 0.005%**
Bull F1	Darling Downs	Susc	Susc	100	100	100	100
Yar F4	Darling Downs	Susc	Susc	100	99	100	100
BG F134	St George	Susc	Susc	98	100	100	100
Yar Rat	Darling Downs	Susc	Susc	100	99	100	100
Lan F4	Darling Downs	Susc	Susc	100	64 RF_{50} = 39 (25-63)	100	81 RF_{50} = 15 (6.3-36)
Kaj F1	St George	Susc	Susc	100	44 RF_{50} = 43 (23-80)	100	84 RF_{50} = 14 (7.2-26)
The Ovr	Darling Downs	Susc	Susc	100	60 RF_{50} = 58 (30-114)	100	91 RF_{50} = 4.6 (2.1-10.2)
Nun B12	Macquarie Valley	Susc	Susc	98	100	100	100
My Pla F11	Macquarie Valley	Susc	Susc	100	100	100	99
F27 F15	St George	Susc	Susc	100	99	100	100
Car	Macintyre	Susc	Susc	100	80	100	89
Pl Far F153	St George	Susc	Susc	100	49 RF_{50} = 69 (45-105)	100	89
West F8	Macquarie valley	Susc	Susc	100	44	98	93
Arm	Macquarie valley	Susc	Susc	100	53 RF_{50} = 39 (20-75)	89 RF_{50} = 4.0 (1.4-11)	95

Table 3. Pirimicarb and dimethoate susceptibility using molecular diagnosis ***(Ssp*I)** plus bioassay determination of clothianidin (Shield®), diafenthiuron (CGA140408)(Pegasus®), and thiamethoxam (Actara or Cruiser®) resistance via percent mortality at the discriminating dose (ie percent susceptible) for cotton aphid collected during season 2010-2011

Strain	Pirimor® (*Ssp*I)	Pegasus® 0.0015%	Shield® 0.005 %	Actara® 0.002%
Alch #	Susc	100%	81%	75%
Ball	Susc	100%	90%	90%
Bel	Susc	100%	95%	85%
Carn	Susc	100%	96%	92%
Car Gin	Susc	100%	98%	92%
Corin #	Susc	100%	95%	85%
Farm 101	Susc	100%	67%	47%
Farm 96 #	Susc	100%	100%	100%
Glen av	Susc	100%	89%	83%
Glen twn orig	Susc	100%	95%	76%
Glen twn shld	Susc	100%	96%	67%
Kilm	Susc	100%	91%	83%
Kilm Unsprd	Susc	100%	93%	87%
Mayf farm #	Susc*	100%	91%	82%
Myal P A	Susc	100%	91%	71%
Myal P B	Susc	100%	96%	15%
Nor Field	Susc	100%	93%	94%
Nor TSM Split	Susc	100%	95%	92%
Pal Lyn	Susc	100%	90%	78%
Plant farm #	Susc	100%	90%	88%
Red M	Susc	100%	89%	93%
S Gin Block #	Susc	99%	89%	85%
The Bore pad	Susc	100%	94%	86%
Wat	Susc	100%	95%	55%
W Gin Block	Susc	100%	92%	86%
Whol	Susc	100%	95%	70%
Wil	Susc	100%	88%	90%

*An initial PCR detected two resistant aphids however a confirmatory re-test did not confirm that resistance

#Subsample for resistance testing taken immediately before strain culturing

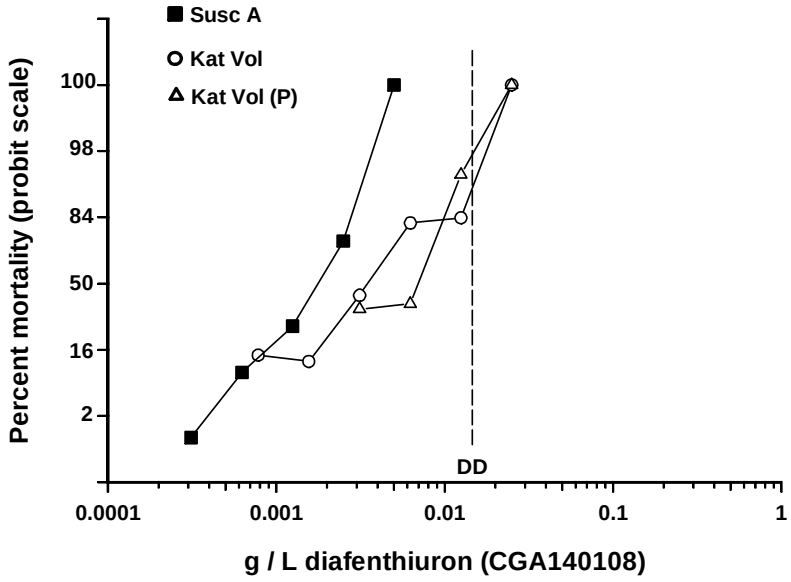

Figure 1. Does response for a susceptible (Susc A) and suspect resistant strain of cotton aphid (Kat Vol) that was subsequently pressured at a discriminating concentration (DD) of diafenthiuron (Pegasus®) and then retested (Kat Vol (P))

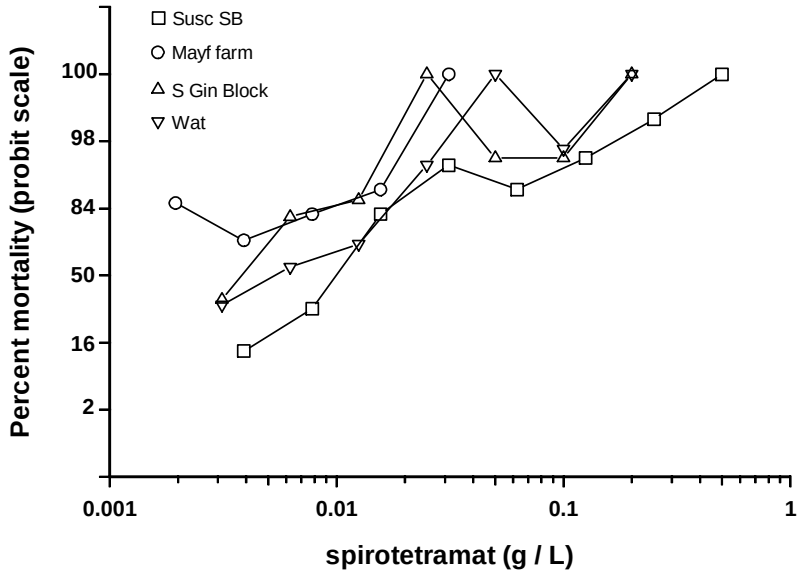

Figure 2. Dose response for three 2010-2011 collected field strains and a reference susceptible strain of cotton aphid against spirotetramat (Movento®)

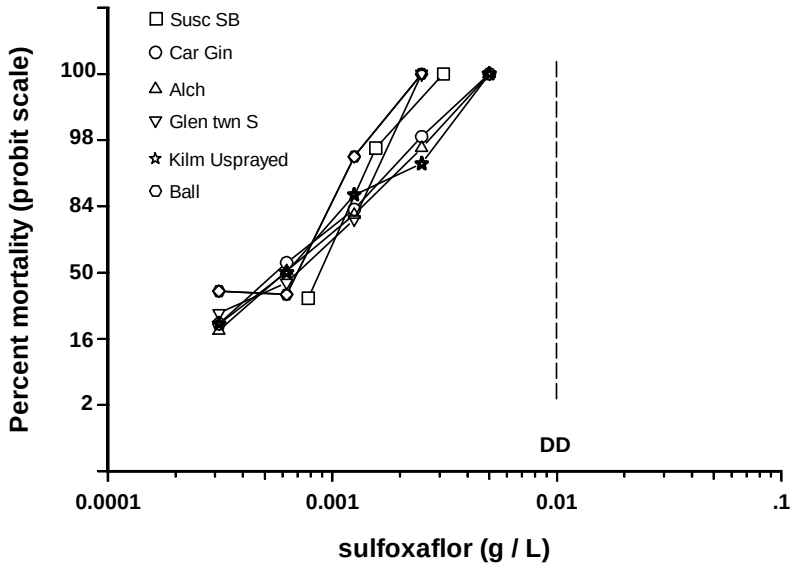

Figure 3. Dose response for five 2010-2011 collected field strains (that were all neonicotinoid resistant, see Table 3) and a reference susceptible strain of cotton aphid against sulfoxaflor (Transform™)

Table 4. Percent mortality at the discriminating dose (ie percent susceptible) for various strains of TSM collected during season 2008-2009 and evaluated for resistance against bifenthrin (Talstar®), chlorfenapyr (Intrepid®), abamectin (Agrimec®), propargite (Comite®) and diafenthiuron (Pegasus® (CGA-140408))

Season	Strain	Area	Chemical				
			Bifenthrin (Talstar®)	Chlorfenapyr (Intrepid®)	Abamectin (Agrimec®)	Propargite (Comite®)	Diafenthiuron (Pegasus® CGA140408)
2008-2009	WL	Narromine	14	100	100#	100	100
	TU8	Namoi	56	98	100	99	100
	MN	Namoi	98	98	100	100	100
	MY	Namoi	76	97	100	100	100
	TU10	Namoi	30	99	100	100	100
	GL	Namoi	17	100	100	100	100

* not replicated

field abamectin control issue not resistance

Table 5. Percent mortality at the discriminating dose (ie percent susceptible) for various strains of TSM collected during season 2010-2011 and evaluated for resistance against bifenthrin (Talstar®), abamectin (Agrimec®), propargite (Comite®) and diafenthiuron (Pegasus® (CGA-140408))

Strain	Region	Bifenthrin (Talstar®)	Abamectin (Agrimec®)	Propargite (Comite®)	Diafenthiuron (Pegasus® CGA140408)
Nor	Moree	89	100	100	100
Quong F 11 (North)	Narromine	85*	90*	100*	100*
Nan F 3	Narromine	53*	98*	100	100*
Nan Back F1	Narromine	40*	97*	100	100*
Quong F 11 (South)	Narromine	^	^	^	^
Tocabil	Hillston	#	#	#	#
Watson	Hillston	#	#	#	#

* not replicated
^ predatory mites ate
# died in transit (Australia Post)

Table 6. Percent mortality for EMAI collected green mirid when treated at various doses of fipronil (Regent®), clothianidin (Shield®) or dimethoate (Rogor®) and withholding periods of 24, 48 or 72 h.

Chemical	Dose	24h Mortality	48h Mortality	72 h Mortality
Regent® rep 1	0.00025	100	100	100
	0	0	0	30
Regent® rep 2	0.00025	100	100	100
	0	0	0	30
Regent® rep 3	0.001	100	100	*
	0.0001	100	100	*
	0.00001	0	0	*

	0	0	0	*
Regent® rep 4	0.0001	100	100	*
	0.00005	33	66	*
	0.000025	0	100	*
	0.0000125	0	0	*
	0	0	100	*
Shield® rep 1	0.001	100	100	*
	0.0001	0	0	*
	0	0	0	
Shield® rep 2	0.001	66	100	*
	0.0005	100	100	*
	0.00025	33	33	*
	0.000125	0	0	*
	0	0	0	*
Shield® rep 3	0.001	100	100	*
	0.0005	100	100	*
	0.00025	33	33	*
	0.000125	100	100	*
	0	50	50	*
Dimethoate rep 1	0.005	100	100	*
	0.0005	0	50	*
	0.00005	0	0	*
	0	0	0	*
Dimethoate rep 2	0.005	100	100	*
	0.0025	66	100	*

0.00125	33	66	*
0.000625	0	33	*
0.0003125	0	0	*
0	0	0	*

* not tested

Extension Opportunities

8. Detail a plan for the activities or other steps that may be taken:
   (a) to further develop or to exploit the project technology.

   - Reference susceptible and resistant strains need to be maintained so they can be used in future cross resistance studies

   (b) for the future presentation and dissemination of the project outcomes.

   - Data to be included into a significant review article covering the successes and failures of IPM that will include chemical control and resistance management of secondary sucking pests including aphids, mites and mirids.

   (c) for future research.

   - Resistance in cotton aphid against newer chemistries such as spirotetramat (Movento®) will continue to be studied with bioassay but organophosphate and carbamate insecticides will be monitored with molecular genetic techniques previously developed. Further, molecular genetic techniques will be developed to detect neonicotinoid resistance in cotton aphid that will support a PhD study on that topic. Initially this will include full bioassay dose response assays with synergists to characterise resistance as either likely target site or detoxification. The PhD study will aim to find the point mutation for neonicotinoid resistance in cotton aphid that will simultaneously elucidate the underlying resistance mechanism and thus cross resistance implications that are essential for management. Once the mechanism is known its genetic sequence will be fully characterised and that will provide the first step in the development of a molecular based test for neonicotinoid resistance monitoring. The PhD study will also further boost the human capacity available to Australian cotton to manage the ongoing problem of insecticide resistance. Importantly, the PhD study will train a young scientist in both bioassay and

molecular genetic methodology for resistance detection so bridging the gap between these two different but essential sciences.

8. A. List the publications arising from the research project and/or a publication plan. (NB: Where possible, please provide a copy of any publication/s)

- Wilson, L., Herron, G., Smith, T., Franzmann, B. and Heimona, S. (2008) Cotton Insects. Aphid Ecology in Cotton. Cotton Catchment Communities CRC. pp. 6.

- Wilson, L., Herron, G., Smith, T. and Heimona, S. (2008) Cotton Insects. Strategies to Manage Aphids in Cotton. Cotton Catchment Communities CRC. pp. 8.

- Martin O. M^{c}Loon and Grant A. Herron (2008) PCR detection of pirimicarb resistance in Australian field isolates of *Aphis gossypii* Glover (Aphididae: Hemiptera). In 14th Australian Cotton Conference, Broadbeach Queensland, 12-14 August 2008.

- Grant A. Herron, Martin O. M^{c}Loon, & Lewis J. Wilson (2008) Resistance testing summary for the 2006-2007 and 2007-2008 cotton seasons: cotton aphid *Aphis gossypii* and two-spotted mite *Tetranychus urticae.* In 14th Australian Cotton Conference, Broadbeach Queensland, 12-14 August 2008.

- Grant A. Herron and Jeannette Rophail (2008) Resistance development a possibility in mirids from Australian cotton In 14th Australian Cotton Conference, Broadbeach Queensland, 12-14 August 2008.

- McLoon MO, Carletto J, Herron GA, Vanlerberghe-Masutti F, Smith, T and Wilson LJ (2008) Microsatellite typing identifies genotypic variability in Australian isolates of *A. gossypii.* Australian Entomological Society 39th Annual General Meeting and Scientific Conference 2008, 28 September 2008 - 1 October 2008, Orange Agricultural Institute Orange, New South Wales.

- *McLoon MO and Herron GA (2009) PCR detection of Pirimicarb resistance in Australian field isolates of *Aphis gossypii* Glover (Aphididae: Hemiptera). *Australian Journal of Entomology* **48**: 65-72.

- Herron G (2009) Insecticide resistance monitored, p 28. In: Spotlight on Cotton R&D, Autumn 2009. CRDC, Narrabri.

- Herron GA and Wilson LJ (2010) Aphids – Where to From Here? Proceedings of the 15th Australian Cotton Conference, 10-12th August 2010, Gold Coast, Australia. http://www.australiancottonconference.com.au/resources.php?ContentID=3&PresenterID=19 pp. 1-4.

- *Herron, GA and Wilson LJ. (2011) Neonicotinoid resistance in Aphis gossypii Glover (Aphididae: Hemiptera) from Australian cotton. *Australian Journal of Entomology* **50**: 93-98.

- Herron, GA (2011) Challenges for better aphid management. pp. 56-59 In: CCA Cropping Solutions Seminar, 3-4 May 2011, Moree. Crop Consultants Australia.

- *Marshall, K., Moran, C., Chen, Y. and Herron G. (2011) Detection of *kdr* Pyrethroid Resistance in the Cotton Aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) using an PCR-RFLP Assay. *Journal of Pesticide Science*, Submitted.

- *Yizhou Chen, Jerome Carletto, Martin O. McLoon, Flavie Vanlerberghe-Masutti, Tanya Smith, Lewis J. Wilson & Grant A. Herron. Insecticide resistance associated with the *ACE*1 mutation and genetic structure in Australian *Aphis gossypii* Glover (Aphididae: Hemiptera). In preparation.

NB. * indicates a refereed scientific journal contribution – a copy is given in Appendix 1 at the end of the report if it is published and reprints are available.

B. Have you developed any online resources and what is the website address?

Outcomes from the research feed directly into the annual Cotton Pest Management Guide available from http://www.crdc.com.au/emags/PMG10_11/

Part 4 – Final Report Executive Summary

Provide a one page Summary of your research that is not commercial in confidence, and that can be published on the World Wide Web. Explain the main outcomes of the research and provide contact details for more information. It is important that the Executive Summary highlights concisely the key outputs from the project and, when they are adopted, what this will mean to the cotton industry.

Since the introduction of Bt-cotton secondary pests such as aphids, mites and bugs have become more prominent requiring targeted insecticide control. These sprays have lead to resistance in some pest species that have caused the chemical control to fail. Spray failures increase grower costs and the likely hood of unforseen environmental consequences but very importantly can permanently tarnish Australia's reputation for producing high quality lint if

failures cause ‘sticky cotton’. Recently in Australian cotton, there has been control failure against aphids with chemicals belonging to the group known as neonicotinoids. This group includes the mainstay cotton seed treatment thiamethoxam (Cruiser®) and the cost effective foliar spray clothianidin (Shield®). The neonicotinoid insecticides control several problematic pest species in cotton including aphids and mirids and their loss due to resistance puts increased pressure on remaining control products by severely limiting control options.

In the 2007-2008 cotton season neonicotinoid resistance was detected for the first time in cotton aphid but control failures were not experienced. During the following 2008-2009 season neonicotinoid resistance increased in both level and abundance (82% of the stains tested) and for the first time neonicotinoid resistance caused control failures and cross resistance to other neonicotinoid products was additionally implicated. Interestingly, the proportion of strains showing neonicotinoid resistance in 2009-2010 remained approximately static at 78% but for season 2010-2011 the frequency of strains showing resistance increased to an extreme 96% of strains tested. Such resistance makes the neonicotinoids unreliable for cotton aphid control and growers may well be better off using alternative foliar products for aphid control if neonicotinoid seed dressings have been used. It is noteworthy that Integrated Pest Management friendly pirimicarb (Pirimor®) is currently resistance free so it would be a good first choice for an aphid specific foliar spray. Other alternatives are also available including, diafenthiuron (Pegusus®), spirotetramat (Movento®), pymetrozine (Chess®) an OP or spray oil. If foliar neonicotinoids are to be used against aphids I recommend the following to help manage resistance. Try to limit in-season use of foliar neonicotinoids for aphid control if they have been used as a seed treatment. If foliar sprays are required for aphid control and neonicotinoid seed treatments have been used it is most important that the first foliar spray is not from the neonicotinoid chemical group. Finally, consider the implications of coincident aphids if spraying neonicotinoids against other pests such as mirids. Aphids will develop resistance even if the sprays are not targeted directly against them and control failures could result.

Appendix 1: Published refereed scientific journal reprints

AUSTRALIAN JOURNAL of ENTOMOLOGY

Australian Journal of Entomology (2009) **48**, 65–72

PCR detection of pirimicarb resistance in Australian field isolates of *Aphis gossypii* Glover (Aphididae: Hemiptera)

Martin O McLoon and Grant A Herron*

New South Wales Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle 2568, NSW, Australia.

Abstract *Aphis gossypii* Glover (cotton aphid) is a major secondary pest of Australian cotton that readily develops resistance to the carbamate insecticide pirimicarb (Pirimor®) and to organophosphates generally. To test the pirimicarb resistance status of Australian strains of *A. gossypii*, a polymerase chain reaction (PCR) assay followed by restriction enzyme assay (REA) was designed to identify the *Ace*I polymorphism S431F known to be responsible for resistance. The method was tested against reference and 33 field strains collected over two consecutive seasons. Both methods confirmed pirimicarb resistance in two field strains, one from each cotton season, giving credence to the molecular technique described. The PCR assay proved specific for the *Ace*I gene. This PCR REA assay has the potential to replace bioassay for the routine pirimicarb resistance monitoring in *A. gossypii*. With the molecular assay providing results in 48 h, compared with 4–8 weeks for bioassay, such an assay could be used before insecticide control.

Key words bioassay methods, insecticide resistance, molecular methods.

INTRODUCTION

Resistance in both *Aphis gossypii* Glover (cotton aphid) and *Myzus persicae* (Sulzer) (green peach aphid) to the carbamate insecticide pirimicarb (Pirimor®) and to organophosphates in general are a major threat to Australian cotton production (Herron *et al.* 2001). To better manage aphids and prevent control failures because of resistance, routine insecticide monitoring was undertaken via a conventional discriminating dose bioassay (Herron *et al.* 2001). The information gained from monitoring contributes directly to the aphid component of the Insecticide Resistance Management Strategy for Australian cotton (Farrell 2006).

Until recent Australian insecticide control failures (Herron *et al.* 2001) pirimicarb was very effective at controlling cotton aphid. Because of chemical failure, growers often tried to manage resistant populations by alternating organophosphates such as omethoate with pirimicarb. However, crop failures continued until it was confirmed that the two insecticides cause cross-resistance to one another (Herron *et al.* 2003). Moores *et al.* (1996) previously documented the existence of at least two insecticide-insensitive forms of acetylcholinesterase (AChE) resistant *A. gossypii* aphid that conferred different resistance spectra to pirimicarb and specific organophosphates.

The mechanism of resistance to pirimicarb in *A. gossypii* has been shown to be via target site insensitivity in the acetylcholinesterase enzyme encoded by the *Ace*I gene (Nabeshima *et al.* 2003; Benting & Nauen 2004; Toda *et al.* 2004). The target site insensitivity is caused by a non-synonymous DNA polymorphism that causes the replacement of a serine with a phenylalanine (S431F) proximal to the enzymes' active site gorge (at the acyl pocket) and removes a *Ssp*I restriction site. (Andrews *et al.* 2004; Toda *et al.* 2004; Oh *et al.* 2007). The substitution of serine with phenylalanine at the acyl pocket creates steric hindrance and an increased hydrophobicity at the entrance to the active site preventing access to pirimicarb but allowing acetylcholine entry (Andrews *et al.* 2004; Oh *et al.* 2007). The loss of the *Ssp*I restriction site indicates a phenylalanine substitution in the acetylcholinesterase and hence a pirimicarb resistant phenotype. Andrews *et al.* (2004) designed a diagnostic nested polymerase chain reaction (PCR) and *Ssp*I restriction enzyme assay (REA) to determine pirimicarb resistance or susceptibility in *A. gossypii* and *M. persicae*.

Here we refine Andrews *et al.* (2004) method using a single PCR with primers approximately equally distant from the S431F DNA polymorphism(s) and a post PCR *Ssp*I REA. To design the new primers and validate any S431F DNA polymorphisms the *Ace*I gene sequence amplified using the primers of Andrews *et al.* (2004) were determined for five reference strains of *A. gossypii* in which the pirimicarb resistance status was known. These were aligned against the Genbank database sequences provided by Andrews *et al.* (2004) and Toda *et al.* (2004). Primers were then designed such that the *Ssp*I restriction site altered by the DNA polymorphism was at the centre of the amplicon. Thus if *Ssp*I digests the PCR amplicon it will create two fragments of the

*grant.herron@dpi.nsw.gov.au

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same size. These will co-migrate on an agarose gel and present as a single intense band half the size of an undigested amplicon. As removal of the *Ssp*I site indicates a phenylalanine substitution, an undigested amplicon indicates pirimicarb resistance and a cut amplicon, pirimicarb susceptibility.

The results of this PCR REA are presented here concurrently with conventional discriminating dose bioassay to validate the method.

MATERIAL AND METHODS

Aphid strains

The reference strains insecticide resistance profiles have been previously determined with some published in Herron *et al.* (2003). Reference strains are maintained as live cultures that are pressured (sprayed) with the appropriate insecticide(s) on an *ad hoc* basis. The field isolates of aphid were collected by researchers, Cotton CRC Industry Development Officers, consultants and growers from cotton fields (Table 1). They were then sent by overnight courier to the bioassay laboratory at Camden (Elizabeth Macarthur Agricultural Institute) and each field isolates cultured separately on pesticide-free cotton (Deltapine 90) at 25 ± 4°C under natural light. Isolate integrity was assured by maintaining populations in purpose built insect proof cages.

Bioassay

Aphids were sprayed with insecticide using the methods described by Herron *et al.* (2000). Briefly, batches of 10 adult female aphids were placed in a 35-mm Petri dish that had in it an excised cotton plant leaf disc fixed in agar. The Petri dish with aphids in place were then sprayed with the aid of a Potter spray tower that produced an aqueous deposit of 1.6 ± 0.07 mg cm^{-2} with a 2-mL spray sample. Each test was replicated once and included a water only sprayed control that did not exceed 10% natural mortality. After spraying, Petri dishes were covered with ventilated (to limit condensation) clear plastic film and maintained at 25 ± 0.1°C in 16:8 L : D for 24 h after which mortality was assessed with the aid of a stereo microscope

Table 1 Discriminating dose bioassay results and molecular assay results (*Ssp*I REA) for field isolates of *Aphis gossypii* collected during the 2005/2006 and 2006/2007 cotton seasons and evaluated for pirimicarb resistance

Year	Strain	Area	Bioassay	Molecular *Ssp*I REA
2005/2006	ACRI R6 1	Namoi	8	R
	CH	Gwydir	100	S
	Tell F 33	Gwydir	100	S
	Wood	Gwydir	100	S
	Yar	Downs	Did not establish	S
	Oak F 1	Downs	100	S
	Aru F 3	Downs	100	S
	War M7	Downs	100	S
	Over F 4	Downs	100	S
	Sin F 23	Downs	Did not establish	S
	Eden	Downs	100	S
	War F 2	U Namoi	100	S
	Bell F 15	U Namoi	100	S
	Mer†	U Namoi	99	S
	Cur F 25	U Namoi	100	S
	Cur F 22	U Namoi	100	S
	Car F 2–7	MacIntyre	100	S
	My D Cr	MacIntyre	100	S
	Alch C 4–5	MacIntyre	100	S
	Rio G	Mungindi	100	S
	Har	St George	100	S
	Cal	Namoi	100	S
	Uya	Namoi	100	S
	Mill 1	Downs	Did not establish	S
2006/2007	Aus Mid 23	Gwydir	100	S
	Car 34	M°Intyre	100	S
	War 20–22	M°Intyre	100	S
	Alch 007	M°Intyre	100	S
	Car 13	M°Intyre	100	S
	Nor 4	Gwydir	100	S
	Byr 55	Macquarie	100	S
	Bur 4	Macquarie	100	S
	Wil 21B	Macquarie	6	R

†See results section.
R, pirimicarb resistant; S, pirimicarb susceptible.

DNA extraction

DNA was isolated from field isolates and reference strains of *A. gossypii* (10 aphids per strain) using Chelex – 100 resin (BioRad). Basically, an individual aphid is placed in a 1.5-mL labelled microcentrifuge tube containing 70 µL of 5% Chelex – 100 resin. DNA is extracted by grinding the aphid with a sterile micro pestle. Then heating the microcentrifuge tube at 56°C for 30 min followed by 100°C for 5 min. The crude DNA sample is then used for PCR (2 µL per reaction) or stored at –20°C until needed.

PCR amplification of *Ace*I

Polymerase chain reaction was performed using iQ Sybr green supermix (BioRad) and the primers AceF (CAAGCCATC ATGGAATCAGG) and AceR (TCATCACCATGCATCAC ACC) with the RotaGene 2000 real time PCR machine (Corbett Research). Cycling parameters were an initial 5-min denaturation at 94°C followed by 40 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 45 s. Melt curves analysis was determined between 75°C and 95°C.

DNA sequencing

The five reference strains of *A. gossypii* had DNA sequencing performed on the real time PCR products amplified using primers RESF1and RESR1 (Andrews *et al.* 2004). The sequencing was out sourced to Newcastle DNA (University of Newcastle, NSW, 2308). It was carried out using an ABI 377 sequencer and the PCR primers. DNA sequence analysis was carried out using the software BioEdit (Hall 1999)

Primer design

Primers AceF and AceR were designed from DNA sequence alignments of the five reference strains plus the GenBank sequences supplied by Andrews *et al.* (2004) and Toda *et al.* (2004). The primers were designed to be equally distant from the polymorphic *Ssp*I restriction site. AceF and AceR amplify a 667-bp product. A *Ssp*I digested PCR product (pirimicarb susceptible) will generate two DNA fragments of the same size (331 bp and 336 bp) that co-migrate on a 2% agarose gel and present as a single intense band half the size of the undigested PCR product (pirimicarb resistant).

Restriction enzyme digests of *Ace*I products

The *Ssp*I (New England; BioLabs) REA was initially performed on the reference strain's PCR products. Their insecticide resistance profiles were known allowing their use as experimental controls. *Ace*I PCR products from the field collected aphid isolates were digested with *Ssp*I to detect mutations associated with pirimicarb resistance. *Ssp*I REA was performed by incubating AceF and AceR generated PCR products at 37°C for 3 h with 4U of enzyme and the manufacturers supplied buffer. The total reaction volume was 20 µL.

Gel electrophoresis

Electrophoresis was performed using an Easy Cast apparatus (Owl Scientific Instruments). Agarose (Progen) gels were made with fresh 1 X TBE buffer, supplemented with ethidium bromide (5 µg/mL), run at 94 volts for 1–3 h in 1 X TBE buffer. Gels were seen and documented with the Bio-Rad Gel Doc system.

RESULTS

Bioassay analysis

Bioassay results for the reference strains were in complete agreement between previous and current bioassay data and those data produced via PCR REA. All field strains (Table 1) showed a susceptible phenotype to pirimicarb except for the strains, ACRI R6 (2006) and Wil 21B (2007) which were resistant. Another strain, Mer, also gave a single aphid survivor in the pirimicarb bioassay but the strain is still considered susceptible. This is due to the bioassay procedure using a dose set at the $LC_{99.9}$ level so giving a small chance of a single susceptible survivor.

DNA sequencing results

DNA sequencing was carried out on both strands of a reference strains' PCR product, resulting in 705 bp of DNA sequence that could be directly compared between strains and with GenBank database. The DNA sequence covers the mutations responsible for pirimicarb and organophosphate resistance in *A. gossypii*. Strain 171B (GenBank accession GI:48714782), strain 968E (GenBank accession GI:48714786) and strain 1081K (GenBank accession GI:48714784) are sequences from Andrews *et al.* (2004), Strain 171B is pirimicarb susceptible, strain 968E and strain 1081K are pirimicarb resistant having polymorphisms at 1290–1293 bp (strain 171B numbering) that result in the S431F mutation. Strains GSM and H-16 are also pirimicarb resistant strains but have two alleles at the S431F locus; GSM-1 (GenBank accession GI:52313423), GSM-2 (GenBank accession GI:52313425), H-16-1 (GenBank accession GI:52313419) and H-16-2 (GenBank accession GI:52313421) (Toda *et al.* 2004). Strains 968E and H-16-1 also have a G-to-T mutation at 904 bp (strain 171B numbering) causing the mutation A302S thought to confer a level of organophosphate resistance (Andrews *et al.* 2004; Oh *et al.* 2007).

The *Ace*I DNA sequence of strains Sus A and AW were identical to that of the susceptible strain 171B (Fig. 1). The *Ace*I DNA sequence of Adam was the same as strain 968E at both the S431F and A302S loci (Fig. 1). Strain JQ *Ace*I DNA sequence was identical to that of strain GSM-2 at the S431F locus. Two DNA alleles were evident from the DNA chromatographs of Togo at the S431F locus (Fig. 6); one the same as strain 968E (termed Togo H1) and one the same as GSM-2 (termed Togo H2). The dual peaks seen on the chromatograph is present on both DNA strands and in a second

```
                890       900       910       920       930       940       950       960
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
[1]171B   ATGTGACACTTTTCGGTGAATCGGCCGGCGCCGTTTCGGTTTCACTACACTTGCTATCTCCATTGAGTAGGAACCTTTTT
[1]968E   ........................T.......................................................
[1]1081K  ........................................................................
[2]GSM-1  ........................................................................
[2]GSM-2  ........................................................................
[2]H-16-1 ........................T.......................................................
[2]H-16-2 ........................................................................
[3]SusA   ........................................................................
[3]AW     ........................................................................
[3]Adam   ........................T.......................................................
[3]JQ     ........................................................................
[3]Togo H1 ......................T.......................................................
[3]Togo H2 ......................T.......................................................

                970       980       990       1000      1010      1020      1030      1040
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
[1]171B   AACCAAGCCATCATGGAATCAGGATCCTCAACAGCACCTTGGGCAATTTTGTCACGGGAAGAAAGTTTTAGTAGAGGACT
[1]968E   ........................................................................
[1]1081K  ..................................T.....................................
[2]GSM-1  ........................................................................
[2]GSM-2  ........................................................................
[2]H-16-1 ........................................................................
[2]H-16-2 ........................................................................
[3]SusA   ........................................................................
[3]AW     ........................................................................
[3]Adam   ........................................................................
[3]JQ     ........................................................................
[3]Togo H1 ......................................................................
[3]Togo H2 ......................................................................

                1050      1060      1070      1080      1090      1100      1110      1120
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
[1]171B   TAAACTAGCAAAAGCAATGGGATGTCCAGATGACAGAAACGAAATACATAAAACAGTCGAGTGCTTAAGAAAGGTTAACA
[1]968E   ........................................................................
[1]1081K  ........................................................................
[2]GSM-1  ........................................................................
[2]GSM-2  ........................................................................
[2]H-16-1 ........................................................................
[2]H-16-2 ........................................................................
[3]SusA   ........................................................................
[3]AW     ........................................................................
[3]Adam   ........................................................................
[3]JQ     ........................................................................
[3]Togo H1 ......................................................................
[3]Togo H2 ......................................................................

                1130      1140      1150      1160      1170      1180      1190      1200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
[1]171B   GTTCAGCAATGGTTGAAAAAGAATGGGACCATGTGGCTATATGTTTCTTCCCGTTTGTTCCGGTGGTCGATGGCGCTTTT
[1]968E   ........................................................................
[1]1081K  ........................................................................
[2]GSM-1  ........................................................................
[2]GSM-2  ........................................................................
[2]H-16-1 ........................................................................
[2]H-16-2 ........................................................................
[3]SusA   ........................................................................
[3]AW     ........................................................................
[3]Adam   ........................................................................
[3]JQ     ........................................................................
[3]Togo H1 ......................................................................
[3]Togo H2 ......................................................................
```

Fig. 1. DNA sequence alignment of *Ace*I PCR products from the five reference strains and those of Andrews *et al.* (2004) and Toda *et al.* (2004). The sequences cover the three DNA polymorphisms creating the S431F mutation located at 1294 bp (171B numbering). As well as the region containing the single polymorphism (G→T) responsible for the A302S mutation. A302S is located at 904 bp (171B numbering) in strains 968E, H-16-1, Adam, Togo H1 and H2, it purportedly confers resistance to a select group of organophosphates. [1]Andrews *et al.* (2004). [2]Toda *et al.* (2004). [3]Current study.

```
                1210       1220       1230       1240       1250       1260       1270       1280
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
[1]171B    CTTGACGATCATCCTCAAAAGTCTTTATCAACAAACAATTTTAAAAAAACCAATATACTCATGGGTAGTAACTCCGAAGA
[1]968E    ....................................................................................
[1]1081K   ....................................................................................
[2]GSM-1   ....................................................................................
[2]GSM-2   ....................................................................................
[2]H-16-1  ....................................................................................
[2]H-16-2  ....................................................................................
[3]SusA    ....................................................................................
[3]AW      ....................................................................................
[3]Adam    ....................................................................................
[3]JQ      ....................................................................................
[3]Togo H1 ....................................................................................
[3]Togo H2 ....................................................................................

                1290       1300       1310       1320       1330       1340       1350       1360
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
[1]171B    GGGTTACTATTCAATATTTTATTATTTGACGGAGCTTTTCAAAAAGGAGGAAAATGTGGTGGTGTCTCGTGAGAATTTTA
[1]968E    ...........TT.......................................................................
[1]1081K   ...........TT.......................................................................
[2]GSM-1   ...........TC.......................................................................
[2]GSM-2   .........C.TC.......................................................................
[2]H-16-1  ...........TT.......................................................................
[2]H-16-2  ....................................................................................
[3]SusA    ....................................................................................
[3]AW      ....................................................................................
[3]Adam    ...........TT.......................................................................
[3]JQ      .........C.TC.......................................................................
[3]Togo H1 ...........TT.......................................................................
[3]Togo H2 .........C.TC.......................................................................
```

Fig. 1. Continued.

DNA sequencing run of Togo's S431F locus. Togo H1 has the sequence TTTT and Togo H2 the sequence CTTC. The G-to-T polymorphism causing the A302S mutation seen in strain 968E is present in both Togo H1 and H2 (Fig. 1).

Real time PCR

The real time PCR products from the five reference strains had a melt curve of 83.23 ± 0.5°C (Fig. 2). Half melt temperature (1/2Tm) curve analysis with SYBR green was not sensitive enough to distinguish the different polymorphisms occurring at the S431F locus (Fig. 2). The few polymorphic nucleotides do not change the guanine plus cytosine molar per cent concentration (%GC) enough to alter the different PCR products 1/2Tm (the basis of melt curve variation). To ensure a melt curve of 83.23 ± 0.5°C was indicative of *Ace*I amplification, PCR products were size confirmed using agarose gel electrophoresis to see the 667-bp size expected from the gene sequence. All 33 field strains amplified a real time PCR product with a melt curve of 83.23 ± 0.5°C indicating a successful amplification of *Ace*I.

Restriction enzyme digests

The *Ssp*I REA performed on the reference strains correctly indentified the pirimicarb resistant strains (Fig. 3). The results for the 33 field isolates from the 2005/2006 and 2006/2007 cotton seasons can be seen in Figure 4. In the *Ssp*I REA on the 33 field isolates only ACRI R6 (2005/2006) and Wil 21B (2006/2007) gave a result representing pirimicarb resistance (uncut by *Ssp*I) The remaining field isolates all gave a result representing pirimicarb susceptibility (cut by *Ssp*I).

DISCUSSION

The *Ace*I DNA sequences of the two pirimicarb susceptible reference strains (Sus A and AW) were the same as strain 171B (pirimicarb susceptible) coding for a serine (S) at position 431. In contrast, the three pirimicarb resistant reference strains (Adam, JQ and Togo) all had DNA sequence coding for a phenylalanine (F) at position 431. Adam and JQ have homozygous polymorphisms at the S431F locus although they have different sequences. Adam's sequence is the same as 968E, 1081K and H-16-1 where as JQ has the same as GSM-2 (Fig. 5). The DNA chromatographs for two independent sequencing runs of Togo indicated it was heterozygous at the S431F locus. The DNA chromatographs consistently showed the presence of two overlapping nucleotide peaks (C or T) at positions 1291 and 1294 resulting in two different alleles (Fig. 6). One allele, Togo H1, has the same sequence as Adam and the majority of the resistant strains (TTTT). The second allele, Togo H2, is the same as JQ and GSM-2 (CTTC). However unlike JQ and

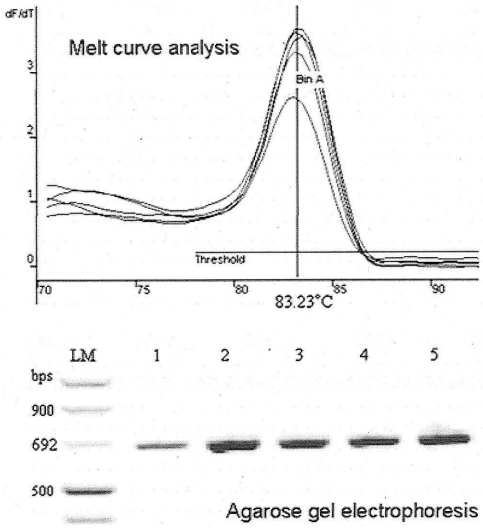

Fig. 2. Real time PCR products amplified using primers AceF and AceR from the five reference strains. The lower image shows the five products after agarose gel electrophoresis. The bands seen are equivalent to the anticipated size of 667 bp. The upper image shows the five melt curves produced by the PCR. The curves all show a half melting temperature ($^1/_2$Tm) of 83.23 ± 0.5°C. No difference is seen between the $^1/_2$Tm temperatures from the pirimicarb resistant and pirimicarb susceptible strains. A product amplified by standard PCR will have an agarose gel band size of 667 bp whereas a product amplified by real time PCR will have a melt curve of 83.23 ± 0.5°C. 1, Sus A; 2, AW; 3, JQ; 4, Adam; 5, Togo. LM, lane marker.

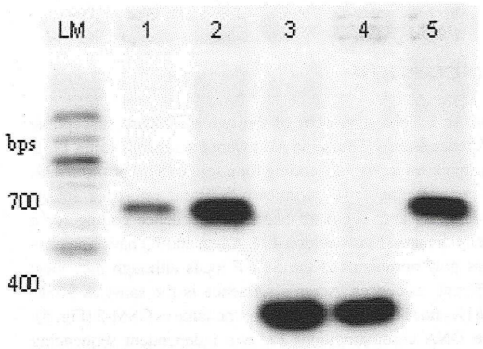

Fig. 3. *Ssp*I REA of *Ace*I real time PCR products from the five reference strains. The pirimicarb susceptible strains Sus A and AW show a single intense band at 336 bp (cut by *Ssp*I), whereas the pirimicarb resistant strains Adam, JQ and Togo show a single intense band at 667 bp (uncut by *Ssp*I). 1, JQ; 2, Adam; 3, Sus A; 4, AW; 5, Togo. LM, lane marker.

GSM-2, Togo H2 also has the G-to-T polymorphism at position 904 bp creating the A302S mutation. Both Togo's alleles code for phenylalanine at S431F. Confirming the heterozygous nature of Togo's S431F locus would require cloning and sequencing the two alleles.

The PCR assay proved to be specific for the *Ace*I gene without the need for a nested PCR thereby simplifying the assay of Andrews *et al.* (2004) reducing the cost, labour and time inputs. Comparisons of the real time PCR melt curves and their corresponding bands on agarose gel electrophoresis shows that a melt curve of 83.23 ± 0.5°C is representative of an *Ace*I PCR product. This negates the need for agarose gel electrophoresis after PCR to confirm amplification as real time PCR products with a melt curve of 83.23 ± 0.5°C are considered to have originated from *Ace*I and can be used directly for *Ssp*I REA. In the absence of real time PCR capabilities a standard thermo cycler can be used with the inclusion of an agarose gel electrophoresis step to confirm amplification before *Ssp*I REA.

The PCR REA for pirimicarb successfully identified the three resistant reference strains and the two susceptible strains (Fig. 3). Of the 33 field isolates tested only two failed to cut with *Ssp*I indicating their resistance to pirimicarb. Both isolates have since been confirmed pirimicarb resistant through bioassay (Table 1). Conversely, the remaining field isolates in which both bioassay and PCR REA data were available, proved susceptible to pirimicarb by both methods. The results confirm the validity of the PCR REA for detecting pirimicarb resistance.

This work highlights the ability of PCR REA to replace bioassay for the routine resistance monitoring of pirimicarb resistance in cotton aphid. This molecular assay has several other advantages over bioassay, including an ability to obtain a definitive result within a few days. In contrast, the bioassay method requires strains to be first cultured; a process that can take several weeks and is not always successful as was seen for three strains which failed to establish culture preventing bioassay testing (2005/2006 cotton season, Table 1); however, the three strains were able to be tested by PCR and *Ssp*I REA.

As the PCR assay uses both the aphids' *Ace*I alleles as template, it can also detect heterozygous resistant aphids. In such aphids one *Ace*I allele has the S431F mutation (resistant) and the other *Ace*I allele is the wild type susceptible. When the *Ace*I PCR product from a heterozygous resistant aphid is cut with *Ssp*I the profile contains two bands distinguishing it from either a homozygous resistant or homozygous susceptible REA profile which have only a single band differing in size (667 and 336 bp, respectively). As DNA is collected from each aphid (or pool of aphids) as the testing material (and unlike bioassay or biochemical assays, dead aphids can be used as a DNA source) the same sample can be used for a multitude of genomic screening including; additional insecticide resistant mechanism such as sodium channel mutations (kdr and super kdr), GABA receptor polymorphisms (rdl), esterase gene duplications or resistant esterase isoforms and those identified in the future, microsatelite typing and even

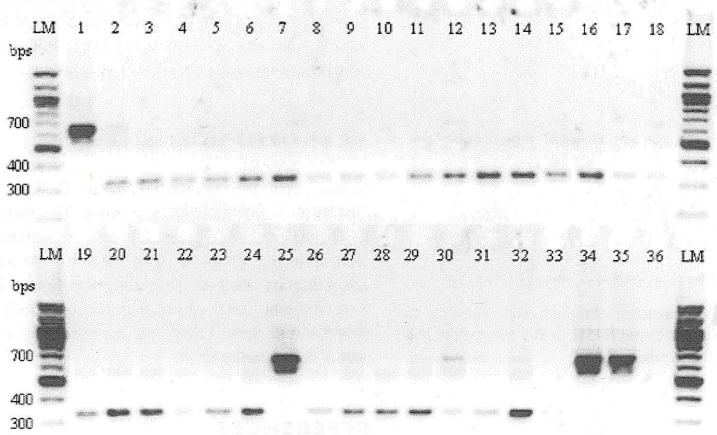

Fig. 4. *Ssp*I REA of *Ace*I real time PCR products from the 33 *Aphis gossypii* field strains collected during the 2005/2006 and 2006/2007 cotton seasons. The two pirimicarb resistant isolates can be seen in lanes 1 and 25. 1, ACRI R6; 2, Sin F 23; 3, Aru F 3; 4, CH; 5, Tell F 33; 6, War M7; 7, Yar; 8, Eden; 9, Oak F 1; 10, Over F 4; 11, Wood; 12, War F2; 13, Bell F15; 14, Mer; 15, Cur F25; 16, Cur F22; 17, Cur F 2–7; 18, My D Cr; 19, Alch C 4–5; 20, Rio G; 21, Har; 22, Cal; 23, Uya; 24, Eden; 25, Wil 21B; 26, Aus Mid 23; 27, Car 34; 28, War 20–22; 29, Alch 007; 30, Car 13; 31, Nor 4; 32, Byr 55; 33, Bur 4; 34, Adam; 35, Togo; 36, MQW; LM, lane marker.

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                280       290       300       310       320       330       340       350
               ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
[1]171B        ALQWVHENIKLFGGNPNNVTLFGESAGAVSVSLHLLSPLSRNLFNQAIMESGSSTAPWAILSREESFSRGLKLAKAMGCP
[1]968E        ..........................S.....................................................
[1]1081K       ..................................................V.....................
[2]GSM-1       ..........................................................................
[2]GSM-2       ..........................................................................
[2]H-16-1      ..........................S.....................................................
[2]H-16-2      ..........................................................................
[3]SusA        ..........................................................................
[3]Adam        ..........................S.....................................................
[3]AW          ..........................................................................
[3]JQ          ..........................................................................
[3]Togo        ..........................S.....................................................

                360       370       380       390       400       410       420       430
               ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
[1]171B        DDRNEIHKTVECLRKVNSSAMVEKEWDHVAICFFPFVPVVDGAFLDDHPQKSLSTNNFKKTNILMGSNSEEGYYSIFYYL
[1]968E        ...............................................................................F.....
[1]1081K       ...............................................................................F.....
[2]GSM-1       ...............................................................................F.....
[2]GSM-2       ...............................................................................F.....
[2]H-16-1      ...............................................................................F.....
[2]H-16-2      ..........................................................................
[3]SusA        ..........................................................................
[3]Adam        ...............................................................................F.....
[3]AW          ..........................................................................
[3]JQ          ...............................................................................F.....
[3]Togo        ...............................................................................F.....
```

Fig. 5. Amino acid sequence alignment of the *Ace*I PCR products the five reference strains and those of Andrews *et al.* (2004) and Toda *et al.* (2004). It shows the S431F (serine to phenylalanine) and A302S (alanine to serine) substitutions at amino acid positions 431 and 302, respectively (171B numbering). [1]Andrews *et al.* (2004). [2]Toda *et al.* (2004). [3]Current study.

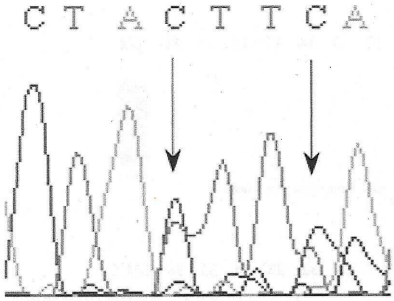

Fig. 6. Togo's DNA sequencing chromatograph showing the heterozygous locus present at the S431F locus (arrows indicate the two polymorphic nucleotides).

viral profiling. Furthermore, the DNA is kept as an archival source which can be screened in the future for new genomic markers as they become available. Finally, the assay can be used to validate bioassay data by testing aphid DNA post bioassay for the presence or absence of resistant acetylcholinesterase genes. This can be performed using either surviving aphids (resistant) or dead aphids (susceptible).

As the molecular assay can provide results within 48 h compared with 4–8 weeks for bioassay such tests could be used before insecticide control. The assay could be used to determine if resistant aphids were present so eliminating the risk of expensive spray failures and environmental comtamination caused by resistance.

Initially allele discriminating real time PCR methodologies were considered as they offer enormous potential to molecular diagnostics, unfortunately the S431F locus is not conducive to most of the current single nucleotide polymorphism (SNP) detection methods. As Figure 1 shows there are two SNPs that can create the S431F mutation and therefore would require at least three labelled DNA primers differing only at the terminal 3′ base (plus one common primer) or three dual labelled probes for Taqman techniques (two for resistant and one for susceptible) both are expensive options. As Togo is heterozygous for the resistant alleles (two different SNPs) and H-16 is heterozygous for susceptible and resistant alleles, detection would require a multiplex assay. Further to this, the %GC immediately 3′ to the *Ssp*I restriction site is only 20% making primer design for multiplex PCR extremely difficult. On the 5′ side of the *Ssp*I restriction site there are additional polymorphisms that vary among the isolates creating primer instability on this side. Such polymorphisms also prevent suitable probe design for Taqman assays designed to detect SNPs. The only methodology that is currently suited to detect the polymorphisms present at, and 5′ to, the S431F locus is high resolution melt (HRM) curve analysis and our method could be refined for HRM by redesigning the primers to produce an amplicon approximately 100 bp that covers the SNPs at the S431F loci but excludes any SNPs that may occur in the adjacent DNA which have no affect on pirimicarb resistance.

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Neonicotinoid resistance in *Aphis gossypii* Glover (Aphididae: Hemiptera) from Australian cotton

Grant A Herron[1]* and Lewis J Wilson[2]

[1]*Industry and Investment New South Wales, Elizabeth Macarthur Agricultural Institute, PMB 4008, Narellan, NSW 2567, Australia.*
[2]*CSIRO, Plant Industry and Cotton Catchment Communities Cooperative Research Centre, Australian Cotton Research Institute, Locked Bag 59, Narrabri, NSW 2390, Australia.*

Abstract Cotton or melon aphid, *Aphis gossypii* Glover, is an important pest of cotton, and recently the neonicotinoid group of insecticides has provided a key option for control where they are used as seed treatments and foliar sprays. Here we document for the first time in Australian cotton, resistance to three neonicotinoids (acetamiprid, clothianidin and thiamethoxam) in *A. gossypii* via diagnostic discriminating concentration assays that were also associated with field control failure. Subsequent full log dose probit analysis on strains with discriminating dose survivors confirmed acetamiprid, clothianidin and thiamethoxam resistance at 6.4-, 10- and 22-fold, respectively. Further laboratory pressuring of strains caused acetamiprid resistance to significantly increase to 22-fold but resistance factors to clothianidin and thiamethoxam were unchanged. Clearly there is a need to reduce overall neonicotinoid selection to prevent or slow any increase in neonicotinoid resistance. An effective method to contain resistance would be to move away from the more persistent neonicotinoid seed dressings to either organophosphate or carbamate-based products and to limit the use of neonicotinoid foliar sprays.

Key words acetamiprid, clothianidin, insecticide resistance, thiamethoxam.

INTRODUCTION

Aphis gossypii Glover (cotton or melon aphid) is an important pest of cotton because of its ability to reduce yield through feeding damage (Godfrey *et al.* 1997) and spread plant diseases such as blue disease in South America, South-East Asia and Africa (Correa *et al.* 2005) and cotton bunchy top disease in Australia (Reddall *et al.* 2004). Additionally, uncontrolled aphids on cotton late in the season cause contamination of the cotton lint with aphid honeydew. This downgrades the lint value as a result of post harvest problems in processing and spinning.

In Australian cotton systems, the emergence of the aphid-vectored disease 'cotton bunchy top' in the 1998–1999 growing season led to a reduction in aphid tolerance levels by producers, resulting in more targeted insecticide applications for aphid control. During subsequent growing seasons resistance levels in *A. gossypii* increased to organophosphate (dimethoate and omethoate) and some carbamate (pirimicarb) insecticides with high level resistance causing control failures in many Australian cotton growing regions (Herron *et al.* 2001).

Until the introduction of *Bt*-cotton (Cry1Ac) to Australia in the mid-1990s and the stacked gene *Bt*-cotton (Cry1Ac and Cry2Ab) in 2002, cotton aphids were considered late season pests. This was because for most of the early part of the season they were suppressed by insecticides used against other pests, predominantly *Helicoverpa* spp. As spraying for other pests declined late in the season and with only low numbers of beneficial insects (as a result of spraying) aphid populations were able to establish and increase quickly, often requiring control. With the introduction of transgenic cotton there were dramatic reductions in insecticide applications against *Helicoverpa* spp. (Naranjo *et al.* 2008). However, early season application of broad-spectrum insecticides such as fipronil or dimethoate to control pests not controlled by Bt-cotton, such as the green mirid (*Creontiades dilutus* Stål), often reduced beneficial populations and allowed aphid populations to increase earlier in the season in a transgenic crop.

Currently, control of aphids relies on insecticides from several modes of action, including carbamates, organophosphates, endosulfan, the neonicotinoids, pymetrozine, diafenthiuron and paraffinic oils (InfoPest 2008). However, the major group used for aphid control in Australian cotton is the neonicotinoids (Farrell 2008). Since their commercial release in 1991 the neonicotinoids have proved relatively resilient to resistance but it has developed in select species and now exceeds 1000-fold in silverleaf whitefly *Bemisia tabaci* type B (Gennadius) (Nauen & Denholm 2005). The neonicotinoid seed treatments have been very popular because of their ease of use, pricing and pest spectrum controlled. This includes thrips (predominantly *Thrips tabaci* Lindeman (Wilson &

*grant.herron@industry.nsw.gov.au

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Bauer 1993)), *A. gossypii*, cotton flea beetles (the redheaded flea beetle, *Nisotra* sp. and the brown flea beetle, *Chaetocnema* sp.) and the larvae of various true and false wireworms (false wire-worm, e.g. *Pterohelaeus darlingensis* Carter, and wire-worm, e.g. *Agrypnus variabilis* (Candèze)) (Farrell 2008). Estimates of market share are very difficult to obtain, but it is likely that through the last 5 to 6 years approximately 60% of planted cotton seed is treated with a neonicotinoid insecticide, and this has increased to about 80% in 2008 (P Steele, Cotton Seed Distributors, pers. comm. 2009). With such common broad-scale use there can be significant selection pressure through the early part of the cotton season i.e. first 4–6 weeks as insecticide concentrations in plant tissue decline to sub-optimal levels. Use of foliar applications of neonicotinoids varies from year to year, targeting either aphids or mirids, but in the period up to 2007–2008 would be far lower in comparison to seed treatment use, probably less than 0.1 sprays per hectare. In 2008–2009 the registration of clothianidin in cotton with competitive pricing and robust efficacy has led to increased foliar neonicotinoid use against aphids and green mirids, probably about 0.5 sprays per hectare.

Loss of the neonicotinoid group for aphid control due to resistance would be a significant setback to the cotton industry because other options have significant limitations. For instance, the cotton resistance management strategy prohibits the use of endosulfan after 15 January, aldicarb and phorate are only used at planting, diafenthiuron is more expensive than foliar neonicotinoid sprays and not suitable for use on young cotton where the canopy has not closed over the rows, the efficacy of organophosphates and pirimicarb can be compromised by resistance, some products are not readily available, e.g. pymetrozine and, finally, others will not control heavy aphid infestations, e.g. paraffinic oils.

Here we present our neonicotinoid resistance monitoring data for the 2006–2007, 2007–2008 and 2008–2009 Australian cotton seasons and discuss some options for future chemical management.

MATERIALS AND METHODS

Sample collection and maintenance

Aphis gossypii were collected by researchers, CRC Regional Extension Officers, consultants and growers from commercial cotton fields and volunteer or ratoon cotton plants in the vicinity of commercial crops. Collections were made in the 2006–2007, 2007–2008 and 2008–2009 cotton seasons, usually in mid-March, at the end of the cotton season. They were sent to the bioassay laboratory at Camden (Elizabeth McArthur Agricultural Institute) and each field strain was cultured separately on pesticide-free cotton (Deltapine 90) at 25 ± 4°C under natural light. A reference laboratory susceptible strain collected from an unsprayed source was maintained under similar insecticide-free conditions and its response to several chemicals has been previously documented (Herron *et al.* 2001). Strain integrity was assured by maintaining populations in purpose-built insect-proof cages.

Chemicals tested

Aphids were treated with proprietary commercial neonicotinoid insecticide formulations of acetamiprid, thiamethoxam or clothianidin (2008–2009 only) (Table 1). The neonicotinoid insecticide group is mode of action group 4A (InfoPest 2008).

Bioassay

Aphids were tested by placing them in a 35 mm Petri dish on an excised cotton plant leaf disc germinated from insecticide-free seed that was fixed in agar (Herron *et al.* 2001). Discriminating dose testing required three batches of approximately 25 adult female aphids per leaf disc to be sprayed with insecticide at the discriminating dose with the aid of a Potter spray tower. Discriminating doses sprayed included 0.002 g/L acetamiprid, 0.02 g/L thiamethoxam and 0.05 g/L clothianidin. Each test included the three sprayed leaf discs, each with 25 aphids, plus a water-only sprayed control leaf disc, again with 25 aphids. The whole process was replicated giving a total of six insecticide sprayed and two control discs in which mortality did not exceed 10%. If control mortality did exceed 10% the replicate was abandoned and the test repeated. After spraying, clear plastic film was used to cover the Petri dishes, which were then maintained at 25 ± 0.1°C in 16:8 L:D for 24 h after which mortality was assessed and control-corrected mortality calculated.

For each of the three chemicals tested, the three populations with the highest proportion of survivors at the discriminating dose were then further studied by spraying serial concentrations (selected to achieve $0 < x < 100\%$; see Figs 1–3) of insecticide using methods outlined above to yield full log dose probit regressions for the purpose of resistance factor calculation. Each full log dose probit regression was replicated two to four times and each replicate included a control that did not exceed 10% mortality.

Table 1 Common name, trade name, formulation and supplier of chemicals tested

Common name	Trade name	Formulation	Supplier
Acetamiprid	Intruder®	225 g/L SL	Dupont (Australia) Ltd
Clothianidin	Shield®	200 g/L SC	Sumitomo Chemical Australia Pty Ltd
Thiamethoxam	Actara®	250 g/kg WG	Syngenta Crop Protection Ltd

SC, suspension concentrate; SL, soluble concentrate; WG, water dispersible granule.

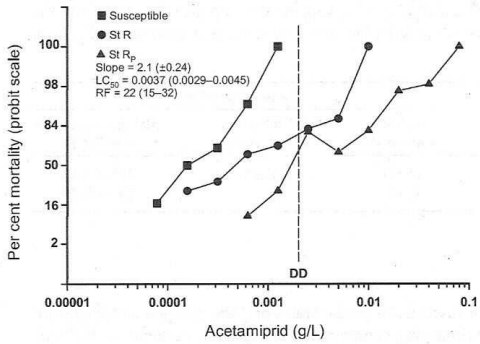

Fig. 1. Dose–response for acetamiprid against *Aphis gossypii* strains susceptible, field-collected (St R) and field-collected following three acetamiprid discriminating concentration sprays (ST R$_P$).

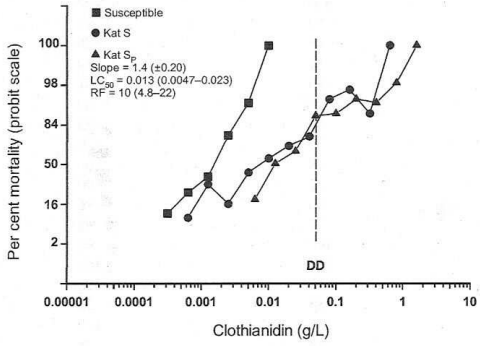

Fig. 2. Dose–response for clothianidin against *Aphis gossypii* strains susceptible, field-collected (Kat S) and field-collected following three clothianidin discriminating concentration sprays (Kat S$_P$).

The strain with the highest proportion of survivors at the discriminating dose was pressured to evaluate if further selection would increase resistance factors. This strain was not necessarily the one with the highest resistance factor. The strain was divided into three subcultures, one for each insecticide. Each subculture was sprayed with its designated insecticide three times (at approximately 3 week intervals) at the discriminating dose. This new '*pressured*' strain, identified with a (P), was then subjected as above to full log dose probit analysis and resistance factors and their 95% confidence interval calculated again relative to the susceptible strain.

Data analysis

All discriminating dose tests were control mortality corrected (Abbott 1925). For dose–response data probit regressions were calculated (Finney 1971) and resistance factors were derived

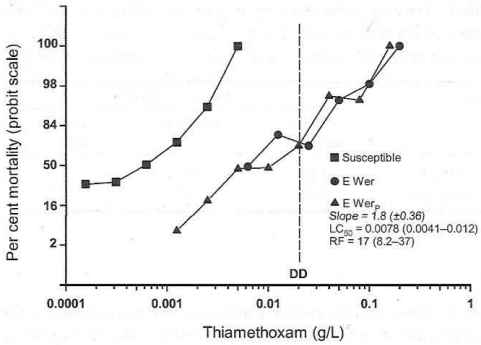

Fig. 3. Dose–response for thiamethoxam against *Aphis gossypii* strains susceptible, field-collected (St R) and field-collected following three thiamethoxam discriminating concentration sprays (ST R$_P$).

by dividing the calculated LC$_{50}$ of the field-collected population by the value of a reference susceptible strain. The 95% confidence interval (Robertson & Preisler 1992) for each resistance factor was then calculated using a Genstat computer software routine (Barchia 2001).

RESULTS

Unexpectedly, there were acetamiprid and thiamethoxam survivors at the discriminating concentration of insecticide in season 2007–2008 with associated maximum resistance of 9.0- and 8.8-fold, respectively (Table 2). There was an increase in the frequency of neonicotinoid resistance from 2007–2008 to 2008–2009 with 2 out of 15 strains (13%) (Table 2) showing resistance in 2007–2008 and 18 out of 22 strains (82%) in 2008–2009 (Table 3). From 2007–2008 to 2008–2009 maximum levels of thiamethoxam resistance increased from 8.8- to 22-fold while acetamiprid resistance remained statistically constant (Table 4). Individual strains were often resistant to all the neonicotinoid insecticides but not always. Resistance was widespread as it was found in strains from all regions surveyed (Table 3). Pressuring the field-collected populations caused acetamiprid (as indicated by non-overlap of the 95% confidence interval) to increase to 22 (16–32) fold (Fig. 1). Unexpectedly, resistance factors of 10 (4.8–22) fold for clothianidin and 17 (8.2–37) fold for thiamethoxam remained statistically constant after pressuring (Figs 2,3).

DISCUSSION

Despite the overall reduction in sprays associated with the stacked gene *Bt*-cotton (Bollgard II®), resistance causing control failure against *A. gossypii* remains an issue. During the 2007–2008 season, we detected for the first time, *A. gossypii*

Table 2 Dose–response summary over two sampling seasons 2006–2007 and 2007–2008 giving number of populations tested (*n*), the number of populations with discriminating concentration (DC) survivors in brackets, DC mortality range and maximum LC_{50} level resistance factor (RF) with its associated 95% confidence interval (CI)

Chemical	Season 2006–2007			Season 2007–2008		
	n (with survivors)	DC range	Maximum LC_{50} RF (95% CI)	*n* (with survivors)	DC range not including 100	Maximum LC_{50} RF (95% CI)
Acetamiprid	9 (0)	100	–	15 (2)	78–96	9.0 (6.4–13)
Thiamethoxam	9 (0)	100	–	15 (1)	79	8.8 (4.2–18)

Table 3 Strain specific discriminating concentration results (i.e. per cent susceptible) for 24 strains of *Aphis gossypii* collected from Australian cotton during the 2008–2009 season and tested against a discriminating concentration of 0.002 g/L acetamiprid, 0.02 g/L thiamethoxam and 0.05 g/L clothianidin

Strain	Location	Acetamiprid (%)	Thiamethoxam (%)	Clothianidin (%)
The Ovr 08	Darling Downs	100	100	100
Ros R2	Darling Downs	100	80	93
Brook	St George	100	100	100
War F1	Namoi Valley	100	100	100
W Lag V	Namoi Valley	76	86	95
Elra	Darling Downs	82	44	99
Bin	Gwydir Valley	77	57	89
The My	Namoi Valley	100	100	91
Kat Vol	St George	74	70	80
St R	Darling Downs	49	42	98
Kat S	St George	76	73	70
The Ovr 09	Darling Downs	100	98	100
Cav	Darling Downs	100	72	90
Ros R9	Darling Downs	100	66	86
Tull	Namoi Valley	92	71	98
Wam	Darling Downs	98	100	91
War Vol	Namoi Valley	100	100	100
Bal	St George	92	100	100
Nar	Darling Downs	91	80	50
Went F6	Namoi Valley	97	100	100
W Lag F5	Namoi Valley	98	98	95
Yar	Namoi Valley	99	100	98
Fair	Darling Downs	87	100	100
EWer	Darling Downs	94	36	80

Table 4 Full log dose probit regression summary for the three strains of *Aphis gossypii* with the highest proportion of discriminating concentration survivors for each neonicotinoid chemical tested during cotton season 2008–2009

Chemical	Strain	Slope (±SE)	LC_{50} (95% FL) (g/L)	RF (95% CI)
Acetamiprid	Susceptible	2.8 (±0.26)	0.00017 (0.00012–0.00021)	–
	Kat Vol	2.0 (±0.37)	0.0011 (0.00062–0.0017)	6.4 (3.8–10.6)
	St R	1.4 (±0.25)	0.00051 (0.00023–0.00084)	3.1 (1.7–5.6)
	Kat S	1.6 (±0.21)	0.00020 (0.00011–0.00030)	1.2 (0.7–2.1)
Thiamethoxam	Susceptible	1.7 (±0.42)	0.00045 (0.00014–0.00079)	–
	Elra	1.3 (±0.20)	0.010 (0.0043–0.017)	22 (9.5–53)
	St R	1.2 (±0.10)	0.0077 (0.0048–0.011)	17 (8.3–36)
	EWer	0.9 (±0.26)	0.0017 (0.000017–0.0067)	3.9 (0.6–24)
Clothianidin	Susceptible	2.3 (±0.44)	0.0012 (0.00078–0.0018)	–
	Kat S	1.1 (±0.17)	0.0074 (0.0032–0.012)	5.9 (2.9–12)
	Nar	0.8 (±0.27)	0.013 (0.00027–0.054)	10 (1.8–56)
	EWer	0.9 (±0.15)	0.0024 (0.0011–0.0043)	1.9 (1.0–4.0)

CI, confidence interval; FL, fiducial limit; RF, resistance factor: LC_{50} of field strain/LC_{50} of susceptible; SE, standard error.

surviving the discriminating dose of acetamiprid and thiamethoxam giving a *prima facie* detection of resistance. Although imidacloprid is registered for use in Australian cotton we did not test it because experience suggested that reliable data could not be obtained with a 24 h bioassay holding period and control mortality became unacceptably high if the holding period was extended. During the following season (2008–2009) 20% of aphids in strain E Wer survived a discriminating concentration of clothianidin that was associated with a control failure. Some strains were resistant to all neonicotinoid products tested so we consider it prudent to assume cross-resistance for the purpose of resistance management including imidacloprid. Cross-resistance has been established between several group 4A mode of action insecticides in the study of Wang *et al.* (2007) who demonstrated a relationship between imidacloprid and acetamiprid resistance in cotton aphid and that of Alyokhin *et al.* (2007) that linked imidacloprid resistance to thiamethoxam resistance in Colorado potato beetle. It seems reasonable then to conclude that the high use of neonicotinoid insecticide seed dressings in Australian cotton (about 80% of the planting seed was treated in 2008, P Steele, Cotton Seed Distributors, pers. comm. 2009) may have contributed substantially to selection for the widespread neonicotinoid resistance detected in this study. Consequently, Australian populations of cotton aphid should also be considered a single cross-resistance group within the 4A group for the purpose of resistance management as suggested by Nauen and Denholm (2005).

Neonicotinoid resistance causing control failure was noted in a single strain (E Wer) against clothianidin despite a low 1.9-fold LC_{50} level resistance factor to the chemical. Such levels of resistance are much lower than those found for other species including the 1000-fold neonicotinoid resistance detected against *B. tabaci* type B from Israel (Nauen & Denholm 2005). The low LC50 level resistance causing control failure in this present study implies the strain was very heterogeneous for resistance against clothianidin and indeed $LC_{99.9}$ level resistance was 294-fold. LC_{50} resistance levels should significantly increase with further field selection. However, when field strains were pressured with discriminating concentration sprays of insecticide in the laboratory, only acetamiprid resistance increased significantly (see Figs 1–3). Thany (2010) noted that neonicotinoid resistance could be caused by multiple mechanisms so Australian *A. gossypii* may not have the same mechanism that caused the very high resistance levels seen in silverleaf whitefly *B. tabaci* type B or the Colorado potato beetle *Leptinotarsa decemlineata* (Say). This gives some hope to the possibility that resistance levels against the other compounds may not increase significantly with additional field selection. Alternatively, neonicotinoid resistance in *A. gossypii* may be relatively unstable making laboratory selection difficult. There is anecdotal evidence for this; when we pressured the *A. gossypii* strains the discriminating dose often caused higher initial mortality than expected, suggesting some loss of resistance.

The development and apparent increase in neonicotinoid resistance in recent years indicate the need to reduce overall neonicotinoid selection pressure and to use alternative insecticide groups to slow further resistance increase. Resistance to neonicotinoids could compromise aphid management late and early season, when control is believed to reduce the risk of cotton bunchy top disease (though this is unproven). Options to decrease selection would be: (1) the use of non-neonicotinoid seed treatments; (2) the use of alternative mode of action groups against aphids when foliar sprays are used; or (3) managing the use of neonicotinoid insecticides on cotton crops where a neonicotinoid seed treatment was used. Given the value of the neonicotinoids for management of other pests, such as green mirids, excluding foliar use entirely is not a practical option. Further, another insecticide, spirotetramat, from a different mode of action group (23) was registered in 2010 for control of *A. gossypii* which provides another alternative. We recommend a compromise: limit in-season use of foliar neonicotinoid treatments if they have been used as a pre-germination seed treatment on the same crop. This will have implications for control of other pests such as *B. tabaci* type B and mirids. If foliar sprays are required for aphid control and neonicotinoid seed treatments have been used it is most important that the first foliar spray, targeting aphids, is from a different chemical group.

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