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Investigating pyrethroid resistance in UK cabbage stem flea beetle populations and developing a PCR-based assay for detecting turnip yellows virus in aphids

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CONTENTS

1. Abstract

On 1 December 2013, a restriction was enforced by the European Commission (EC) on the use of the neonicotinoids clothianidin, imidacloprid and thiamethoxam. These actives were used to treat winter and spring oilseed rape (OSR) seed to protect the crop during the first 6–8 weeks of growth from peach–potato aphids, which transmit turnip yellows virus (TuYV) and cabbage stem flea beetles (CSFB). This project assessed concerns regarding insecticide resistance in CSFB (Part A) and the transmission of TuYV by the peach–potato aphid (Part B).

CSFB, *Psylliodes chrysocephala*, is a major pest of winter OSR in several European countries, particularly attacking young emerging plants in autumn. The EU-imposed restriction on neonicotinoids has left growers with only one alternative of applying a pyrethroid spray. Recent reports of knock-down resistance (kdr) to pyrethroids in CSFB in Germany in the last few years demonstrate that this pest has evolved resistance which would potentially undermine this alternative control strategy. Furthermore, there are growing numbers of reports from UK OSR growers that pyrethroids are failing against CSFB suggesting that resistance is now present in this country. To address this, UK CSFB samples were collected from oilseed rape in 2014 (from July to September) and tested for the presence of the kdr mutation. Live adults were also screened in a topical glass vial assay with lambda-cyhalothrin to measure the level of resistance, including resistance to the recommended field rate for CSFB (7.5 g ai/ha). kdr was found but this did not equate well with the resistance phenotype. Bioassays using a synergist disclosed that there was an unknown metabolic-based resistance mechanism present in this species which confers strong pyrethroid resistance.

The transmission of TuYV by the peach–potato aphid, *Myzus persicae,* is also a major concern for OSR growers. It is already estimated that 60% of UK OSR is affected by TuYV with average yield losses in untreated crops of 15–30%. The loss of the neonicotinoid seed treatment (which is currently very effective for controlling UK populations of *M. persicae*), combined with existing strong resistance to alternative compounds (pyrethroids and pirimicarb) will undoubtedly result in a significantly increased threat of TuYV transmission for OSR in future. At present, it is difficult to monitor both resistance status (kdr/super-kdr and MACE) and likelihood of virus transmission in *M. persicae* populations in real time. Although rapid, high throughput PCR-based tests are available for detecting resistance mutations in aphid samples, a similar DNA-based assay is not currently available for detecting TuYV in the same samples. Instead, TuYV and other plant viruses are generally monitored retrospectively at the end of the season using more labour intensive enzyme-linked immunosorbent assay (ELISA) assays. We addressed this by developing a rapid PCR-based diagnostic assay for TuYV that can be run alongside the insecticide resistance assays that gives a more complete ‘real-time’ picture of both resistance status and the virus transmission potential of *M. persicae* populations collected from OSR.

2. Part A. Investigating pyrethroid resistance in UK cabbage stem flea beetle populations

2.1. Introduction

Pyrethroid insecticides have been used as foliar sprays for the control of CSFB on OSR in the UK and Europe for many years. Although normally very effective, there have been reports of poor control from Germany over recent years (Heimbach & Muller, 2013) and these have been followed by similar reports of pyrethroid control failure in the UK in 2013. In Germany, resistance has now been confirmed by the identification of the kdr mutation (L1014F) in the sodium channel of resistant beetle populations that show reduced efficacy for lambda cyhalothrin and a range of other pyrethroids (Zimmer *et al*., 2014). We investigated whether the same mutation is also now present in UK CSFB populations (by polymerase chain reaction (PCR) and sequencing of the same region of the sodium channel target). This was done by collecting CSFB adults from OSR fields, mainly at harvest, and extracting RNA and sequencing the region of the sodium channel where mutations (such as L1014F) are known. We also carried out bioassays with lambda cyhalothrin (previously developed for testing pollen beetles and grain aphids) to quantitate the level of resistance in the UK CSFB population.

2.2. Materials and methods

2.2.1. Cabbage stem flea beetle testing

Adult samples

Eighteen adult samples were tested. These were collected from oilseed rape between July and September (Table 1).

Table 1. Origins of UK cabbage stem flea sample.

Sample	*Collection site*	*Date*
1	Feltwell, Norfolk	13/07/14
2	Harpenden, Herts	15/07/14
3	Great Wilbraham, Cambs	16/07/14
4	Ousden, Suffolk	17/07/14
5	Wickhambrook, Suffolk	17/07/14
6	Great Wilbraham, Cambs	20/07/14
7	Barrow, Suffolk	20/07/14
8	Tadlow, Cambs	25/07/14
9	Ryton, Yorks	29/07/14
10	Driffield, Yorks	06/08/14
11	Harpenden, Herts	08/09/14
12	Royston, Herts	08/09/14
13	Harpenden, Herts	10/09/14
14	Driffield, Yorks	11/08/14
15	Ongar, Essex	13/09/14
16	Harpenden, Herts	17/09/14
17	Harpenden, Herts	19/09/14
18	Sandy, Beds	16/10/14

Mutation analysis

Standard methods for RNA extraction and RT-PCR were used to obtain and sequence fragments of the voltage-gated sodium channel where kdr mutations are known to occur (these methods are routine at Rothamsted Research). This focussed on the region where the kdr mutation (L1014F) has already been reported in German CSFB samples.

Lambda-cyhalothrin bioassays

Bioassays were used to establish a suitable bioassay end point, criteria for scoring and screening dose for resistance.

Bioassays were done at 20^{o}C under a 16/8 h light/dark photoperiod. Information on the baseline susceptibility of CSFBs to lambda-cyhalothrin was not available prior to this study. However, a method for assessing pyrethroid susceptibility of pollen beetles (*Meligethes aeneus* Fabricius) (Slater *et al.*, 2011) and grain aphids (*Sitobion avenae* Fabricius) (Foster *et al*., 2014), was available using insecticide-coated glass vials and this was adapted for *P. chrysocephala*. An initial stock solution (10 g ai/hectare representing 133% recommended field rate for this pest) was made by diluting technical-grade lambda-cyhalothrin in 100% technical grade acetone. This was then diluted using acetone to various concentrations (down to 0.04% of the field rate). Then, 0.5 ml of each of these was then pipetted into individual 14 ml glass vials (7 cm tall/ 2 cm diameter) which were then placed horizontally (without lids) and rotated on a roller for 2 h until all of the acetone had evaporated.

Control vials were treated with acetone only. Vials were then stored upright overnight, after which screw caps were attached. They were then stored at 4^{o}C in the dark prior to use.

Bioassays used adult beetles, which had been collected up to a few days before from oilseed rape, either at harvest or from OSR seedlings in autumn. This involved placing each beetle sample, which sometimes included seeds and/or plant material, on a white tray inside a large three-sided Perspex cage. Beetles that were capable of walking/jumping were collected using a hand-held, battery-powered insect pooter. They were then knocked out of the inverted pooter through a small funnel placed into each vial and re-sealed with their screw cap lids to prevent any escape. Pilot assays showed that up to 20 beetles could be assayed in each replicate vial. A control and up to seven lambda-cyhalothrin doses were used: 3, 15, 25, 37.5, 50, 75 (field rate) and 100 ng ai/cm^2. After 24 h, the beetles were transferred to smaller untreated glass vials (without lids) under upturned transparent plastic cups to allow potential recovery from treatment (a phenomenon seen in some insects carrying metabolic-based resistance). After a further 24 h, beetles were then scored with the naked eye. At this 48 h end-point, control mortality remained generally low. Individuals were categorised as 'mobile' (capable of walking and/or jumping in a coordinated way), 'affected' (incapable of coordinated movement) or 'dead'. Each replicate was scored for up to five minutes to distinguish beetles that were 'playing dead' (a characteristic of this species). The beetles at each of the doses and in each of the categories were then transferred to eppendorfs and snap frozen for kdr testing and potential future testing for metabolic-based resistance.

2.3. Results

2.3.1. Cabbage stem flea beetle testing

Lambda-cyhalothrin bioassays

The response of the adult samples to lambda-cyhalothrin in the glass vial bioassays are shown in Table 3. There were 13 (72%) of the 18 CSFB samples contained beetles that were fully resistant to the recommended field rate of lambda-cyhalothrin (7.5 g ai/ha). The location of these samples is shown in Figure 1. There is a suggestion that samples without mobile (resistant) beetles tended to be located further north.

Table 3. Response of CSFB adults to lambda-cyhalothrin applied at the recommended field rate (7.5 g ai/ha). Samples containing mobile (resistant) beetles are shown in **bold**.

Sample	*% Response*			*N*[a]	*Controls*[b]
	Mobile	*Immobile*	*Dead*		
1	0	91	9	11	0
2	0	50	50	16	0
3	**53**	47	0	15	0
4	**71**	29	0	7	0
5	**44**	50	6	16	8
6	**31**	69	0	13	18
7	**50**	50	0	8	6
8	**33**	67	0	15	0
9	0	92	8	12	17
10	0	100	0	11	20
11	**25**	65	10	20	0
12	**100**	0	0	9	0
13	**67**	33	0	21	0
14	0	100	0	12	45
15	**58**	17	25	12	0
16	**100**	0	0	12	0
17	**47**	40	13	15	11
18	**40**	47	13	15	0

[a] Total number of adults tested.

[b] % immobile + dead adults in acetone controls.

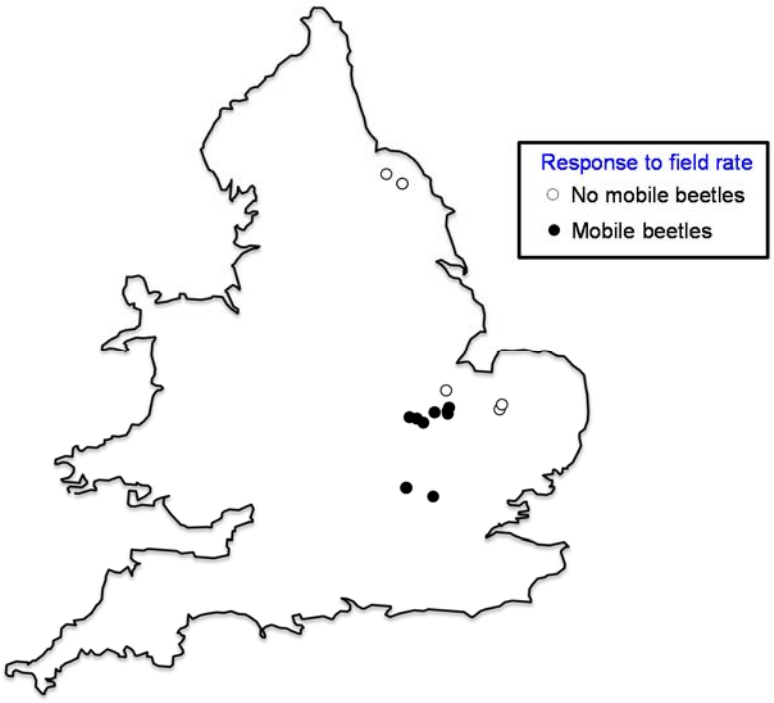

Figure 1. Origin of UK CSFB samples containing mobile (resistant) & susceptible beetles when exposed to a dose equivalent to field rate in the lambda-cyhalothrin bioassays.

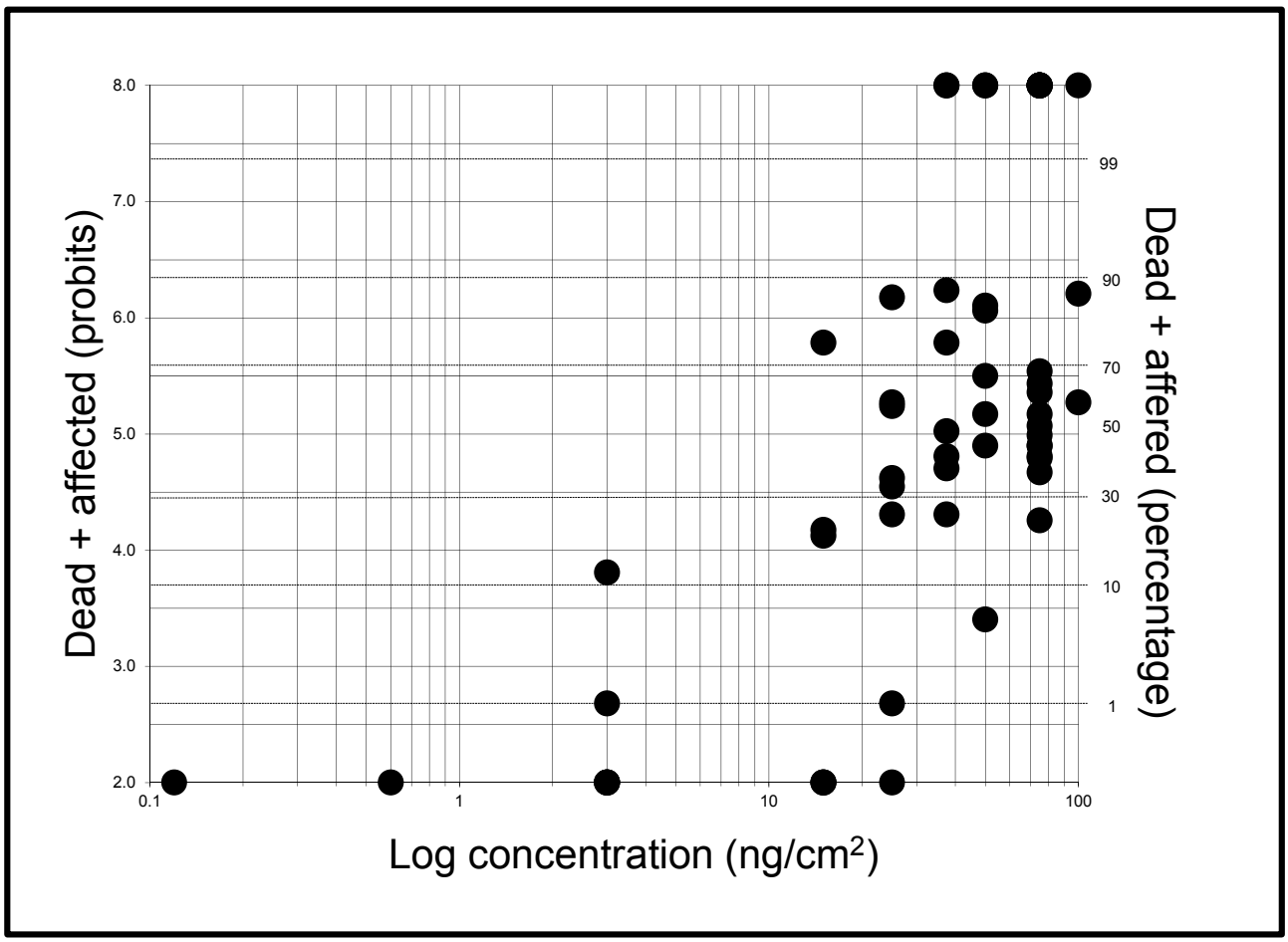

Figure 2. Dose-response of cabbage stem flea beetle samples in lambda-cyhalothrin bioassays.

kdr frequency in adult samples

Table 4 shows the frequency of kdr in the CSFB samples. All three genotypes were present in the UK population in 2014 (SS: 29%, SR: 17%, RR: 54%) with kdr-RR (homozygotes) prevailing. Interestingly, kdr-SS and -SR genotypes were not restricted to the 'affected' or 'dead beetle' phenotypes, i.e., they were also found in beetles that were scored as mobile (resistant) in the lambda-cyhalothrin bioassays. For example, in sample 13, six of the 12 adults that were scored as being mobile (resistant) were genotyped as kdr-SS and therefore not carrying the kdr target site mutation. This suggests that kdr is not the only resistance mechanism present in CSFB in the UK.

Table 4. Percentage of cabbage stem flea beetles scoring as homozygous susceptible (SS), heterozygous (SR) or homozygous (RR) for kdr in the CSFB samples.

Sample	*% kdr genotype*			*N*[a]
	SS	*SR*	*RR*	
1	72	9	19	32
2	29	16	55	31
3	33	12	55	33
4	27	18	55	11
5	39	7	54	13
6	16	22	62	37
7	32	20	48	25
8	10	40	50	10
9	0	0	100	9
10	0	0	100	13
11	33	22	45	18
12	22	11	67	9
13	38	29	33	83
14	0	0	100	36
15	12	0	88	8
16	25	25	50	28
17				NT
18				NT

[a]Total number of adults genotyped. NT: Not Tested.

To investigate this further, in bioassays on samples 17 and 18, some beetles were pre-treated in coated vials for 1 h, with the xenobiotic metabolism inhibitor, piperonyl butoxide (PBO), a compound that is used to test for the presence of metabolic-based resistance. They were then bioassayed alongside adults treated as normal by being moved to either untreated vials or vials coated with lambda-cyhalothrin at the 100% dose rate. The resulting data are shown in Table 5. PBO alone had little effect on adults and 43% and 36% mobile beetles were present when exposed to lambda-cyhalothrin at 100% and 133% field rates, respectively. However, all 26 adults, most likely containing resistant individuals, pre-treated with PBO prior to exposure to lambda-cyhalothrin at the 100% rate were killed. This is strong evidence that resistant beetles carry a metabolic-based mechanism which confers the ‘lion’s share’ of resistance to lambda-cyhalothrin. This needs to be investigated further to establish which particular enzyme is involved. However, this is not a trivial piece of work as the enzyme responsible could be one of many candidates.

Table 5. Response of CSFB adults in samples 17 and 18 in bioassays including pre-treatment with PBO.

% Response			N[a]
Mobile	*Immobile*	*Dead*	
Acetone control			
95	0	5	19
PBO moved to acetone control			
94	0	6	16
PBO moved to 7.5 g ai/ha lambda-cyhalothrin			
0	0	100	26
7.5 g ai/ha lambda-cyhalothrin			
43	43	14	30
10 g ai/ha lambda-cyhalothrin			
36	18	46	11

3. Part B. Developing a PCR-based assay for detecting turnip yellows virus in aphids

3.1. Introduction

Although PCR-based assays have been developed for detecting luteovirids, such as TuYV, these have tended to be generic (non-specific) for virus type (Chomic *et al*., 2010) or are complicated with additional selection steps (e.g. immune-capture) that make them unsuitable for rapid, high-throughput screening of aphid samples (Vigano & Stevens, 2007). To address this, we developed a high throughput reverse transcriptase PCR (RT-PCR) TaqMan assay for detecting TuYV in individual *M. persicae* that can be run alongside existing molecular insecticide resistance assays, to provide a complete analysis of resistance status and virus transmission potential of individual aphids.

3.2. Materials and methods

3.2.1 TuYV RT-PCR Taqman assay

Individual aphids were placed in the wells of a 96 well immunoplate along with 50µl sucrose buffer solution (0.3M sucrose, 0.3M NaCl, 60mM Tris.Cl pH 8) and gently ground using a multi-

homogeniser (Bukard Scientific, UK). The plate was heated to 96°C for 10 min and 2µl aliquots of each aphid homogenate transferred to the wells of a 96 well PCR plate for the reverse transcription (RT) reaction. 8µ reaction mix, containing 50ng primer Virus_R1 (Table 2), 0.5mM dNTP and 100U Revertaid reverse transcriptase (ThermoFisher, UK) was added to each 2µl homogenate and incubated at 50°C for 1 hour. RT reactions were then tested for the presence of TuYV and BMYV by TaqMan assay, using fluorescent dye labelled oligonucleotide probes for allele-selective amplification and detection of the virus sequences in a modified real-time PCR assay (Livak 1999). Primer and probe sequences for the assay were designed using Primer Express software v.2.0 and purchased from Life Technologies (see Table 2). Primers 1F and 1R are standard oligonucleotides with no modification. The TuYV_M probe is labelled with 6-FAM at the 5' end for the detection of TuYV fragments, and the BMYV_V probe is labelled with VIC for detection of BMYV fragments. Each probe also has a 3' non-fluorescent quencher and a minor groove binder at the 3' end. PCR assay reactions (15µL) contained 1.5µL each RT reaction, 7.5 µL of SensiMix Probe mix (Bioline Reagents Ltd), 800 nM of each primer and 200 nM of each probe. Reactions were run on an ABI 7900HT (Applied Biosystems) using temperature cycling conditions of 10 min at 95°C, followed by 40 cycles of 95°C for 10 s and 62°C for 45 s. The increase in VIC and 6-FAM fluorescence was monitored in real time by acquiring each cycle on the yellow channel (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 emission) of the 7900HT, respectively.

Table 2. Oligonucleotide primer and probe sequences.

Name	*Sequence*
Virus_R1	GAACCATTGCCTTTGTAGAGG
primer_1F	TCGGACAACACAACGCCG
primer_1R	GGAACTTCCCGCGAGATTGTC
TuYV_M probe	6FAM-CGAGACATTTGTTTTC
BMYV_V probe	VIC-CGAGACATTCATTTTC

3.3. Results

3.3.1. Taqman assay for TuYV detection in *Myzus persicae*

Identifying virus selective sequences for assay design

Virus sequences for the closely related Luteovirids – turnip yellows virus (TuYV), beet mild yellows virus (BMYV) and beet chlorosis virus (BClV) – were downloaded from public databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and aligned using Geneious software (http://www.geneious.com). The ORF-3 region of the virus encoding the coat protein was selected for the alignments as this is one of the most highly conserved regions across the luteovirids. The aim of the alignments was to identify sequences that are almost identical between TuYV and BMYV isolates (to allow the design of generic primers that will PCR amplify both virus types), but with occasional conserved nucleotide differences (SNPs) that could be exploited for differentiation between TuYV and BMYV isolates using allele selective TaqMan probes. An alignment of ORF-3 sequences highlighting the SNP that was chosen for assay development is shown in Figure 3A. Generic primers 1F and 1R (Table 2) flank this region and were shown to amplify both TuYV and BMYV fragments from infected aphids (Figure 3B). Allele selective probes (TuYV_M and BMYV_V, Table 2) were then designed against the SNP highlighted in 3A. These probes will not amplify sequences for other related luteovirids (eg BClV) as the flanking sequence contains several other SNP differences within this region (Figure 3A).

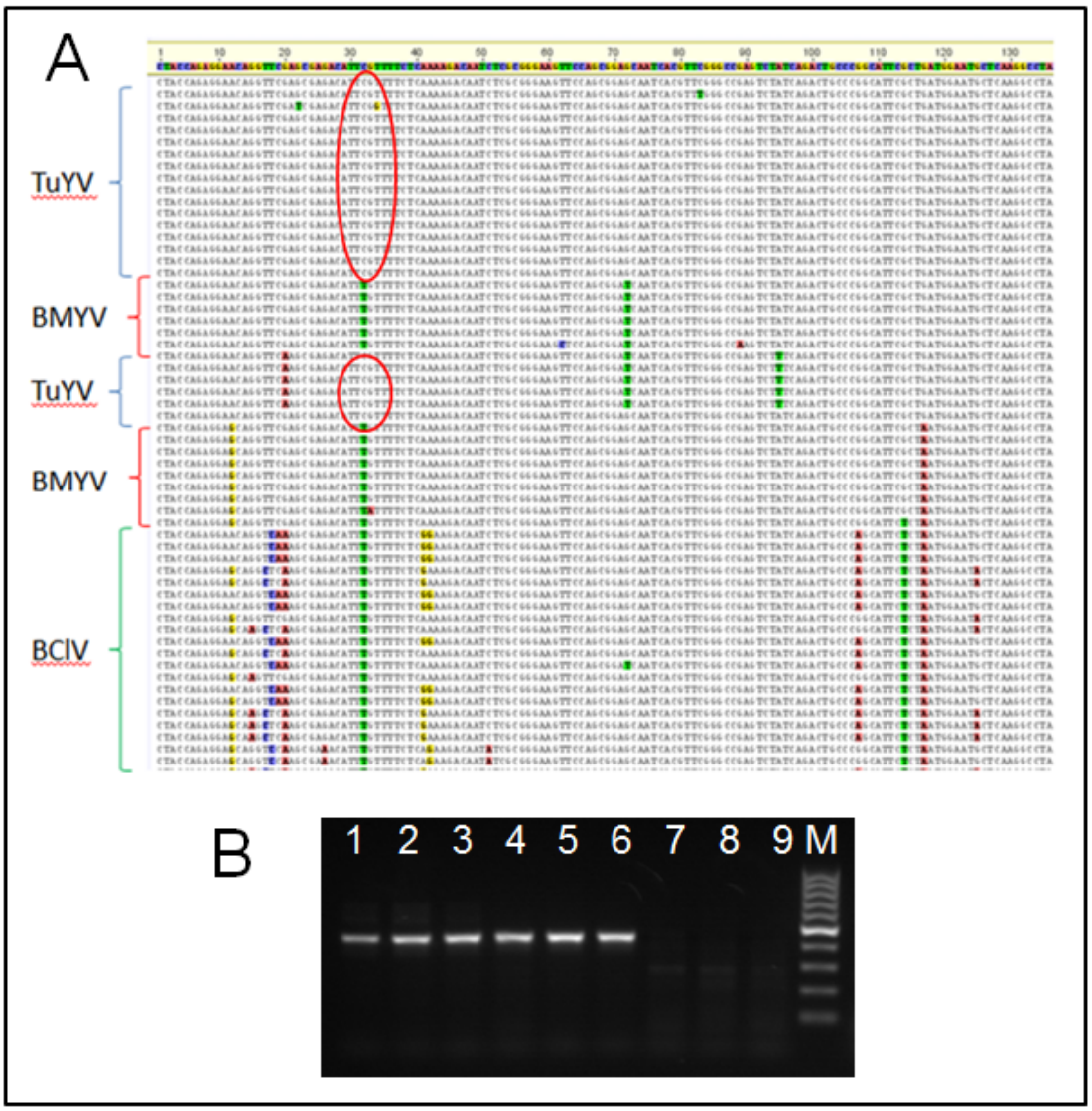

Figure 3. Alignment of virus sequences (A) highlighting the nucleotide difference (C/T) between TuYV and BMYV isolates that was used for allele-selective probe design. B) shows the result of a conventional PCR that confirms that the generic primers do amplify TuYV (lanes 1–3) and BMYV (lanes 4–6) fragments from infected aphids. Lanes 7–9 are non-infected control aphids.

4.2.2 Testing the assay and analysis of field samples

The assay was initially tested on aphids from BMYV- and TuYV-infected *Myzus persicae* cultures at Rothamsted Research (positive controls), a culture of uninfected aphids in culture at Rothamsted (negative controls), and some 'field' aphids taken from the Brooms Barn suction trap (kindly provided by the Rothamsted Insect Survey, November 2014). A total of 40 aphids were processed and assayed according to the method described in section 3.2.1 and the output of the assay is shown in Figure 4. Eight BMYV infected aphids were placed in lane 1 of the plate (1A – 1H) and all scored strongly for the BMYV probe (increased VIC fluorescence, as scored on the Y axis in figure 4). Similarly, the eight TuYV infected aphids (lane 2, 2A-2H) all scored strongly for TuYV (increased 6-FAM fluorescence, scored on the X axis in Figure 4). The eight uninfected control aphids (lane 3) scored negative as expected (no significant increase in VIC or 6-FAM

fluorescence), while the 16 field aphids (lanes 4 and 5) scored as 8 negative and 8 with TuYV infection (Figure 4). This result was reassuring – all the controls scored correctly, with an estimated TuYV infection rate of 50% in the 16 field aphids tested.

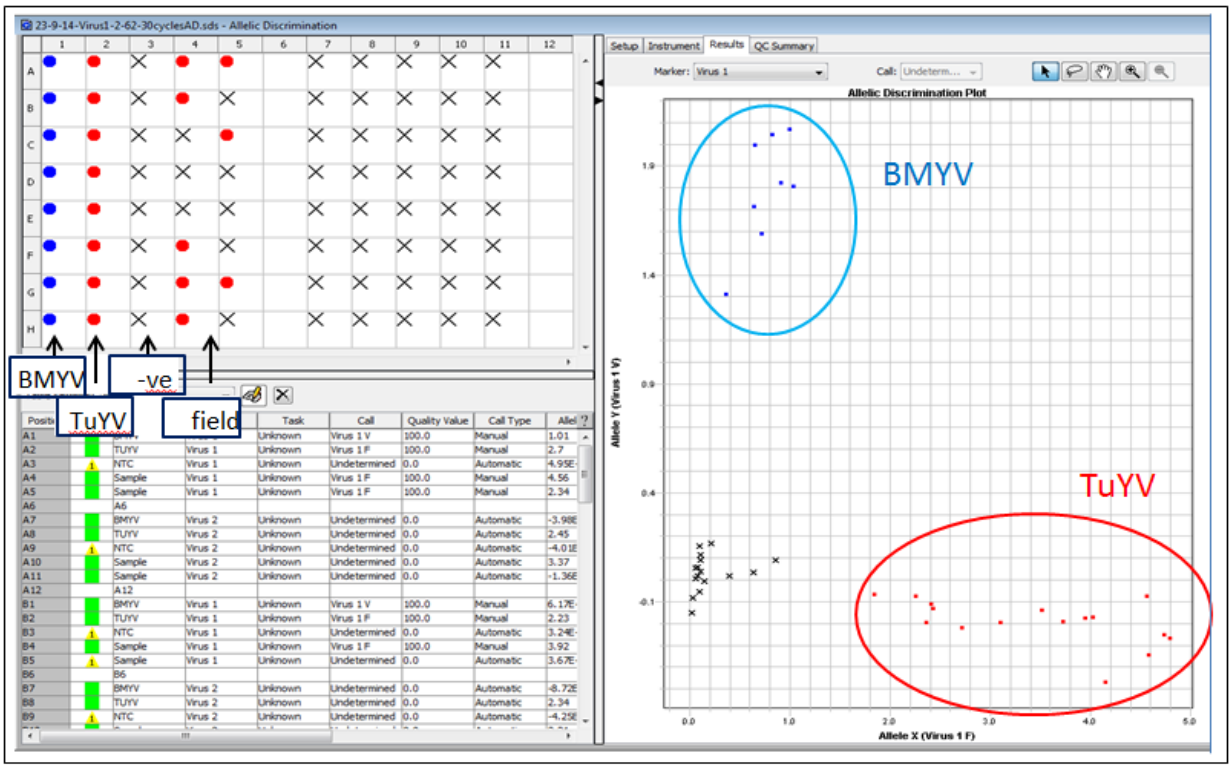

Figure 4. Analysis of control aphids (BMYV infected, lane 1; TuYV infected, lane 2; uninfected, lane 3) and field-collected aphids (lanes 4 and 5) using the Taqman assay. The figure shows the output produced by the TaqMan software programme – the increases in VIC (Y axis) and 6-FAM (X axis) fluorescence are shown on the right, with aphids scored as BMYV infected (blue dots) or TuYV infected (red dots) in the plate template, top left.

The assay was further evaluated by testing a selection of suction trap samples derived from the Wellesbourne and Kirton traps during May, June and July 2015. This testing was carried out at Rothamsted Research as part of a new project 'developing integrated approaches for pest and disease control in horticultural field crops (IAPAD)', led by Dr John Walsh at Warwick University and funded by the BBSRC Horticulture and Potato Initiative (HAPI). A total of 176 aphids were tested (99 from Wellesbourne and 77 from Kirton), with 69 and 62 respectively scoring positive for virus infection (see Table 6). This indicates an overall infection level of 75% across the two trap sites, which is consistent with previous reports of TuYV infection in winged *M. persicae* affected oil seed crops in the UK (Stevens et al. 2008).

Table 6. Levels of TuYV infection in *Myzus persicae* samples collected from Rothamsted Insect Survey suction traps at Wellesbourne and Kirton during June/July 2015. Aphids were tested for TuYV using the new taqman assay protocol described in this report.

Suction Trap	*No tested*	*TuYV +*	*TuYV -*	*% TuYV*
Wellesbourne	99	69	30	70%
Kirton	77	62	15	80%
Total	176	131	45	75%

The new TaqMan assay for TuYV detection reported here is faster than the traditional ELISA-based method for TuYV detection in *M. persicae*. A plate of 96 aphids can be processed in around 5 hours using the TaqMan assay, whereas similar testing by ELISA is normally spread over 2 days. However, the main advantage of the new TaqMan assay is that it uses the same assay platform as established tests for the detection of resistance mutations in this species, which means that it is now possible to test individual aphids for infection with TuYV and combine this with simultaneous testing for a range of known resistance mutations (MACE, kdr, super-kdr and nicR) on the same aphid homogenates. This was not possible previously because the ELISA testing protocol requires the use of complete, individual aphids for virus detection.

4. Discussion

Part A of the project developed a bioassay method for quickly testing CSFB adults for resistance to pyrethroids. This was used to test eighteen UK CSFB samples, collected from different counties in 2014. Most of these samples contained mobile (resistant) beetles after exposure to doses equivalent to the field rate (7.5 g ai/ha) or above (10 g ai/ha) of lambda-cyhalothrin. This demonstrates widespread, strong resistance in this pest in the UK in 2014. The kdr resistance mutation (L1014F), known to confer pyrethroid resistance in other pests, such as aphids and pollen beetles, was found, respectively, in the heterozygous and homozygous form in 17% and over 50% of the beetles tested. However, resistance in the bioassays did not compare well with kdr genotype, as some kdr-susceptible (SS) adults were scored as being fully mobile after being treated with 100% lambda-cyhalothrin. This suggested CSFB were carrying at least one other form of resistance, a hypothesis that was verified in bioassays pre-treating beetles with PBO, which circumvent the presence of metabolic-based resistance. Further research is needed to identify this mechanism which could be based on the over-expression of a P450, as seen in pollen beetles (Slater *et al*., 2011). In the meantime, using kdr frequencies alone will not accurately describe pyrethroid resistance in CSFB in the UK and bioassays on live beetles will continue be needed to diagnose resistance phenotype in this pest.

Oilseed rape growers and agronomists have been advised through a range of KT activities (presentations, press articles, Resistance Alerts; see below) that control-busting pyrethroid resistance is present in CSFB in the UK. Growers should therefore not use repeat applications of these compounds if they do not work initially as this will only exacerbate the situation by selecting for resistant beetles. Furthermore, repeat applications will kill off any beneficial insects in the crop, such as predatory beetles and spiders, which would otherwise reduce the pest population.

Part B of the project developed a rapid PCR-based taqman assay for the detection of TuYV in individual *Myzus persicae*. The full assay protocol from collection of aphids through to scoring of the assay (for a plate of 96 samples) can be completed in 5 hours. Additionally, the same aphids can be simultaneously tested for a range of mutations (MACE, kdr, super-kdr, nicR) that are known to confer resistance in this species. A preliminary analysis of *M. persicae* samples collected from the Insect Survey suction traps at Kirton and Wellesbourne in June/July 2015 indicates high infection levels with TuYV, ranging between 70% (Wellesbourne) and 80% (Kirton).

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