VIRGINIA CORN BOARD

STEVENS INTERIM REPORT FOR 2024/2025 PROJECT PROPOSAL

Title:

Identifying Corn Xylem Fluid Nutrients Important for Microbial Phytopathogen Growth

Project Leader: **Dr. Ann M. Stevens**

5036 Derring Hall, 926 West Campus Drive, Virginia Tech

Blacksburg VA 24061

Project Objectives:

Aim 1: Determine the nutrients present in corn xylem fluid through both untargeted and targeted metabolomics approaches

Aim 2: Develop and utilize synthetic xylem fluid for laboratory-based growth experiments of microbial corn pathogens

Results:

Executive Summary: From May 2024 through December 2024 we have determined sole sources of carbon or nitrogen used as nutrient sources by the xylem-dwelling corn wilt pathogen *Pantoea stewartii* subsp. *stewartii* (Phase 1) and used targeted metabolomics to define the major organic nutrient sources present in corn xylem fluid (Phase 2). Work to generate synthetic xylem medium (Phase 3) is in progress. Project outputs (i.e., presentations, grant proposals, manuscripts) to date are listed at the end of the report.

Phase I: Analyze the ability of *P. stewartii* to use sole sources of carbon or nitrogen for metabolism *in vitro*

Abstract: The bacterium *P. stewartii* causes Stewart's wilt disease in maize grown in the USA. *P. stewartii* is introduced into maize via the corn flea beetle vector, *Chaetocnema pulicaria*,

when beetle feces enter wounds created during feeding. The infection begins in the apoplast of the leaf where *P. stewartii* causes leaf blight. Subsequently, the bacteria move to the xylem and form a biofilm, preventing water transport. This causes wilting and leads to necrosis, consequently affecting both crop yield and survival. Biolog plates were used to examine the capacity of *P. stewartii* to grow using sole carbon or nitrogen sources *in vitro*. This work has provided insights into how *P. stewartii* exploits nutrients in the maize xylem environment during its growth there.

Materials and Methods:

Bacterial strains and growth medium. Wild-type *P. stewartii* DC283 (3) was used for experiments. The bacteria were grown in Luria Bertani broth (LB; 10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract) supplemented with 30 μg/ml nalidixic acid (NA).

Growth assays in Biolog PM1 and PM3 Microplates. Biolog plates contained a negative control well and 95 sole source carbon (PM1) or nitrogen (PM3) substrates. The microplates were prepared following the manufacturer's protocol. However, the volumes were adjusted to prepare 10 mL of inoculating buffer for just one plate at a time. For PM1 plates, 8.33 mL of 1.2x IF-0 (Biolog), 1.57 mL of sterile water, 0.1 mL of 100x tetrazolium indicator dye (Biolog) were combined. For PM3 plates, 8.33 mL of 1.2x IF-0 (Biolog), 1.37 mL of sterile water, 0.1 mL of 100x tetrazolium indicator dye (Biolog), and 0.2 mL of 1 M sodium-succinate were combined. Overnight LB liquid cultures of *P. stewartii* DC283 were diluted to an OD_{600} of 0.02 and grown to an OD_{600} of 0.2. Then two 1.25 mL aliquots of culture were washed twice with an equal volume of phosphate buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.4). Cells were centrifuged at 15,000 RPM for 5 min in an Eppendorf microcentrifuge 5424 with rotor FA-45-24-11. The washed cell pellets were resuspended in the

inoculating buffer to achieve an OD_{600} of 0.05 and 100 µL was pipetted into each well on the microplate. A BreatheEasy membrane (Diversified Biotech) was added to the top of the microplate, then the plates were incubated in a Synergy HTX multimode plate reader at 30°C for 24 hr with orbital shaking and an absorbance reading at 590 nm was taken every 20 min. Absorbance readings from the final 24 hr time point for the three experimental trials were averaged and standard error was calculated. A t-test was performed using two-tailed distribution and equal variance parameters to determine significant differences ($p<0.05$) between the negative control versus each sole source carbon or nitrogen source.

Results and Discussion:

Ability of *P. stewartii* to metabolize sole carbon and nitrogen sources *in vitro*. Biolog plates were used to measure *P. stewartii* DC283 growth after 24 hr on sole carbon and nitrogen sources, as indicated by the relative absorbance (590 nm) of the tetrazolium dye. Statistical t-tests were used to the growth of wild-type DC283 growth for a given substrate to the negative control to determine significantly higher growth (Table 1).

Out of the 95 carbon sources on the PM1 microplate, there were 41 that DC283 metabolized significantly better than the negative control, based on the average absorbance values (Table 1). Some of these findings confirmed previous work using defined medium to establish the metabolic capabilities of the bacterium. Malate, succinate, glycerol, fumarate, and pyruvate were sole carbon sources highly abundant in the apoplast and *P. stewartii* grew efficiently on them *in vitro* (6).

Out of the 95 nitrogen sources on the PM3 microplates, there were 31 that DC283 metabolized significantly better than the negative control (Table 1). Thus, specific sole organic

acids, sugars, and amino acids can be used as nutrient sources by *P. stewartii* DC283 *in vitro* and also likely within a corn plant, if they are available.

Table 1: Biolog carbon and nitrogen sources used by wild-type *P. stewartii* significantly more than the negative control, arranged from highest absorbance at 590 nm to lowest

Carbon source	DC283 mean	DC283 STE	t-test		Nitrogen source	DC283 mean	DC283 STE	t-test
D-Glucose-6-phosphate	2.165	0.036	2.9E-06		Adenosine	1.844	0.096	3.1E-04
L-Arabinose	2.109	0.025	8.8E-07		Cytidine	1.842	0.082	1.7E-04
L-Glutamine	2.077	0.037	3.8E-06		Ala-Glu	1.816	0.100	4.0E-04
L-Asparagine	2.053	0.049	1.2E-05		Ala-Gln	1.752	0.027	3.1E-06
myo-Inositol	2.039	0.066	3.8E-05		Gly-Asn	1.722	0.018	8.0E-07
α-Methyl-D Galactoside	1.907	0.031	3.5E-06		Ala-Asp	1.711	0.037	1.3E-05
D-xylose	1.891	0.040	9.6E-06		L-Methionine	1.673	0.075	2.3E-04
D-gluconic acid	1.888	0.045	1.4E-05		Ala-Leu	1.652	0.010	1.3E-07
L-Serine	1.873	0.074	1.1E-04		Gly-Gln	1.645	0.039	2.1E-05
D-mannose	1.872	0.028	2.9E-06		Ala-Gly	1.629	0.029	6.7E-06
D-Fructose-6- Phosphate	1.844	0.039	1.0E-05		D-Glucosamine	1.618	0.159	5.0E-03
D-Ribose	1.827	0.067	8.6E-05		L-Serine	1.584	0.048	6.0E-05
D-Glucose-1- Phosphate	1.817	0.038	9.9E-06		N-Acetyl-D Glucosamine	1.521	0.015	1.0E-06
Fumaric Acid	1.799	0.045	2.1E-05		L-Glutamine	1.507	0.060	2.1E-04
D-Mannitol	1.781	0.051	3.6E-05		Ala-His	1.460	0.010	3.2E-07
D-Fructose	1.777	0.033	7.3E-06		L-Asparagine	1.432	0.045	9.7E-05
D-galactose	1.770	0.051	3.5E-05		Guanine	1.431	0.122	4.5E-03
N-Acetyl-D Glucosamine	1.745	0.068	1.2E-04		Gly-Met	1.403	0.051	1.9E-04
D-Melibiose	1.740	0.035	9.9E-06		Met-Ala	1.358	0.015	2.5E-06
Pyruvic Acid	1.730	0.063	9.4E-05		L-Alanine	1.339	0.075	1.2E-03
succinic acid	1.678	0.044	3.0E-05		Allantoin	1.329	0.027	2.4E-05
L-Malic Acid	1.673	0.051	5.3E-05		L-Aspartic Acid	1.327	0.066	8.3E-04
glycerol	1.651	0.131	2.1E-03		L-Glutamic Acid	1.271	0.031	6.8E-05

D, L-Malic Acid	1.605	0.063	1.6E-04		Uric Acid	1.230	0.051	6.3E-04
L-Glutamic Acid	1.475	0.067	3.7E-04		Gly-Glu	1.116	0.072	5.8E-03
Adenosine	1.437	0.150	8.9E-03		Ala-Thr	1.088	0.021	8.1E-05
ß-Methyl-D Glucoside	1.383	0.035	5.7E-05		Adenine	1.026	0.016	6.2E-05
α-D-Glucose	1.381	0.058	3.6E-04		Ammonium Formate	0.998	0.038	2.2E-03
Sucrose	1.331	0.021	1.4E-05		D-Asparagine	0.880	0.016	9.2E-04
L-Alanyl-Glycine	1.314	0.033	6.7E-05		Urea	0.846	0.018	3.6E-03
D-trehalose	1.284	0.064	9.8E-04		Glycine	0.845	0.010	4.2E-04
L-Alanine	1.253	0.038	1.8E-04		Negative control	0.729	0.005	NA
Glycyl-L Glutamic Acid	1.206	0.054	9.2E-04					
D-Malic Acid	1.195	0.065	2.1E-03					
Methyl Pyruvate	1.090	0.092	1.7E-02					
Glycyl-L-Aspartic Acid	1.085	0.057	3.5E-03					
Bromo Succinic Acid	1.036	0.022	2.1E-04					
L-Aspartic acid	1.014	0.027	5.6E-04					
m-Tartaric Acid	0.962	0.034	2.5E-03					
Glycyl-L-Proline	0.941	0.045	9.5E-03					
Citric Acid	0.802	0.014	1.1E-02					
Negative control	0.723	0.011	NA					

Phase 2: Determine the key metabolites present in xylem fluid via targeted metabolomics

Abstract: *In vitro* assays require extracted xylem fluid from maize seedlings for use as a growth medium for *P. stewartii*. However, this extraction process has limitations in the volume that can be recovered. Synthetic plant fluid media is an emerging strategy to study phytopathogens.

Targeted metabolomics analysis of the sugars, amino acids, and organic acids present in PBS-inoculated (naïve) and *P. stewartii*-inoculated extracted maize xylem fluid is being used in order to construct a synthetic xylem media (SXM) to better study *P. stewartii* physiology in the xylem environment.

Materials and Methods:

Xylem fluid volume and colony forming units (CFUs) in naïve vs inoculated plants. To determine the ideal timepoints for targeted metabolomics analysis, corn plants grown in a plant growth chamber were scratched with a needle 1-2 cm above the soil line, and inoculated with 5 µL of PBS or *P. stewartii* on day 5 of growth (2, 4, 5). From 2-8 days post inoculation (dpi), xylem fluid was extracted and pooled from 2-4 plants. Fluid was massed to determine total volumes and serially diluted for spread plating on LB agar to count *P. stewartii* CFUs.

Plant inoculations and xylem fluid extraction. Nine maize seedling stems were scratched with a needle 1-2 cm above the soil line, and either 5 µL of PBS (naïve) or *P. stewartii* was injected into the vasculature of 5-day old seedlings (4, 5). Either 3- or 6-dpi stems are cut with a sterile blade and drops of xylem fluid are collected for 90 min. Samples were centrifuged at 15,000 RPM for 5 min in an Eppendorf centrifuge 5424 to remove bacterial cells. A 200 µL volume of each supernatant was transferred to a sterile tube, flash frozen in liquid nitrogen, and the levels for 31 sugars, 77 organic acids, and 29 amino acids were measured using liquid chromatography targeted tandem mass spectrometry (University of North Texas).

Inorganic nitrogen analysis (in progress). Xylem fluid samples were sent to Woods Hole Oceanographic Institution in November 2024 for measurement of ammonium and nitrate levels.

Construct synthetic xylem media (SXM) (planned). The top ~90% of metabolites along with the inorganic nitrogen levels found from the targeted metabolomic and inorganic nitrogen analysis of

6 dpi extracted xylem fluid will be used to construct SXM, as previously done for apoplast fluid (6). To ensure the SXM contains all of the necessary metabolites for *P. stewartii* growth, assays comparing extracted xylem fluid to SXM will be conducted. Once comparable growth rates of *P. stewartii* are achieved in SXM, it will be used as a primary growth medium for *P. stewartii* for *in vitro* assays to study its physiology.

Results and Discussion:

Xylem fluid volume and CFUs in inoculated vs naïve plants. There was little difference in xylem fluid volume and bacterial CFUs and extracted for each trial condition until 6 dpi, where volumes decreased in inoculated plants (Figure 1). Overall, less xylem fluid was extracted from *P. stewartii*-inoculated plants than naïve (Figure 1A). Although only performed one time, *P. stewartii* CFU counts increased until about 6 dpi (Figure 1B). The goal of this experiment was to find an ideal second time point for harvesting xylem fluid from infected plants for future metabolomics analysis. Day 3 was chosen based on its prior use for Tn-Seq and RNA-Seq analyses (46, 75), while day 6 was chosen for future experimental work based on the higher volumes extracted and the stabilization of CFU counts.

Targeted metabolomics. The concentrations of select sugars, amino acids, and organic acids were analyzed for 3 dpi and 6 dpi naïve and *P. stewartii*-inoculated plants. The 3 dpi samples had unexplained variability that is being further studied, but may be related to the residual usage of nutrients from the seed, rather than generation of nutrients from photosynthesis (6, 7). The 6 dpi samples were consistent and follow expected trends of previous xylem studies (1, 5). The 6 dpi naïve (Table 2) and *P. stewartii*-inoculated (Table 3) metabolites were analyzed to identify the top ~90% of each type of metabolite. The metabolite composition was similar between naïve and inoculated samples, with the additional detection of pyruvate and valine in inoculated samples

(Table 3). A more detailed comparison of metabolite concentrations from naïve and *P. stewartii*-inoculated extracted xylem fluid reveals a trend of higher concentrations of compounds in inoculated samples than naïve (Table 2 vs. Table 3; Figure 2). Out of the 118 compounds analyzed, there were 16 significantly higher metabolite concentrations from *P. stewartii*-inoculated samples compared to naïve, using naive metabolite concentrations above 1 pmol/µL (Figure 2).

Table 2: Top ~90% of each type of metabolite in naïve xylem fluid.

Category	Sample	Avg Naïve (pmol/µL)	Std Error	% Composition
Sugars (~92%)	Sucrose	234.75	30.39	36.17
	Glucose	214.33	14.14	33.03
	Fructose	148.57	13.07	22.89
Amino Acids (~90%)	Glutamine	3278.48	287.51	65.01
	Serine	432.11	28.54	8.57
	Alanine	229.44	31.62	4.55
	Asparagine	162.33	17.55	3.22
	Arginine	161.44	11.65	3.20
	Lysine	150.54	8.31	2.99
	Ornithine	132.39	10.11	2.63
Organic Acids (~92%)	Citrate	150.09	8.63	51.46
	Malate	77.46	1.80	26.56
	trans-Aconitate	17.28	0.74	5.93
	Succinate	10.30	0.35	3.53
	Glucose-6P	6.74	0.45	2.3
	Quinic acid	5.85	0.30	2.01

Table 3: Top ~90% of each type of metabolite in *P. stewartii*-inoculated xylem fluid.

Category	Sample	Avg *P. stewartii* (pmol/µL)	Std Error	% Comp
Sugars (~94%)	Sucrose	280.77	28.16	34.68
	Glucose	277.64	25.25	34.30
	Fructose	199.50	19.85	24.64
Amino Acids (~92%)	Glutamine	3997.42	429.24	62.99
	Serine	484.15	45.54	7.63
	Alanine	324.28	70.11	5.11
	Asparagine	283.83	85.19	4.47
	Arginine	212.51	38.88	3.35
	Ornithine	190.71	41.72	3.00
	Lysine	183.75	30.23	2.90
	Valine	153.06	9.99	2.41
Organic Acids (~92%)	Citrate	186.66	18.38	44.26
	Malate	102.15	14.91	24.22
	trans-Aconitate	38.91	7.27	9.23
	Succinate	22.31	5.54	5.29
	Pyruvate	19.00	3.40	4.50
	Glucose-6P	9.01	0.80	2.14
	Quinic acid	8.78	0.48	2.08

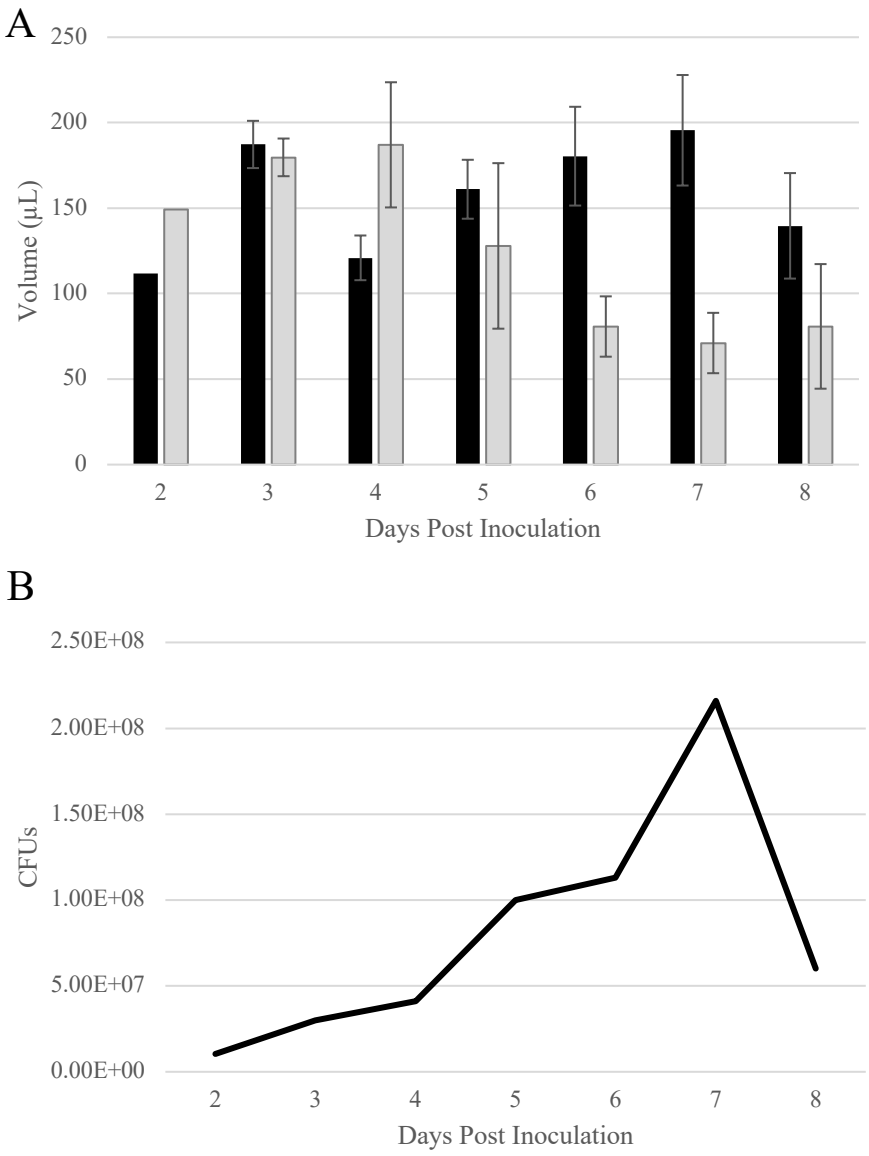

Figure 1: Volume of xylem fluid extracted and corresponding *P. stewartii* CFU counts from maize seedlings. A: Average volume of xylem fluid extracted from naïve (black) and *P. stewartii*-inoculated (gray) plants. Bars represent standard error (n=4, except for day 2 where n=2). **B:** CFU count of *P. stewartii*-inoculated xylem fluid. Data represents one replicate.

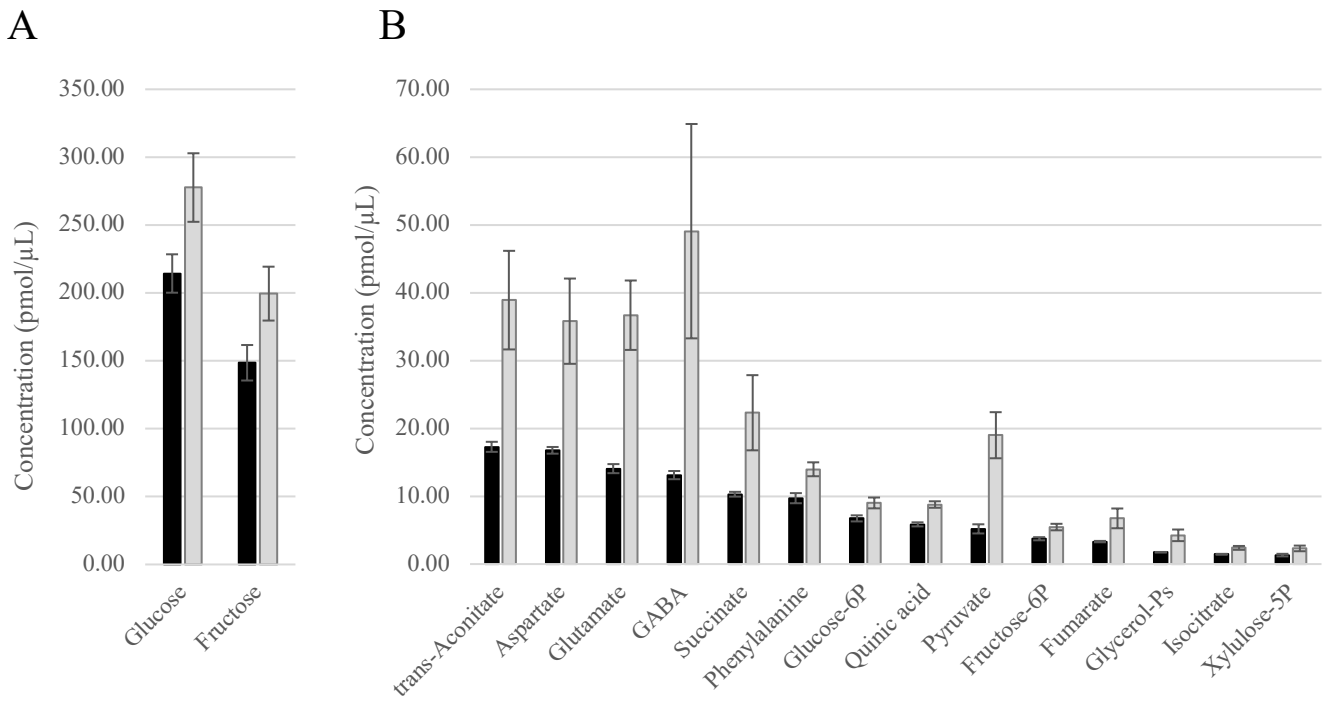

Figure 2: Comparison of 6 dpi naive (black) and *P. stewartii*-inoculated (gray) metabolite concentrations from extracted xylem fluid. A: Metabolites with concentrations above 50 pmol/µL. **B:** Metabolites with concentrations below 50 pmol/µL. Concentrations greater than 1 pmol/µL for the naïve samples with significant ($p<0.05$) differences are shown. Bars represent standard error (n=4).

Phase 3: Develop synthetic xylem medium (SXM) for corn.

Work has just initiated on this phase of work. We have calculated the concentrations for the sugars, amino acids and organic acids that comprise ~85-90% of the nutrient composition in corn xylem fluid. The necessary chemical and supplies have been ordered to make stock solutions. Compilations of SXM tested for their ability to support *P. stewartii* growth early in 2025. In a change to the order of the research originally proposed, untargeted metabolomics will be utilized

to identify additional compounds in xylem fluid, if the initial formulations of SXM do not permit levels of *P. stewartii* growth comparable to that of xylem fluid extracted from plants.

Project outputs:

- Farthing WM, Heimbach AM, Stevens AM. Gene regulation impacting the growth of *Pantoea stewartii* subsp. *stewartii* in corn. Poster presentation. International Conference on Plant Pathogenic Bacteria. Blacksburg, VA July 7-12, 2024.
- Ann M. Stevens submitted a NSF-USDA Plant-Biotic Internation Proposal with David Mackey and Jessica Cooperstone (The Ohio State University) (August 9, 2024) for $983,869 ($343,739 to Stevens), based on preliminary results from this research.
- Farthing WM, Heimbach AM, Stevens AM. The role of *Pantoea stewartii* subsp. *stewartii* leucine-responsive regulatory protein (Lrp) during maize xylem growth. Poster presentation. Joint CeZAP and Virginia ASM conference. Blacksburg, VA October 11-12, 2024.
- Abigail M. Heimbach submitted a NSF Graduate Research Fellowship Proposal (GRFP) proposal October 15, 2024 for $159,000, based on preliminary results from this research.
- Farthing WM, Heimbach AM, Stevens AM. The role of *Pantoea stewartii* subsp. *stewartii* leucine-responsive regulatory protein (Lrp) during maize xylem growth. Manuscript in preparation for January 2025 submission to Applied and Environmental Microbiology.

References:

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5. Farthing WM, Heimbach AM, Stevens AM. 2025. The role of *Pantoea stewartii* subsp. *stewartii* leucine-responsive regulatory protein (Lrp) during maize xylem growth. Appl Environ Microbiol. In preparation.

6. Gentzel I, Giese L, Ekanayake G, Mikhail K, Zhao W, Cocuron J-C, Alonso AP, Mackey D. 2022. Dynamic nutrient acquisition from a hydrated apoplast supports biotrophic proliferation of a bacterial pathogen of maize. Cell Host & Microbe 30:502-517.e4.

7. Guo Y, Yang X, Chander S, Yan J, Zhang J, Song T, Li J. 2013. Identification of unconditional and conditional QTL for oil, protein and starch content in maize. The Crop Journal 1:34–42.