Proposal Title: Screening kasugamycin resistance in *Erwinia amylovora* on pears

Report Type: Final report

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Project Duration: 1 Year

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WTFRC Collaborative Costs: None

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Item	2024		
Salaries[1]	$14,400.00		
Benefits[1]	$5,778.00		
Salaries[2]	$1,250.00		
Benefits[2]	$513.00		
RCA Room Rental			
Shipping[3]	$292.00		
Supplies	$2,805.00		
Travel[4]	$848.00		
Plot Fees			
Miscellaneous			
Total	$25,886.00	$0.00	$0.00

Footnotes: [1]Postdoc salary (Zhao lab) for 3 months at $4,800/month and postdoc benefit rate at 40.1%. [2]Technician salary (Tianna lab) for 0.25 month at $5,000/month and benefit rate at 41%. [3]shipping cost and materials (Tianna). [4]Milage for collecting samples (Tianna $348; Zhao $500).

Objectives:

1. To collect and screen kasugamycin (will also include streptomycin and oxytetracycline) resistance in pear orchards throughout the state and possibly determine the resistant/tolerant nature;
2. To deliver results to growers and provide guidance on kasugamycin use in orchards.

Significant Findings:

- No *Erwinia amylovora* isolates exhibited resistant to streptomycin and oxytetracycline in 2024;
- 7 *Erwinia amylovora* isolates exhibited resistance/tolerance to kasugamycin in 2024;
- The resistant/tolerant isolates were isolated from orchards in Sunnyside and Dryden.
- Mutation in the kasugamycin target *ksgA* gene was found in five of the resistant/tolerant *E. amylovora* isolates.
- This is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington or elsewhere.
- These results suggest that growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease.
- Based on our findings, we recommended that growers should mix kasugamycin with oxytetracycline or be in rotation with streptomycin for fire blight control.

Significance to the industry and potential economic benefits.
Since the identification of streptomycin-resistant strains of *E. amylovora* by Loper et al. in 1991, there has been limited data in evaluating the status of antibiotic resistance throughout the central Washington regions. The significance of this research to the industry lies in two aspects. First, this is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington or elsewhere and the isolates were from orchards in two different locations, Sunnyside, and Dryden. Growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease. Based on our findings, growers should rotate kasugamycin with other antibiotics such as

streptomycin/products to treat fire blight or should mix kasugamycin with oxytetracycline. In summary, the findings of the current project directly benefit the growers of Washington state by providing instant feedback to growers in antibiotics resistance situation in orchards and growers should take immediate actions to avoid control failure.

Methods and Procedures:

In 2024, we either collected symptomatic samples in central Washington by our own field trips to local area growers or samples were sent to us via mail by growers or consultants or extension specialists. We also collected asymptomatic blossom samples. Samples were placed in plastic bags and held on ice or in a refrigerator until they were processed. Samples were processed by cutting into small pieces with a sterile knife, washed briefly with sterile water, soaked in 900 µl 10 mM PBS, vortexed, and streaked for isolation onto five types of media: LB, CCT, LB + Sm 100 µg/mL, LB + Kg 100 µg/mL, LB + Tc 20 µg/mL and incubated at 82.5 Fº (28 °C) for 48 - 72 h. Colonies that appeared white in color on CCT media, slightly raised and nonfluorescent were suspected to be *E. amylovora*. Screening for resistance was performed by observing the presence of individual colonies on antibiotic media. Isolates of known resistant *E. amylovora* strains were obtained from culture collections for use as positive controls. Isolates were then confirmed by PCR using *E. amylovora* specific primers G1-F and G2-R.

Spot dilution test was performed for selected resistant/tolerant strains (**Figure 1**). Bacteria were grown on LB plates and a single colony was inoculated in LB broth and grown for 24 hr with shaking at 250 rpm. Bacterial suspensions were adjusted to an absorbance of OD_{600} = 1 in PBS and 10-fold serial dilution was made in PBS. For each dilution, 5 µL was spotted onto plates: LB and LB + Kg 50, 75, 100 125, and 150 µg/mL and incubated at 82.5 Fº (28°C) for 48 - 72 h. Bacterial growth was visually observed on plates with or without antibiotics. Growth on plates without antibiotics was used as a control to compare to the plates with antibiotics.

In addition, the minimum inhibitory concentration (MIC) was determined. Bacteria were grown on LB plates and a single colony was inoculated in LB broth with shaking at 250 rpm. Overnight bacterial suspensions were adjusted to an initial concentration of OD600 = 0.1 and 2-fold serial dilutions were performed, starting with LB + Kg1000 µg/mL and ending with LB + Kg 0.976 µg/mL. IC_{50} was defined as the concentration of antibiotics at which growth of the bacterium was 50 % less of that of the control without antibiotics. IC_{95} was defined as the concentration of antibiotics at which growth of the bacterium was 95 % less of that of the control without antibiotics.

Selected resistant/tolerant *E. amylovora* isolates were used to amplify the kasugamycin target *ksgA* gene by primers KsgA-F and KsgA-R. PCR products were then sequenced by Eton Biosciences Inc, San Diego, CA and compared to those of known sensitive strains.

Results and Discussion:

Samples were collected from three pear varieties in central Washington, i.e. Bosc, Anjou, and Bartlett. *E. amylovora* isolates were confirmed by PCR. Seven isolates collected in 2024 from Bartlett pears were shown to be resistant or tolerant to kasugamycin at 100/150 ppm, respectively. However, no isolates were found to be resistant to streptomycin or oxytetracycline. Among the resistant/tolerant isolates, colony size was significantly smaller as compared to growth of the same isolate on LB medium and spot dilution assay showed similar growth for resistant/tolerant isolates at LB with antibiotics and without antibiotics (**Figures 1 and 2**). These resistant/tolerant isolates were able to grow on plates with kasugamycin at 50, 75, 100, 125 and 150 ppm. These findings indicated that these isolates from 2024 were shown to be resistant/tolerant to kasugamycin.

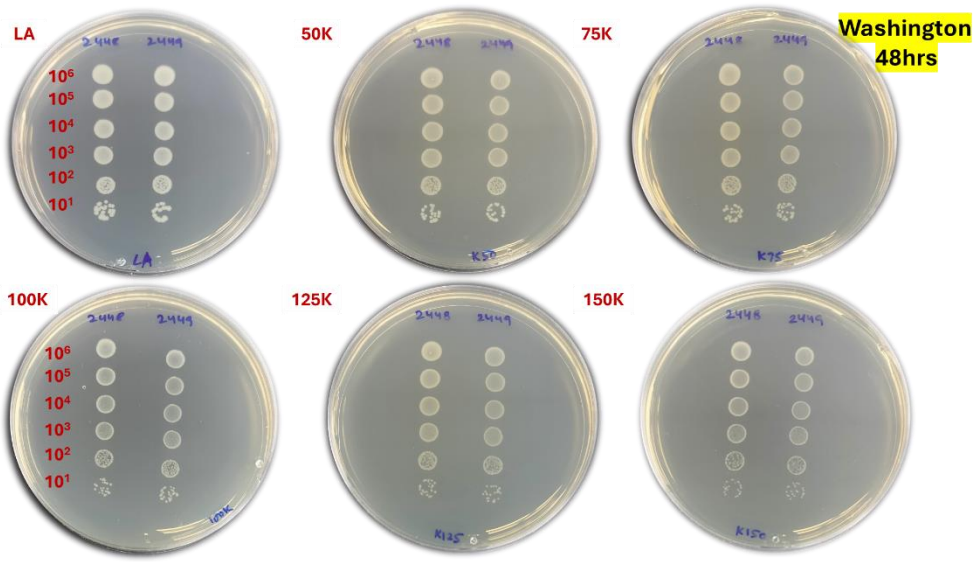

Figure 1. Spot dilution assay for two representative resistant isolates. Serial 10-fold dilutions were made in PBS. For each dilution, 5µL was spotted on LB plates containing no antibiotics or kasugamycin at 0, 50, 75, 100, 125, and 150 µg/ml. Pictures were taken 48 hours post inoculation. Both 2448 and 2449 are resistant to kasugamycin.

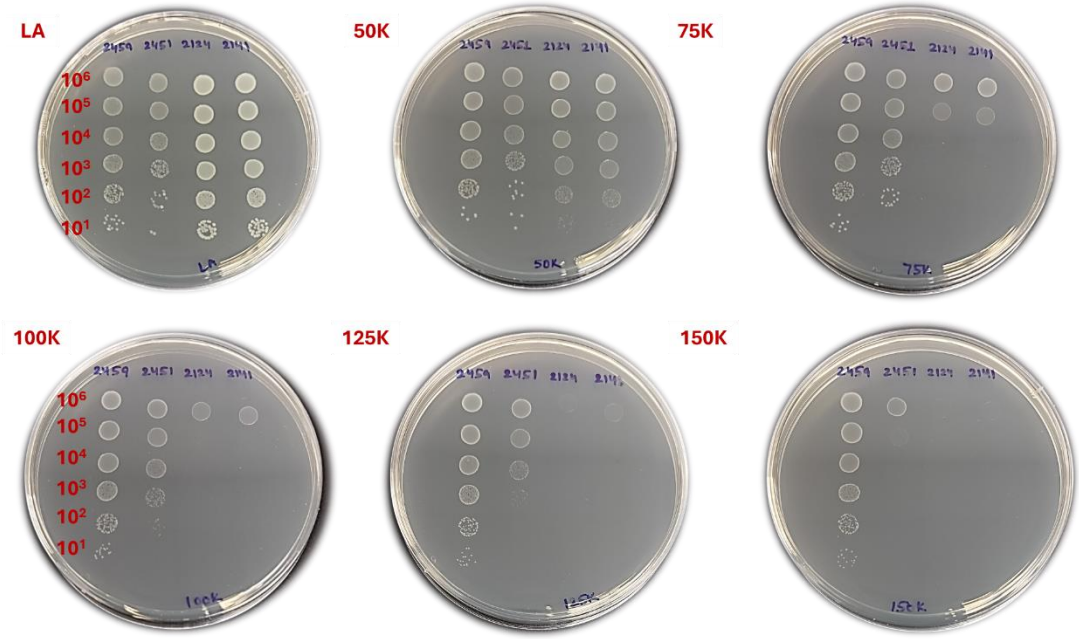

Figure 2. Spot dilution assay for one representative resistant isolate as compared to three tolerant/sensitive isolates. Serial 10-fold dilutions were made in PBS. For each dilution, 5µL was spotted on LB plates containing no antibiotics or kasugamycin at 0, 50, 75, 100, 125, and 150 µg/ml. Pictures were taken 48 hours post inoculation. Isolate 2459 is resistant; whereas isolate 2451 is tolerant and 2124 and 2141 are sensitive.

Next, we determined MIC_{50} and MIC_{95} for 7 isolates from WA. The five resistant strains isolated from 2024 had the highest MIC_{50} of more than 150 µg/ml and the MIC_{50} for two tolerant isolates was above 60 µg/ml. Similarly, the five resistant strains isolated from 2024 had the highest MIC_{95} of about 400 µg/ml and the MIC_{95} for two tolerant isolates was above 200 µg/ml. Sequence comparison of the *ksgA* gene showed mutations in five resistant isolates as compared to known type strains (data not shown). Based on previous studies, resistance to kasugamycin arises from mutations of its target gene *ksgA*, encoding an adenine demethylase. Our results indicate that resistance to kasugamycin of the five *E. amylovora* isolates is due to mutations in the *ksgA* gene. This is the first time we found mutations in the *ksgA* gene in *Erwinia amylovora*.

Executive Summary

Project Title: Comprehensive monitoring and mapping antibiotics resistance in orchards

Key words: Fire blight, antibiotics resistance, streptomycin, tetracycline, kasugamycin

Abstract: Antibiotics remain one of the best tools for managing blossom blight of apple and streptomycin remains the better choice in terms of cost and efficacy in killing pathogens as compared to tetracycline and kasugamycin. The occurrence of streptomycin resistance of the fire blight pathogen in WA pear orchards in 1980s results in increased use of tetracycline and kasugamycin. However, there has been limited data evaluating the existence and extent of antibiotic resistance of *Erwinia amylovora* in central WA since then. The purpose of the current study was to comprehensively monitor and map antibiotics resistance in orchards in WA. In 2024 growing seasons, diseased samples were collected from pear orchards and *E. amylovora* isolates were examined for their resistance to streptomycin, oxytetracycline and kasugamycin. Although no *E. amylovora* isolates exhibited resistance to streptomycin and oxytetracycline, 7 isolates exhibited resistance or tolerance to kasugamycin in 2024. WA isolates from 2024 had the highest MIC_{50} as compared to previous years. Mutation was found in the kasugamycin target *ksgA* gene in five of the resistant/tolerant *E. amylovora* isolates. This is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington. These results suggest that growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease. Based on our findings, we recommended that growers should mix kasugamycin with oxytetracycline or be in rotation with streptomycin.