Research Article

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Insecticide resistance management of *Bemisia tabaci* (Hemiptera: Aleyrodidae) in Australian cotton – pyriproxyfen, spirotetramat and buprofezin

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Abstract

BACKGROUND: *Bemisia tabaci* is a globally significant agricultural pest including in Australia, where it exhibits resistance to numerous insecticides. With a recent label change, buprofezin (group 16), is now used for whitefly management in Australia. This study investigated resistance to pyriproxyfen (group 7C), spirotetramat (group 23) and buprofezin using bioassays and available molecular markers.

RESULTS: Bioassay and selection testing of *B. tabaci* populations detected resistance to pyriproxyfen with resistance ratios ranging from 4.1 to 56. Resistance to spirotetramat was detected using bioassay, selection testing and sequencing techniques. In populations collected from cotton, the A2083V mutation was detected in three populations of 85 tested, at frequencies ≤4.1%, whereas in limited surveillance of populations from an intensive horticultural region the frequency was ≥75.8%. The baseline susceptibility of *B. tabaci* to buprofezin was determined from populations tested from 2019 to 2020, in which LC_{50} values ranged from 0.61 to 10.75 mg L^{-1}. From the bioassay data, a discriminating dose of 200 mg L^{-1} was developed. Recent surveillance of 16 populations detected no evidence of resistance with 100% mortality recorded at doses ≤32 mg L^{-1}. A cross-resistance study found no conclusive evidence of resistance to buprofezin in populations with high resistance to pyriproxyfen or spirotetramat.

CONCLUSIONS: In Australian cotton, *B. tabaci* pest management is challenged by ongoing resistance to pyriproxyfen, while resistance to spirotetramat is an emerging issue. The addition of buprofezin provides a new mode-of-action for whitefly pest management, which will strengthen the existing insecticide resistance management strategy.

Supporting information may be found in the online version of this article.

Keywords: insecticide resistance; pyriproxyfen; spirotetramat; buprofezin; target-site mutation; pest management

1 INTRODUCTION

The whitefly cryptic species complex, *Bemisia tabaci* are pests of food, fibre and ornamental crops, through direct feeding damage, deposition of honeydew and as a vectors of plant viruses.[1] Two invasive members of the complex are: *B. tabaci* Middle East-Asia Minor 1 (MEAM1) and *B. tabaci* Mediterranean (MED). The species complex is widely distributed globally from tropical to temperate climates.[2] In Australia, *B. tabaci* MEAM1 is a well-established pest of horticultural crops – both field and protected cropping as well as broad acre crops – primarily cotton and soybeans.[3,4]

As a result of their pest status and a history of developing resistance to insecticides,[5] *B. tabaci* MED and *B. tabaci* MEAM1 have been the focus of several insecticide resistance management (IRM) strategies[6–10] and integrated pest management (IPM) programs.[11–15] Adoption of selective insecticides, such as insect growth regulators (IGRs), the use of rotations between different insecticide modes-of-action (MoAs),[16] and the conservation of natural enemies are common themes in these programs. The common aim of these programs is achieving effective pest

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management while reducing the exposure of *B. tabaci* to insecticide resistance selection.

However, even with IRM strategies, rapid evolution of insecticide resistance in *B. tabaci* is a recurring problem globally to many MoAs,[5] including in Australia to the selective insecticides pyriproxyfen (group 7C) and spirotetramat (group 23).[17,18] Pyriproxyfen has been used regularly to control *B. tabaci* MEAM1 since 2002, and incidences of resistance in cotton and horticulture were reported in the mid-2000s.[17] The registration of spirotetramat occurred in 2009 for a range of pests, in fruit, vegetable and cotton crops. Spirotetramat-resistant *B. tabaci* MEAM1 were first detected in 2016.[18]

The IGR, buprofezin (group 16) was developed in the early 1980s,[19] but it was not until 2020 that it was registered for the control of *B. tabaci* in Australia. Prior use had been restricted to the control of scale, leafhopper and mealybug pests of tree and vine crops. Buprofezin acts specifically against homopteran pests, via contact, vapour activity and limited translaminar movement.[20] The insecticidal activity of buprofezin inhibits chitin biosynthesis, resulting in nymphal death during ecdysis[21] and the suppression of egg hatch, following female whitefly exposure to treated leaves.[20]

In response to the inclusion of buprofezin in the cotton IRM strategy, we surveyed *B. tabaci* populations to determine their resistance status to pyriproxyfen and spirotetramat, owing to the likelihood of these insecticides being used interchangeably or as part of a rotation with buprofezin. This study tested the baseline susceptibility of *B. tabaci* to buprofezin using a bioassay and from that data developed a discriminating dose. Finally, to explore the potential of cross-resistance,[22] populations with known resistance to pyriproxyfen and spirotetramat were assayed with buprofezin.

2 MATERIAL AND METHODS

2.1 Insect collections and plants

Whitefly (*B. tabaci* species complex) populations were collected from field crops (primarily cotton) grown in Queensland (QLD) and New South Wales (NSW), following insecticide use between 2017 and 2021 (Fig. 1; Supporting Information Table S1).[17,23] Adult whiteflies were collected directly from crops using a handheld vacuum (modified BG75 leaf blower; Stihl, Waiblingen, Germany), transported to the laboratory in small cages and then aspirated into larger rearing cages containing clean cotton plants. A small number of populations were established from leaf collections infested with late instar whitefly nymphs sent to the laboratory by agronomists. Whitefly populations then were reared under glasshouse conditions [25 °C; 60% relative humidity (RH)] for several discrete generations until they were used in bioassays. Our study included a susceptible reference strain (SU07-1) of *B. tabaci* MEAM1, which has been in continuous culture since 1995, with no exposure to insecticides.

Reference *B. tabaci* MEAM1 strains with resistance to pyriproxyfen (AY09-1R) and spirotetramat (AY16-1R) were established in 2009 and 2016, respectively, from field populations in Ayr, QLD with incipient resistance. Each strain was kept under constant selection pressure – AY09-1R with 320 mg L^{-1} pyriproxyfen and AY16-1R with 1000 mg L^{-1} spirotetramat

Cotton plants, *Gossypium hirsutum*, variety Sicot 714B3F were used in bioassays and as a host plant to maintain the whitefly populations. Plants were grown from seed in pots for a month in a controlled environment room (29 °C, 70% RH, 16 h:8 h, light:dark photoperiod) with artificial light, then transferred and grown in a glasshouse (25 °C, 60% RH) with natural light, until used in assays or whitefly rearing. Plants not utilized for experiments within a month of transfer to the glasshouse were disposed of to reduce the risk of other pests (such as mites and aphids) establishing.

2.2 Insecticides and dose–response bioassays

The toxicity of buprofezin, 440 g L^{-1} (Applaud®; Corteva, Frenches Forest, NSW), pyriproxyfen 100 g L^{-1} (Admiral® Advance; Sumitomo Chemical Australia, Epping, NSW) and technical grade spirotetramat (Bayer CropScience, Hawthorn East, VIC) to *B. tabaci* MEAM1 was determined in dose–response bioassays. A stock solution of technical grade spirotetramat was prepared in acetone and then diluted to the required concentration for bioassays.[24] A nonionic surfactant (Agral®) at 100 mg L^{-1} was included in all bioassays. Data from Crop Consultants Australia's market audit, a survey that covers 30–40% of the industry, were used to calculate the amount (g ha^{-1} ai) of pyriproxyfen, spirotetramat and buprofezin that was applied between 2014 and 2021.

2.2.1 *Bioassay for screening B. tabaci resistance to pyriproxyfen and spirotetramat*

Bemisia tabaci MEAM1 populations were screened for the presence of resistance to pyriproxyfen and spirotetramat using leaf-dip bioassays as described previously.[17,25] The toxicity of pyriproxyfen against whitefly eggs was tested in a dose–response bioassay with five treatment doses ranging from 0.001 to 10 mg L^{-1}, replicated five times with a minimum of 150 eggs tested per replicate and mortality assessed at 10 days post-treatment. As determined from an earlier study,[17] survival at the discriminating dose of 10 mg L^{-1} indicated a resistant population. The spirotetramat bioassay tested toxicity against 2nd instar whitefly nymphs at five treatment doses from 1 to 100 mg L^{-1}, replicated five times with a minimum of 150 nymphs tested per replicate, with an assessment of mortality at 11 days after treatment. From 2019, the discriminating dose for spirotetramat was set at 100 mg L^{-1}.[25] A control consisting of diluent only (100 mg L^{-1} Agral®) was included in all assays.

2.2.2 *Resistance selection testing of populations with pyriproxyfen and spirotetramat*

In order to further investigate resistance, populations with survivors at the discriminating dose were placed under selection pressure with the relevant insecticide, either pyriproxyfen or spirotetramat. For each selection test, an additional plant was placed into the rearing cage for 2 days so adult whitefly could oviposit on it. The plant then was placed into a separate cage, and an initial dose higher than the discriminating dose was applied. Pyriproxyfen selection started at 30 mg L^{-1} and was sprayed while eggs were present, whereas for spirotetramat selection testing, formulated insecticide (Movento®, 240 g L^{-1}) was sprayed at 1000 mg L^{-1} approximately 10 days later, targeting the 2nd instar nymph stage. If there was significant population survival, the response to selection was evaluated via a laboratory bioassay using a higher dosage range. For some populations, the glasshouse selection continued for several generations, with increasing doses, up to 300 mg L^{-1} for pyriproxyfen and 3000 mg L^{-1} for spirotetramat.

2.2.3 *Susceptibility of B. tabaci to buprofezin, evaluation of cross-resistance to pyriproxyfen and spirotetramat*

In order to determine the baseline susceptibility of *B. tabaci* MEAM1 to buprofezin, concentrations in the range 0.32–100 mg L^{-1} were tested, to create a mortality range of 10–100%.

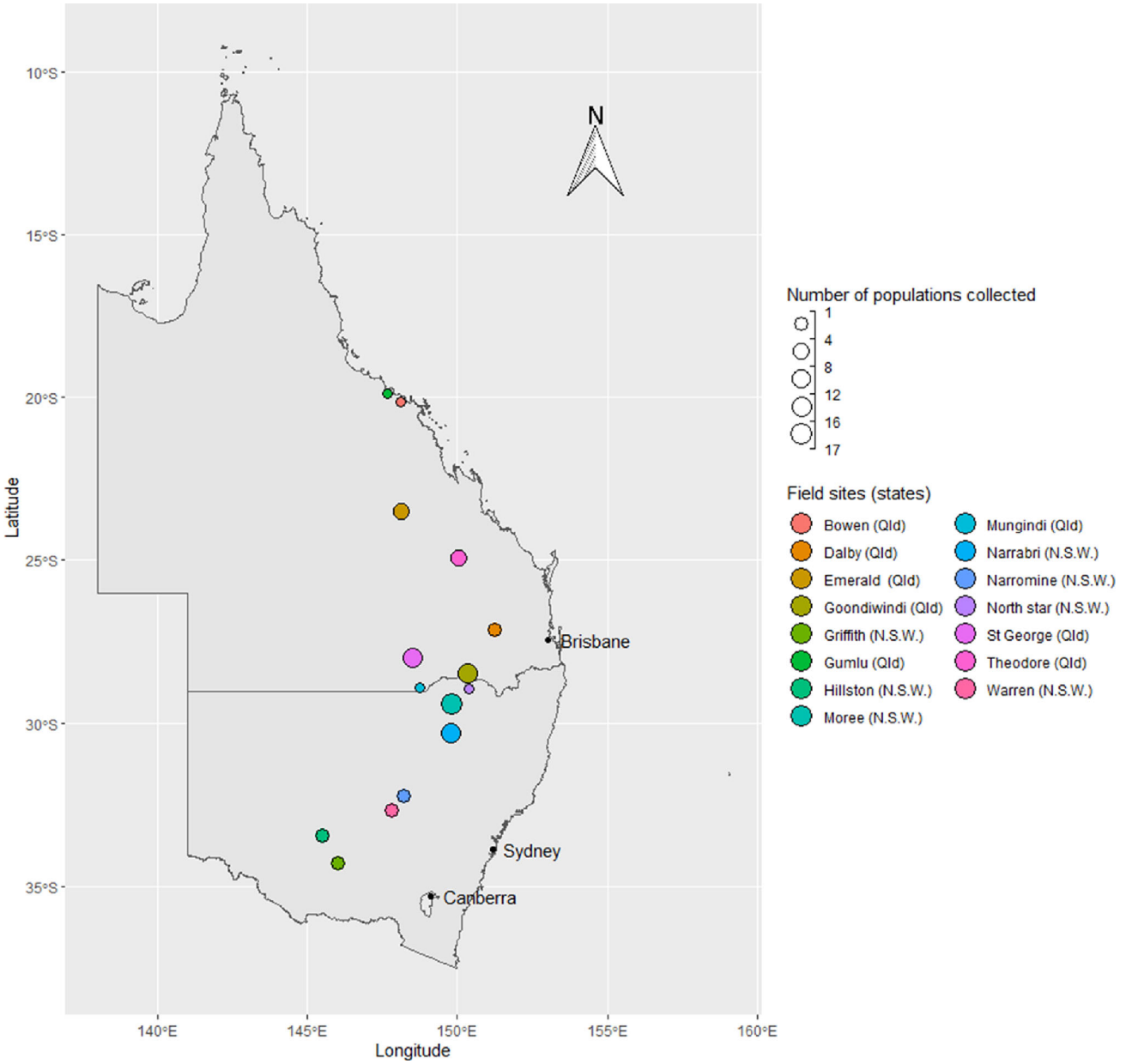

Figure 1. *Bemisia tabaci* collection sites (2017–2021) from Queensland (QLD) and New South Wales (NSW).

Each treatment was replicated five times and each bioassay included a control of 100 mg L^{-1} Agral®, also replicated five times. The minimum number of insects tested per replicate ranged from 100 to 150, in general 20–30 insects were tested per experimental unit. The bioassay procedure followed a modified version of the Insecticide Resistance Action Committee (IRAC) test method for whitefly nymphs (number 16).[26] In place of whole plants for each concentration, detached leaves were used, similar to a bioassay already used for spirotetramat.[25] The bioassay tests susceptibility of nymphs at an early stage of development; settled 1st and 2nd instar nymphs. Bioassays were kept in a constant temperature room (27 °C, 60% RH, 14 h:10 h, light:dark photoperiod) and mortality of nymphs was assessed at 10 days post-treatment, using a stereo microscope to record live and dead nymphs. After 2 years (2019–2020) of evaluating the baseline susceptibility of buprofezin, 1 year (2021) of resistance testing was conducted using the same bioassay methodology.

In order to evaluate if resistance to pyriproxyfen or spirotetramat confers cross-resistance to buprofezin, the susceptibility of reference laboratory resistant strains was tested. The laboratory populations included AY09-1R which is resistant to pyriproxyfen and AY16-1R with resistance to spirotetramat.

2.3 Screening for A2083V and A2151V mutations

From each population, a subsample of insects was screened for the presence of two known mutations linked to spirotetramat resistance in *B. tabaci*, A2083V and A2151V.[18] Insects were preserved in 90% ethanol and kept in a freezer at −20 °C until DNA extraction. For each population, DNA was extracted from 30 to 120 individuals using DNeasy Blood & Tissue kit as per the manufacturer's instructions (Qiagen, Hilden, Germany). The amplicons for screening the resistance alleles were generated by modified gene-specific primers.[18] The modified gene-specific primers for generating A2083V and A2151V amplicons have two

components, the linker sequences (in bold) and the gene-specific primer. The linker sequence **5′-[i5]TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′** is attached to the forward primers, whereas **5′-[i7] GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′** is attached to the reverse primers ([i5] and [i7] are the attachment sites for the Illumina adapters). The gene-specific primers are; A2151Vfor 5′-TGTGCTTGAGCCGGAAGGAA-3′ and A2151Vrev 5′-CTGGTGATACATGGGTTTCAAGT-3′; A2083Vfor 5′-AGTAATGAAATTCGGTGC-3′ and A2083Vrev 5′-ATCGGCGTACATTTCCATATGTC-3′. All PCRs were performed using Platinum Taq (Invitrogen, Carlsbad, CA, USA). The thermal cycles for the amplicons were 95 °C for 3 min followed by 45 cycles of 95 °C for 30 s, 54.5 °C for 45 s, 72 °C for 30 s and 72 °C for 5 min for a final extension. High throughput sequencing libraries were prepared as per the Illumina protocol (# 15044223 Rev. B; Illumina, San Diego, CA, USA) with modifications as described previously.[23,27]

2.4 Data analysis

Nonpooled bioassay data were analyzed using GENSTAT 19.[28] Lethal concentration values (LC_{50}, LC_{99} and $LC_{99.9}$) plus their 95% fiducial limits were calculated using the probit method outlined by Finney[29] with control mortality correction.[30] Heterogeneity was checked during analysis using a chi-square test and if significant at the 5% level, the variance of the estimated parameter was scaled by the corresponding heterogeneity factor equal to the residual mean deviance.[29] The mortality response of populations was considered different if there was no overlap between their corresponding 95% fiducial limits. This method was used to evaluate vigour tolerance in field populations and the presence of cross-resistance in the reference resistant strains. The minimum effective concentration (MEC) was determined from the bioassay data and is defined as the lowest concentration required to kill 100% of insects in all replicates.[31] Discriminating doses[17,25] were used to distinguish resistant from susceptible populations. To compare resistance levels between populations, the resistance ratio (RR) and the 95% confidence interval were calculated using the methodology outlined by Robertson and Jones.[32] The level of resistance was classified by using RRs in terms described by Torres-Vila *et al.*[33]: susceptibility (RR = 1), tolerance to low resistance (RR = 2–10), moderate resistance (RR = 11–30), high resistance (RR = 31–100), very high resistance (RR > 100).

High-throughput sequencing data analysis was completed using GENEIOUS PRIME 2021.2.2 (https://www.geneious.com) following the analysis pipeline described in Tay *et al.*,[27] Raw FASTQ reads were imported as paired reads and quality trimmed using BBDuk Adapter/Quality Trimming Version 38.84[34] with parameters set to minimum quality of Q = 30, minimum overlap of 25 bp and minimum length of 135 bp. The trimmed paired reads then were merged, contigs were verified using TBLASTN[35] to confirm that the correct gene regions were amplified. The merged reads were mapped to the reference acetyl-CoA-carboxylase gene (ACCase) (MN567039.1), using GENEIOUS MAPPER with sensitivity set to Medium Sensitivity/Fast. After mapping, single nucleotide polymorphisms (SNPs) were called when >1% (i.e. variants observed at less than this frequency will not be called).

3 RESULTS

3.1 Screening populations for resistance to pyriproxyfen and spirotetramat

Using a discriminating dose of 10 mg L^{-1}, 18 of 69 populations tested from cotton production regions (Table S2) were resistant to pyriproxyfen, with resistance ratios from 4.1 to 56 (Table 1). Control mortality across all assays ranged from 0% to 6% ($\bar{x}$ = 1.4%). In resistant populations, at 10 mg L^{-1} mortality ranged from 96% to 99.6%, with viable nymphs always present. At 1 mg L^{-1}, a dose that kills 100% of the susceptible laboratory

Table 1. Log-dose probit–mortality data and percentage mortality data for *Bemisia tabaci* MEAM1 with resistance to pyriproxyfen

Population	Year of collection	LC_{50} (mg L^{-1})	95% FL	Slope (SE)	Mortality (%) at 1 mg L^{-1} (*n*)	Mortality (%) at 10 mg L^{-1} (*n*)	RR* (95% CI)
Susceptible		0.019	0.014–0.024	2.64 (0.34)	100.0 (150)	-	
St George (a)	2018	0.693	0.447–1.099	1.07 (0.11)	51.7 (143)	96.0 (150)	37 (23–61)
St George (b)	2018	0.217	0.134–0.355	0.93 (0.09)	77.3 (150)	98.6 (150)	12 (6.9–20)
Goondiwindi	2018	0.075	0.046–0.123	0.80 (0.09)	78.7 (150)	99.3 (150)	4.1 (2.4–6.9)
Moree (a)	2018	0.265	0.189–0.374	1.22 (0.10)	79.3 (150)	99.3 (150)	14 (9.5–21)
Moree (c)	2018	0.334	0.214–0.530	0.97 (0.09)	64.7 (150)	98.0 (148)	18 (11–29)
Mungindi	2019	0.248	0.147–0.425	0.89 (0.01)	66.0 (150)	99.3 (148)	13 (7.7–23)
Narrabri	2019	0.184	0.142–0.239	1.05 (0.06)	83.3 (150)	99.3 (150)	9.9 (7.0–14)
Goondiwindi	2020	0.255	0.164–0.374	1.32 (0.12)	86.4 (150)	99.2 (241)	14 (10–20)
Moree	2020	0.268	0.208–0.337	1.46 (0.09)	77.1 (131)	99.3 (152)	14 (8.6–22)
Goondiwindi (b)	2021	0.199	0.136–0.278	1.42 (0.12)	84.1 (170)	99.6 (250)	11 (7.0–16)
Goondiwindi (c)	2021	0.097	0.075–0.124	1.04 (0.05)	87.9 (165)	98.4 (250)	5.2 (3.7–7.4)
Moree (a)	2021	1.048	0.738–1.391	1.89 (0.22)	32.7 (150)	97.2 (247)	56 (38–83)
Moree (b)	2021	0.461	0.376–0.557	1.51 (0.08)	74.0 (150)	97.5 (200)	25 (18–34)
Moree (c)	2021	0.565	0.355–0.831	1.42 (0.16)	40.7 (150)	99.2 (250)	30 (19–49)
Narrabri (a)	2021	0.219	0.134–0.334	1.09 (0.03)	69.4 (134)	97.2 (250)	12 (7.1–19)
Narrabri (b)	2021	0.278	0.154–0.463	0.94 (0.10)	76.2 (105)	99.5 (190)	15 (8.4–27)
Narrabri (c)	2021	0.185	0.128–0.258	1.13 (0.08)	76.0 (114)	99.2 (243)	10 (6.6–15)
Hillston	2021	0.671	0.367–1.100	1.23 (0.16)	28.3 (120)	99.2 (249)	36 (21–64)

* The resistance ratio (RR) is calculated by dividing the LC_{50} of the field populations by LC_{50} of the susceptible population (SU07-1).

Table 2. Phenotypic and genetic response of *Bemisia tabaci* MEAM1 following a single generation of selection with 1000 mg L^{-1} spirotetramat

Population	Selection	Year of collection	LC_{50} (mg L^{-1})	95% FL	Slope (SE)	Mortality (%) 100 mg L^{-1} (*n*)	RR* (95% CI)	Frequency (%) of A2083V
Laboratory Susceptible (SU07-1)			4.08	3.32–4.99	2.62 (0.27)	100 (140)		< 1
Field populations								
Emerald (b)	field	2019	6.09	3.68–9.48	1.65 (0.27)	97.3 (150)	1.5 (0.9–2.4)	1.4
	selected		41.2	16.09–96.22	0.58 (0.10)	62 (150)	10 (4.4–23)	94.7†
Emerald	field	2020	7.89	5.52–11.11	1.28 (0.14)	98.7 (149)	1.9 (1.3–2.8)	1.2
	selected		115.9	68.7–196	0.95 (0.12)	40.1 (137)	28 (17–48)	57.4
Dalby	field	2020	7.04	5.64–8.77	2.05 (0.18)	98.7 (150)	1.7 (1.3–2.3)	< 1
	selected		182.5	110.2–321.9	0.89 (0.11)	33 (150)	45 (26–77)	72.0
Narrabri (c)	field	2021	5.71	3.59–8.65	1.43 (0.21)	93 (143)	1.4 (0.9–2.2)	4.1
	selected		799.9‡	356.3–2946	0.50 (0.09)	28.8 (66)	196 (71.8–535.7)	89.8‡

* The resistance ratio (RR) is calculated by dividing the LC_{50} of the field populations by LC_{50} of the susceptible population (SU07-1).
† Sequencing based on sample collected after 3 rounds of selection (1000, 2000 and 3000 mg L^{-1}) over five generations.
‡ Bioassay completed after 2 rounds of selection (1000 and 1000 mg L^{-1}) over five generations.

strain, the mortality response of resistant populations was variable, ranging from 28.3% to 87.9%. Selection testing with pyriproxyfen also produced variable responses (Table S3). With two exceptions, populations that survived the discriminated dose of 10 mg L^{-1} survived selection at 30 mg L^{-1}, but only five populations showed higher resistance, surviving exposure to 100 mg L^{-1}. Only one population [St George (b) 2018] survived selection testing at 300 mg L^{-1}.

Since the 2016–17 season, a total of 71 populations were tested by bioassay for spirotetramat resistance, with seven populations from cotton growing regions surviving the discriminating dose of 100 mg L^{-1} spirotetramat.[25] Control mortality was between 0% and 14.7% ($\bar{x}$ = 6.3%) (Table S4). Applying the discriminating dose developed in 2019 to dose–response data collected in 2017 and 2018 retrospectively detected spirotetramat resistance in three populations: St George 2017, Dalby 2017 and Griffith 2018 had 1.4%, 2.7% and 2.3% survival (respectively) at the discriminating dose. These populations were not subjected to selection testing.

Since 2019, resistance was detected in four populations from cotton-growing regions: Emerald 2019, Emerald 2020, Dalby 2020 and Narrabri 2021. When the populations were selected for resistance, a shift to a more resistant phenotype was detected by a subsequent bioassay of the survivors' descendants (Table 2). In each bioassay, the LC_{50} of the selected population was higher (with nonoverlapping fiducial limits) than the LC_{50} of the field population.

In 2019, two populations collected from the horticultural regions of Bowen and Gumlu were assayed with spirotetramat and found to be resistant, with 58.1% and 34.7% survival (respectively) at the discriminating dose (Fig. 2).

3.2 Frequency of A2083V and A2151V mutations

Screening for the presence of the acetyl-CoA carboxylase mutations, A2083V and A2151V (Fig. 3) was conducted in 87 field populations collected between 2017–2021 (Table S5). The A2083V mutation was detected above a frequency of 1% in five field populations, with three originating from cotton-growing regions (Table 2) and the remaining two from horticultural regions. In the populations collected from cotton, the frequency of A2083V ranged from 1.2% to 4.1%, whereas those from the horticultural regions Bowen and Gumlu had frequencies of 75.8% and 83.7% (respectively). The A2151V mutation was not confirmed in any of the populations. A single detection at a frequency of 1.5% was not considered credible, owing to the low total reads recorded in that sample (Table S3).

Sequencing of the four populations following spirotetramat selection showed the presence of the A2083V mutation at moderate to high frequencies (Table 2). The laboratory reference strains were used as controls during the sequencing study. In the reference resistant strain, AY16-1R, the A2083V mutation was present at a frequency of 97.2%, whereas in all other reference populations including the susceptible reference strain (SU07-1), the mutation frequencies were below the 1% sample call threshold (Table S2).

3.3 Susceptibility of B. tabaci to buprofezin and determining a discriminating dose

The baseline susceptibility of *B. tabaci* was determined from populations collected from 2019 to 2020 in QLD and NSW (Table 3). In total, 21 populations were tested, with bioassay control mortality ranging from 0% to 10.6% ($\bar{x}$ = 4.1%), with up to an

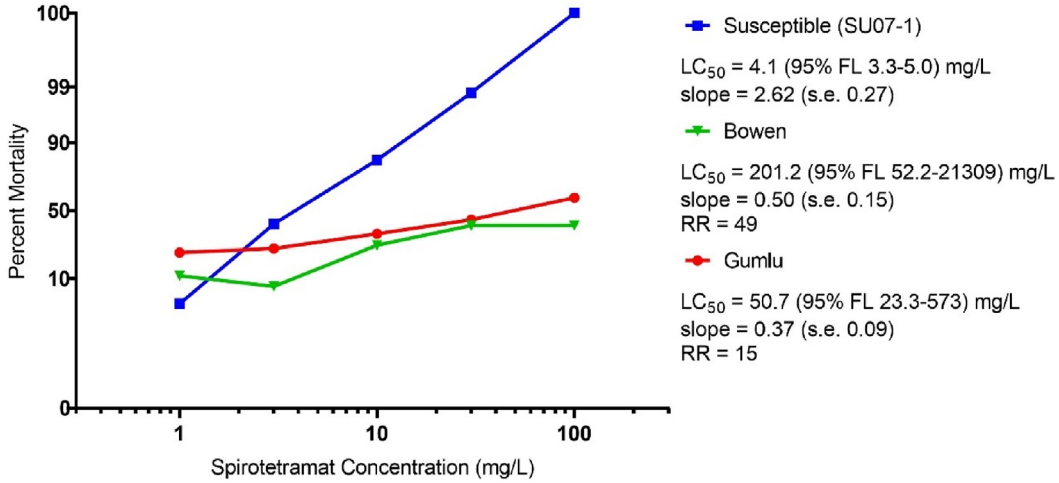

Figure 2. Dose–response of *Bemisia tabaci* MEAM1 to spirotetramat, as measured by percentage mortality for the susceptible reference population (SU07-1), as well as two field populations collected in 2019 from Gumlu and Bowen. LC, lethal concentration; FL, fiducial limit; RR, resistance ratio.

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MEAM1 A2083V resistant MN567039.1 TCATAATCTACATTCCTCCTAATGGTGAGTTGAGAGGAGGAGCCTGGGTCGTCGTTG
MEAM1 A2083V sus                  TCATAATCTACATTCCTCCTAATGGTGAGTTGAGAGGAGGAGCCTGGGCCGTCGTTG

MEAM1 A2151V resistant MN567039.1 CTTAAACAGAGGCTGTCCAGTACAGATATCAGTCCGGAGGAAAAGGTAGACGTAGA
MEAM1 A2151V sus                  CTTAAACAGAGGCTGTCCAGTACAGATATCAGTCCGGAGGAAAAGGCAGACGTAGA
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Figure 3. Alignment of partial *ACCase* gene from *Bemisia tabaci* MEAM1. Nucleotide sequences from the reference sequence (MN567039.1) with resistant allele GTC and GTA (both code for Valine); nucleotide sequences from the MEAM1 field populations with susceptible allele GCC and GCA (both code for Alanine).

18-fold difference in dose–response between populations (at LC_{50}). The most susceptible field population was from Narromine in 2020 with an LC_{50} value of 0.61 mg L^{-1}, whereas the most tolerant field population was from Mungindi in 2019, with an LC_{50} value of 10.75 mg L^{-1} (Table 3). Slopes of the field populations ranged between 1.38 (± 0.20) to 3.14 (± 0.33). Based on nonoverlapping 95% fiducial limits, four field populations in 2019 and two in 2020 had LC_{50} values greater than the susceptible strain.

In order to select a discriminating dose for annual resistance surveillance, we evaluated two bioassay parameters. The first was the lowest concentration of buprofezin required to achieve 100% mortality across all the dose–response assays, which was 100 mg L^{-1}, and the second parameter, the highest recorded $LC_{99.9}$, which was 161.9 mg L^{-1}. Based on these parameters the discriminating dose was set at double the minimum effective concentration (i.e., 200 mg L^{-1}), balancing the risk of not detecting low level resistance but limiting the risk of reporting false positives.

In 2021, 16 *B. tabaci* populations were tested for buprofezin resistance (Table 4). All populations were susceptible with 100% mortality recorded at 32 mg L^{-1}, well below the discriminating dose. Control mortality in these populations ranged from 1.4% to 11.3% ($\bar{x}$ = 6.5%). The highest LC_{50} was 1.26 mg L^{-1}, recorded in a population from Narrabri, which resulted in an RR of 1.1. Usage of buprofezin during the 2020/21 season was low at 2.5 g ai ha^{-1} (Source: Crop Consultants Australia, Annual Market Audit).

3.4 Screening for cross-resistance between buprofezin and pyriproxyfen or spirotetramat

Two reference populations with resistance to pyriproxyfen and spirotetramat were assayed with buprofezin. The AY09-1R with a RR of >2000 to pyriproxyfen, was susceptible to buprofezin with an LC_{50} of 2.26, resulting in a RR of 0.98 (0.7-1.4) (Table 5). The minimum effective concentration for the AY09-1R population was 100 mg L^{-1}, which is within the range observed in field populations. As the LC_{50} fiducial limits of the AY09-1R population overlapped the corresponding values from the susceptible reference population, there is no indication of cross-resistance between pyriproxyfen and buprofezin.

When the AY16-1R population (spirotetramat RR of >1200), was assayed with buprofezin an LC_{50} of 2.33 was estimated with a corresponding RR of 2.34 (1.5-3.6). The assay resulted in nonoverlapping LC_{50} fiducial limits with the susceptible reference population, which is an indicator of potential cross-resistance between spirotetramat and buprofezin, but as the dose–response of the AY16-1R fits within the range observed in the field populations it does not provide strong evidence of cross-resistance.

4 DISCUSSION

Since the early 2000s, pyriproxyfen has been used widely to control *B. tabaci* in both cotton and horticulture. Reports of resistance started within a few years of use,[17] but the emergence of widespread resistance in cotton did not occur until 2017.[17] The growing season of 2016/17 experienced sustained season-long pest pressure, triggering frequent insecticide use. The reduced abundance of natural enemies coupled with late season hot and dry climatic conditions meant there was a rampant proliferation of *B. tabaci* in most production valleys and, consequently, an overreliance on the use of pyriproxyfen. In response to resistance, the cotton industry introduced a 30-day application window, with the aim of reducing the selection pressure that *B. tabaci* was experiencing. In subsequent seasons as documented in this study a positive change

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Table 3. Buprofezin lethal dose–response data for reference susceptible strain (tested in 2019, 2020) and field-collected *Bemisia tabaci* MEAM1

Population (G†)	Collection period	χ2 (df)	Slope (SE)	LC_{50} (mg L^{-1}) (95% FL)	LC_{99} (mg L^{-1}) (95% FL)	$LC_{99.9}$ (mg L^{-1}) (95% FL)	MEC	Control mortality
Susceptible (2019‡)		52.80 (18)	1.58 (0.25)	1.65 (0.99–2.36)	47.7 (23.0–189.0)	143.8 (53.7–952.1)	32	6.5
Susceptible (2020‡)		47.77 (23)	2.37 (0.21)	1.11 (0.91–1.35)	10.7 (7.2–18.4)	22.4 (13.6–45.3)	10	2.1
Emerald (6)	Jan 2019	47.38 (18)	2.36 (0.25)	1.48 (1.18–1.87)	14.3 (9.1–28.6)	30.2 (16.9–73.7)	10	5.3
Theodore (5)	Jan 2019	73.43 (23)	2.78 (0.34)	3.10 (2.46–3.92)	21.2 (13.5–43.7)	39.9 (22.6–100.9)	100	0.0
Dalby (6)	Mar 2019	15.47* (18)	1.95 (0.14)	3.22 (2.76–3.73)	50.4 (35.9–78.1)	124.4 (80.0–221.0)	32	4.7
St George (5)	Mar 2019	94.05 (23)	1.69 (0.23)	1.85 (1.29–2.65)	44.5 (20.8–165.2)	126.5 (47.6–696.0)	100	4.7
Mungindi (4)	Mar 2019	22.31* (23)	3.14 (0.33)	10.75 (9.42–12.65)	59.3 (41.2–102.2)	103.9 (65.8–205.8)	100	0
Moree A (4)	Mar 2019	111.00 (23)	1.81 (0.25)	2.82 (1.97–4.14)	54.3 (25.0–217.6)	143.4 (53.5–858.6)	100	3.3
Moree B (4)	Mar 2019	67.55 (23)	1.90 (0.22)	1.62 (1.23–2.13)	27.3 (15.2–68.6)	69.03 (32.5–228.7)	100	2.8
Warren (3)	Apr 2019	46.81 (23)	2.29 (0.24)	6.24 (5.06–7.92)	64.8 (38.9–141.1)	139.8 (73.7–375.1)	100	0.7
Bowen (1)	Oct 2019	49.54 (17)	2.23 (0.34)	3.45 (2.36–4.74)	38.0 (21.1–113.3)	83.57 (38.9–357.3)	32	6.8
Dalby (3)	Apr 2020	83.00 (28)	1.52 (0.15)	1.47 (1.09–2.01)	50.4 (26.1–132.3)	160.6 (69.3–559.1)	32	3.5
St George A (3)	Mar 2020	12.36* (13)	1.38 (0.20)	0.92 (0.59–1.24)	45.2 (22.8–148.3)	161.9 (62.4–865.6)	10	8.8
St George B (3)	Mar 2020	12.32* (12)	2.12 (0.32)	1.60 (1.12–2.10)	20.2 (11.7–52.8)	46.3 (22.5–170.6)	32	0.0
St George C (3)	Mar 2020	23.14* (18)	2.02 (0.20)	1.64 (1.30–1.98)	23.3 (15.9–40.0)	55.6 (33.4–116.3)	32	2.4
Goondiwindi (2)	Mar 2020	40.97 (18)	1.92 (0.29)	2.14 (1.34–2.98)	34.9 (19.1–105.0)	87.2 (39.1–396.0)	32	3.5
Moree A (4)	Mar 2020	55.04 (23)	2.17 (0.21)	1.06 (0.84–1.33)	12.5 (7.9–24.3)	28.0 (15.6–66.1)	10	4.9
Moree B (4)	Mar 2020	82.18 (18)	2.35 (0.37)	0.95 (0.68–1.30)	9.2 (5.1–28.9)	19.5 (9.0–87.1)	10	3
Moree C (1)	Mar 2020	32.48 (13)	1.61 (0.36)	0.91 (0.36–1.40)	25.7 (10.5–307.8)	76.7 (22.3–2580)	10	8.9
Narrabri A (2)	Mar 2020	30.84 (18)	1.88 (0.22)	2.04 (1.52–2.60)	35.1 (20.4–83.2)	89.3 (43.8–284.6)	32	5.2
Narrabri B (2)	Mar 2020	47.84 (23)	1.64 (0.19)	1.82 (1.29–2.41)	47.5 (27.1–114.4)	138.7 (65.33–458.6)	100	2.4
Narrabri C (2)	Mar 2020	57.05 (17)	1.93 (0.30)	1.90 (1.23–2.64)	30.5 (16.1–101.7)	75.8 (32.5–388.8)	32	4.4
Narromine (4)	Apr 2020	19.83* (13)	2.03 (0.19)	0.61 (0.51–0.71)	8.6 (5.8–14.8)	20.4 (12.2–42.5)	3.2	10.6

* Chi-square test significant at $P < 0.05$.
† Generation tested.
‡ Year of test.

was observed, with a steady decline in the detection of resistant populations. That was until the 2020/21 season, where our survey has detected numerous resistant populations, contrary to the low use of pyriproxyfen in that season (Fig. 4).

Evaluating the effectiveness of using a 30-day application window to reduce selection pressure has been difficult as the observed decline in the frequency of populations with resistance to pyriproxyfen coincided with several years of reduced insect pest pressure most likely linked to a decline in area planted with cotton as a consequence of drought (https://cottonaustralia.com.au/statistics). The regions most affected by drought included the areas where resistance previously

Table 4. Buprofezin lethal dose–response data of field populations of *Bemisia tabaci* collected in 2021

Population (G*)	Collection period	LC_{50} (mg L^{-1})	95% FL	Slope (SE)	Mortality (%) at 10 mg L^{-1} (*n*)	Mortality (%) at 32 mg L^{-1} (*n*)	RR‡ (95% CI)
Susceptible (2021†)		1.18	0.94–1.47	2.13 (0.20)	99.3 (149)	100 (129)	
Emerald (1)	Dec 2020	1.10	0.81–1.46	1.14 (0.09)	98.5 (133)	100 (138)	0.9 (0.7–1.3)
Theodore (1)	Jan 2021	1.02	0.72–1.43	1.14 (0.11)	95.7 (116)	100 (140)	0.9 (0.6–1.3)
St George A (3)	Mar 2021	0.7	0.49–0.99	1.78 (0.23)	100 (150)	100 (135)	0.6 (0.4–0.9)
St George B (3)	Mar 2021	0.78	0.56–1.08	1.46 (0.16)	99.2 (123)	100 (128)	0.7 (0.5–1.0)
St George C (3)	Mar 2021	0.72	0.49–1.03	1.40 (0.17)	95.1 (102)	100 (104)	0.6 (0.4–0.9)
Goondiwindi B (3)	Mar 2021	0.43	0.26–0.65	1.45 (0.21)	100 (127)	100 (121)	0.4 (0.2–0.6)
Goondiwindi C (3)	Mar 2021	0.59	0.44–0.77	2.21 (0.25)	100 (137)	100 (120)	0.5 (0.4–0.7)
Moree A (3)	Mar 2021	0.59	0.35–0.91	1.10 (0.15)	99.1 (117)	100 (126)	0.5 (0.3–0.8)
Moree B (3)	Mar 2021	0.58	0.46–0.74	2.63 (0.32)	100 (118)	100 (135)	0.5 (0.4–0.7)
Moree C (3)	Mar 2021	0.54	0.33–0.82	1.21 (0.15)	99.3 (138)	100 (149)	0.5 (0.3–0.7)
Narrabri A (2)	Mar 2021	1.03	0.82–1.32	1.90 (0.18)	100 (127)	100 (121)	0.9 (0.6–1.2)
Narrabri B (2)	Mar 2021	1.26	0.95–1.67	1.93 (0.20)	98.0 (150)	100 (150)	1.1 (0.8–1.5)
Narrabri C (3)	Mar 2021	1.05	0.84–1.32	1.80 (0.20)	98.7 (150)	100 (150)	0.8 (0.5–1.1)
Narromine (3)	Mar 2021	0.80	0.53–1.15	1.04 (0.10)	99.3 (139)	100 (131)	0.7 (0.4–1.0)
Griffith (3)	Mar 2021	0.75	0.56–1.00	1.60 (0.17)	100 (150)	100 (150)	0.6 (0.4–0.9)
Hillston (3)	Mar 2021	0.65	0.49–0.87	2.38 (0.32)	100 (150)	100 (147)	0.6 (0.4–0.8)

* Generation tested.
† Year of test.
‡ The resistance ratio (RR) is calculated by dividing the LC_{50} of the field populations by LC_{50} of the susceptible population (SU07-1).

Table 5. Buprofezin lethal dose–response data of *Bemisia tabaci* reference strains resistant to pyriproxyfen (AY09-1R) and spirotetramat (AY16-1R)

Laboratory strain	Tested	$\chi 2$ (df)	LC50 (mg L^{-1}) (95% FL)	Slope (SE)	MEC	RR† (95% CI)
Pyriproxyfen						
SU07-1	2017	26.26* (23)	2.72 (2.26–3.28)	1.80 (0.13)	32	-
AY09-1R	2017	79.91 (33)	2.26 (1.79–2.85)	1.99 (0.18)	100	0.98 (0.70–1.38)
Spirotetramat						
SU07-1	2022	55.98 (23)	0.99 (0.66–1.39)	1.27 (0.14)	32	-
AY16-1R‡	2022	61.26 (28)	2.33 (1.74–3.062)	1.24 (0.10)	100	2.34 (1.50–3.64)

* Chi-square test significant at $P < 0.05$.
† The resistance ratio (RR) is calculated by dividing the LC_{50} of the field populations by LC_{50} of the susceptible population (SU07-1).
‡ Resistant to both pyriproxyfen and spirotetramat.

had been most frequently detected. In 2019 and 2020 there was a correlation between decreased use of pyriproxyfen and resistance detection, but in 2021, even though usage remained low, there were several detections of resistance, including two populations with RRs >30. To an extent this finding is supported by the modelling of Crowder *et al.*,[36] who found a

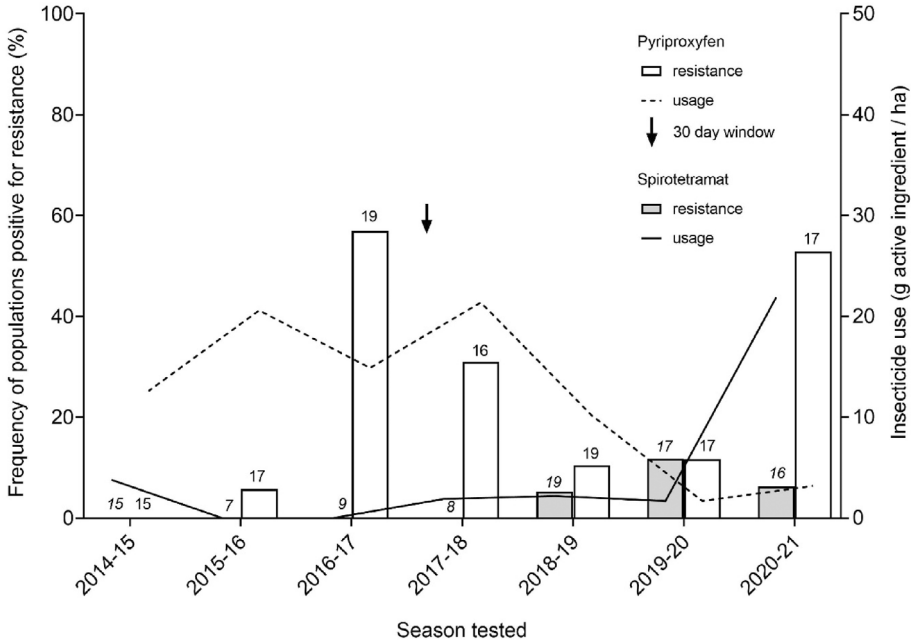

Figure 4. Seasonal frequency of resistance detection to pyriproxyfen and spirotetramat in *Bemisia tabaci* MEAM1 populations collected from Australian cotton. Number of populations tested against each insecticide is shown above each column. Lines show the amount (g ai ha^{-1}) of pyriproxyfen and spirotetramat applied to Australian cotton from 2014 to 2021. Data: Crop Consultants Australia Annual Market Audit.

combination of traits including the lack of fitness costs linked to pyriproxyfen resistance in *B. tabaci* MEAM1 would result in the retention of a resistant population even with spatial and temporal refuges in place.

Applying selection pressure did not result in a highly pyriproxyfen-resistant phenotype in any of the tested populations with only a modest increase in resistance ratios. This may be a result of the relative effectiveness of the detoxification enzymes present in these populations. In Arizona, metabolic detoxification due to increased activity levels of cytochrome P450 monooxygenase and glutathione S-transferase has been linked to pyriproxyfen-resistant *B. tabaci* MEAM1.[37] Pyriproxyfen resistance in *B. tabaci* MEAM1 is thought to be controlled by two loci.[36] This may explain why resistance to pyriproxyfen tends to be dominant under field conditions[38] but can be recessive at high concentrations.[39] From our selection studies, we surmise that the populations collected from Australian cotton may contain only one mutation linked to metabolic resistance and as suspected by Crowder *et al.*,[39] the concentrations that populations are exposed to in the field are too low or residue deposition is too discontinuous to select for recessive inheritance. In comparison, the resistant reference strain (AY09-1R) collected from a different cropping system, routinely survives exposure to 320 mg L^{-1} pyriproxyfen, which may be an indication that it contains either more than one resistance mutation or has more effective detoxification enzymes.

Further research into the underlying genetic makeup of field resistant populations would clearly be beneficial. Metabolic detoxification mechanisms can often be elucidated by examining the expression profiles, either using QPCR for candidate genes or by transcriptome sequencing to identify and quantify differentially expressed genes. The resistant (AY09-1R), susceptible (SU07-1) and field populations referenced in this study may present the opportunity for further investigation, including the sequencing of candidate target sites.

The detection of several populations with very low-level resistance to spirotetramat in cotton production regions was not unexpected as resistance previously had been detected in *B. tabaci* MEAM1 collected from horticultural crops in north QLD.[18] The Lueke *et al.*[18] study found that resistance to spirotetramat was linked to a genetic change at the target site in a highly conserved region of the acetyl-CoA carboxylase gene and was demonstrated to have dominant inheritance.

In our study, the presence of the mutation A2083V was detected in only a small number of the field populations tested, indicating that resistance to spirotetramat is currently rare in Australian cotton production regions, reflecting usage which until 2020 had been minor (Fig. 4). Our sampling of populations from horticultural regions is too restrictive to comment on the current distribution of resistance within that industry, yet it would appear that there is a moderate-to-high frequency of resistance present in the Burdekin district of North QLD. Spirotetramat has been commonly used in this region on vegetable crops since 2009 to control hemipteran pests including *Myzus persicae* in which resistance has recently been detected.[40]

The sequencing results for the A2083V mutation aligned well with the phenotypic screening for spirotetramat resistance which is in agreement with a recent study that demonstrated a phenotype-by-genotype correlation between mortality to a ketoenol insecticide and the mutation frequency of A2083V.[41] Based on our sequencing results the discriminating dose of 100 mg L^{-1} is performing satisfactorily; although it may have occasionally reported false positives, no false negatives were recorded in this study. A simple validation step to confirm resistance would be to sequence individuals that survive the discriminating dose for the presence of the A2083V mutation. Where populations were placed under selection pressure there was corresponding increase in the frequency of A2083V, which is a strong confirmation that the mutation is the primary source of resistance in *B. tabaci* MEAM1 to spirotetramat.[18] The A2151V mutation was

not definitively detected in any of the populations tested, which is likely to be a result of the absence of *B. tabaci* MED in local populations.[18,23]

The current very low-level of resistance is not an immediate concern as it is unlikely to impact field efficacy, yet it does indicate the risk of resistance increasing, particularly if whitefly management becomes too reliant on spirotetramat alone (Table 2). The populations from Gumlu and Bowen clearly demonstrate the capacity of *B. tabaci* to evolve a high level of field resistance. In 2019, with the aim of delaying the development of resistance to spirotetramat, the cotton industry made a proactive change to the IRM strategy, reducing the number of annual sprays targeting *B. tabaci* from two to one per field.

The *B. tabaci* MEAM1 populations tested during the evaluation of baseline susceptibility and the first year of resistance surveillance were susceptible to buprofezin, with no evidence of resistant individuals. The dose–responses observed in our study were similar to results recorded in California and Arizona during the late 1990s,[42] but not as susceptible as the SUD-S and PAK populations tested by Cahill *et al.*[43] Similar to the populations tested by Toscano *et al.*,[42] our populations showed considerable variability in response at LC_{50} with some field populations having a greater tolerance to buprofezin compared to the reference susceptible strain. This highlights the value in completing baseline studies with high spatial and temporal distribution before the widespread usage of an insecticide.

Resistance to buprofezin has been detected internationally with responses ranging from declining efficacy to extreme levels of resistance in glasshouse populations of *B. tabaci* in Israel, the Netherlands and Spain.[43–45] One of the more extreme cases of resistance to buprofezin was detected in an invasive population of *B. tabaci* MED collected from poinsettia growing in a glasshouse in Tucson, Arizona.[46] When tested, only 34.5% of the population was controlled at 1000 mg L^{-1}, compared to the 99.8% control recorded in field populations in the same year.[46] In Arizona and Florida, *B. tabaci* generally are susceptible to buprofezin,[10,42] whereas in Spain, Turkey and Pakistan, resistant *B. tabaci* have been collected from field crops.[43,47,48]

Our cross-resistance study provided no evidence for a shared resistance mechanism between pyriproxyfen and buprofezin. This is in contrast to a laboratory study that detected significant cross-resistance to pyriproxyfen in a buprofezin-selected strain.[22] Based on relative changes in susceptibility, there is limited evidence of cross-resistance in field crops and glasshouses where both IGRs have been used extensively.[44] Although nonoverlapping fiducial limits theoretically indicates cross-resistance, the dose–response of the AY16-1R population to buprofezin is within the bounds of the field populations tested in this study. Our conclusion is that the bioassay result does not provide sufficiently strong evidence to make a claim of cross-resistance between buprofezin and spirotetramat. Owing to its history of insecticide exposure, the AY16-1R population may retain a capacity to metabolically detoxify a range of toxins including buprofezin, but this will require further investigation to confirm. Given the discovery of separate target site mechanisms for both spirotetramat and buprofezin,[49] strong cross-resistance between the two insecticides seems unlikely.

At the time of registration, given the globally documented cases of resistance, buprofezin was included in the cotton industry IRM strategy with annual use restricted to a single application per field targeting *B. tabaci*. The addition of buprofezin for *B. tabaci* management strengthens the IRM strategy by adding a new MoA with proven efficacy against whitefly[50] and low impact on natural enemies.[11] Annual resistance surveillance will continue to monitor the susceptibility of *B. tabaci* to pyriproxyfen, spirotetramat and buprofezin.

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CONFLICT OF INTEREST DECLARATION

The authors declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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