NYWGF RESEARCH - FINAL REPORT TEMPLATE

Please fill in by typing over the red directions in each section and change font to black.

Funding for fiscal year: 2022-2023

SECTION 1:

Project title: Development of a high throughput assay to detect insecticide resistance in *Drosophila melanogaster*

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New Research ☒ **Continued Research** ☐ (CHECK APPROPRIATE BOX)

Amount Funded **$** 49,585

SECTION 2: (This section should be in depth and akin to an academic report)

Project Summary Impact Statement: Sour rot is a devastating disease of wine grapes in NY. Management is achieved by late season control of vectors (fruit flies) of the disease. Insecticide resistance in *Drosophila melanogaster* is severe and widespread in NY. Current assays for resistance are time consuming and of low resolution. We seek to develop a rapid, high throughput assay to detect resistance providing the knowledge needed to better protect NY vineyards.

Objectives: Our goal is to improve control of *Drosophila melanogaster,* and hence sour rot, in NY vineyards. To obtain our goal, we will pursue four objectives that focus on the only insecticide to which resistance is not yet widespread (spinetoram, the active ingredient in Delegate).

1. Create a strain of *Drosophila melanogaster* that is congenic to a susceptible strain, but is resistant to spinetoram (the active ingredient in Delegate).
2. Conduct a bulk segregant analysis of the resistant and susceptible strains to determine the candidate mutations responsible for resistance.
3. Modify candidate mutations (via CRISPR) to confirm the causal resistance mutation(s) is identified.
4. Develop allele specific PCR to detect resistance in field populations *of D. melanogaster* which will greatly enhance our ability to detect resistance.

Materials & Methods: *2.1. Drosophila melanogaster*

Drosophila melanogaster populations were collected from each of 9 vineyards and 1 orchard in New York, United States in 2019 (Mertz et al. 2021). One additional strain, originating from NY, was also used. SpR was the strain resulting from selection of the NY18 population against spinosad for two generations (Mertz et al. 2023). Canton-S was the laboratory susceptible strain used in all experiments. *D. melanogaster* were reared on standard fly medium under a standard laboratory environment (~23 ℃) with a photoperiod of about 12L: 12D as previously described (Sun et al. 2019).

2.2. Insecticide Bioassays

Spinetoram (96.4%) was from Chem Service, West Chester, PA, USA. Spinetoram was dissolved in acetone (Fisher Scientific, Fair Lawn, NJ) and 0.5 mL was applied evenly to the inside of a scintillation vial (Wheaton Scientific, Millville, NJ, USA) with an internal surface area of 38.6 cm^2 and allowed to evaporate on a hot dog rolling machine (Gold Medal Products Co., Cincinnati, OH, USA) at room temperature for at least 30 min before flies were placed inside. Controls were treated with acetone only. Stoppers were made with a piece of cotton covered by white nylon tulle and 10% sugar water was applied with a syringe to saturate the stoppers. Each treated vial containing 20 female flies (3- to 7-d old) was laid on its side and held in a chamber at 25℃ with a photoperiod of 16 L: 8 D. Mortality was assessed after 24 and/or 48 h of exposure for all insecticides, and flies were considered dead if they were ataxic.

LD_{50} values were determined by probit analyses conducted with at least three replicates from two different cohorts for each concentration. Each replicate included a control concentration (acetone only), the highest concentration that gave a zero percent mortality, the lowest concentration that gave 100% mortality, and at least three concentrations in-between. Bioassay data were pooled, corrected for control mortality using the method of Abbott (Abbott 1925) and analyzed by standard probit analysis using an R script (**https://github.com/JuanSilva89/Probit-analysis**) version 4.

2.3. Selections

A series of five selections were conducted starting with the populations listed above, using the residual exposure method. Thirty flies were placed in vials treated as described above. The populations and concentrations used in the selections are shown in Table 1. Flies from each selection were preserved at -70°C.

2.4. DNA Extraction

DNA was extracted from *D. melanogaster* from the Ulster Co, Chautauqua, Schuyler, Saint Genevieve, and Suffolk populations that had been collected in 2019 and stored at -70℃. A total of 94 or 96 individuals, half female and half male, were used for each population. Selected flies were put in 10 µL 0.2 M NaOH along with three or four zircon beads in individual wells of a 96-well PCR plate. Plates were vortexed at top speed for one minute. Lysis was performed by incubating at 70℃ for 10 minutes. Finally, 90 µL of a 1:9 10X TE and ddH_2O was added to neutralize the solution.

2.5. Cloning and sequencing of cDNAs.

Total RNA was extracted from a pool of 30 SpinR adults (whole bodies, 15 male, 15 female), 1-2 d old that had been frozen at -80 °C overnight prior to RNA extraction. Total RNA was isolated as described elsewhere (Green and Sambrook 2020) with the following modifications: flies were initially homogenized for 1-2 min in 1 mL of TRIzol (Life Technologies, Carlsbad, CA) with a pestle in a 1.5 mL microcentrifuge tube, and the purified RNA was resuspended in 50 µL water. Nucleic acid purity and concentrations were assessed on a NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA). Total RNA samples were DNAse treated to remove residual genomic DNA (Turbo DNA-*free*, Invitrogen, Waltham, MA). cDNA libraries were prepared with the GoScript kit (Promega, Madison, WI). First-strand cDNA synthesis reactions were prepared according to manufacturer's recommendations with 2.5 mM $MgCl_2$ final concentration, equal amounts of random primer and oligo(dT) primer, and 1.0 µg total RNA in a 20 µL reaction. PCR was carried out with GoTaq polymerase (Promega) in an iCycler thermalcycler (Bio-Rad, Hercules, CA) and the following thermalcycler conditions: 5 min, 95 °C; 35 cycles of PCR (30 s, 55 °C; 30 s, 95 °C; 2 min 72 °C); 10 min, 72 °C. The amplicon was purified by gel electrophoresis in 1.0% agarose and extracted with the Wizard SV gel extraction kit (Promega) prior to cloning. Clones were obtained from two different pools of flies.

Clones of the amplified cDNAs were prepared with the pGEM-T Easy kit (Promega) according to manufacturer's instructions. Insert and vector were combined at equimolar ratio and ligated overnight at 4 °C. Ligation reactions were transformed into competent JM109 cells (Promega) by heat shock, and transformants were plated on LB-agar with 100 µg/mL ampicillin, 80 µg/mL X-gal, and 0.5 mM IPTG for blue/white colony screening and incubated at 37 °C. Individual colonies were cultured in LB with 100 µg/mL ampicillin at 37 °C with shaking overnight and miniprepped with the Wizard Plus SV miniprep kit (Promega). Purified plasmids were screened for full-length insert by digestion with EcoRI (Thermo Fisher) and visualization by gel electrophoresis. Plasmids were submitted to Plasmidsaurus (Eugene, OR) for long read sequencing. Sequenced clones were analyzed by alignment to transcript sequences annotated in FlyBase.

2.6. Genotyping by allele-specific PCR

A multi-primer amplification refractory mutation system (ARMS) was used for genotyping by PCR (Little 1995). Allele-specific primers were designed to amplify an allele-specific 674-676 bp fragment of *alpha 6* that includes parts of exons 8 and 9. The external (allele-specific) reverse primers were complementary to either the resistant or susceptible allele at their 3' base. The internal control primers were designed to amplify a 445 bp fragment of *Ace* fully complementary to either strain's sequence. Primers were ordered from IDT DNA (Morrisville, NC). The primer sequences are given in Supplementary Table 1. The reaction mixture was 12.5 µL GoTaq Green (Promega, Madison WI), 9.5 µL H2O, 0.5 µL Dace-F1, 0.5 µL Dace-

R1, 0.5 µL of either Da6-R12 (resistant) or Da6-R11 (wild type), 0.5 µL of either Da6-F14 (resistant) or Da6-F12 (wild type), and 1 µL of DNA extraction. Empirically optimized PCR conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 58°C for 30 seconds, and 72°C for 30 seconds, with a final extension time of 2 minutes at 72°C. After amplification, 3 µL of each reaction were electrophoresed on a 2% agarose-TBE gel stained with ethidium bromide. A 96-well plate was used to hold samples, and all wells were used for each population with 48 females and 48 males. Each gel resulted in at least 70 clear samples per population.

Results/Outcomes/Next Steps:

3.1 Selection of spinetoram resistance
The average mortality across all the populations in the first selection was 62% for a mix of males and females at 48 h (Table 1). The concentration of spinetoram was increased ~8-fold for the second selection and caused a 75% mortality at 24 h. A third selection at another ~8-fold increase killed 81% at 24 h. The fourth selection was carried at a 2-fold increase killing 46% at 48 h. The final selection used 26,500 ng/cm^3 for unmated females and males. This resulted in 63% and 93% mortality in females and males, respectively.

3.2. Characterization of resistance in the SpinS5 strain
By topical application, the SpinS5 stain had a spinetoram resistance ratio of 265 for females, and 194 for males, in comparison to the susceptible strain Canton-S (Table 2). Neither PBO nor DEF reduced the resistance observed in SpinR, suggesting that CYPs and hydrolases are not involved in the resistance (Table 3).

3.3 Cloning and sequencing of α6nAChR
In order to identify mutations that could be responsible for spinetoram resistance in SpinR, we obtained six full length cDNAs. We identified a single mutation associated with resistance.

3.4 Frequency of the resistance mutation in field collected and laboratory selected lines.
The SpinR strain was selected from field populations across five generations. Genotyping flies from each of the selections shows the rapid increase in the frequency of the resistance allele from the first selection until the final selection, after which the population contained only the resistance allele (Figure 1). The frequency of the resistance allele was low in the populations collected in 2019 (i.e. those from which SpinR was selected) (Mertz et al. 2021). The resistance allele was not detected in either the Chautauqua (n=80) or Suffolk (n=70) populations. A single individual heterozygous for the resistance allele (n=90) was found in the Ste. Genevieve (MO) population. The resistance allele was detected at frequencies of 18 and 3.6 % in the Ulster (n=86) and Schuyler #1 n=83) populations, respectively.

Technology Transfer Plan:

<u>Presentations</u>

Gilrein, D. 2022. Discussed in numerous Fruit and Vegetable Update newsletters (Suffolk County, goes to agriculture producers in the region): 6/23, 7/7, 7/21, 7/28, 8/4, 8/18, 89/1, 9/8, 9/15, 9/22 and 9/29.
Loeb, G. 2023. Update on managing pests of grapes: both the new and the old. 45 minute in-person presentation in the Viticulture session at the 2023 Long Island Ag Forum. Session was held in Riverhead, NY on 12 January 2023. Approx. 30 in attendance. Mostly growers but some extension educators. Contact hours = 22.5.

Loeb, G. 2022, Entomology update: insects and grape disease and managing new invasive species. 35-minute talk (in person) for the Lake Erie Regional Grape Program Winter Grape Growers' Conference held in Fredonia, NY on 16 March 2022. Approximately 100 in attendance. Contact hours = 58.3.

Loeb, G. 2022. Program update: management of arthropod pests of grapes. 45-minute talk (virtual) for the Long Island Hort Show, Viticulture Session. Approximately 70 in attendance. Contact hours = 52.5.

Russo, J. 2022. Discussed at our in-person Coffee Pot Meetings throughout the 2022 growing season when discussing IPM strategies, specifically on the following dates: 6/29, 7/6, 7/13, 7/20, 7/27. Discussed at a Twilight meeting on 7/27 and again on 12/9 in a Virtual Spray Program.

Wise, A. and Gilrein, D. 2023. Long Is Agriculture Forum Viticulture Session our Viticulturist Alice Wise spoke about these topics in an update on the berry cuticle enhancer project.

Zaman, F. and Gilrein, D. 2022. Long Island Spring Grape Pest Management Meeting (webinar) on 3/15/22.

Publications
Loeb, G. 2022. Grape and Mite Pests, 2022 Field Season. Posted online at https://bpb-us-e1.wpmucdn.com/blogs.cornell.edu/dist/3/7313/files/2022/11/2022-insects-grapes-review.pdf and published in newsletters from Finger Lakes grape program, Lake Erie grape program, and Long Island grape program.

Bhandari, R., Hesler, S. Scott, J., Gold, K., and Loeb, G. 2022. Understanding berry injury as a risk factor for sour rot and evaluating sustainable management options. Appellation Cornell, Research Focus 2022-1, May 2022.

Attachments: None

SECTION 3: (The goal of this research is to benefit growers and producers across New York State. Result summaries will be shared on the NYWGF website and via email newsletters. To that end, this section should be brief and written in terms understandable for the average grower and producer, as well as consumers and trade interested in our industry.)

Project summary and objectives:
Spinetoram (Delegate) is the only registered insecticide (for control of *Drosophila*

melanogaster in vineyards) to which vinegar flies have not yet evolved high levels of resistance. However, low levels of resistance have been found in vineyard populations, and we were able to select (in the laboratory) a highly resistant strain. We identified the mutation responsible for the resistance and have developed a rapid, high-throughput assay for resistance.

Importance of research to the NY wine industry:
Sour rot is a devastating disease of wine grapes in NY. Management is achieved by late season control of vectors (fruit flies) of the disease. Insecticide resistance in *Drosophila melanogaster* is severe and widespread in NY, limiting our ability to control flies and sour rot. Current assays for resistance are time consuming and of low resolution. We have developed a new, rapid, high throughput assay to monitor resistance to spinetoram (Delegate). Results will provide the information needed to better manage the use of Delegate, prolong its efficacy, and protect NY vineyards.

Project Results/next steps: Our next steps are to develop an effective trap for *D. melanogaster* that will allow us to use the captured flies and determine the frequency of the resistance allele in each population. We will compare the frequency of resistance alleles to use of Delegate in vineyards to improve our understanding of the evolution of resistance.

Supporting attachments: (Choose a maximum of 1 supporting figure or table to demonstrate results if desired)