Agricultural Certification Services, Inc
1030 Lincoln Rd., Fredericton, NB E3B 5X6

16 January 2024

PEI Insecticide Resistance survey 2023
FINAL REPORT

Summary:

- DNA from 234 aphid individuals was extracted to produce 146 high-quality DNA sequences for screening for pyrethroid resistance mutations, representing aphids from all twelve weeks of collection from the 2022 season;
- Of all aphids, 46% had at least one pyrethroid resistance mutation; two distinct peaks of of resistance were evident, one early in the season peaking at 45.5% of aphids resistant in Week 4 (July 18 to 24), and a late peak Week 11 and 12 (Sept 5 to 15th) with 100% of tested aphids resistant;
- Average resistance levels early in the season (Weeks 1 to 6, before Aug 8th) was 17.8%, while late in season (Weeks 7-12, on or after Aug 8th) was 74.0%; a dip in resistance happened between peaks during Weeks 5 and 6 (July 25 to Aug 7), which averaged only 7.1% resistant during this time;
- There was a trend toward lower rates of resistance in PEI agricultural Region 3 aphids compared to very similar levels of resistance in Regions 1 and 2, however, this potential regional difference was not statistically significant;
- Matching Na-channel (*para*) gene sequences to species-specific published sequences showed that 94% of resistant individuals were matched closely to *M. persicae* (Green Peach Aphid), with the remaining 6% of resistant individuals matching *R. padi* (Bird Cherry Oat Aphid); sequence analysis showed an unexpected number of matches in both resistant and non-resistant individuals to *M. persicae* even early in the season.

Background:

Aphids are a significant agricultural pest and have been a major concern in crop production due to their role as vectors for plant pathogens, including the economically damaging Potato Virus Y (PVY). These pests have a direct impact on crop yields through feeding and virus transmission. The potato industry, in particular, has relied heavily on the use of pyrethroid insecticides, such as lambda-cyhalothrin and deltamethrin, to control aphid populations. These insecticides are known for their rapid knock-down effect, safety, cost-effectiveness, and minimal environmental persistence.

However, the increasing prevalence of insecticide-resistant aphid populations poses a significant challenge to agricultural pest control. In recent years, studies have shown a rapid growth in insecticide-resistant aphid populations across various potato-producing regions globally (e.g. North America: Gillespie et al. 2009; Europe: Fontaine et al. 2011). The resistance to pyrethroids in aphids is facilitated by behavioral, metabolic, and genetic factors (Criniti et al. 2008). Of particular interest is the genetic aspect, which includes mutations in the Na-channel (*para*) gene. This gene encodes a voltage-gated sodium transport protein crucial for nerve signal conduction and is the primary target of pyrethroid insecticides. Key mutations, such as the knockdown resistance (*kdr*) and super-knockdown resistance (*skdr*) mutations, have been identified and characterized for their role in conferring resistance to pyrethroids (Fontaine et al. 2011). Other mutations on this gene, like F979S and L932F, have also been found, albeit less frequently and with lesser-understood roles in resistance (Criniti et al. 2008).

In 2015-2016, an extensive survey in New Brunswick (MacKenzie et al. 2018) revealed a worrisome rise in pyrethroid insecticide resistance among aphid populations, particularly in *Myzus persicae* (Green Peach Aphid). In 2015, approximately 76% of *M. persicae* individuals showed one or more resistance mutations in the Na-channel (*para*) gene, which rose to 96% in 2016, indicating a near saturation of resistance within this species. Resistance was also observed in other aphid species but at lower frequencies. For instance, in *Acyrthosiphon pisum* (Pea Aphid), resistance rates ranged up to 20% of the population.

Study Objectives and General Methodology:

Building on the approach and insights gained from the New Brunswick study, the 2023 project described here aimed to conduct a similar genetic survey of preserved aphids from the 2022 weekly aphid collections from across Prince Edward Island (PEI). The objectives were to (1) determine the degree of pyrethroid insecticide resistance conferred by known resistance mutations, (2) quantify changes in rates of insecticide resistance throughout the season and across three main agricultural regions of PEI, and (3) identify the specific aphid species responsible for resistance within the larger aphid community. This analysis will provide crucial information on the evolving dynamics of aphid resistance to pyrethroids in PEI and help develop more effective and sustainable pest management strategies for PEI potato growers.

DNA was to be extracted from a minimum of 200 preserved aphids from the 2022 PEI collection. This DNA was subjected to PCR amplification by primers amplifying either a smaller, generic region of the gene, or a larger region specific only to *M. persicae*, or both. Extracting DNA of sufficient quality and quantity from individual aphids preserved for more than a year is challenging, particularly for the mutation analysis used here. To identify resistance mutations, chromatograms of high quality DNA sequencing runs were inspected manually to identify base assignment at specific loci within the gene; chromatogram peaks either showed only the nucleotide base associated with wild-type (insecticide sensitive) gene sequence, or specific alternative bases that would code for differences in the amino acid structure of the Na-channel protein known to cause resistance, or the more subtle condition of both bases superimposed at the locus. These results need interpretation in the context of the diploid genome of the insect, with chromatograms indicating either homozygous sensitive (strong single peak of wild-type base), homozygous resistant (strong single peak mutant base) or heterozygous resistant (superimposed smaller peaks of both wild type and resistant bases, indicating one copy of each sensitive and resistant gene in the chromosome pair). An example of three individual DNA sequencing chromatograms is shown in Fig. 1.

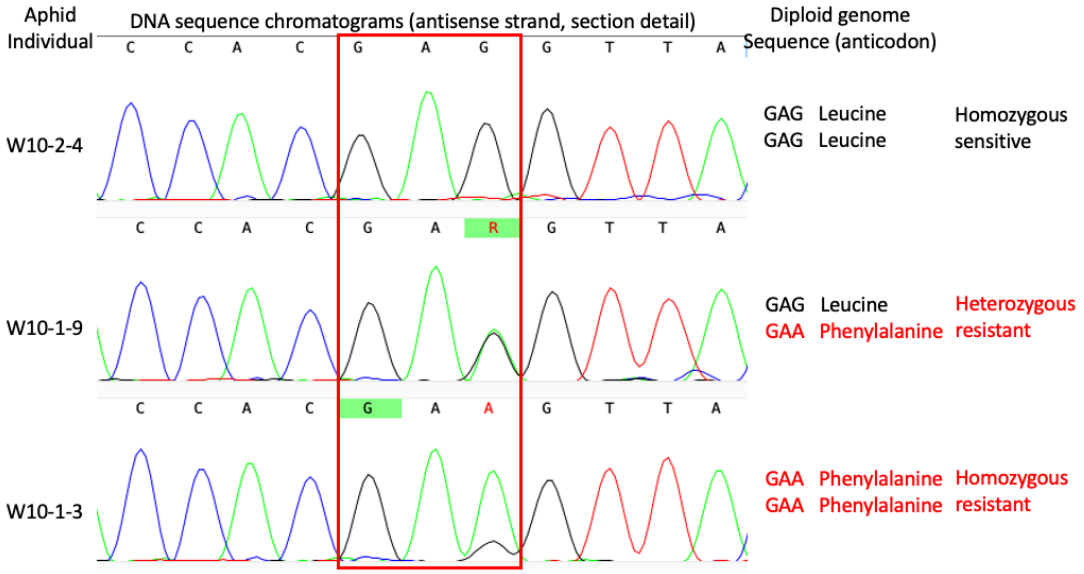

Figure 1. Example DNA sequencing chromatograms for three individual aphids, demonstrating an one with "wild type" sequence GAG (W10-2-4) showing pyrethroid sensitive, another heterozygous resistant GAR (W10-1-9; "R" denoting an ambiguous third base call from overlapping chromatid copies of GAG and GAA) and homozygous resistant GAA genotype (W10-1-3). Criteria for calling the mutant base homozygous (W10-1-3 example) was if mutant base peak was greater than three times height of wild-type base, or opposite for wild-type homozygous (W10-2-4), and background ambiguous peaks in unrelated loci were even smaller (indicates clean sequence); heterozygous if both peaks superimposed, but within 50% of each other in height (W10-1-9); when base assignment was doubtful, an independent confirmatory sequencing reaction was performed.

Results:

DNA extraction and sequencing

In total, we have extracted DNA from 234 preserved aphid individuals balanced across all 12 weeks of collection from the 2022 season (collections running from June 27 to Sept 15, 2022). Most weeks had an initial minimum of 18 individuals extracted (6 from each region when available), though Weeks 8 and 9 only had 12 and 8 individuals respectively because sufficient aphids were not available in the collection for those dates. Some other weeks did not have sufficient aphids to sequence even 6 individuals from all regions as, particularly later in the season, Region 3 had few aphids collected.

Of the extracted individuals, 146 have been successfully sequenced to a level of quality sufficient to provide information on genetically-conferred pyrethroid insecticide resistance, out of 282 attempted sequencing reactions. Achieving a "successful" sequence for this application is more difficult than simply determining a rough base determination sufficient for species barcoding; for the mutation analysis needed here, sequence quality had to be sufficient to distinguish overlapping signals from mutant and wild type in the diploid genome, as described in the Methodology section above.

Rates of pyrethroid resistance mutation through the season and by collection region

From all aphids sequenced in this study, nearly 46% showed genetic resistance to pyrethroid insecticides (Table 1). Many aphids, particularly Green Peach Aphids later in the season, showed two or three independent and redundant mutations. Two peaks of pyrethroid insecticide resistance, as first reported in our Interim Report in August 2023, were confirmed and

better distinguished with additional sequencing; overall, rates of resistance mutations are marginally higher throughout the season in the final analysis, likely because of repeated sequencing of some individuals whose initial assessment was too obscure to accurately determine mutation status.

The early season resistance population peaked in Week 4 (July 18 to 24) with 45.5% of sequenced individuals showing resistance. Resistance was first seen in Week 2 (July 4 to 10), then peaked and declined again to a low but detectable level in weeks 6 and 7 (Aug 1 to 14). Later in the season, in Weeks 10 to 12 (Aug 29 to Sept 15), resistance levels quickly grew to 100% of the population. Resistant individuals were definitively absent only from a single time in the season, the very first week of aphid sampling (late June). While no resistant individuals were found in Week 8, the sample size of sequences was too low do definitively say the rate was zero; instead, the relatively under-sampled weeks 7, 8 and 9 were combined into an average of 19% resistant.

Regionally, there were no significant differences in pyrethroid resistance notable between early season, late season or all season together between the three collection regions (Table 2). While Region 3 appeared to have a lower proportion of resistant aphids over all three time spans, the difference was not statistically significant in the early season, and the rates are not really comparable in the late season (or whole season overall) as very few aphids were available in the collection from Region 3 later in the season in order to make an accurate assessment of resistance rate in that Region alone.

Types of resistance mutations observed

Aphids were screened for mutations at 13 sites on the gene for the Na-channel (*para*) gene that is the target of pyrethroid insecticides. Specific mutations at seven of these sites are known to confer some degree of resistance to pyrethroid insecticides in insects, including aphids. Our observations regarding the types of mutations found have not changed since the August 2023 Interim Report. We observed, almost exclusively, resistance mutations at the “knockdown resistance” (*kdr*; L1014 site) and “super-knockdown resistance” (*skdr*; M918 site) loci, which are the best characterized of these resistance mutations. The *kdr* mutation changes the codon coding for a leucine amino acid at locus 1014 into a phenylalanine amino acid, altering the physico-chemical form of the protein near an interaction point for the pyrethroid molecule, making it insensitive to the insecticide. At the *skdr* site 918, the wild type methionine can be replaced by a leucine, isoleucine or threonine amino acid to confer resistance. All the mutations we observed at *kdr* were the expected L1014F leucine -> phenylalanine change, while at the *skdr* site we observed only the very similar M918L and M918I leucine and isoleucine substitutions, and never the more chemically distinct threonine substitution sometimes reported in the literature. We could only find single mutations in several of the aphids, though others, particularly Green Peach Aphids tested from Weeks 10, 11 and 12 (29 Aug to 15 Sept), had two or even three independent mutations (i.e. the *kdr* phenylalanine substitution and both leucine and isoleucine substitutions carried on alternate chromatids of their diploid chromosomes). This may be important to serve as independently redundant mechanisms of resistance that would complicate efforts to improve the activity of novel pyrethroid formulations, guard against occasional germ line back-mutations or reproduction with non-resistant mates eliminating the resistance of offspring, or simply act as additive degrees of resistance in the individual.

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Table 1: Rates of insecticide resistance mutations through the cropping season on PEI

Dates of week (2022) Start	End	Week	DNA extractions	successful sequences	number of resistance mutations one	two	three	total resistance rate*	
27-Jun	3-Jul	1	31	12	0	0	0	0.00%	
4-Jul	10-Jul	2	22	11	2	0	0	18.2%	17.8% Early season average
11-Jul	17-Jul	3	22	11	3	0	0	27.3%	
18-Jul	24-Jul	4	24	11	4	1	0	45.5%	
25-Jul	31-Jul	5	19	15	1?†	1	0	6.7%	
1-Aug	7-Aug	6	18	13	1	0	0	7.7%	
8-Aug	14-Aug	7	18	11	2	1	0	19.0%	74.0% Late season average
15-Aug	21-Aug	8	12	4	0	0	0		
22-Aug	28-Aug	9	8	6	0	1	0		
29-Aug	5-Sep	10	19	18	2	2	12	88.9%	
5-Sep	9-Sep	11	18	17	0	0	17	100.0%	
10-Sep	15-Sep	12	23	17	1	4††	12	100.0%	
		total	234	146	16	10	41	45.9%	

*rates are calculated from the total number of aphid individuals with at least one resistance mutation per all aphids tested that week, irrespective of the number of independent mutations in each; also, the rate is combined for all aphids in Weeks 7 through 9 as relatively few aphids were sequenced in this period
†a single aphid in Week 5 showed an L925S mutation, cited at a locus known to confer resistance via several types of amino acid substitutions, but of a specific substitution here that has not yet been physiologically characterized

Table 2: Regional distribution of insecticide resistance mutations

		Region 1	Region 2	Region 3
Early season (before 8 Aug)	Resistant individuals	4	6†	3
	All individuals	20	25	28
	Resistance rate	20.0%	24.0%	10.7%
Late season (on/after 8 Aug)	Resistant individuals	26	27	1
	All individuals	39	32	2
	Resistance rate	66.7%	84.4%	50.0%
All season	Resistant individuals	30	32	4
	All individuals	59	57	30
	Resistance rate	50.8%	56.1%	14.8%

We were only able to sequence the *kdr* (L1014) site in Green Peach Aphids, because of the presence of a variable intron sequence immediately adjacent to this mutation. However, as reported in the literature and as we found in the present study and our 2015-16 study in NB, *kdr* mutations appear to always be accompanied by *skdr* mutations, thus the absence of the later – which we can observe in any species – should act as a good indicator for the lack of a *kdr* mutation as well. We could distinguish Green Peach Aphids from others, and gain information directly for *kdr* in this

species, as one of our PCR primers matched a sequence specific to that species alone, situated within the intron region and thus allowing amplification of the nearby *kdr* site.

A single aphid in Week 5 (last week of July) showed a heterozygous mutation of L925S, a leucine -> serine substitution on one copy of the gene at locus 925. A mutation here has been reported to confer physiological resistance to pyrethroids in a range of insect species including aphids. However, literature reports of the mutations here include chemically similar isoleucine, valine or methionine substitutions, which are quite different than the serine substitution we found; therefore, we are not certain that the mutation we discovered would actually confer resistance at the physiological level, and we decided conservatively to leave it out of the calculated rates of pyrethroid insecticide resistance.

Numerous mutations were found in the remaining confirmed-resistance mutation sites and several other mutation sites not yet known to confer resistance. Of those confirmed-resistance sites, mutations were found in three of them (T929, L932 and S989) but not in one of them (I936 – all homozygous wild-type). However, in all cases of DNA mutations in the former three, the mutation produced an alternate codon for the *same amino acid* as the wild-type, thus it was a "silent" mutation that would not confer any functional difference to the aphid. In most cases, these mutations were homozygous isomorphs more likely to be related to evolutionary divergence between species than unusual (i.e. non-wild type) mutations maintained in a population.

Identity of aphid species with resistance mutations

Many of the sample DNA sequences could be identified to match particular aphid species, while others could be identified to the level of Genus (e.g. *Aphis*) or Tribe (e.g. the Macrosiphini tribe of insect subfamily Aphidinae). While sequence comparison of the Na-channel (*para*) gene is not ideal for species assignments because of its less-than-comprehensive coverage in published sequences across all aphid species or genera, several common important species, especially Green Peach Aphid, have extensive published sequences for this gene because of resistance mutation research.

All sequenced individuals were assigned taxonomic identification, as well as possible, to the species or larger group level, with only 19 of 146 sequences of unknown aphid species match. Aphid sequence matches were dominated by *Aphis* species and *M. persicae* (Green Peach Aphid) matching to 33.6% and 46.6% of individuals, respectively. 15 of 49 *Aphis* identities matched closely with *A. gossypii* (Cotton-Melon Aphid, 6 individuals), *A. glycines* (Soybean Aphid, 2 individuals), or *A. fabae* (Black Bean Aphid, 7 individuals). The remaining 34 individuals matched well within the range of the *Aphis* genus, but not closely with any species with published Na-channel (*para*) gene sequences. A notable member of this genus expected to occur in PEI that could be responsible for some of these unidentified "*Aphis* sp." matches would be the Buckthorn aphid (*A. nasturtii*), but this species for example, does not have any published Na-channel (*para*) gene sequences to directly compare our samples to. All *M. persicae* sequences but one slightly ambiguous sequence in Week 1 showed high match confidence, likely as it is the best characterized species for pyrethroid resistance genetics. *R. padi*, on the other hand, was a little less definitive because of limited sequence information, and a few individuals aligning within the aphid tribe Macrosiphini (including common Potato Aphid and Pea Aphid) could not be identified to a more specific level because of very poor published sequence coverage in this group.

Most notable in this sequence analysis is that it could be used to identify what species contained resistance mutations, and when these species were present during the season. The majority of the resistant individuals, unsurprisingly, were definitively identified as *M. persicae*

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Table 3. Species identity based on blastn analysis of Na-channel (*para*) gene sequence. Species identity and confidence is shown for the insecticide resistant individuals, with percentages showing base-matching of sample DNA sequence to known published sequences in the NCBI database, or "(specific)" denoting identification by M. persicae-specific PCR primers. Ranges in percentages indicate lowest and highest match estimates based off infrequent ambiguities in the sequencing reaction results and variation in published sequences. In general, sequence matches are likely within species if >97%, among species within a genus (e.g. Aphis spp.) if ~94% to 97%. "HIGH" confidence distinctions indicate high likelihood of species match, "LOW" are included where the species indicated have a better match than any other, but lower than the expected "within species" level of >97.5%. "Aphis" category includes three identified species, as well as a generic "Aphis sp." where the sample sequence is 94-97% match to several Aphis species; the "Macrosiphini" category notably includes the colonizing Potato Aphid, and common non-colonizer Pea Aphid. Assignment to species cannot be made in this group, however, due to the underrepresented in published sequences. "Unknown" indicates no match better than 92% to any published species nor clear bias toward any one aphid group over others.

	Aphis				R. padi		Macro-siphini	Myzus persicae			
week	Aphis sp.	A. gossypii	A. glycines	A. fabae	HIGH	LOW	LOW	HIGH	LOW	Unknown	Resistant individuals
1	5	2	1	0	1	0	1	0	1	1	(none)
2	5	0	0	1	1	0	0	4	0	0	M. persicae 97.8-100% M. persicae 99.0-99.7%
3	6	1	0	0	0	2	0	1	0	1	2x R. padi (LOW) 92.1-97.3% M. persicae 99.4%
4	3	0	0	1	1	1	1	4	0	0	R. padi (HIGH) 97.5-99.4% R. padi (LOW) 94.0-98.1% 3x M. persicae 98.7-99.7%
5	7	0	0	3	0	0	1	1	0	3	M. persicae 98.2-99.7%
6	4	1	0	1	0	0	1	1	0	5	M. persicae 97.7-99.0%
7	2	0	1	1	0	0	0	3	0	4	3x M. persicae 98.7-100%
8	0	1	0	0	0	0	0	0	0	3	(none)
9	2	1	0	0	0	0	0	1	0	2	M. persicae 97.4-99.4%
10	0	0	0	0	0	0	0	18	0	0	4x M. persicae 98.5-100% 12x M. persicae (specific)
11	0	0	0	0	0	0	0	17	0	0	17x M. persicae (specific)
12	0	0	0	0	0	0	0	17	0	0	2x M. persicae 99.0-100% 15x M. persicae (specific)

(94% of resistant individuals season-wide), and the only other species match found with resistant individuals was *R. padi* (Bird Cherry Oat Aphid; 6% of resistant individuals). Most surprising from this analysis was that substantial numbers of strong *M. persicae* matches occurred even early in the season, despite visual anatomical assessment not identifying these aphids as that species. There is also a bias toward lower rates of resistance in *M. persicae* individuals early in the season (67% in Weeks 1-6) compared to late in the season (96% in Weeks 7 to 12), which is not unexpected if pyrethroid use in the field was selecting for resistant individuals through multiple generations during the cropping season.

Conclusions:

In this study, we analyzed DNA sequences to determine rates of pyrethroid insecticide resistance in preserved aphids collected through the 2022 season in PEI. Our findings indicate that 46% of the tested aphids exhibited at least one pyrethroid resistance mutation. Notably, resistance levels changed significantly over the season, with a distinct peak early (around mid to late July), a decline in early August, then rising to a later peak in early to mid-September where 100% of tested aphids were resistant. Unlike the changes in resistance through time, we did not find convincing differences in rates from different regions of PEI. The vast majority (94%) of the resistance mutations were linked to *M. persicae* (Green Peach Aphid), with the remaining 6% associated with sequences most closely matching *R. padi* (Bird Cherry Oat Aphid). Surprisingly, a substantial number of early-season aphids were genetically matched to *M. persicae*, contrary to visual assessments of the aphids when collected. This study shows that substantial pyrethroid insecticide resistance occurs in the aphids of PEI, even early in the season, but especially late in the season. The levels of resistance observed in this study exceed that found in the two year survey (2015-2016) in NB (MacKenzie et al. 2018), which in itself found an increase in resistance in the second year of the survey compared to the first; this suggests that resistance is similar across our region, and increasing over time. Our research was limited to resistance to the pyrethroid insecticide class, generally among the most commonly used foliar insecticides in the region, and for which the greatest concentration of study and characterization of resistance mutations is known. It is important to note, however, that similar resistance mechanisms have also been seen to other commonly used insecticide classes such as neonicotinoids, organophosphates and others, but they were not studied as a part of this survey.

References:

Criniti, A., Mazzoni, E., Cassanelli, S., Cravedi, P., Tondelli, A., Bizzaro, D., & Manicardi, G. C. (2008). Biochemical and molecular diagnosis of insecticide resistance conferred by esterase, MACE, kdr and super-kdr based mechanisms in Italian strains of the peach potato aphid, Myzus persicae (Sulzer). *Pesticide Biochemistry and Physiology*, *90*(3), 168-174.

Fontaine, S., Caddoux, L., Brazier, C., Bertho, C., Bertolla, P., Micoud, A., & Roy, L. (2011). Uncommon associations in target resistance among French populations of Myzus persicae from oilseed rape crops. *Pest management science*, *67*(8), 881-885.

Gillespie, D. R., Quiring, D. J. M., Foottit, R. G., Foster, S. P., & Acheampong, S. (2009). Implications of phenotypic variation of Myzus persicae (Hemiptera: Aphididae) for biological control on greenhouse pepper plants. *Journal of applied entomology*, *133*(7), 505-511.

MacKenzie, T. D., Arju, I., Poirier, R., & Singh, M. (2018). A genetic survey of pyrethroid insecticide resistance in aphids in New Brunswick, Canada, with particular emphasis on aphids as vectors of potato virus Y. *Journal of economic entomology*, *111*(3), 1361-1368

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