Australian Government
Cotton Research and Development Corporation

FINAL REPORT

"Resistance management of aphids, mites and mirids in cotton"

DAN 184

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NSW DEPARTMENT OF PRIMARY INDUSTRIES

Contents

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Part 1 - Summary Details

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Part 3 – Final Report

(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, sucking insect pests have become more troublesome, requiring increased targeted insecticide control. Two-spotted mite has a proven ability to develop resistance when targeted and has recently developed resistance to chlorfenapyr (Intrepid®) (Herron *et al*. 2004a). Of late, high-level organophosphate and carbamate resistance has developed in cotton aphid (Herron *et al*. 2001). Other sporadic but troublesome sucking pests include green peach aphid *Myzus persicae* (Sulzer), bean spider mite *Tetranychus ludeni* Zacher, thrips (including western flower thrips) and green mirids (Forrester and Wilson 1988). 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 suppressed mirid populations. There has been an increase in spray formulations specifically targeting mirids. Currently there is a high reliance on dimethoate and fipronil for mirid control, inevitably 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.

Mirids
Pre-emptive baseline data proved critical to the successful management of cotton aphid because resistance could quickly be confirmed. However, no baseline data for mirids currently exists, preventing an early confirmation of resistance and subsequent resistance management. This is now a serious concern because mirids required considerable targeted control during the 2003 -2004 cotton season to an extent that the control strategy for aphids was adversely effected (Herron *et al.* 2004). This was because the use of OP's against mirids (especially dimethoate) also selects for resistance to this group of insecticides in aphids. The sustainable chemical control of mirids would be greatly enhanced by the pre-emptive generation of baseline data for resistance monitoring.

Cotton aphid
The pest status of aphids is often related to the contamination of the cotton lint with sugary 'honey-dew'. However, earlier outbreaks can significantly reduce yield and recently cotton aphid was confirmed as a vector for 'Cotton Bunchy Top' disease. These changes in the system mean that the need for effective tools to control aphids and resistance management for those tools is critical to the cotton industry.

Cotton aphid reproduces asexually causing very rapid changes in resistance levels. Management of aphids is further complicated because there is no dilution of resistance by outcrossing to susceptibles, as is used to manage *Bt* resistance in *Helicoverpa* spp. Therefore, aphids can very quickly become a major problem when chemical control fails due to resistance (Herron 2001).

Effective management of cotton aphid will be best achieved by pursuing an integrated approach, including monitoring, cross-resistance studies, resistance mechanism elucidation and evaluation of new chemistry and its effect on beneficial insects. Without this study Australia's reputation as a producer of clean cotton could be dramatically affected.

Two-spotted mite
Two-spotted mite is notorious world-wide for developing resistance, with Australian researchers publishing many first citations (Herron *et al*. 1993, Herron and Rophail 1998).

As each new compound has become available we have pro-actively established baseline resistance levels and cross-resistance profiles and initiated routine resistance monitoring. Unfortunately, resistance continues to evolve as seen most recently to bifenthrin (Herron *et al.* 2001a) and then

chlorfenapyr (Herron *et al*. 2004a). This is occurring largely due to use of these compounds against other pests, rather than mites themselves, and is disturbing, as it reduces the number of chemicals available for two-spotted mite control.

Management of mites is complicated because most chemicals applied against them are also used against other pests such as aphids or *Helicoverpa* spp. and this has contributed to resistance development to organophosphates, bifenthrin and chlorfenapyr in mite populations. Effective management of two-spotted mite will be best achieved by pursuing an integrated approach that includes resistance management, based on a sound understanding of their resistance and cross-resistance spectra. Continued resistance monitoring, and the timely inclusion of new chemistry, is essential for effective ongoing management of this pest species.

Western flower thrips

This new thrips pest of Australian cotton has to date only required very limited targeted control. Consequently, it does not require specific inclusion into this current study at this time. However, recent horticulture studies, where western flower thrips are a serious pest, have shown they are not controllable with aldicarb and resistance to newer products such as spinosad and fipronil is likely (Herron and James 2005).

Molecular diagnosis of resistance

The development of resistance in aphids and mites threatens the sustainability of their control. Resistance monitoring underpins their effective resistance management with those results used to refine their resistance management strategies. Resistance is currently monitored using bioassay with an established discriminating dose technique. The bioassay discriminating dose method is very robust as it can highlight resistance before it has established. However, bioassay is very labour intensive and in comparison to biochemical or molecular methods is also slow and laborious. Once resistance is established molecular methods particularly have significant advantages in speed to diagnosis with potential for 'real time' resistance detection. Therefore the need to develop a molecular capability for detecting common established resistances in cotton aphid and two-spotted mite is compelling.

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Objectives

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

The project aims to achieve sustainable Australian cotton production through ongoing and effective resistance management of cotton aphid, two-spotted mite and mirids. This will be achieved by:

Aphids and mites

- Monitoring resistance levels in mites and aphids to current products used for their control.

  Aim achieved: populations of mites and aphids were collected annually and screened for resistance against a standard suite of chemicals used for their control (also see Appendix 1-3)

- Establishing baseline resistance levels in cotton aphid and two-spotted mite to develop reliable discriminating doses for new acaricides and aphicides.

  Aim achieved: Baseline data generated was used to confirm acetamiprid resistance in cotton aphid and additional baseline data generated against thiacloprid used to produce a more reliable discriminating dose

- Defining efficacy-resistance profiles for experimental chemicals that may be useful to industry

  Aim not achieved: With the introduction of Bollgard® II cotton fewer insecticides are being made available by industry.

- Application of this knowledge to develop and or refine resistance management strategies for mites and aphids in cotton

  Aim achieved: Outputs from the resistance monitoring are relayed at the annual TIMS technical meeting to feed directly into the new season's resistance management strategy

Mirids

- To culture an Australian population of mirids.

  Aim achieved: After several attempts mirids were collected and maintained continuously in culture without supplemental top up from field collections (also see Appendix 4)

- To investigate mirid bioassay

  Aim achieved: A bioassay using a Potter spray tower was successfully completed with control mortality at acceptable levels (also see Appendix 5)

Supplement to DAN 184 "Molecular methods to detect resistance in cotton aphid and TSM"

The supplement aims to develop a molecular capability for detecting common established resistances in cotton aphid and two-spotted mite.

- Capability to detect carbamate resistance in cotton aphid

  Aim achieved: An established method was successfully used and refined to eliminate an additional nested PCR step with more precise primers additionally developed (also see Appendix 1)

- Capability to detect cyclodiene resistance in cotton aphid

  Aim not achieved: Cyclodiene resistant aphids were not found during the course of the study

- Capability to detect organophosphate resistance in cotton aphid and two-spotted mite

Aim achieved: An established method was successfully used and refined (see above)

- Capability to detect pyrethroid resistance in two-spotted mite

Aim not achieved: Very few two-spotted strains were collected significantly diminishing the requirement for molecular methods development

Methods

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

Chemicals tested

Mites and aphids were treated with proprietary commercial insecticide formulations. For aphids these included chlorpyrifos (Lorsban®), acetamiprid (Intruder®), thiacloprid (Calypso®), endosulfan (Thiodan®), thiamethoxam (Cruiser®) and pirimicarb (Pirimor®) 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 acetamiprid (intruder®), thiacloprid (Calypso®) and thiamethoxam (Cruiser®) are all from the same neonicotinoid group. Mite treatments were, bifenthrin (Talstar®), abamectin (Agrimec®), propargite (Comite®), chlorfenapyr (Intrepid®) and diafenthiuron (Pegasus® as CGA140408). With the introduction to Australia of Bollgard II® cotton the use of insecticides to control pests has dramatically reduced. For this reason the organophosphate profenofos (e.g. Curacron®) is no longer available in Australia and is no longer included in our resistance monitoring.

Cotton aphid

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) and each field strain cultured separately on pesticide-free cotton (Deltapine 90) at 25 ± 4 ^{o}C under natural light. Strain integrity is assured by maintaining populations in purpose built insect proof cages. A small subpopulation of each field strain was collected for use in the molecular assays. The subpopulations were taken from the original field submissions, and as such, the results are indicative of farm level resistance.

Aphid Bioassay. Aphids 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 female aphids per leaf disc were then sprayed with the aid of a Potter spray tower. Each test was replicated 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 after which mortality was assessed.

Aphid Molecular Assay. Pirimicarb and organophosphate resistance were detected using methods developed during the study and previously published (M^{c}Loon and Herron 2006). Briefly, DNA is isolated from a pool of 20 aphids in addition to 10 individual aphids from each of the different

field strains. Both the pool of DNA (from the 20 aphids) plus the 10 individual aphid DNA extractions were subject to PCR amplification of the *Ace*I gene (covering the mutation responsible for resistance) using real time 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 chlopyrifos) 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 were collected from a range of cotton fields in NSW and Qld late in each cotton season 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 insecticide with the aid of a Potter spray tower as above. Each test was replicated and included a water only sprayed control. 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

Mirids were collected from lucerne at EMAI on the 13/09/07. From this collection 9 adult mirids were stunned briefly (about 20 seconds) with carbon dioxide gas, to make handling easy and added to the cage. The cage was placed in a growth cabinet to maintain constant conditions of 27^{o}C and 10L:14D. After 7 days and subsequently twice weekly adult mirids were removed from the bean pods and the old pods plus agar transferred to a new cage to which additional fresh pods were added. The original cage(s) with adult mirids then had fresh bean pods in agar were added thus repeating the process. The process was repeated with old pods being removed from the adult mirids to new cages with additional food. These were left until adult mirids developed that could be used to sustain the culturing process or be used for testing.

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Results

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

Season 2005-2006

In season 2005-2006 Agrimec®, Comite® and Pegasus® resistance was not detected (Table 1). Intrepid® and Talstar® resistance was detected in 4 and 3 of the 6 TSM strains tested respectively. Curacron resistance frequencies were very high in all six strains tested with strain KI being nearly all resistant.

Aphids were collected more widely than mites with samples from the Namoi and Upper Namoi, the Gwydir, the Darling Downs, the MacIntyre, Mungindi and St George (Table 2) No resistance was detected against Intruder®, Actara®, or Pegasus®. Pirimor®, Rescue® Curacron® and Thiodan® resistance was detected at very low frequencies except in strain ACRI R1 that had a high frequency of Pirimor® and Rescue® resistance. Bioassay and molecular tests were similar in their strain specific characterisation of Pirimor® and organophosphate resistance (Table 2).

Table 1. Percent mortality at the discriminating dose (ie percent susceptible) for various strains of TSM collected during season 2005-2006 and evaluated for resistance against Talstar®, Intrepid®, Agrimec®, Comite®, Pegasus® (CGA-140408) and Curacron®

Strain	Area	Chemical					
		Talstar®	Intrepid®	Agrimec®	Comite®	Pegasus	Curacron®
AN	St George	100	100	100	100	100	2
KI	St George	100	99	100	100	100	1
BE	U Namoi	53	93	100	100	100	4
CU	U Namoi	92	97	100	100	100	14
M	U Namoi	98	98	100	100	100	4
CA	Not recorded	100	100	100	100	100	6

Results for Curacron® against two-spotted mite show quite a contrast to the previous seasons result. In 2004-2005 two strains showed percent mortality at the discriminating dose of 43 and 79% (Herron 2005). However, in the 2005-2006 seasons, only one strain had more than 10% survivors at the discriminating dose. The result indicates that Curacron® resistance continues to increase in level and the resistant genotype may eventually become fixed in the population.

Results for Talstar® against two-spotted mite were more encouraging than Curacron® with frequencies decreasing from the previous 2004-2005 season. Previously resistance was detected in four out of the seven strains tested with discriminating dose values less than or equal to 50% (Herron 2005). In the 2005-2006 results the most resistant strain showed 53% mortality at the discriminating dose and the remaining two being virtually susceptible. It is unknown if the current Talstar® result is a one off aberration or the start of a return to viability for the compound.

Against Intrepid®, two-spotted mite survivors were detected for the first time during season 2001-2002 (Herron 2003). Season 2002-2003 produced an alarming trend of increasing level and abundance of Intrepid® resistance (Herron 2005). In response the mite management strategy for Intrepid® was modified from season 2003-2004 with a reduction in total Intrepid® sprays to one per season for either *Helicoverpa* spp or two-spotted mite. Unfortunately, during season 2004-2005 resistance was detected in 3 out of 7 strains tested but encouragingly resistance frequencies in each strain were generally less than in seasons 2003-2004 (Herron 2005). For season 2005-2006 Intrepid® was not available for use in Australian cotton yet resistance continued to be detected but at frequencies less than season 2004-2005.

Table 2. Pirimor® and Organophosphate (OP) susceptibility using molecular diagnosis and percent mortality at the discriminating dose (ie percent susceptible) using bioassay for various strains of cotton aphid collected during season 2005-2006

Strain	Area	Molecular		Bioassay						
		*Ssp*I (Pirimor®)	*Nae*I (OP)	Pirimor®	Rescue®	Curacron®	Thiodan®	Intruder®	Pegasus® (CGA140408)	Actara®
ACRI R6 1	Namoi	R	R	8.1	54*	Na	Na	Na	Na	Na
CH	Gwydir	S	S	100	100	99	100	100	100	100
Tell F 33	Gwydir	S	S	100	100	100	100	100	100	100
Wood	Gwydir	S	S	100	100	100	100	100	100	100
Yar	Downs	S	S	did not establish into culture						
Oak F 1	Downs	S	S	100	100	100	100	100	100	100
Aru F 3	Downs	S	S	100	100	100	100	100	100	100
War M7	Downs	S	S	100	100	100	100	100	100	100
Over F 4	Downs	S	S	100	100	100	100	100	100	100
Sin F 23	Downs	S	S	did not establish into culture						
Eden	Downs	S	S	100	100	100	100	100	100	100
War F 2	U Namoi	S	S	100	100	100	100	100	100	100
Bell F 15	U Namoi	S	S	100	100	100	100	100	100	100
Mer	U Namoi	S	S	99	100	100	100	100	100	100
Cur F 25	U Namoi	S	S	100	100	100	100	100	100	100
Cur F 22	U Namoi	S	S	100	100	100	100	100	100	100
Car F 2-7	MacIntyre	S	S	100	100	100	99	100	100	100
My D Cr	MacIntyre	S	S	100	100	100	100	100	100	100
Alch C4-5	MacIntyre	S	S	100	100	97	100	100	100	100
Rio G	Mungindi	S	S	100	100	100	100	100	100	100
Har	St George	S	S	100	100	100	100	100	100	100
Cal	Namoi	S	S	100	100	100	100	100	100	100
Uya	Namoi	S	S	100	100	100	100	100	100	100
Mill 1	Namoi	S	S	did not establish into culture						

Na = Not available
* = Not replicated
S = Susceptible
R = Resistant

The results form the resistance testing for cotton aphid were particularly encouraging for season 2005-2006 with Pirimor® resistance only detected at high frequencies in a single strain. In comparison, in the previous 2004-2005 cotton season Pirimor® resistance was detected in 61% of cotton aphid populations (Herron 2005). As Pirimor® is known to cause cross resistance to Folimat® / Rogor® those products would also now be susceptible to control failure because of the Pirimor® resistance. It would be encouraging to consider that the loss of Pirimor® resistance was in part due to the changes made to the management strategy in 2003-2004 when Pirimor® and Folimat® use was restricted by the implementation of chemical use windows. However, major contributing factors are undoubtedly the worsening drought and the subsequent lack of over-wintering aphid harbourages. None-the-less the practical outcome is growers now have a resistance reprieve with cotton aphid but they need to continue to use their chemical options wisely or resistance will certainly return.

For the first time resistance in cotton aphid was diagnosed with both molecular and conventional bioassay methods. There was general agreement between both methods and molecular tests were therefore included as part of the routine resistance monitoring, especially for Pirimor®. Both bioassay and molecular methods detected Pirimor® resistance in strain ACRI R6 yet only bioassay detected 1% Pirimor® resistance in strain MER. This is not a failure of the molecular diagnosis but rather a lack of sample size processed for the molecular assay. Simply more insects were bioassayed so there was more chance of detecting a very low frequency of resistant aphids. The same was true for detecting resistance to the two organophosphate insecticides Rescue® and Curacron® that also showed a very low frequency of resistant aphids in isolated strains.

These results raise the question of what is an appropriate sample size for the molecular diagnostic techniques. Discussion with a biometrition has revealed the need for a short series of experiments using known reference strains before a definitive sample size can be determined. These experiments are currently underway.

Having a validated molecular method for determining Pirimor® resistance will enhance the ability of the entomology unit to detect resistance in the field since the technique doesn't require culturing of the samples. Therefore strains that fail to establish as a cage culture (which is necessary for bioassay) are still able to give a result, as was the case for strains Yar, Sin F23 and Mill 1 (Table 2).

Season 2006-2007

Two-spotted mite was collected from the Gwydir and M^{c}Intyre valleys and resistance detected against Talstar®, Intrepid® and Comite® (Table 3). Cotton aphid was collected more widely than two-spotted mite with samples collected from the Gwydir, M^{c}Intyre and Macquarie Valleys with Pirimor® resistance restricted to the Macquarie Valley (Table 2). Molecular testing was used in conjunction with bioassay to detect resistance in cotton aphid with both methods yielding equivalent results (Table 4)

Table 3. Percent mortality at the discriminating dose (ie percent susceptible) for various strains of TSM collected during season 2006-2007 and evaluated for resistance against Talstar®, Intrepid®, Agrimec®, Comite® and Pegasus® (CGA-140408)

Strain	Area	Chemical				
		Talstar®	Intrepid®	Agrimec®	Comite®	Pegasus® CGA140408
AU	Gwydir	99	100	100	97	100
NO	Gwydir	45	94	100	100	100
W	M^{c}Intyre	100	98	100	100	100

Table 4. Pirimor® and Organophosphate (OP) susceptibility using molecular diagnosis and percent mortality at the discriminating dose (ie percent susceptible) using bioassay for various strains of cotton aphid collected during season 2006-2007

Strain	Area	Molecular		Bioassay					
		SspI (Pirimor®)	NaeI (OP)	Pirimor®	Rescue®	Thiodan®	Intruder®	Pegasus® CGA140408	Calypso®
Aus Mid 23	Gwydir	S	S	100	100	100	100	100	100
Car 34	McIntyre	S	S	100	100	100	100	100	100
War 20-22	McIntyre	S	S	100	100	100	100	100	100
Alch 007	McIntyre	S	S	100	100	100	100	100	100
Car 13	McIntyre	S	S	100	100	100	100	100	100
Nor 4	Gwydir	S	S	100	100	100	100	100	100
Byr 55	Macquarie	S	S	100	100	100	100	100	100
Bur 4	Macquarie	S	S	100	100	100	100	100	100
Wil 21B	Macquarie	R & S – see discussion	R & S – see discussion	6 & 100 – see discussion	100	100	100	100	100

Na = Not available
* = Not replicated
S = Susceptible
R = Resistant

With the introduction to Australia of Bollgard II® cotton the use of chemical sprays to control pests has dramatically reduced. For this reason the organophosphate Curacron® (profenofos) is no longer available in Australia and was deleted from the 06/07 resistance monitoring. However, the loss of Curacron® monitoring has been offset by the inclusion of Calypso® that was evaluated for the first time during 06/07.

Despite the overall reduction in sprays associated with Bollgard II®, resistance causing control failure was still an issue in the Macquarie Valley. One strain, namely Wil 21B, was shown to be highly Pirimor® resistant with associated resultant control failure. The strain was confirmed both Pirimor® and Folimat® resistant via molecular testing and Pirimor® resistant via bioassay. However, when strain Wil 21B was retested some four months later the resistance had completely disappeared and was not detected with either bioassay or molecular methods. Reversion of pirimicarb resistance is unlikely however since the mutation giving rise to it is particularly stable. It is more likely a case of mixed aphid cultures present at the collection site or an overall change in the clonal dominance within the strain. The first bioassay result identified 6% of pirimicarb susceptible individuals (either intra or inter strain variants). It is this pool of aphids that has given rise to the next dominant clone, which when tested four months later (in the absence of selection) was pirimicarb susceptible.

Comite®, Intrepid® and Talstar® resistance were again detected in two-spotted mite for season 06/07. None the less the result is encouraging with only a single Talstar® result having a discriminating dose mortality of less then 50%.

For the second time resistance in cotton aphid has been diagnosed with both molecular and conventional bioassay methods. There is good agreement between the methods and molecular tests will soon be included as part of the routine resistance monitoring.

Season 2007-2008

Two-spotted mite was collected from Macquarie and Namoi Valleys during 2007-2008 (Table 5). Resistance was detected against propargite (Comite®), abamectin (Agrimec®) and bifenthrin (Talstar®) with high frequency resistance restricted to bifenthrin (Talstar®) only (Table 1).

Cotton aphid strains were collected more widely than TSM and in season, 2007-2008 pirimicarb (Pirimor®) resistance was restricted to the Gwydir (strain Bin WF) and St George (strain Rvlnd Mo) only (Table 6). Unexpectedly, survivors at the discriminating dose were also detected in strain Bin WF against acetamiprid (Intruder®), thiamethoxam (Cruiser®) and thiacloprid (Calypso®). Thiamethoxam (Cruiser®) survivors were also seen in strain Blan F3. Survivors at the discriminating dose were detected against thiacloprid (Calypso®) in strains Ovr, St G F 134 and Bin WF. Any discriminating dose survivors suggest *Prima Facie* resistance to the insecticide. Cross-resistance (neonicotinoids) and multiple resistance (carbamate and neonicotinoids) in strain Bin WF is suspected since resistance to pirimicarb (Pirimor®), thiamethoxam (Cruiser®), thiacloprid (Calypso®) and acetamiprid (Intruder®) was evident. Subsequent full log dose probit analysis with the survivors of the Bin WF discriminating dose test confirmed acetamiprid (Intruder®) resistance at 9.1 fold at the LC_{50} level (Figure 1).

The molecular testing for pirimicarb and general organophosphate (OP) resistance identified two strains with a pirimicarb resistant profile (Table 6). One strain, Rvlnd Mo, had a definitive pirimicarb resistance profile. The other strain, Bin WF, had a mixed profile indicative of a strain with low levels of pirimicarb resistance (approximately 2.5%).

Table 5. Percent mortality at the discriminating dose (ie percent susceptible) for various strains of TSM collected during season 2007-2008 and evaluated for resistance against bifenthrin (Talstar®), chlorfenapyr (Intrepid®), abamectin (Agrimec®), propargite (Comite®) and diafenthiuron (Pegasus® (CGA-140408))

Strain	Area	Chemical				
		Bifenthrin (Talstar®)	Chlorfenapyr (Intrepid®)	Abamectin (Agrimec®)	Propargite (Comite®)	Diafenthiuron (Pegasus® CGA140408)
WA	Macquarie	46	100	94	99	100
WI	Namoi	40	100	100	100	100

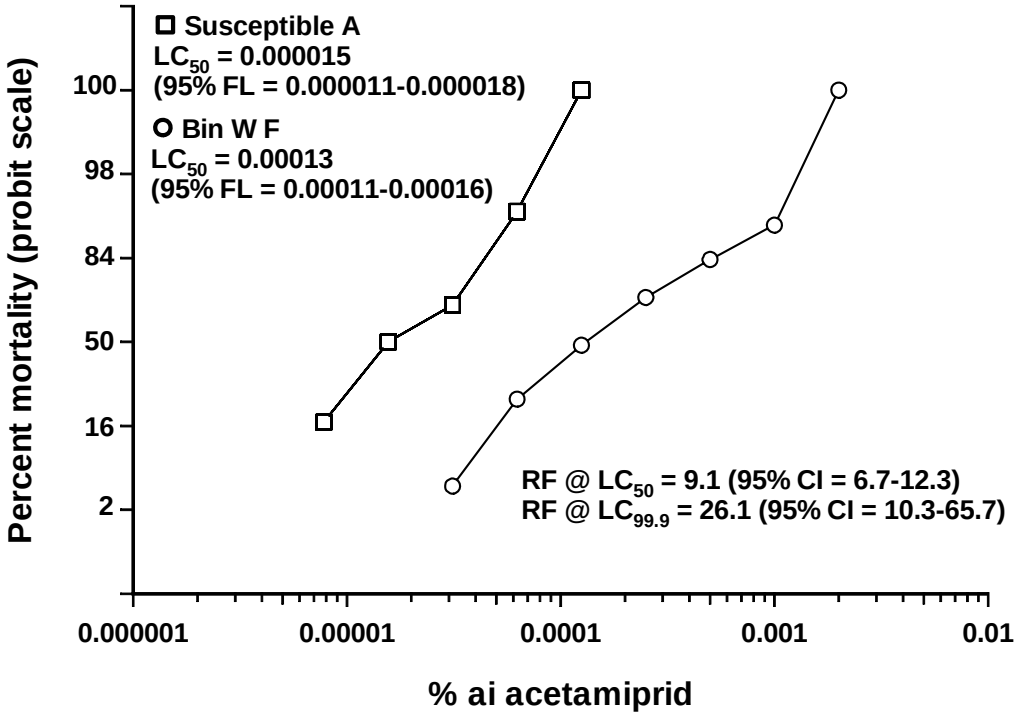

Figure 1. Dose response for Susceptible A (baseline) and field strain Bin WF against acetamiprid (Intruder®)

Despite the overall reduction in sprays associated with Bollgard II®, resistance causing aphid control failure remains an issue with cotton aphid. The 2007/2008 season produced a single highly pirimicarb resistant strain from St George and a single strain from the Gwydir showing a low level of resistance. Alarmingly, a Gwydir strain was also shown, via bioassay, to have acetamiprid (Intruder®), thiamethoxam (Cruiser®) and thiacloprid (Calypso®) survivors. Discriminating dose survivors indicate a *Prima Facie* detection of resistance.

Several strains showed thiacloprid (Calypso®) survivors but it is unlikely that those strains are all resistant. The discriminating dose was previously shown to be too low and false positive results were likely. In fact, additional baseline data generated during season 2006-2007 indicated the minimum effective dose required to kill a single tolerant strain (Car 13) was equivalent to the discriminating dose. For that reason the discriminating dose for thiacloprid (Calypso®) was increased for season 2007-2008 to 0.05 g / L to avoid false positive results. It is plausible, even likely, that the thiacloprid

Table 6. Pirimicarb and Organophosphate (OP) susceptibility using molecular and bioassay diagnosis plus bioassay determination of endosulfan (Thiodan®), acetamiprid (Intruder®), diafenthiuron (Pegasus® (CGA140408)), thiacloprid (Calypso®) and thiamethoxam (Cruiser®) via percent mortality at the discriminating dose (ie percent susceptible) for various strains of cotton aphid collected during season 2007-2008

Strain	Area	Molecular		Bioassay						
		*Ssp*I (Pirimicarb)	*Pdi*I (OP)	Pirimor®	Lorsban®	Thiodan®	Intruder®	Pegasus® CGA140408	Calypso®	Cruiser®
Bel P	St George	S	S	-	-	100	100	100	100	100
Glen vol	Upper Namoi	S	S	-	-	100	100	NA	100	100
War vol	Upper Namoi	S	S	100*	100*	100	100	NA	100	100
Gos vol	Darling Down	S	S	-	-	100	100	100	100	100
Ovr	St George	S	S	-	-	100	100	100	91	100
St G F 134	St George	S	S	-	-	100	100	100	98	100
Red vol	Gwydir	S	S	-	-	100	100	100	100	100
Wil F5 vol	Lower Namoi	S	S	-	-	100	100	100	100	100
Ros F3 vol	DarlingDown	S	S	-	-	100	100	100	100	100
Bin W F	Gwydir	S[#]	S	98.5	-	100	78	100	92	79
Blan F3	St George	S	S	-	-	100	96	100	100	100
Ash vol	St George	S	S	-	-	100	100	100	100	100
M rocks	St George	S	S	-	-	100	100	NA	100	100
Oak C vol	Darling Down	S	S	-	-	100	100	100	100	100
BrkGlenF3	St George	S	S	-	-	100	100	100	NA	100
Plan Fa F3	St George	S	S	100	-	100	100	100	100	100

Strain	Area	Molecular		Bioassay						
		*Ssp*I (Pirimicarb)	*Pdi*I (OP)	Pirimor®	Lorsban®	Thiodan®	Intruder®	Pegasus® CGA140408	Calypso®	Cruiser®
Rvlnd Mo	St George	R	R	6.7	30	100	100	100	100	100
Brk F133-1	St George	S	S	-	-	100	100	100	100	100

* = Not replicated
NA = Not available
S = Susceptible
R = Resistant
# = Low level of resistance (<5%)
- = Not tested unless molecular assay detects resistance (War vol and Plan Fa F3 tested as negative controls)

(Calypso®) discriminating dose is still too low and survivors seen in strains Ovr, St G F 13 and Bin WF are a result of vigour tolerance. For this reason, the discriminating dose for thiacloprid (Calypso®) in season 2008-2009 will be increased to 0.10 g / L

For the first time in Australia cotton aphids have survived a discriminating dose of acetamiprid (Intruder®) and thiamethoxam (Cruiser®) giving putative *Prima Facie* detection of neonicotinoid resistance. To confirm acetamiprid (Intruder®) resistance discriminating dose survivors were used to create a new strain that was subsequently subjected to full log-dose probit analysis. This yielded aphids with a 9.1 fold resistance at the LC_{50} level confirming acetamiprid (Intruder®) resistance in strain Bin WF (Figure 1) as well as providing a new laboratory reference strain (kept under acetamiprid (Intruder®) pressure) for subsequent monitoring and research studies. The discriminating dose data concurrently suggest cross resistance to the seed treatment thiamethoxam (Cruiser®) (Table 6) but interestingly, not to the related neonicotinoid compound imidacloprid (Gaucho®)(unpublished data). The result is also not consistent with the study of Wang *et al*. (2007) that demonstrated a relationship between imidacloprid (Gaucho®) and acetamiprid (Intruder®) resistance in cotton aphid or that of Alyokhin *et al*. 2007 that linked imidacloprid (Gaucho®) resistance to thiamethoxam (Cruiser®) in Colorado potato beetle. Clearly, more research is required on Australian populations of cotton aphid to better understand the likely cross resistance implications and its affect on the relative positioning of neonicotinoids for cotton aphid within the insecticide resistance management strategy. Until more quantitative data is available for Australian cotton aphid on cross resistance, neonicotinoids should continue to be thought of as a single cross resistance group as suggested by Nauen and Denholm (2005). Clearly there is a need to reduce overall neonicotinoid selection to prevent or slow a resistance increase. Currently the most consistent use of neonicotinoids in cotton is as seed treatments e.g. imidacloprid (Gaucho®, Genero®, Amparo®) or thiamethoxam (Cruiser®) which account for approximately 50% of the insecticides used at-planting. Some options to help manage resistance could include:

1. Preventing any in-season use of foliar neonicotinoids if they have been used as a seed treatment. This will have implications for control of other pests such as sliver leaf whitefly and mirids. However, as information is developed on cross-resistance within the neonicotinoid group it may be possible to modify the strategy to allow use of non-cross-resistant neonicotinoids (if this indeed occurs).
2. Developing a seasonal rotation system for the seed treatments e.g. avoiding year-to-year reliance on neonicotinoid seed treatments. This would need to be co-ordinated within regions to avoid a special mosaic effect (which is what we currently have – aphids may be selected for neonicotinoid resistance on one farm in a particular year, then on an adjacent farm the next year)
3. Remove the use of neonicotinoids as seed treatments altogether. This will place greater reliance on the remaining seed treatments thiodicarb/fipronil, aldicarb and phorate, and restrict grower choice.

Propargite (Comite®), abamectin (Agrimec®) and bifenthrin (Talstar®) resistance were again detected in two-spotted mite for season 2006/2007. It is not encouraging that bifenthrin (Talstar®) resistance was detected at a discriminating dose mortality of less then 50% despite the rather small sample. Clearly bifenthrin (Talstar®) resistance is persisting despite changes to the resistance

management strategy and the overall reduction of insecticide use associated with the introduction of Bollgard® II cotton.

Baseline data generated

During season 2006-2007 additional baseline generation for Calypso® showed the minimum effective dose required to kill strain Car 13 was equivalent to the discriminating dose (Figure 2).

The discriminating dose used for Calypso® was interpolated from the dose response for cotton aphid strain Susceptible A. The 0.04 g / L chosen was midway between the calculated $LC_{99.9}$ and $LC_{99.99}$ level of response (ie 0.02- 0.054 respectively). However, the additional baseline data showed the minimum effective dose required to kill strain Car 13 was equivalent to the discriminating dose. For that reason the discriminating dose for Calypso® should be increased for season 07/08 to avoid false positive results.

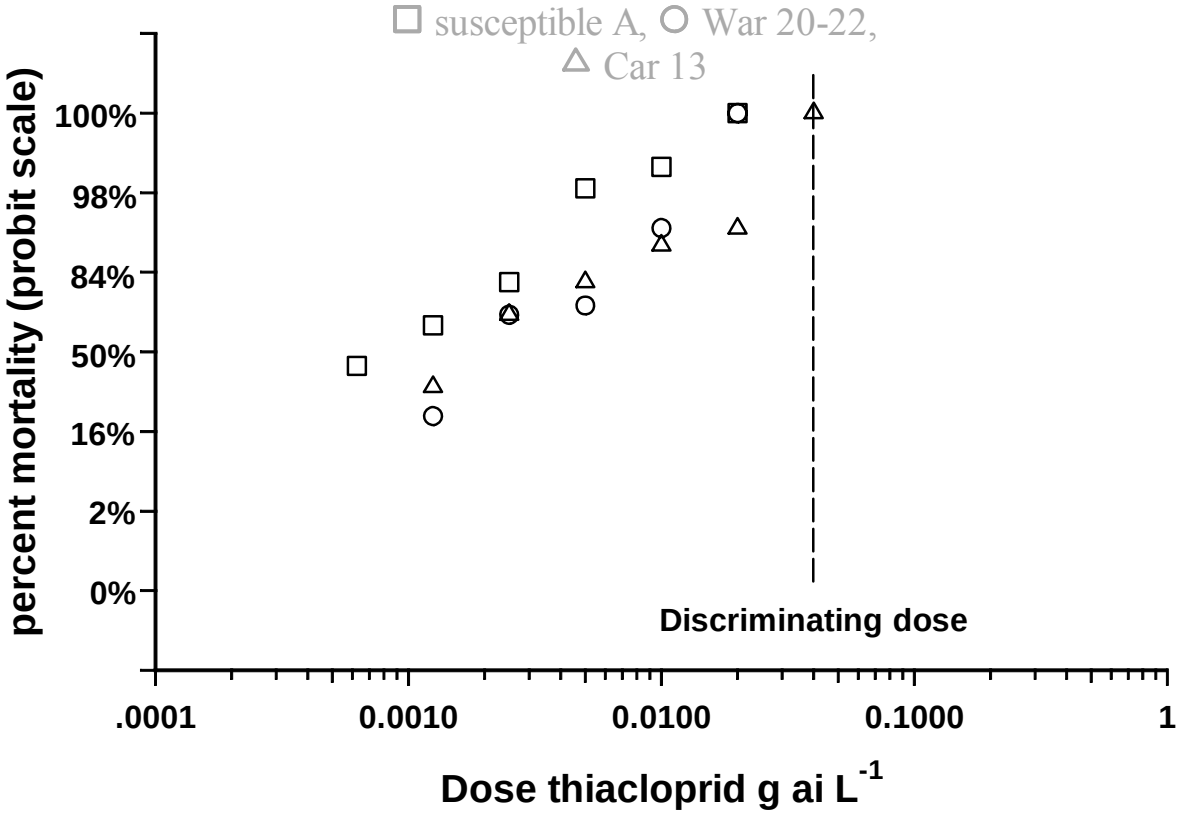

Figure 2. Dose response for Susceptible A, and field strains War 20-22 and Car 13 against Calypso® (thiacloprid) with the 06/07 discriminating dose superimposed

Baseline data for acetamiprid (Figure 3) were generated in advance so that resistance could be quickly diagnosed if a suspect resistant strain was found.

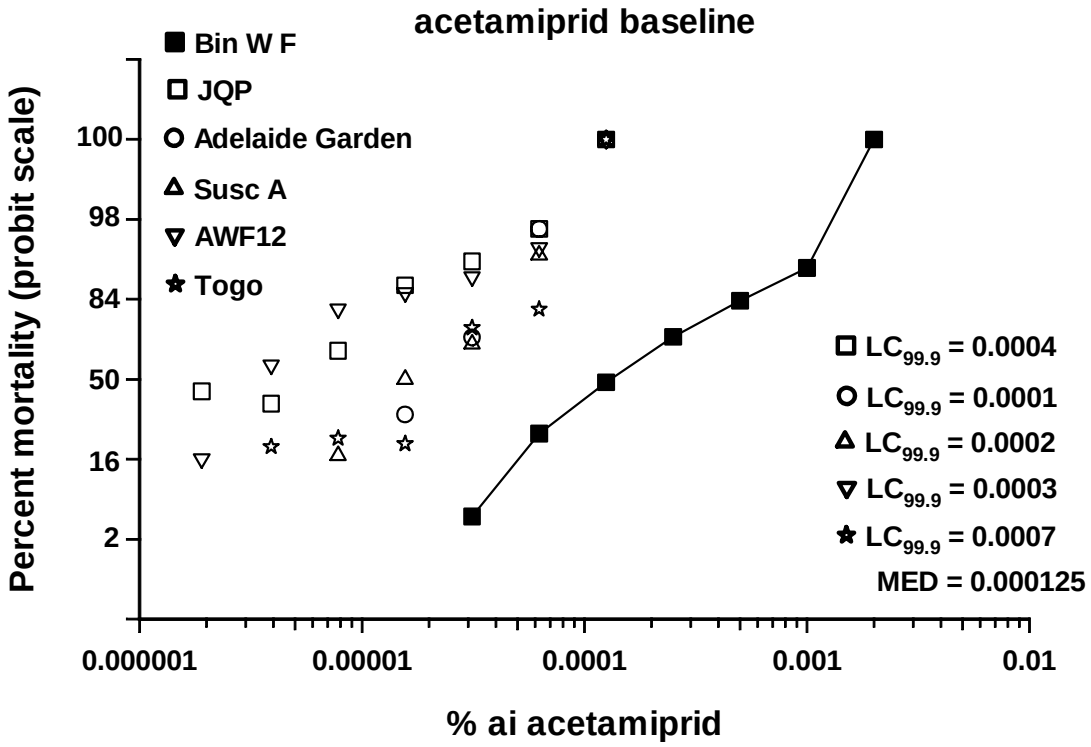

Figure 3. Baseline dose response data for strains Susceptible A, AWF12, Togo, Adelaide Garden and JQP relative to the suspect acetamiprid (Intruder®) resistant strain Bin WF.

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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.

    - *The project outcome is the sustainable chemical management of aphids, mites and mirids in Australian cotton underpinned by via resistance monitoring. Monitoring outputs identified a newly emerging issue with the neonicotinoid insecticides that caused suggested changes to the IRMS for aphids to aid sustainability.*

6. Please describe any:-
   a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);
   - *Nothing of commercial significance*

   b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and
   - *New methodology developed to allow carbamate and organophosphate resistance in cotton aphid to be detected via molecular methods*
   - *Baseline data for acetamiprid (Intruder®) available allowing resistance to be unequivocally diagnosed.*
   - *Development of a method to rear mirids and successful bioassay.*

   c) required changes to the Intellectual Property register.
   - *None required*

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?

- Methods were developed to breed mirids under laboratory conditions and evaluate them for insecticide resistance
- Cotton aphid and two-spotted mite were collected from Australian cotton growing regions and tested in the laboratory for insecticide resistance.
- Propargite (Comite®), chlorfenapyr (Intrepid®), abamectin (Agrimec®) and bifenthrin (Talstar®) resistance was detected in two-spotted mite strains with high frequency resistance restricted to bifenthrin (Talstar®) only.
- Molecular testing is now used to detect Pirimicarb (Pirimor®) and organophosphate (Lorsban®) resistance in field collected cotton aphid strains that significantly reduce the time required from sample collection to resistance diagnosis.
- Aphids have become scarcer in recent years but none-the-less strains showing pirimicarb (Pirimor®) resistance were still detected and field control failures recorded.
- *Prima facie* acetamiprid (Intruder®) and thiamethoxam (Cruiser®) (both neonicotinoids) resistance has been detected in cotton aphid for the first time. Acetamiprid (Intruder®) resistance was confirmed using full log dose probit analysis in one strain to be 9.1 fold. Full log dose analysis with thiamethoxam (Cruiser®) is yet to be completed.
- Clearly there is a need to reduce overall neonicotinoid selection to prevent or slow any increase in neonicotinoid resistance. There are several options available, though each will necessarily reduce flexibility in the use of this group. One 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 limit neonicotinoid use to foliar sprays.

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.
   - *Project outputs will continue to feed into the IRMS and molecular methods will continue to be developed to replace routine bioassay techniques.*

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

- *Via the annual IRMS, TIMS and the annual resistance extension tour and via the Australian CottonGrower magazine, Cottontales magazine and Cotton CRC Pest Reviews (which are included on the CRC website)*

(c) for future research.

- *Aphid and mite resistance monitoring should continue and baseline data generated against green mirids.*

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)

Published

- Lewis Wilson, Mary Whitehouse, Grant Herron, and Simone Heimoana (2005) How is Bollgard II affecting management of mites and aphids. In: Variety Trial Results 2005. Cotton Seed Distributors, Wee Waa and Dalby.
- Herron G. (2005) Resistance monitoring of aphids and mites in cotton pp 15-18. In: (Scott Vaessen Ed) Southern New South Wales Cotton Trial Book. NSW Department of Primary Industries and Cotton CRC, Griffith.
- Herron, G.A. and Wilson, L.J. (2006) Insecticide resistance in cotton aphid and two-spotted mite: seasons 2004-2005 and 2005-2006. 13th Australian Cotton Conference, Gold Coast Queensland, 8-10 August 2006. CD ROM. Australian Cotton Growers Research Association.
- M^{c}Loon, MO and Herron, G.A. (2006) Real time PCR detection of pirimicarb and organophosphate resistance in Australian field isolates of cotton aphid, *Aphis gossypii*. 13th Australian Cotton Conference, Gold Coast Queensland, 8-10 August 2006. CD ROM. Australian Cotton Growers Research Association.
- Smith, T., Wilson, L., Heimoana, S., Herron, G. and Franzmann, B. (2006) overwinter host plants of cotton aphid (*Aphis gossypii*) and implications for managing abundance and resistance. 13th Australian Cotton Conference, Gold Coast Queensland, 8-10 August 2006. CD ROM. Australian Cotton Growers Research Association.
- Wilson, L. and Herron, G. (2006) Thrips on Cotton. Facts on Friday, 24 November 2006. Cotton Seed Distributors, Wee Waa.
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- Herron, G. (2007) The changing face of secondary pests in Australian cotton. *Tarsus*, **576** July 2007: 94-95.
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- 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.
- 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). *Australian journal of Entomology*, In Press

Planned

- Martin O M^{c}Loon, Jerome Carletto, Tanya Smith, Lewis J. Wilson, Grant A Herron and Flavie Vanlerberghe-Masutti. The relationship between pirimicarb resistance, host plant origin and aphid linage in *Aphis gossypii* Glover (Homoptera : Aphididae).

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

- Some of the above references are available online. See www.cotton.crc.org.au

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.

With the introduction of transgenic cotton, sucking insect pests have become more troublesome, so requiring increased targeted insecticide control and resistance development. Pre-emptive baseline data proved critical to the successful management of cotton aphid because resistance could quickly be confirmed. However, no baseline data for mirids currently exists, preventing an early confirmation of resistance and subsequent resistance management. For this reason methods were developed to breed mirids under laboratory conditions and test them to insecticides.

Cotton aphid and two-spotted mite were collected from Australian cotton growing regions and tested in the laboratory for insecticide resistance. Two-spotted mite was tested against Propargite (Comite®), chlorfenapyr (Intrepid®), abamectin (Agrimec®) and bifenthrin (Talstar®) and resistance detected but high frequency resistance was restricted to bifenthrin (Talstar®) only.

Molecular testing is now used to detect Pirimicarb (Pirimor®) and organophosphate (Lorsban®) resistance in field collected cotton aphid strains that significantly reduce the time required from sample collection to resistance diagnosis. Although aphids have become scarcer in recent years pirimicarb (Pirimor®) resistance was still detected and field control failures recorded. *Prima facie* acetamiprid (Intruder®) and thiamethoxam (Cruiser®) (both neonicotinoids) resistance has been detected in cotton aphid for the first time. Acetamiprid (Intruder®) resistance was confirmed using full log dose probit analysis in one strain to be 9.1 fold. Full log dose analysis with thiamethoxam (Cruiser®) is yet to be done to confirm resistance and provide a resistance level. 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 (the down side being growers are then locked into rather toxic chemicals such as aldicarb and phorate) based products and limit neonicotinoid use to foliar sprays.

Appendix 1

M^{c}Loon MO and Herron GA (2008) PCR detection of pirimicarb resistance in Australian field isolates of Aphis gossypii Glover (Aphididae: Hemiptera). Australian Journal of Entomology, In Press

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

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Running title: PCR resistance monitoring in aphids

Abstract
Aphis gossypii Glover (cotton aphid) is a major secondary pest of Australian cotton which 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 hours, compared to 4 to 8 weeks for bioassay, such an assay could be used prior to insecticide control.

Key Words insecticide resistance, bioassay methods, 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 due to 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, T. 2006).
Until recent Australian insecticide control failures (Herron *et al*. 2001) pirimicarb was very effective at controlling cotton aphid. Following 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*1 gene (Nabeshima *et* al 2003; Benting, J & Nauen, R. 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 PCR and *Ssp*I 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*1 gene sequence amplified using the primers of Andrews *et al* (2004) were determined for 5 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 same size. These will co-migrate on an agarose gel and present as a single intense band half the size of an undigested amplicon. Since 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 (EMAI) 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 ten 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 aliquot. 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

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 minutes followed by 100°C for 5 minutes. 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

PCR was performed using iQ Sybr green supermix (BioRad) and the primers AceF (CAAGCCATCATGGAATCAGG) and AceR (TCATCACCATGCATCACACC) with the RotaGene 2000 real time PCR machine (Corbett Research). Cycling parameters were an

initial 5 minute denaturation at 94 °C followed by 40 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds and 72 °C for 45 seconds. 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 done using the software BioEdit (Hall, T.A. 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 hours with 4U of enzyme and the manufacturers supplied buffer. The total reaction volume was 20 µL.

Gel electrophoresis

Electrophoresis was done using an Easy Cast apparatus (Owl Scientific Instruments). Agarose (Progen) gels were made with fresh 1 X TBE buffer, supplemented with ethidium bromide (5µg/mL), run at 94 volts for 1-3 hours in 1 X TBE buffer. Gels were visualised 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 done on both strands of a reference strains' PCR product, resulting in 705bp 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 -1293bp (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 904bp (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 (Figs.1). The *Ace*I DNA sequence of Adam was the same as strain 968E at both the S431F and A302S loci (Fig. 1). 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 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. 3). Half melt temperature (½Tm) curve analysis with SYBR green was not sensitive enough to distinguish the different polymorphisms occurring at the S431F locus (Fig. 3). The few polymorphic nucleotides don't change the guanine plus cytosine molar percent concentration (%GC) enough to alter the different PCR products ½Tm (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 visualise 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. 4). The results for the33 field isolates from the 2005 / 2006 and 2006 / 2007 cotton seasons can be seen in Fig. 5. 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 (SusA 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. 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 GSM-2, Togo H2 also has the G to T polymorphism at position 904 bps 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 since 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 prior to *Ssp*I REA.
The PCR REA for pirimicarb successfully identified the three resistant reference strains and the two susceptible strains (Fig. 4). 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 was 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.
Since 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 bps and 336 bps respectively). Since 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 viral profiling. Further, 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 done using either surviving aphids (resistant) or dead aphids (susceptible).

Since the molecular assay can provide results within 48 hours compared to 4 to 8 weeks for bioassay such tests could be used prior to 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 3 labelled DNA primers differing only at the terminal 3' base (plus one common primer) or 3 dual labelled probes for Taqman techniques (2 for resistant and 1 for susceptible) both are expensive options. Since Togo is heterozygous for the resistant alleles (2 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 bps 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.

ACKNOWLWDGMENTS

Swami Thalavaisundaram, Graeme Gullick and Carolyn Griffin provided technical assistance. The many researchers, CRC Industry Development Officers, consultants and growers who collected aphids or who helped us to locate sites for collecting are thanked. This study was funded by the Cotton Research and Development Corporation (DAN184c). Louise Rossiter and Steve Djordjevic are thanked for critical comments on an earlier draft.

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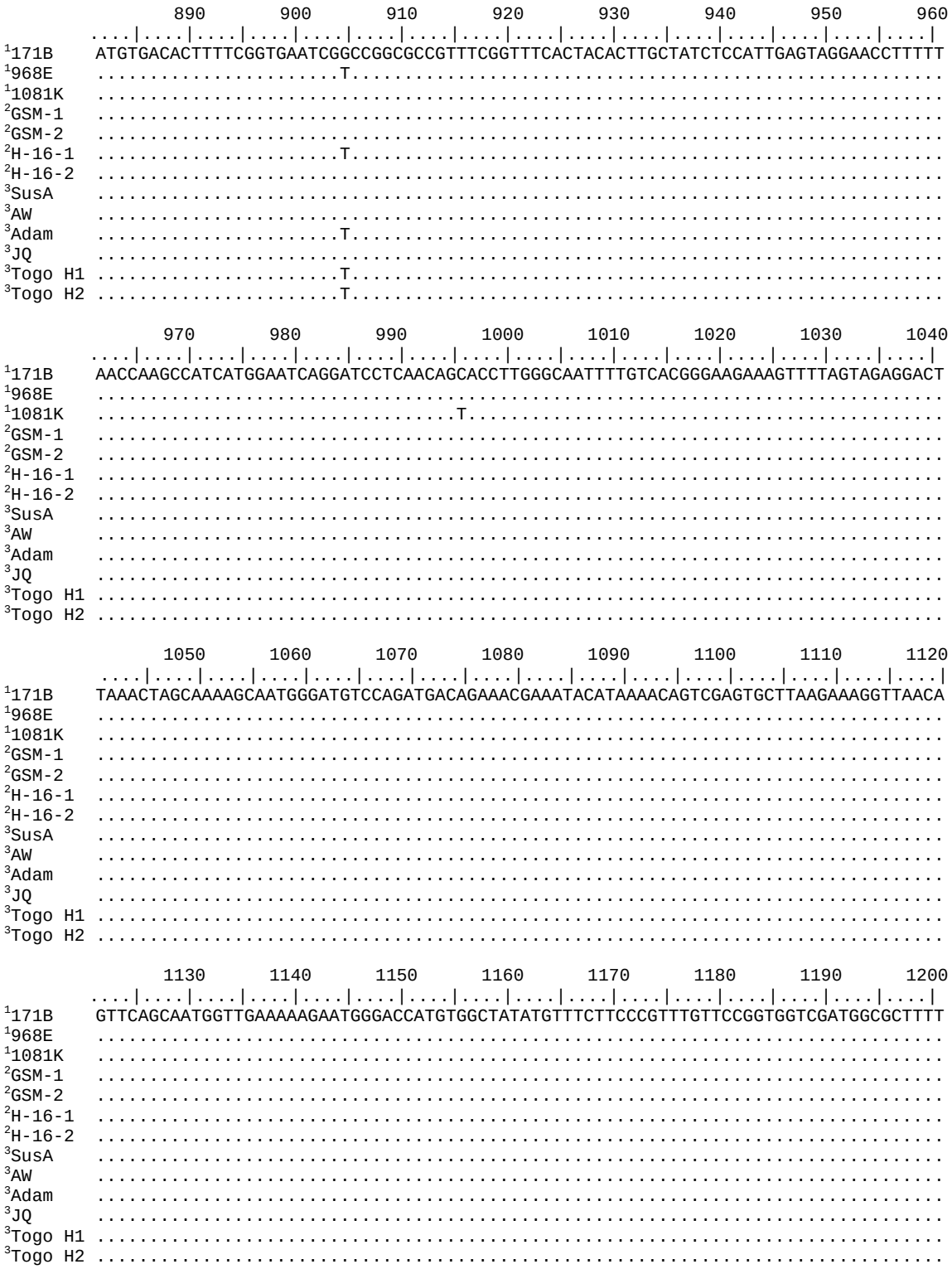
Table 1 Discriminating dose bioassay results and molecular assay results (*Ssp*I REA) for field isolates of *Aphis gossypii* collected during the 2005-2006 and 2006-2007 cotton seasons and evaluated for pirimicarb resistance.

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 C4-5	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^{c}Intyre	100	S
	War 20-22	M^{c}Intyre	100	S
	Alch 007	M^{c}Intyre	100	S
	Car 13	M^{c}Intyre	100	S
	Nor 4	Gwydir	100	S
	Byr 55	Macquarie	100	S
	Bur 4	Macquarie	100	S
	Wil 21B	Macquarie	6	R

R = Pirimicarb resistant
S = Pirimicarb susceptible.
[a] see results section

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 bps (171B numbering). As well as the region containing the single polymorphism (G→T) responsible for the A302S mutation. A302S is located at 904 bps (171B numbering) in strains 968E, H-16-1, Adam, Togo H1 and H2, it purportedly confers resistance to a select group of organophosphates.



```
                1210      1220      1230      1240      1250      1260      1270      1280
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
[1]171B    CTTGACGATCATCCTCAAAAGTCTTTATCAACAAACAATTTTAAAAAAAACCAATATACTCATGGGTAGTAACTCCGAAGA
[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...................................................................
```

[1] Andrews *et al* (2004)

[2] Toda *et al* (2004)

[3] current study

Fig. 2. 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).

```
          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.....
```

[1] Andrews *et al* (2004)

[2] Toda *et al* (2004)

[3] current study

Fig. 3. 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 (½Tm) of 83.23 ± 0.5 °C. No difference is seen between the ½Tm 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 whilst a product amplified by real time PCR will have a melt curve of 83.23 ± 0.5 °C.

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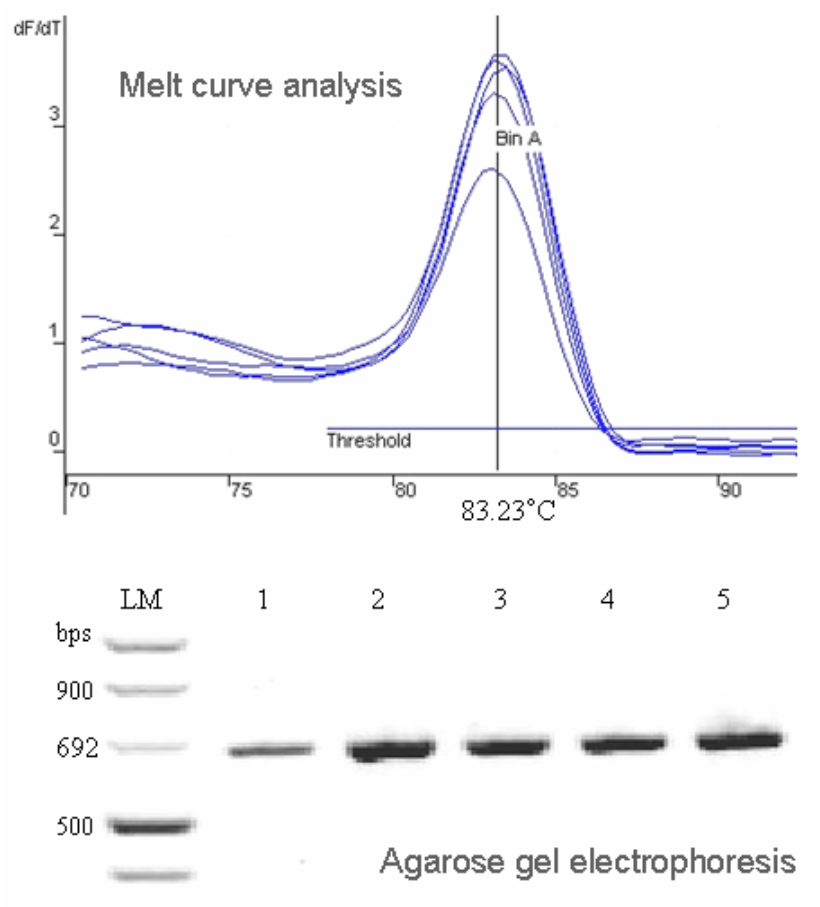
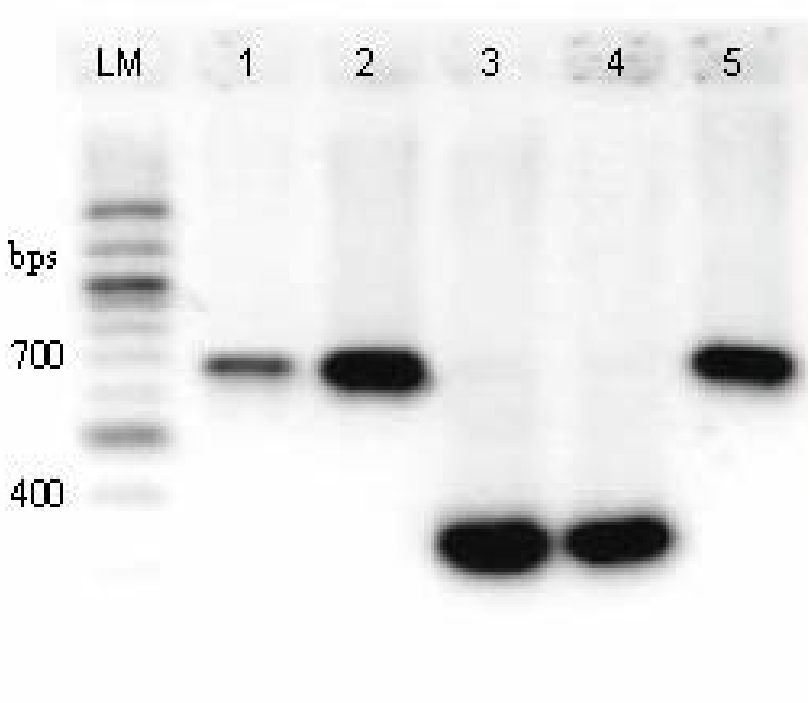


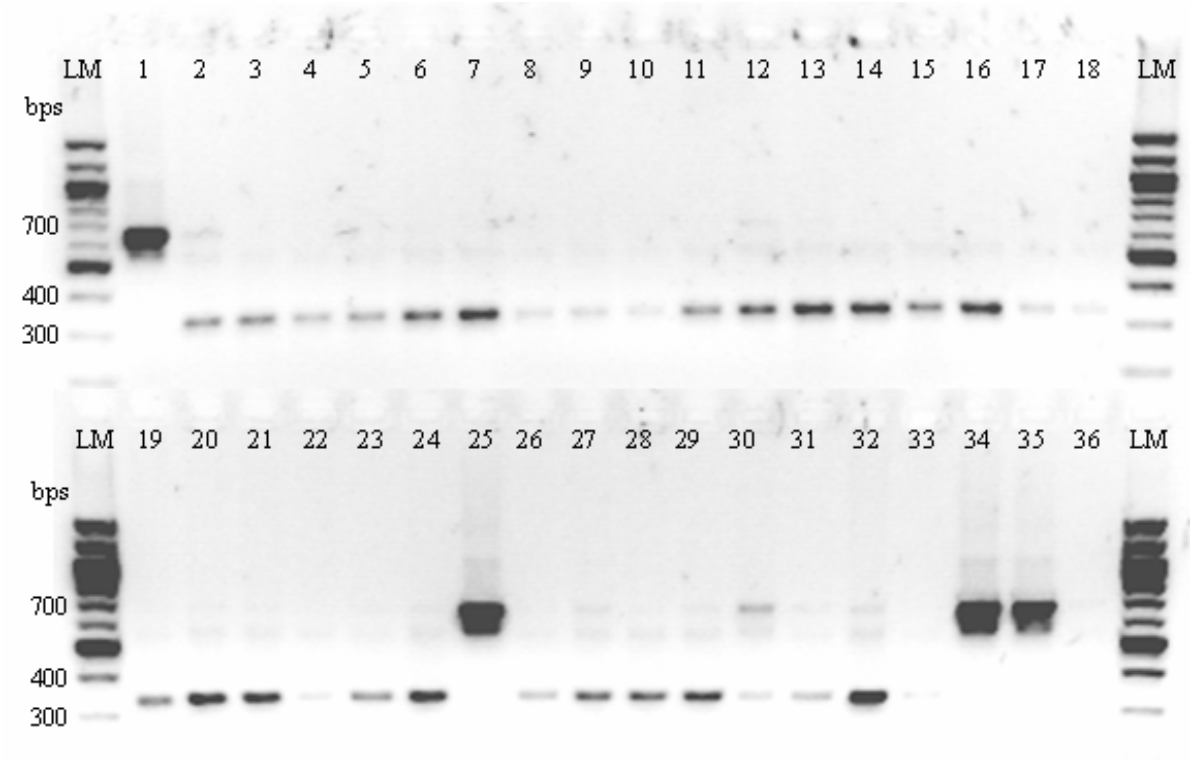
LM- Lane marker, 1- Sus A, 2- AW, 3- JQ, 4- Adam, 5- Togo.

Fig. 4. *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 bps (cut by *Ssp*I), whilst the pirimicarb resistant strains Adam, JQ and Togo show a single intense band at 667 bps (uncut by *Ssp*I).



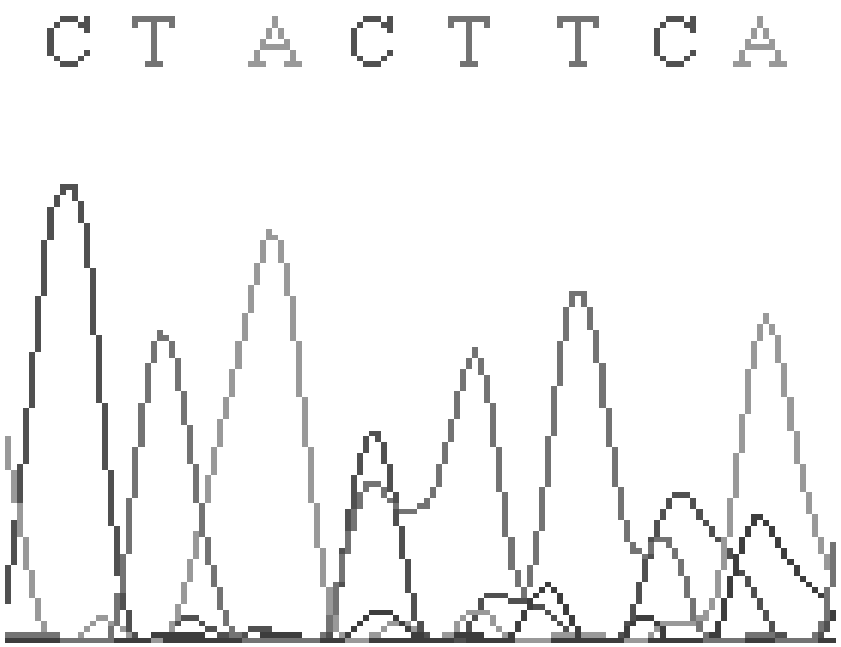
LM- Lane marker, 1-,JQ, 2- Adam, 3- Sus A, 4- AW, 5- Togo.

Fig. 5. *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.



LM- Lane marker, 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 F2-7, 18- My D Cr, 19- Alch C4-5, 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.

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



Appendix 2

Herron, G.A. and Wilson, L.J. (2006) Insecticide resistance in cotton aphid and two-spotted mite: seasons 2004-2005 and 2005-2006. 13th Australian Cotton Conference, Gold Coast Queensland, 8-10 August 2006. CD ROM. Australian Cotton Growers Research Association.

Insecticide resistance in cotton aphid and two-spotted spider mite: seasons 2004-2005 and 2005-2006

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Abstract
For season 2004-2005, sixty-one percent of cotton aphid populations were Pirimor® resistant but Intruder®, Actara®, Thiodan® and Pegasus® susceptible. Two-spotted mite was Agrimec®, Comite® and Pegasus® susceptible. Intrepid® resistance was detected in 43 and Talstar® 57 percent of mite stains. Curacron® resistance in two-spotted mite was ubiquitous and often at high frequencies. At the time of writing testing for season 2005-2006 was incomplete but Pirimor® resistance seemed less than in season 2004-2005. Intrepid® resistance was still detected in 67% of the two-spotted mite strains although the product was not available for use in cotton. We conclude that the resistance management strategy for cotton aphid and two-spotted mite should remain 'as is' for season 2006-2007.

Introduction
With the introduction of transgenic cotton, sucking insect pests have become more troublesome, so requiring increased targeted insecticide control. Two-spotted mite has a proven ability to develop resistance and recently developed resistance to chlorfenapyr (Intrepid®)(Herron *et al*. 2004). A few years earlier, high-level organophosphate and carbamate resistance has developed in cotton aphid (Herron *et al*. 2001).

The pest status of aphids is often related to the contamination of the cotton lint with sugary 'honey-dew'. However, earlier outbreaks can significantly reduce yield and recently cotton aphid was confirmed as a vector for 'Cotton Bunchy Top' syndrome. These changes in the system mean that the need for effective tools to control aphids and resistance management for those tools is critical to the cotton industry.

Cotton aphid reproduces asexually causing very rapid changes in resistance levels. Management of aphids is further complicated because there is no dilution of resistance by outcrossing to susceptibles, as is used to manage *Bt* resistance in *Helicoverpa* spp. Therefore, aphids can very quickly become a major problem when chemical control fails due to resistance (Herron *et al*. 2001).

Two-spotted mite is notorious world-wide for developing resistance. As new compounds have become available we have pro-actively established baseline resistance levels and cross-resistance profiles and initiated routine resistance monitoring. Unfortunately, resistance continues to evolve as seen most recently to Talstar® (Herron *et al.* 2001a) and then Intrepid® (Herron *et al*. 2004). This is occurring largely due to use of these compounds against other pests, rather than mites themselves, and is disturbing, as it reduces the number of chemicals available for two-spotted mite control. Management of mites is complicated because most chemicals are also targeted against other pests such as aphids or *Helicoverpa* spp. and this has contributed to resistance development to organophosphates, Talstar® and Intrepid®.

Effective management of two-spotted mite and cotton aphid will be best achieved by pursuing an integrated approach that includes resistance management, based on a sound understanding of their resistance and cross-resistance spectra (reported separately in M^{c}Loon and Herron). Continued resistance monitoring, and the timely inclusion of new chemistry, is essential for effective ongoing management of this pest species. Here we report our

resistance monitoring data for cotton aphid and two-spotted mite for seasons 2004-2005 and 2005-2006.

Materials and methods

Chemicals

Proprietary commercial formulations of pirimicarb (Pirimor®), thiamethoxam (Actara®), acetamiprid (Intruder®), profenofos (Curacron®), bifenthrin (Talstar®), abamectin (Agrimec®), propargite (Comite®), chlorfenapyr (Intrepid®) was used, except difenthiuron (Pegasus®). The UV activated carbodiimide derivative of diafenthiuron, CGA-140408 was tested instead.

Cotton aphid

Aphids were collected by researchers, CRC Industry Development Officers, consultants and growers from cotton fields. They were then sent by overnight courier to the bioassay laboratory at Camden (EMAI) and each field strain cultured separately on pesticide-free cotton (Deltapine 90) at 25 ± 4^{o}C under natural light. Strain integrity was assured by maintaining populations in purpose built insect proof cages. Aphids were the tested by placing them in a 35 mm Petri dish on an excised cotton plant leaf disc fixed in agar (Herron *et al* 2001). Batches of ten adult female aphids per leaf disc 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 aliquot (Herron *et al* 2001). Each test was replicated and included a water only sprayed control. After spraying, Petri dishes were covered with ventilated clear plastic film and maintained at 25 ± 0.1 ^{o}C in 16:8 L:D for 24 h after which mortality was assessed.

Two-spotted mite

Strains of two-spotted mite were randomly collected late in each cotton season 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.* 2001a). Mites and leaf disc are then sprayed with insecticide with the aid of a Potter spray tower as above. Each test was replicated and included a water only sprayed control. 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.

Results

During season 2004-2005 strains of mites were collected from the Namoi, Gwydir and Griffith areas (Table 1). Agrimec®, Comite® and Pegasus® resistance was not detected. Intrepid® resistance was detected in 3 of the 7 strains and Talstar® resistance in 4 of the 7 stains. Curacron® resistance was ubiquitous and often at high frequencies. In the following 2005-2006 season Agrimec® and Comite® resistance was again not detected but Pegasus® testing was not yet complete. Intrepid® and Talstar® resistance was detected 4 and 2 of the 6 strains tested respectively. Curacron resistance frequencies were very high in the two strains tested (AN and KI)

Aphids were collected more widely than mites in season 2004-2005 with samples from the Gwydir, the Namoi, Goondiwindi, Dalby, Bourke, St George and Hillston (Table 2) No resistance was detected against Intruder®, Actara®, Thiodan® or Pegasus®. Pirimor® resistance was detected in 61% of the populations surveyed. Testing during season 2005-2006 was still very much at initial stages at the time of writing with only Pirimor® data available. Although not complete, Pirimor® resistance was less abundant than in the previous season.

Discussion

During season 2001-2002 Intrepid® survivors were detected for the first time. Season 2002-2003 produced an alarming trend of increasing level and abundance of Intrepid® resistance. In response the mite management strategy for Intrepid was modified from season 2003-2004

with a reduction in total Intrepid sprays to one per season for either *Helicoverpa* spp or *T. urticae*. Unfortunately during season 2004-2005 resistance was detected in 3 out of 7 strains tested but encouragingly resistance frequencies in each strain were generally less than in seasons 2003-2004. For season 2005-2006 Intrepid® was not available for use in Australian cotton yet resistance continued to be detected but at frequencies less than season 2003-2004.

Pirimor® resistance was detected in 61% of cotton aphid populations. As Pirimor® resistance is extremely high level in cotton aphid control failure would likely result. Unfortunately Pirimor® is known to cause cross resistance to Folimat® / Rogor® and so those products would also be compromised by resistance. Pirimor resistance remains despite a change to the management strategy in 2003-2004 when Pirimor® and Folimat® use was restricted by the implementation of chemical use windows. During season 2004-2005 it is possible that control of mirids with Folimat® or Rogor® may be selecting concurrent aphids so producing Pirimor®, Folimat® and Rogor® resistance in aphids. During season 2005-2006 aphids were difficult to find and those populations collected were nearly all Pirimor® susceptible. It is unknown if the lack of Pirimor resistance was due to low aphid pressure or grower avoidance of Pirimor with the substitution of newer chemistry such as Intruder®

Table 1. Percent mortality at the discriminating dose (ie percent susceptible) for various strains of TSM collected during season 2004-2005 and 2005-2006 and evaluated for resistance against Talstar®, Intrepid®, Agrimec®, Comite®, Pegasus® (CGA-140408) and Curacron®

Year	Strain	Area	Chemical					
			Talstar®	Intrepid®	Agrimec®	Comite®	Pegasus®	Curacron
04-05	AN	Namoi	29	96	100	100	100	12
	G	Namoi	31	84	100	100	100	19
	TG	Griffith	100	100	100	100	100	43
	GL	Gwydir	100	100	100	100	100	10
	NH	Namoi	44	90	100	100	100	17
	RAV	Griffith	100	100	100	100	100	79
	PU	Namoi	50	100	100	100	100	10
05-06	AN	StGeorge	100	100	100	100	TBC	2
	KI	StGeorge	100	99	100	100	TBC	1
	BE	U Namoi	58	93	100	100	TBC	TBC
	CU	U Namoi	93	97	100	100	TBC	TBC
	M	U Namoi	100	98	100	100	TBC	TBC
	CA	?	100	100	100	100	TBC	TBC

TBC=to be completed

Table 2. Percent mortality at the discriminating dose (ie percent susceptible) for various strains of cotton aphid collected during season 2004-2005 and 2005-2006 and evaluated for resistance against Pirimor®, Pegasus® (CGA-140408), Thiodan®, Actara® and Intruder®

Year	Strain	Area	Chemical				
			Pirimor®	Pegasus®	Thiodan®	Actara®	Intruder®
04-	Norw	Gwydir	92	100	100	100	100

Year	Strain	Area	Chemical				
			Pirimor®	Pegasus®	Thiodan®	Actara®	Intruder®
05							
	Lamer	Gwydir	87	100	100	100	100
	F2#1 AC	Namoi	did	not	establish	into	culture
	Mapl F1	Goondi	100	100	100	100	100
	Carring	Goondi	100	100	100	100	100
	Pall F4	Goondi	100	100	100	100	100
	McD K3	S George	95	100	100	100	100
	Alc F007	Goondi	did	not	establish	into	culture
	Mor Win	Goondi	did	not	establish	into	culture
	Car F2-7	Goondi	did	not	establish	into	culture
	Mor E4	Goondi	did	not	establish	into	culture
	War18-22	Goondi	did	not	establish	into	culture
	Tuck	Goondi	did	not	establish	into	culture
	Car20-25	Goondi	99	100	100	100	100
	Caffery	Dalby	100	100	100	100	100
	Lat F15	Bourke	100	100	100	*	*
	Lat F17	Bourke	96	100	100	100	*
	Lat F18	Bourke	100	100	100	100	100
	Lat LM3	Bourke	90	100	100	100	100
	Bee F16	Gwydir	did	not	establish	into	culture
	Tara	Namoi	95	100	100	100	100
	Hava F4	Namoi	91	100	100	100	100
	Beech	Namoi	did	not	establish	into	culture
	Purl F42	Gwydir	91	100	100	100	100
	Milo	Gwydir	did	not	establish	into	culture
	Glen F6B	Namoi	100	100	100	100	100
	Mira F2	Namoi	100	100	100	100	100
	Veth F5	Gwydir	93	100	100	100	100
	LFL Fl B	Hillston	97	100	100	100	100
	LFL30/2015	Hillston	90	100	100	100	100
	LFLG16-26	Hillston	100	100	100	100	100
	LFLY12-15	Hillston	did	not	establish	Into	culture
	LFL B lat	Hillston	92	100	100	100	100
	LFL dr lat	Hillston	93	100	100	100	100
05-06	ACRI R6 1	Namoi	8.1	TBC	TBC	TBC	TBC
	CH	Gwydir	100	TBC	TBC	TBC	TBC
	Tell F 33	Gwydir	TBC	TBC	TBC	TBC	TBC
	Wood	Gwydir	100	TBC	TBC	TBC	TBC
	Yar	Downs	did	not	establish	into	culture
	Oak F 1	Downs	100	TBC	TBC	TBC	TBC
	Aru F 3	Downs	TBC	TBC	TBC	TBC	TBC
	War M7	Downs	100	TBC	TBC	TBC	TBC
	Over F 4	Downs	TBC	TBC	TBC	TBC	TBC
	Sin F 23	Downs	did	not	establish	into	culture
	Eden	Downs	100	TBC	TBC	TBC	TBC
	ACR1 R6 2	Namoi	TBC	TBC	TBC	TBC	TBC
	War F 2	U Namoi	TBC	TBC	TBC	TBC	TBC
	Bell F 15	U Namoi	TBC	TBC	TBC	TBC	TBC
	Mer	U Namoi	TBC	TBC	TBC	TBC	TBC
	Cur F 25	U Namoi	TBC	TBC	TBC	TBC	TBC
	Cur F 22	U Namoi	100	TBC	TBC	TBC	TBC

Year	Strain	Area	Chemical				
			Pirimor®	Pegasus®	Thiodan®	Actara®	Intruder®
	Car F 2-7	MacIntyre	TBC	TBC	TBC	TBC	TBC
	My D Cr	MacIntyre	100	TBC	TBC	TBC	TBC
	Alch C4-5	MacIntyre	TBC	TBC	TBC	TBC	TBC
	Rio G	Mungindi	TBC	TBC	TBC	TBC	TBC
	Har	St George	TBC	TBC	TBC	TBC	TBC
	Cal	Namoi	TBC	TBC	TBC	TBC	TBC
	Uya	Namoi	TBC	TBC	TBC	TBC	TBC
	Mill 1	Namoi	did	not	establish	into	culture

*=Not tested
TBC=to be completed

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Appendix 3

Herron GA, M^{c}Loon MO, & Wilson LJ (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.

Resistance testing summary for the 2006-2007 and 2007-2008 cotton seasons: cotton aphid *Aphis gossypii* and two-spotted mite *Tetranychus urticae*

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- Cotton aphid and two-spotted mite were collected from Australian cotton growing regions and tested in the laboratory for insecticide resistance.
- For the third consecutive season molecular testing was used to detect pirimicarb and organophosphate resistance in field collected cotton aphid strains. Bioassay and molecular testing were conducted in parallel. Similar results where obtained by both methods in their characterisation of pirimicarb and organophosphate resistant aphid strains.
- Pirimicarb and organophosphate resistance associated with control failure was detected in one cotton aphid sample from the Macquarie Valley in 2006-2007. In 2007-2008 aphids were scarce in the Macquarie Valley so no samples were collected. However one strain from St George is pirimicarb resistant and one strain from Gwydir has produced resistant isolates (a mix of susceptible and resistant).
- *Prima facie* acetamiprid resistance has been detected in cotton aphid for the first time but additional testing is required for confirmation.
- Propargite, chlorfenapyr (Intrepid®), abamectin and bifenthrin (Talstar®) resistance were detected in two-spotted mite strains but resistance patterns to the specific chemicals were not the same across seasons. High frequency resistance was restricted to bifenthrin with resistance detected in both seasons 2006-2007 and 2007-2008.

Key Words: Aphids, cotton, resistance, spider mites, molecular, bioassay, monitoring

Introduction

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, control of which with broad-spectrum insecticides depletes beneficial populations and leads to outbreaks of secondary pests such as two spotted mite (TSM) and cotton aphid. Control of these secondary pests inevitably selects for insecticide resistant strains. Dealing with this issue requires on-going monitoring for resistance in pests to key insecticides if future control problems are to be averted (Herron 2007).

Two-spotted mite is notorious world-wide for developing insecticide resistance including Australia where resistance in cotton continues to evolve, as most recently seen with Talstar® (Herron *et al*. 2001b) and subsequently Intrepid® (Herron *et al*. 2004).

Similarly, cotton aphid is resistant to a range of insecticides in many crops and countries. Of late, high-level resistance to organophosphates (omethoate and dimethoate) and some carbamates (pirimicarb) has developed in cotton aphid (Herron *et al*. 2001a). In Australia the cotton aphid reproduces almost exclusively asexually, essentially they clone themselves. With this method of reproduction very rapid fixing of genotypic changes can occur since there is no influx of alleles from the male of the species. This is particularly evident with insecticide resistance genes and the rapid appearance of resistant strains seen shortly after insecticide usage.

Both TSM and aphids are important pests of cotton. TSM is capable of causing dramatic losses of yield and reductions in fibre quality. Cotton aphid is a vector of cotton vein mosaic virus (Dos Santos, K. B *et al*, 2004), citrus tristeza virus (Yokomi, R. K and DeBorde, R. L, 2005), cotton bunchy top (Reddall, A *et al*, 2004) and promotes bacterial or fungal contaminations via its honey dew excretions which contaminate the lint. Hence, both TSM and cotton aphid would become major problems in the cotton industry if the capacity to control them was limited by insecticide resistance (Herron *et al*. 2001a).

Continued insecticide resistance monitoring, including generation of baseline data for new chemistry, is essential for effective ongoing management of resistance in these pests. Here we present our monitoring data for seasons 2006-2007 and 2007-2008.

Methods

Chemicals tested
Mites and aphids were treated with proprietary commercial insecticide formulations. For aphids these included acetamiprid (Intruder®), thiacloprid (Calypso®), endosulfan (Thiodan®), thiamethoxam (Cruiser®) and pirimicarb (Pirimor®) 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 acetamiprid, thiacloprid and thiamethoxam are all from the same neonicotinoids group. Mite treatments were, bifenthrin (Talstar®), abamectin (Agrimec®), propargite (Comite®), chlorfenapyr (Intrepid®) and diafenthiuron (Pegasus® as CGA140408). With the introduction to Australia of Bollgard II® cotton the use of insecticides to control pests has dramatically reduced. For this reason the organophosphate profenofos (e.g. Curacron®) is no longer available in Australia and is no longer included in our resistance monitoring.

Cotton aphid
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) and each field strain cultured separately on pesticide-free cotton (Deltapine 90) at 25 ± 4 ^{o}C under natural light. Strain integrity is assured by maintaining populations in purpose built insect proof cages.

Aphid Bioassay. Aphids were tested by placing them in a 35 mm Petri dish on an excised cotton plant leaf disc fixed in agar (Herron *et al*. 2001a). Briefly, batches of ten adult female aphids per leaf disc were then sprayed with the aid of a Potter spray tower. Each test was replicated 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 after which mortality was assessed.

Aphid Molecular Assay. Pirimicarb and organophosphate resistance were detected via established methods (M^{c}Loon and Herron 2006). Briefly, DNA is isolated from a pool of 20 aphids in addition to 10 individual aphids from each of the different field strains. Both the pool of DNA (from the 20 aphids) plus the 10 individual aphid DNA extractions were subject to PCR amplification of the *Ace*1 gene (covering the mutation responsible for resistance) using real time 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 chlopyrifos-methyl) based on a second mutation within the *Ace*I gene. Agarose gel electrophoresis was performed to visualise the result of the 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 were collected from a range of cotton fields in NSW and Qld late in each cotton season 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 insecticide with the aid of a Potter spray tower as above. Each test was replicated and included a water only sprayed control. 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.

Results

Two-spotted spider mite. TSM was collected from the Gwydir and M^{c}Intyre Valleys during 2006-2007 season and Macquarie and Namoi valleys during 2007-2008 (Table 1). Resistance was detected against bifenthrin, chlorfenapyr and propargite during the 2006-2007 season. Resistance to chlorfenapyr was not detected in the following 2007-2008 season however resistance was detected to abamectin (Table 1).

Aphid Bioassay. Cotton aphid strains were collected more widely than TSM with samples isolated from the Gwydir, M^{c}Intyre and Macquarie Valleys in 2006-2007. In this season, pirimicarb resistance was restricted to the Macquarie Valley only (Table 2). In 2007-2008 survivors at the discriminating dose were detected against pirimicarb in one strain and against acetamiprid in two cotton aphid strains (Bin WF and Blan F3) suggesting *Prima Facie* resistance to this insecticide..

Aphid Molecular Assay. The molecular testing for pirimicarb and general organophosphate (OP) resistance of the 2006/2007 aphids identified resistance in a single strain (Wil 21B) in agreement with the strains' bioassay data. However, when the strain was retested months later (using molecular and bioassay) it had lost the resistance profile to both insecticides. The remaining aphid strains all had a susceptible profile for pirimicarb and general OP resistance. Testing of the 2007/2008 aphid strains identified two with a pirimicarb resistant profile (Table 2). One strain, RvlndMo, had a definitive pirimicarb resistance profile. The other strain, Bin WF, had a mixed profile indicative of a strain with low levels of pirimicarb resistance (approximately 5%).

Additional baseline generation for Calypso® showed the minimum effective dose required to kill strain Car 13 was equivalent to the discriminating dose (Figure 1).

Discussion

Despite the overall reduction in sprays associated with Bollgard II®, resistance causing aphid control failure was still an issue in the Macquarie Valley. One strain, Wil 21B, was shown to be highly pirimicarb resistant with associated resultant control failure. The strain was confirmed to have both pirimicarb and chlopyrifos-methyl (Rescue®) resistance via molecular testing and pirimicarb resistance via bioassay. However, when strain Wil 21B was re-tested some four months later the resistance had completely disappeared and was not detected with either bioassay or molecular methods. Reversion of pirimicarb resistance is unlikely however since the mutation giving rise to it is particularly stable. It is more likely a case of mixed aphid cultures present at the collection site or an overall change in the clonal dominance within the strain. The first bioassay result identified 6% of pirimicarb susceptible

individuals (either intra or inter strain variants). It is this pool of aphids that has given rise to the next dominant clone, which when tested four months later (in the absence of selection) was pirimicarb susceptible. The 2007/2008 season has produced a single highly pirimicarb resistant strain from St George and a strain from Gwydir showing a low level of resistance. The Gwydir strain was also shown, via bioassay, to have some acetamiprid resistance.

For the first time cotton aphid has survived a discriminating dose of acetamiprid giving a *Prima Facie* detection of resistance. However, additional research is required to confirm the *Prima Facie* acetamiprid resistance that will require survivors from each of the strains being transferred to new cultures and allowed to breed. These new strains will then be subjected to full log-dose probit analysis and their response compared to established baseline data. Only if significantly different will acetamiprid resistance in cotton aphid be confirmed. Any strains showing resistance to acetamiprid will also be evaluated fully against the other neonicotinoids to evaluate the degree of cross resistance. This is important as another neonicotinoid, imidacloprid, is widely used in cotton as a seed treatment (Gaucho) and thiamethoxam is also a seed treatment (Cruiser). This use pattern may be important in selecting for resistance in aphids. If neoncotinoid resistance is confirmed then we may need to reconsider the positioning of the products and use patterns in the insecticide resistance management strategy.

The discriminating dose used for thiacloprid was interpolated from the dose response for cotton aphid strain Susceptible A. The 0.04 g / L chosen was midway between the calculated $LC_{99.9}$ and $LC_{99.99}$ level of response (ie 0.02- 0.054 respectively). However, the additional baseline data showed the minimum effective dose required to kill strain Car 13 was equivalent to the discriminating dose. For that reason the discriminating dose for thiacloprid has been increased for season 2007-2008 to 0.05 g / L to avoid false positive results.

For the third time resistance in cotton aphid has been diagnosed with both molecular and conventional bioassay methods. There is good agreement between the methods and molecular tests will soon be included as part of the routine resistance monitoring.

Propargite, chlorfenapyr and bifenthrin resistance were again detected in two-spotted mite for season 2006/2007 with abamectin resistance detected the following season as well, though chlorfenapyr resistance was not. However this probably reflects the limited number of strains that were collected. It is not encouraging that bifenthrin resistance was detected in both seasons at a discriminating dose mortality of less then 50% despite the rather small sample. Clearly bifenthrin resistance is persisting despite changes to the resistance management strategy and the overall reduction of insecticide use associated with the introduction of Bollgard® II cotton

Resistance to propargite or abamectin tends to be unstable and resistance will continue to be detected however as long as the current strategy is adhered to resistance generally disappears. Abamectin is the 3rd most common insecticide used on Bollgard II and Emamectin is the second most common insecticide used on conventional cotton this probably exposes mites to reasonably consistent selection hence resistance occurs. Propargite resistance is more difficult to understand it's hardly used at all and may be a consequence of cross resistance or from high propargite selection on the field it was collected from.

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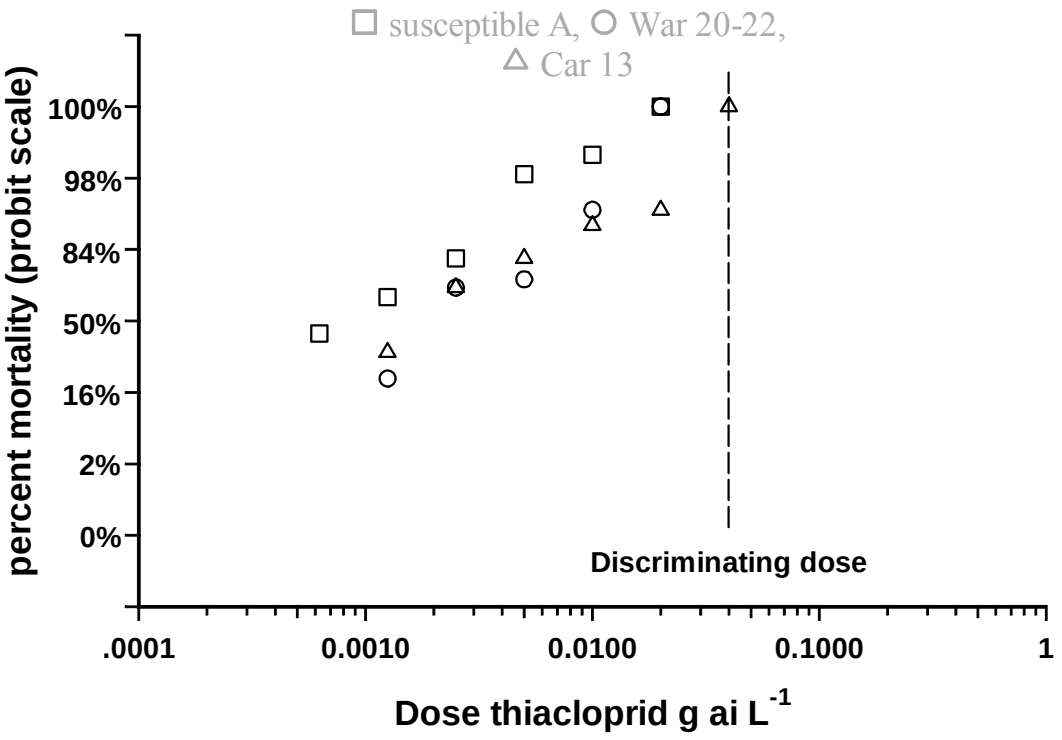

Figure 1. Dose response for Susceptible A, and field strains War 20-22 and Car 13 against Calypso® (thiacloprid) with the 2006/2007 discriminating dose superimposed

Table 1. Percent mortality at the discriminating dose (ie percent susceptible) for various strains of TSM collected during season 2006-2007 and 2007-2008 and evaluated for resistance against Talstar®, Intrepid®, Agrimec®, Comite® and Pegasus® (CGA-140408)

Season	Strain	Area	Chemical				
			Talstar®	Intrepid®	Agrimec®	Comite®	Pegasus® CGA140408
2006-2007	AU	Gwydir	99	100	100	97	100
	NO	Gwydir	45	94	100	100	100
	W	M^{c}Intyre	100	98	100	100	100
2007-2008	WA	Macquarie	46	100	94	99	100
	WI	Namoi	40	100	100	100	100

Table 2. Pirimicarb and Organophosphate (OP) susceptibility using molecular diagnosis and percent mortality at the discriminating dose (ie percent susceptible) using bioassay for various strains of cotton aphid collected during season 2006-2007 and 2007-2008

Season	Strain	Area	Molecular	Test	Bioassay	Chemical					
			*Ssp*I (Pirimicarb)	*Pdi*I (OP)	Pirimor®	[a] Rescue®	Thiodan ®	Intruder®	Pegasus® CGA140408	Calypso®	Cruiser®
2006-2007	Aus Mid 23	Gwydir	S	S	100	100	100	100	100	100	Not tested
	Car 34	M^{c}Intyre	S	S	100	100	100	100	100	100	Not tested
	War 20-22	M^{c}Intyre	S	S	100	100	100	100	100	100	Not tested
	Alch 007	M^{c}Intyre	S	S	100	100	100	100	100	100	Not tested
	Car 13	M^{c}Intyre	S	S	100	100	100	100	100	100	Not tested
	Nor 4	Gwydir	S	S	100	100	100	100	100	100	Not tested
	Byr 55	Macquarie	S	S	100	100	100	100	100	100	Not tested
	Bur 4	Macquarie	S	S	100	100	100	100	100	100	Not tested
	Wil 21B	Macquarie	R & S$^{\Psi}$	R & S$^{\Psi}$	6 & 100$^{\Psi}$	100	100	100	100	100	Not tested
2007-2008	Bel P	St George	S	S	-	-	100	Nf	Not tested	100	100*
	Glen vol	Upper Namoi	S	S	-	-	100	100	Not tested	100	100
	War vol	Upper Namoi	S	S	100*	100*	100	100	Not tested	100	100
	Gos vol	Darling Down	S	S	-	-	100	Nf	Not tested	100	Nf
	Ovr	St George	S	S	-	-	100	100*	Not tested	100	100*
	St G F 134	St George	S	S	-	-	100	100*	Not tested	100	Nf
	Red vol	Gwydir	S	S	-	-	100	100*	Not tested	100	100*

Season	Strain	Area	Molecular	Test	Bioassay	Chemical					
			*Ssp*I (Pirimicarb)	*Pdi*I (OP)	Pirimor®	[a] Rescue®	Thiodan ®	Intruder®	Pegasus® CGA140408	Calypso®	Cruiser®
	Wil F5 vol	Lower Namoi	S	S	-	-	100	100*	Not tested	100	100
	Ros F3 vol	DarlingDown	S	S	-	-	100	Nf	Not tested	100	Nf
	Bin W F	Gwydir	S[#]	S	100	-	100	78	Not tested	100	100
	Blan F3	St George	S	S	-	-	100	96	Not tested	100	100
	Ash vol	St George	S	S	-	-	100	100*	Not tested	100	100*
	M rocks	St George	S	S	-	-	100	100	Not tested	100	100
	Oak C vol	Darling Down	S	S	-	-	100	100*	Not tested	100	100*
	BrkGlenF3	St George	S	S	-	-	100	100	Not tested	100	100*
	Plan Fa F3	St George	S	S	100	-	100	100	Not tested	100	100
	Rvlnd Mo	St George	R	R	7	30	100	100	Not tested	100	100*
	Brk F133-1	St George	S	S	-	-	100	100	Not tested	100	100*

Nf = Not finished
* = Not replicated
[a] = Lorsban® used in 2007-2008
[Ψ] = See discussion
S = Susceptible
R = Resistant
[#] = Low level of resistance (<5%)
- = Not tested unless molecular assay detects resistance (War vol and Plan Fa F3 tested as negative controls)

Appendix 4

Synopsis of preliminary thrips culturing experimentation

Method 1

- Mirids sourced from Mozza Khan QDPI Kingaroy
- Mirids were dispatched by overnight courier from Qld. Mirids were in converted takeaway food containers with whole loose beans as a supplementary food source.
- On arrival the mirids were put into an insect proof cage in a research mass culture insectary at 26 deg with a whole cotton plant and the containers opened.
- Most mirids died close to the open containers suggesting damage in transit. Possible physical damage from the loose bean pods.
- Culture did not establish with all mirids dead within a few days of arrival

Method 2

- Mirids sourced from Judy Nobilo CSIRO Narrabri
- Mirids were dispatched by overnight courier and where in small plastic containers with cotton wicks
- On arrival insects were put into muslin covered 30 cm dia cages in an insectary container at 26 deg and the transport containers opened. Mirids were then maintained on bean pods set in water agar plus an aqueous honey solution with wick.
- Deaths from transport were much less than method 1.
- Beans were replaced weekly and honey solution as required.
- Mirids survived in the cages for 2 weeks but although two juveniles were seen none survived.
- Possibly juveniles escaped through the muslin or were trapped in the mould encrusted water agar.

Method 3

- Mirids sourced from Lee Austin DPI Narrabri
- Mirids were dispatched by overnight courier on the 18/04/06 with mirids contained in small flat Petri dishes with secured food that could not move. Also some mirids in small jars.
- Transport survival was best so far
- On arrival the mirids were put into an insect proof cage in a research mass culture insectary at 26 deg with a whole cotton plant and whole surface sterilised bean pods and honey solution in a Petri dish covered with Para film. A Lucerne plant also available. Fresh beans added every second day.
- 50% probably died in the first week but juveniles were produced and subsequent generations established. Even so numbers slowly declined over three months when the cage was disassembled and remaining mirids counted.
- One adult and one nymph were found.
- We conclude it impossible to manipulate mirids in big cage because they are so good at hiding

Method 4

- Mirids sourced from method 3 cage
- Two mirids, one adult one juveniles were put in a small glass jar
- Whole bean pods included plus honey solution in a Petri dish covered with Para film.
- Jar covered with 112 micron gauze
- Mirids survived 2 weeks but no juvenile production
- Post mortem confirmed both mirids female- Oops
- This method most promising so far but…….

Method 5

- Green mirid looking insects found infesting an onion thrips culture sourced from Jianhua Mo at Yanco
- Insects sent to Orange and confirmed as green mirid.
- Twelve adult mirids removed from the onion thrips culture using a vacuum pump powered pooter. Four mirids were killed via the pooter by being crushed via excessive vacuum. Therefore a high vacuum pooter may not be a workable method for manipulating mirids yet if vacuum is not relatively high mirids escape the pooter.
- Eight surviving mirids transferred into a large 'thrips' cage with onion as a food source. The onion was supplemented with a sugar solution, pollen and fruit fly eggs.
- Mirid culture slowly died out.

Method 6

- Mirids collected from EMAI lucerne 13/09/07 by Grant Herron
- 8 L plastic container previously prepared by cutting a hole and inserting gauze
- 250 ml of agar prepared 1% w/V
- Agar into a takeaway container and allowed to cool
- Bean pods washed in distilled
- When agar cool but not set then half bean pods added.
- Solid brown sugar placed in a 35 mm petri dish and added to the ventilated cage
- 9 mirids co2 gassed for ca. 20 s and added to the cage
- Cage maintained at 27^{o}C 10L:14D in a growth cabinet
- Cages re-cultured as required
- Numbers increased and successfully maintained in culture without more being added until destroyed on 01/05/08
- A preliminary bioassay using a Potter spray tower was successful

Appendix 5

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

Resistance development a possibility in mirids from Australian cotton

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Abstract

With the introduction of Transgenic Bollgard® II cotton Australian populations of green mirid have required targeted insecticidal control, which may select for insecticide resistance in this pest. Unfortunately, no methods are available in Australia to detect resistance or to establish the baseline data used to confirm resistance. To achieve that a simple method is required to culture and maintain a reference strain(s) of mirids. Here we describe a method to breed and culture green mirids, which is a first step toward development of a resistance monitoring program.

Introduction

With the introduction of transgenic cotton, sucking insect pests have become more troublesome, so requiring increased targeted insecticide control. This brings with it the risk of insecticide (or miticide) resistance. Two-spotted mite has a proven ability to develop resistance if targeted with miticides and has recently developed resistance to chlorfenapyr (Intrepid®)(Herron *et al*. 2004a). Similarly, high-level organophosphate and carbamate resistance has developed in cotton aphid (Herron *et al*. 2004).

Other sporadic, but troublesome, sucking pests include green peach aphid *Myzus persicae* (Sulzer), bean spider mite *Tetranychus ludeni* Zacher, thrips (including western flower thrips) and green mirids, *Creontiades dilutus* (Stal)(Forrester and Wilson 1988). Green mirid in particular are a serious pest in Bollgard II® crops. This is due to the reduction in insecticides used against *Helicoverpa* spp., which formerly also suppressed the mirids. There is now an increase in sprays specifically targeted against green mirids, with high reliance on Regent® (fipronil), which accounts for about 70% of sprays and organophosphates (omethoate and dimethoate) which account for about 20% of sprays. 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.

Pre-emptive baseline data is critical in resistance management as it establishes the natural range in susceptibility of a particular pest to insecticide before the insecticide is used widely. The response of insects collected after the insecticide is used can then be compared back to this baseline data. Such data has been critical to management of other sucking pests, such as cotton aphid because resistance could quickly be confirmed. However, no baseline data for mirids currently exists, preventing an early confirmation of resistance and subsequent resistance management.

This is now a serious concern because mirids have increasingly required targeted control, to the extent that the use of OP's against mirids has adversely affected the resistance management strategy for aphids during the 2003 -2004 cotton season (Herron *et al.* 2004). This was because the use of OP's against mirids also selects for resistance to this group of insecticides in aphids. The sustainable chemical control of mirids would be greatly enhanced by the pre-emptive generation of baseline data

for resistance monitoring. This would enable us to receive mirids collected from cotton crops, establish them in culture to increase numbers, then screen them for resistance to a range of insecticides.If resistance develops Australian growers could face increased control costs and/or loss of yield and delayed maturity with resultant loss of fibre quality that could easily damage Australia's reputation as a producer of quality cotton.

Here we outline a method to culture mirids so that we can produce enough insects for baseline bioassay.

Material and methods

Below we describe a method we have developed to culture green mirids. It has been a long and complicated process, with continual problems with mirids dying in transit or the cultures slowly dying out. The technique we present, though labour intensive, will reliably produce mirids in sufficient numbers for resistance testing.

Rearing cages
Eight litre plastic containers were prepared by cutting a hole and inserting a ventilation gauze into the lid. Mrids were added to the rearing cage and fed with green bean pods that were washed in 1% bleach and rinsed in distilled water to remove any contaminants or pesticides. The bean pods were kept alive by placing them into a take away food container which had 250 ml of water agar (1% w/V) in the base. After the agar was poured into container it was allowed to cool, but before it had set the bean pods were added, pushing the end into the agar. The container with beans was placed into the culture cage. Supplementary food was supplied in the form of a lump of solid brown sugar and yeast that was placed in a 35 mm petri dish and added to the ventilated cage.

Culture of Mirids
Mirids were collected from lucerne at EMAI on the 13/09/07. From this collection 9 adult mirids were stunned briefly (about 20 seconds) with carbon dioxide gas, to make handling easy and added to the cage. The cage was placed in a growth cabinet to maintain constant conditions of 27^{o}C and 10L:14D. After 7 days and subsequently twice weekly adult mirids were removed from the bean pods and the old pods plus agar transferred to a new cage to which additional fresh pods were added. The original cage(s) with adult mirids then had fresh bean pods in agar were added thus repeating the process. The process was repeated with old pods being removed from the adult mirids to new cages with additional food. These were left until adult mirids developed that could be used for testing.

Results

Numbers of mirids increased from 9 adults to hundreds and were successfully maintained in culture without more being added until the mirids were destroyed on 01/05/08. In that time enough mirids were produced for a preliminary bioassay on. The bioassay involved treating leaf discs with fipronil and a water only sprayed control with the aid of a Potter spray tower to deliver a repeatable dose. The results showed that the treated mirids died and the untreated controls survived so validating our initial bioassay trial.

Discussion

Cornford and Simpson (Undated) tested field collected Australian green mirids from cotton to ascertain the relative potency of potential chemicals used for their control. Their method required that mirids be temporarily housed in plastic containers with food for two days prior to use and permanent reference colonies were not established. This has the disadvantage that reference strains cannot be re-evaluated at a later date to confirm results or test new insecticides.

Here we have demonstrated that, with perseverance, it is possible to establish and maintain a reference strain of mirids and produce enough insects for subsequent bioassay. By achieving this we have achieved the first step in the development of a method to establish baseline bioassay data for the purpose of resistance monitoring.

It was clear very early, that mirids are very fragile and do not transport readily. This has implications for future resistance monitoring as specific methods will have to be developed so that suspect resistant strains arrive in good condition. Consequently it is desirable to develop molecular based methods for resistance monitoring as soon as practical to reduce or eliminate the need for routine field strain culturing.

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