Project Title: Isolation and in vitro culturing of the X-disease pathogen

Report Type: Final Project Report

Primary PI: Cameron Peace
Organization: Washington State University – Horticulture
Telephone: (509) 335-6899
Email: cpeace@wsu.edu
Address: Information and Technology Building
City/State/Zip: Pullman/WA/99164

Co-PI 2: Scott Harper
Organization: Washington State University – Plant Pathology
Telephone: (509) 78-9230
Email: scott.harper@wsu.edu
Address: WSU – IAREC
Address 2: 24106 N. Bunn Rd.
City/State/Zip: Prosser/WA/99350

CO-PI 3: Lyndon Porter
Organization: U.S. Department of Agriculture – ARS Plant Pathology
Telephone: (509) 786-9237
Email: lyndon.porter@usda.gov
Address: WSU – IAREC
Address 2: 24106 N. Bunn Rd.
City/State/Zip: Prosser/WA/99350

Cooperators: Alexandra M. Johnson (WSU Postdoctoral Research Associate)

Project Duration: 3-Years (NCE)

Total Project Request for Year 1 Funding: $ 29,000
Total Project Request for Year 2 Funding: $ 30,000
Total Project Request for Year 3 Funding: $0

Other related/associated funding sources:
Funding Duration: 2019 - 2021
Amount: $539,661
Agency Name: WTFRC/OSCC
Notes: "Supporting a robust PNW sweet cherry breeding and genetics program". PI: Per McCord. Co-PIs: Cameron Peace, Bernardita Sallato, Mateus Pasa.

Funding Duration: 2020 - 2021
Amount: $28,000
Agency Name: USDA Germplasm Evaluation Funds, *Prunus*
Notes: "Germplasm evaluation for sweet cherry genetic diversity and disease resistance". PI: Cameron Peace. Co-PIs: John Preece, Stijn Vanderzande, Alexandra Johnson.

WTFRC Collaborative Costs:

Item	**2021**	**2022**	**2023**
Salaries	$15,250.00	$15,950.00	$0.00
Benefits	$2,750.00	$2,900.00	$0.00
Wages	$5,850.00	$6,050.00	$0.00
Benefits			
RCA Room Rental			
Shipping			$0.00
Supplies	$2,150.00	$2,100.00	$0.00
Travel	$2,000.00	$2,000.00	$0.00
Plot Fees	$1,000.00	$1,000.00	$0.00
Miscellaneous			$0.00
Total	$29,000.00	$30,000.00	$0.00

Footnotes:
Graduate student support for Alexandra Johnson
Single use, disposable materials for sample collection and laboratory assays
Pullman-Prosser return for approx. 4-5 multi-day trips during spring and summer each year

Budget 1
Primary PI: Cameron Peace
Organization Name: Washington State University
Contract Administrator: Katy Roberts
Telephone: (509) 335-2885
Contract administrator email address: katy.roberts@wsu.edu

RECAP OF OBJECTIVES

Identify optimum growing conditions for generating and maintaining '*Candidatus* Phytoplasma pruni' colonies, the pathogen responsible for X-disease in sweet cherry

1. Develop a rapid and reliable method for culturing '*Candidatus* Phytoplasma pruni'
2. Optimize the culture medium for year-round live growth of '*Candidatus* Phytoplasma pruni'

SIGNIFICANT FINDINGS

- Bacteria were successfully cultured in a liquid medium and on a solid medium from cherry tissues known to be infected with '*Candidatus* Phytoplasma pruni' (*Ca.* P. pruni)
- Five colonies were isolated from the solid medium that were identified phenotypically as possible *Ca.* P. pruni in 2022; these isolates did not survive re-culturing in 2023 to be genotyped via PCR testing to putatively determine if they were Ca. P. pruni
- One isolate in 2021 was determined by DNA sequencing to not be *Ca.* P. pruni
- Year-round in vitro culturing of the X-disease pathogen is likely possible, although the bacteria are fastidious
- The information gained could be used by plant pathologists to inform and assist in devising new methods to combat the bacterial pathogen behind this devastating disease

METHODS

Three-year plan: **2021** – Collect infected plant material and begin isolating and identifying colonies produced. Maintain pure isolates of colonies found to respond to current genetic detection methods.
2022 – Collect additional plant material for bacterial isolation and identification. Maintain pure isolates of colonies found to be '*Candidatus* Phytoplasma pruni'. **2023** – collect additional plant material for isolation and identification. Confirm colonies putatively identified as *Ca*. P. pruni via PCR testing.

Experimental material: Field work utilized accessible material from sites throughout central Washington known to be infected with X-disease as determined by WSU plant pathologists. Twenty trees in 2021, a different set of 20 trees in 2022, and another different set of 10 trees in 2023 were used in this study. All trees were growing in commercial and research orchards, under standard management practices. In 2021, 2022, and again in 2023, stems, spurs, and fruit from trees with high to moderate levels of infection were harvested and processed as described below. Additionally, stems, spurs, and fruit from asymptomatic trees were collected and processed. Briefly, infected and asymptomatic plant materials were transported back to the laboratory for surface disinfestation (tissues were soaked in a 10% NaOCl solution for 10 minutes, and then thoroughly rinsed with distilled, sterile water). Once rinsed, plant materials were placed in a sterile laminar flow hood to dry and then dissected. Five 25 mm transverse sections were cut from each stem and spur using a sterile razor blade. Additionally, 25 mm sections of cambium were also harvested from stems. Five symptomatic, as well as five asymptomatic fruit from each infected tree were sliced sagittally into 10 mm sections. Each dissected piece of tissue was placed in a separate, sealable 15 mL vial of liquid phytoplasma growth medium and incubated to room temperature (26 °C) for at least 48 hours.

Bacterial cultures: Stems, spurs, and fruit from infected, symptomatic trees were collected and numerous colonies were produced on phytoplasma-selective medium (Contaldo and Bertaccini 2019) and in a selective environment. Generation of selective medium in 2022 was significantly delayed due to equipment failure, but was resumed before the end of the 2022 growing season. Both liquid and solid phytoplasma growth media contained the broad-spectrum antibiotic ampicillin (25 µg/mL) as well as the antimycotic nystatin (50 µg/mL), which inhibited growth of most bacterial and fungal species. Additionally, cultures were kept in an oxygen-restricted environment, which further limited growth of both obligate anaerobic and aerobic bacterial species. The pH reactive pigment phenol red was added to each vial of phytoplasma liquid growth medium. Tubes inoculated with dissected plant material were observed to turn yellow after 24 or more hours, indicating acidification of the medium by bacterial metabolic processes, and were considered positive for bacterial growth. These tubes were quantified for degree of color change and 1 mL of broth was removed from each vial and spread aseptically across a new plate of solid phytoplasma growth medium. Plates of solid medium inoculated with bacterial broth were then incubated for at least 48 hours at room temperature (26 °C) in a low oxygen environment. Isolated colonies observed growing after incubation were picked using a sterile loop, introduced to new liquid growth medium, cultured until indication of pH change, and streaked onto a new plate of solid growth medium. Isolated colonies were then sampled for DNA testing.

DNA testing: The existing PCR-based assay described by Kogej et al. (2020) that detects *Ca.* P. pruni-specific DNA sequences was used to putatively determine which colonies were likely *Ca.* P. pruni. Such colonies with the same genotypic signature as *Ca.* P. pruni were subject to targeted genome sequencing (i.e., specific genomic regions) to confirm identity.

RESULTS & DISCUSSION

Summary: In 2021, 2022, and 2023, bacteria were isolated from plant tissues known to be infected with *Ca.* P. pruni, the causative organism in X-disease. For all three years, most colonies generated were determined to not be *Ca.* P. pruni. However, in 2021, four colonies were putatively identified as *Ca.* P. pruni by PCR-based DNA assays. Genome sequencing of one of these colonies indicated it was not *Ca.* P. pruni. The remaining three colonies that were also putatively identified as *Ca.* P. pruni were transferred to a fresh liquid medium and then a solid growth medium, to generate additional pure colonies. These colonies were maintained in pure culture, in growth medium over 2021 and 2022, but did not survive additional culturing in 2023. Phenotypic observation in 2022 identified five additional colonies as possible *Ca*. P. pruni. These newly identified colonies were isolated and maintained in pure culture until 2023, but did not survive re-culturing on solid growth medium. In 2023, liquid cultures were generated but growth of colonies on solid medium was limited. Of the few colonies produced in 2023, none were phenotypically similar to previously reported *Ca*. species and thus not considered for additional culturing as *Ca*. P. pruni. Issues in 2023 with culturing isolates was determined to be from a defective water purification system introducing ionic contamination which negatively impacted bacterial growth.

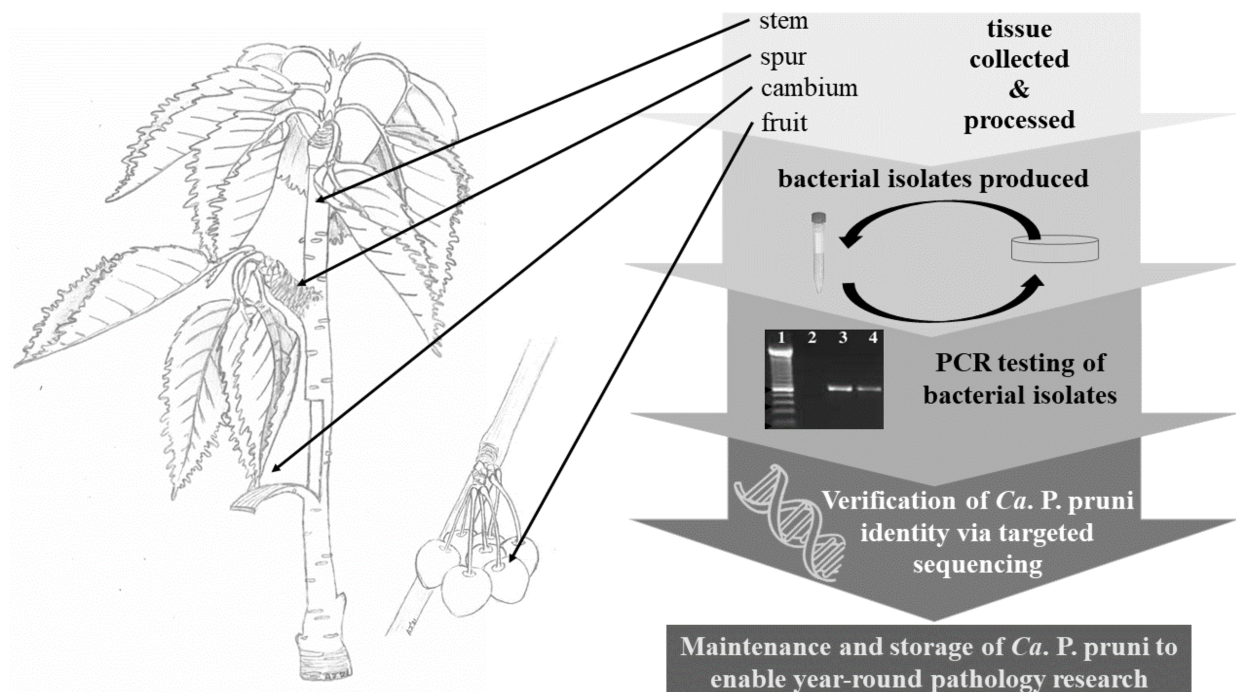

Figure 1: Collection, generation, and identification of bacterial isolates found in trees infected with *Ca.* P. pruni

Bacterial isolation: In 2021, 2022, and 2023, initial plates generated from broth cultures produced individual colonies on solid growth medium. Five to six species of bacteria were identified morphologically in 2021 and 2022, and two species of bacteria were identified morphologically in 2023. Broths from asymptomatic tissues did not produce any bacterial colonies, as expected. Colonies of different bacterial species cultivated in 2022 were phenotypically compared with those produced in 2021 to investigate the hypothesis that a specific subset of other bacteria must be present with *Ca.* P. pruni for X-disease symptoms to appear in sweet cherry trees; however, due to colonies failing to grow on medium produced in 2023, no definitive conclusions regarding presence of other bacterial species could be made.

Bacterial identity confirmation: From PCR-based assays of 60 colonies in 2021, four were putatively identified as *Ca*. P. pruni. One was sequenced to date and was found to be a contaminant. PCR testing of additional colonies produced over all three years was not conducted due to media contamination resulting in colonies failing to grow in 2023.

Bacterial storage: The eight colonies identified as possible *Ca.* P. pruni were maintained on solid phytoplasma growth medium in a low-oxygen environment until 2023. Medium contamination in 2023 rendered additional, long-term storage studies impossible. Colonies grown in 2021 that were putatively determined to be *Ca*. P. pruni indicate that in vitro culturing of this pathogen might be possible. However, results gathered from 2023 also

indicate that *Ca*. P. pruni is a particularly fastidious bacterial species and sensitive to ionic contamination.

EXECUTIVE SUMMARY

PROJECT TITLE: Isolation and in vitro culturing of the X-disease pathogen

Key words: microbiology, '*Candidatus* Phytoplasma pruni', sweet cherry

The objective of this project was to identify optimum growing conditions for generating and maintaining '*Candidatus* Phytoplasma pruni', the causative bacteria responsible for X-disease in sweet cherry, in vitro. Specifically, this project aimed to develop a rapid and reliable method for culturing *Ca*. P. pruni, and to optimize culture medium for year-round live growth of this bacterial species.

Over three growing seasons, different organs, including stems, leaves, and fruit, were gathered from trees known to be infected with *Ca*. P. pruni and from trees determined to not harbor the pathogens that were growing in orchards in Washington state. Organs were transported to a clean laboratory, where they were surface sanitized and dissected into small, ~10 mm sections. Plant sections were then placed in liquid growth medium and incubated for 7-14 days before an aliquot of broth was plated onto solid growth medium, then placed in a reduced oxygen growth chamber. Colonies morphologically similar to previously reported *Candidatus* colonies were subsequently genotyped using a published PCR assay. Of the colonies produced over 2021 and 2022, seven were putatively determined to be *Ca*. P. pruni. Targeted genomic analysis revealed one of these colonies was not *Ca*. P. pruni. Ionic contamination of media in 2023 from a defective water purification apparatus resulted in death of colonies kept from 2021 and 2022. Two colonies produced in 2023 on the contaminated medium were phenotypically dissimilar to previously reported *Candidatus* species and thus not maintained.

While in vitro culturing and maintenance of *Ca*. P. pruni was not successful, useful information was gained from this project. Results from putatively identified colonies produced in 2021 indicate that in vitro culturing of *Ca*. P. pruni is likely possible. Furthermore, colonies maintained from 2021 to 2023 using culturing techniques that alternate between liquid and solid growth mediums demonstrate that *Ca*. P. pruni can likely be grown in vitro year-round. Finally, colony growth failure in 2023 due to ionic contamination underscores the fastidious nature of this bacteria and the need for contaminant-free growth media when culturing *Candidatus* species. The collective information gained from all three years of this project could be used by plant pathologists studying *Ca*. P. pruni to inform and assist in devising new methods to address this bacterial pathogen which threatens the PNW sweet cherry industry.