INVESTIGATION OF BACTERIOPHAGE AS A BIOLOGICAL CONTROL FOR BACTERIAL CANKER OF SWEET CHERRY

By

Erin M. Lauwers

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ABSTRACT

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Bacterial canker of sweet cherry trees, caused by the bacteria *Pseudomonas syringae* pv. syringae (PSS), is an economically important disease in Michigan and effective disease management is not available. For these reasons, bacteriophages (phages) are being investigated as a potential biological control of bacterial canker. A cocktail of *Pseudomonas* bacteriophage was tested as a biological control in Michigan sweet cherry orchards during bloom in 2021 and 2022. Populations of antibiotic-marked pathogenic PSS strains were tracked for the duration of the flowering period. Establishment of PSS populations greater than 5 $\log_{10}$ CFU g^{-1} flowers were detected on all water treated flowers during the experiment. In one field replicate on sweet cherry variety 'Benton', the PSS population on flowers treated with the phage cocktail was lower (P<0.05) 48 hrs after treatment. Environmental conditions during this field experiment were different compared to the other replicates including higher precipitation, higher relative humidity, and lower Daily Light Integral. These observations could indicate conditions in which phage may be an effective biological control. It also corroborates the need for reducing phage degradation by UV in the field. Kaolin clay was tested as a protectant against UV irradiation *in vitro* and *in vivo*. A rate of 4.8 mg ml^{-1} kaolin clay was shown to effectively protect phage from UV degradation in vitro but was not observed when the phage cocktail was supplemented with kaolin clay in the field. This work gives us a better understanding of the potential of phage as a biological control of bacterial canker under field conditions.

This thesis is dedicated to Joseph.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

CFU – colony forming units

diH_2O – distilled water

DLI – Daily Light Integral

INA – ice nucleation activity

KB – King's B

NWMHRC – Northwest Michigan Horticulture Research Center

PAMDB – Plant Associated and Environmental Microbes Database

PB – Phosphate buffer

PFU – plaque forming units

PSM – *Pseudomonas syringae* pv. morsprunorum

PSS – *Pseudomonas syringae* pv. syringae

UVR – Ultraviolet Radiation

CHAPTER 1

LITERATURE REVIEW OF BACTERIAL CANKER OF SWEET CHERRY AND BACTERIOPHAGE USAGE FOR PLANT DISEASE BIOCONTROL

1.1 Bacterial Canker Disease in Michigan

Michigan is the largest producer of tart cherries (*Prunus cerasus*) in the United States and fourth largest producer of sweet cherries (*Prunus avium*) by weight. As of the 2017 census, there are 6,701 acres of bearing sweet cherry orchards, contributing nearly $17 million to Michigan's economy across all markets: fresh, frozen, and processed. In 2016, that number was over $18 million (NASS 2017).

Bacterial canker is a serious disease affecting the cherry industry in Michigan, particularly the sweet cherry sector (Jones and Sutton 1996). The disease is caused by two pathovars of the bacterial pathogen *Pseudomonas syringae* pv. syringae and pv. morsprunorum. *Pseudomonas syringae* pv. syringae (PSS) is more virulent on sweet cherries and *P. syringae* pv. morsprunorum (PSM) is more virulent on tart cherries (Crosse 1966; Latorre and Jones 1979; Jones 1971). Bacterial canker symptoms include blossom blast, spur dieback, leaf lesions, and canker formation (Jones 1971). While disease severity is highly variable between seasons, epidemic years can have lasting impacts in orchards (Jones and Sutton 1996). Some notable years of infection high severity bacterial infection in Michigan include 2021, 2013, 2002, and 1968 (Jones 1971; Renick et al. 2008). These years with high levels of infection are associated with freezing events during sweet cherry bloom, which damages flowers and creates an infection

court for the pathogen (Renick et al. 2008). This avenue of infection typically causes the highest severity of infection of woody tissue (Kennelly et al. 2007).

The impact of these freezing events on bacterial canker is exacerbated by PSS, as this organism is capable of inducing ice formation and thus frost damage through ice nucleation. Ice nucleation activity (INA) was first discovered in *Pseudomonas syringae* in the 1970s and has since been discovered in species of Enterobacteriaceae, Xanthomonadaceae, and Lysinibacillus families (Failor et al. 2017; Kim et al. 1987; Lindow et al. 1978). Ice nucleation active bacteria synthesize an ice nucleating protein that is embedded into the outer cell membrane and functions to arrange water molecules to facilitate ice formation (Lukas et al. 2022). Ice nucleation active bacteria are primarily responsible for ice formation from temperatures 0 to -5°C (Hirano and Upper 2000). For example, ice formation in the intercellular space of tart cherry leaves occurred between -2.2 and -2.5°C in the presence of INA^+ PSS strains, an increase of 0.5-0.8° C compared to INA^- strains (Süle and Seemüller 1987). As the population of INA bacteria on a plant surface increases, the extent of the frost injury at any given subfreezing temperature increases (Lindow 1983). Temperatures in Michigan are projected to increase in the future with impacts on winter seasonality (Ferguson et al. 2015). Warm temperatures occurring earlier in the spring can result in early cessation of dormancy. This increases the risk of frost injury as subfreezing temperatures may still occur while frost sensitive plant tissue are present. For this reason, bacterial canker is a disease of significant concern and importance for the industry.

1.2 Disease Cycle

Colonized bark tissue, healthy buds and leaf scars can serve as the source of PSS inoculum in the spring (Crosse 1959; Roos and Hattingh 1986; Sundin et al. 1988). During

bloom in the spring, growth of PSS is favored by cool and wet weather with populations reaching 10^4 to 10^6 CFU on individual flowers (Jones and Sutton 1996; Kennelly et al. 2007). If a freezing event occurs and flowers are damaged, PSS can enter through those wounds and cause the blossom blast and spur dieback symptoms. Flower clusters and entire fruiting spurs wilt, turn brown, and eventually dry, oftentimes not abscising from the tree (Jones and Sutton 1996). Loss of flowers means a loss in fruit yield for that season (Kennelly et al. 2007) (Fig. 1A, 1B). Freezing temperatures during this time can also weaken trees and predispose woody tissue to infection. Freezing and thawing cycles can cause water soaking and increased invasion and systemic movement of PSS (Cao et al. 1999; Vigoroux 1989). The length of necrosis on sweet cherry shoots inoculated with PSS have been shown to increase with exposure to freezing temperatures (Sobiczewski and Jones 1992). Larger stem diameters have also been associated with an increase in lesion length on various *Prunus* species inoculated with PSS (Cao et al. 1999).

Pseudomonas syringae can survive on healthy plant tissue as an epiphyte throughout the spring, summer, and fall without causing disease (Crosse 1959; Sundin et al. 1988). Epiphytic populations on cherry leaves and shoots are reportedly highest in late spring after bloom (Wimalajeewa and Flett 1985). Leaf surface populations of PSS as large as 10^6-10^7 CFU g^{-1} fresh weight have been detected (Sundin et al. 1988). Leaves are infected through stomata when PSS populations are high, resulting in lesions. Scanning electron microscopy of sweet cherry leaves inoculated with PSM indicate that the bacteria can multiply in sub-stomatal cavities (Roos and Hattingh 1983). Leaf lesions appear as round areas of chlorosis with a purplish-red halo. As the tissue in the center of the lesion becomes necrotic, it turns brown with a yellow halo. This

dried tissue can eventually fall out of the leaf, resulting in the "shot-hole" symptom (Jones 1971; Jones and Sutton 1996; Kennelly et al. 2007) (Fig. 1D).

After the transition of PSS from epiphyte to an endophyte through infected flowers, spurs, fruits, or leaves, the bacteria can colonize woody tissue and initiate cankers – the most devastating symptom of the disease. Cankers appear as water-soaked lesions that become sunken and dark. The outer bark can separate from the wood as it collapses, resulting in a papery, shredded appearance to the bark along the horizontal lenticels. Cankers are often associated with gummosis (Jones 1971; Jones and Sutton 1996; Kennelly et al. 2007) (Fig. 1E, 1F), a non-specific stress response controlled by phytohormones. Gummosis appears as an initially transparent, sticky, sap-like tree exudate that becomes amber colored as it hardens (Saniewski et al. 2006). Canker formation is the most severe symptom as it has repercussions in an orchard for years. Cankers can spread leading to the girdling of scaffold limbs and trunks and eventually tree death (Jones and Sutton 1996). Tissue water soaking, which can occur because of high humidity or heavy rain, has been shown to facilitate the ingress and spread of bacteria in various *Prunus* species (Johnson 1945; Vigoroux 1989). Young trees are particularly vulnerable to girdling and subsequent death due to having smaller trunk diameters. Even if a tree is not killed by bacterial canker, yield and photosynthetic capacity is reduced long term through loss of fruiting spurs, shoots, branches, and scaffold limbs (Kennelly et al. 2007).

Populations of PSS decrease with increasing temperatures and drier conditions in the summer. However, during periods when conditions are cool and wet, the bacterial population can grow and cause infection again, resulting in new lesions on leaves and fruit. Fruit lesions begin as water-soaked tissue with a reddish halo. The tissue becomes sunken and chocolatey brown to black in color over time as the lesion dries out (Jones 1971; Jones and Sutton 1996;

Kennelly et al. 2007) (Fig. 1C). Fruit infection causes a decrease in yield and lower fruit quality, which means a lower price per pound for the remaining harvestable crop.

As temperatures drop and precipitation increases in the fall conditions are again favorable for PSS population growth (Crosse 1957; Crosse 1966; Sundin et al. 1988). When leaves abscise, leaf scars become susceptible to infection and can remain susceptible for weeks (Crosse 1957). Wind and driving rain wash bacterial cells to leaf scars where the bacteria is drawn through leaf-trace vessels into the plant by negative tension in the vascular system and the bacteria can overwinter as an endophyte (Crosse 1956; Crosse 1966). *Pseudomonas syringae* pv. syringae can also colonize and overwinter in healthy dormant buds, sometimes causing the dead-bud symptom the following spring, where buds do not begin developing when the tree breaks dormancy after winter (Jones and Sutton 1996; Roos and Hattingh 1986; Sundin et al. 1988) (Fig. 1E). The bacteria also overwinter in previously infected dead buds and cankers (Crosse 1959).

1.3 Chemical Control

Strategies for disease management of bacterial canker in sweet cherry orchards are extremely limited. Copper compounds are labelled for use on sweet cherry trees; however, copper can be phytotoxic (Montgomery and Shaw 1942; Olson and Jones 1983). PSS control is most needed during bloom when trees have recently broken dormancy, however, flowers are particularly susceptible to phytotoxicity. Copper efficacy is also limited in that it is a contact bactericide and has no mode of action for penetrating plant tissue to control endophytic populations of PSS in buds, leaf scars, or cankers (Kennelly et al. 2007). Copper resistance has

also been reported in PSS populations in Michigan cherry orchards (Renick et al. 2008; Sundin et al. 1989).

As of 2020, the antibiotic kasugamycin has been labelled for use on sweet cherry trees for controlling bacterial canker (Kasumin 2L; UPL, King of Prussia, PA). Kasugamycin is an aminoglycoside antibiotic that interferes with the binding of aminoacyl-tRNA to ribosomal subunit complexes mRNA-30S and mRNA-70S. This prevents the incorporation of amino acids into proteins, thus inhibiting protein biosynthesis (Copping and Duke 2007). A study of chemical efficacy of kasugamycin on green sweet cherries inoculated with PSS showed some control of infection as an eradicant where the chemical was applied after inoculation but little control as a protectant where the chemical was applied before inoculation with PSS (Carroll et al. 2010).

Kasugamycin was originally developed in 1965 as a fungicide for controlling rice blast caused by *Pyricularia oryzae*. Resistance developed within three years of its commercial introduction (Copping and Duke 2007). Kasugamycin is also registered for use on apple for controlling fire blight disease caused by *Erwinia amylovora*. The potential for resistance development has been demonstrated in *E. amylovora* and other gram-negative bacteria, including *P. syringae*. Of 224 isolates of *Pseudomonas* spp. from Michigan apple orchards where Kasumin 4L was applied, 143 were resistant to kasugamycin. Resistance to streptomycin in Michigan apple orchards has already limited control of fire blight disease. Transferrable resistance genes for kasugamycin have not yet been identified in Michigan. However, the potential resistance of PSS, *E. amylovora*, or non-target bacteria to the antibiotic and loss of control for both diseases is a major concern. It has also been reported that Kasumin causes

phytotoxicity on apple trees, which has not been studied on sweet cherries and could limit benefits of its use (McGhee and Sundin 2011).

1.4 Bacteriophage as Biological Control

As efficacy of antibiotics for controlling certain plant pathogenic bacteria has been reduced with the development of resistance and use of antibiotics has been banned in certain countries, interest in using bacteriophage (phage) as a biological control has increased (Jones et al. 2012; Svircev et al. 2018). Bacteriophage are viruses that utilize bacterial cells to replicate and in the process kill (lyse) the host bacterium; the term bacteriophage means "bacteria eater" (Stone et al. 2019).

Phages have two main types of infection cycles: a lytic cycle and lysogenic cycle. In the lytic cycle, a phage irreversibly attaches to a host cell and injects its genetic contents into the bacterium. The host cells metabolism is then redirected to produce new phage particles. After transcription, translation, and replication of the phage DNA or RNA, new phage particles are assembled. Through phage encoded proteins, the bacterium is then lysed, killing the cell, and releasing the phage progeny into the environment to infect other bacterial cells (De Smet et al. 2017). This is an advantage of phage over antibiotics in that the phage therapy is naturally amplified in the presence of the host pathogen (Frampton et al. 2012). During the lysogenic cycle, the phage genome either exists as a plasmid in the cytoplasm or integrates into the bacterial chromosome as a prophage and is replicated along with the host chromosome. Some phage, called temperate phage, can undergo either lifecycle. Phage that are strictly lytic are of greatest interest for use as biological control agents since the host cell is killed in the lytic cycle (De Smet et al. 2017, Hesse and Adhya 2019).

Phage have advantages over antibiotics in many ways. For one, phage are highly host specific, oftentimes with a host range of only a few strains within a bacterial species, meaning phage are nontoxic to humans, plants, and animals. This specificity also means less non-target effects on other microbes in the environment, unlike antibiotics which are less selective (Hesse and Adhya 2019; Svircev et al. 2018). Phage are also able to infect and kill antimicrobial resistant strains of bacteria (Frampton et al. 2012). Phage therapies have been successfully used in human medicine for treating antibiotic resistant bacterial infections (Kortright et al. 2019). A phage biological control could potentially offer a solution to control plant diseases where all other chemical efficacy has been lost with the development of resistance.

A particular challenge for phage as a biological control for plant diseases is the sensitivity of phage to degradation under various environmental conditions. The phyllosphere is an extremely harsh environment. Plant surfaces can undergo major and often rapid changes in temperature, moisture, radiation, and humidity every minute, hour, and day (Hirano and Upper 2000). Phage can be inactivated by high temperatures, high and low pH, sunlight radiation, and even dislodged by rainfall. UV radiation is the most destructive (Jones et al. 2007). Unlike bacteria exposed to UV, phages are unable to repair genetic material (Yasbin et al. 1997). Different methods of mitigating degradation by UV include adding protective formulations. Formulations tested have included pregelatinized corn flour, casein, skim milk, kaolin clay, peptone, and carrot juice (Balogh et al. 2003; Dobbins 2020; Vettori et al. 2000).

1.5 Field Research with Phage

Compared to the number of studies on the isolation, characterization, and *in vitro* efficacy of phage for various plant pathogenic bacteria, relatively few studies have investigated phage

efficacy on plant diseases in the field. In 1993, Saccardi et al. (1993) conducted a phage study in peach orchards for controlling leaf and fruit spot caused by *Xanthomonas campestris* pv. pruni. They applied a single phage isolate, F_8, at three intervals: weekly, every ten days, and every two weeks. All three of the phage treatment schedules were effective at reducing the percentage of diseased fruit and diseased surface per fruit in one of the three locations.

In 2003, Balogh et al. tested phage on leaf surfaces in the field and greenhouse for controlling bacterial spot disease of tomato caused by *Xanthomonas campestris* pv. vesicatoria. They investigated several formulations to increase phage longevity including skim milk, casecrete, and pregelatinized corn flour. They found that all phage treatments reduced disease severity and that the casecrete phage treatment was the most effective formulation in both locations of field trials. Evening applications were also shown to have better disease control than morning applications (Balogh et al. 2003).

There have also been some field studies on lytic phages for pathovars of *Pseudomonas syringae*. In 2016, Rombouts et al. isolated and characterized five phages from soil in a leek field, followed by a field study of these phage for controlling *Pseudomonas syringae* pv. porri, causal agent of bacterial blight in leek. They first tried submerging leek transplants in the phage cocktail before planting in a *P.s.* pv. porri infested field and tracking disease incidence. Successful infection was only observed in one of the three locations and the phage treatment was not effective at preventing infection with 92.5 and 82% symptomatic plants in the untreated and treated plants, respectively, three months after planting. In a second field trial, plants were inoculated three months after planting then sprayed one day later with the phage cocktail. Disease incidence was slightly lower in the phage treatment but was not statistically significant.

Other treatment schedules were tested however no trial demonstrated any disease control (Rombouts et al. 2016).

1.6 Phage and Bacterial Canker

Since effective disease control of bacterial canker on sweet cherries is not achievable with copper compounds and the use of antibiotics is being discouraged, utilizing phage as a biological control for this disease is an area of interest. It is possible that phages may even be able to decrease INA, a critical phenotype impacting frost injury and disease severity. Kozloff et al. (1983) studied the INA of a strain of *Erwinia herbicola* infected with a T4 bacteriophage. INA was decreased by 99% just 55 min after infection (Kozloff et al. 1983). While not tested with *P. syringae* exhibiting INA, these results suggest that a phage biological control may be able to reduce INA of PSS in orchards, thus preventing or decreasing frost injury and infection of bacterial canker.

There are no published field studies on phage for controlling bacterial canker disease, however there are a few with *in vivo* assays. Bacterial canker caused by *Pseudomonas syringae* pv. actinidiae has become a serious problem in kiwifruit production. Pinheiro et al. (2020) tracked bacterial populations and phage titer of bacteria-inoculated kiwifruit leaves treated with phage φ6. They found that populations of the two bacterial strains tested were 1.1 and 1.8 CFU ml^{-1} lower on the phage treated leaves after 24 hrs of incubation. Phage counts also increased when inoculated with the bacteria as opposed to phage counts on uninoculated leaves, which remained constant (Pinheiro 2020). This demonstrates how the phage therapy can be amplified in the presence of the host pathogen.

Two *in vivo* studies have investigated phage for controlling bacterial canker of cherries specifically. Rabiey et al. (2020) conducted trials on inoculated bean plants and cherry trees in a growth chambers. On bean leaves, PSS populations were reduced by 50% after 96 hrs by two of the phages, 15% by the two phage cocktails, and 20% by eight of the phages tested. Phage treatments on cherry leaves showed some reduction of PSS population and populations on inoculated cherry twigs were reduced by 20, 60, 30, and 15% by four individual phage and 10 and 30% reduction by two phage cocktails (Rabiey et al. 2020). Akbaba et al. (2021) measured disease incidence on micropropagated cherry plantlets treated with five phage and a phage cocktail in a growth chamber. Four of the five individual phage and the phage cocktail reduced disease incidence of PSS. The four individual phages exhibited over 50% disease control. Efficacy with the phage cocktail was slightly lower at 42.3% control (Akbaba et al. 2021).

1.7 Future Work

The preliminary *in vivo* studies of phage for controlling bacterial canker on kiwifruit and cherries shows promise in the development of biological controls for this disease. More research on field efficacy of phage treatments needs to be completed before adoption of this practice. Protective formulations should be investigated to mitigate UVR inactivation or otherwise increase stability and efficacy of phage for this disease and each cropping system. Future work could involve foliar applications for controlling epiphytic populations or directly into plant tissue to control endophytic PSS. Optimization for field application will likely be necessary and will depend on the environmental conditions and location and behavior of the target population i.e. epiphytic populations on flowers in spring vs. endophytic populations in cankers in fall. The

development of effective control for PSS is essential for the future of stone fruit and kiwifruit production.

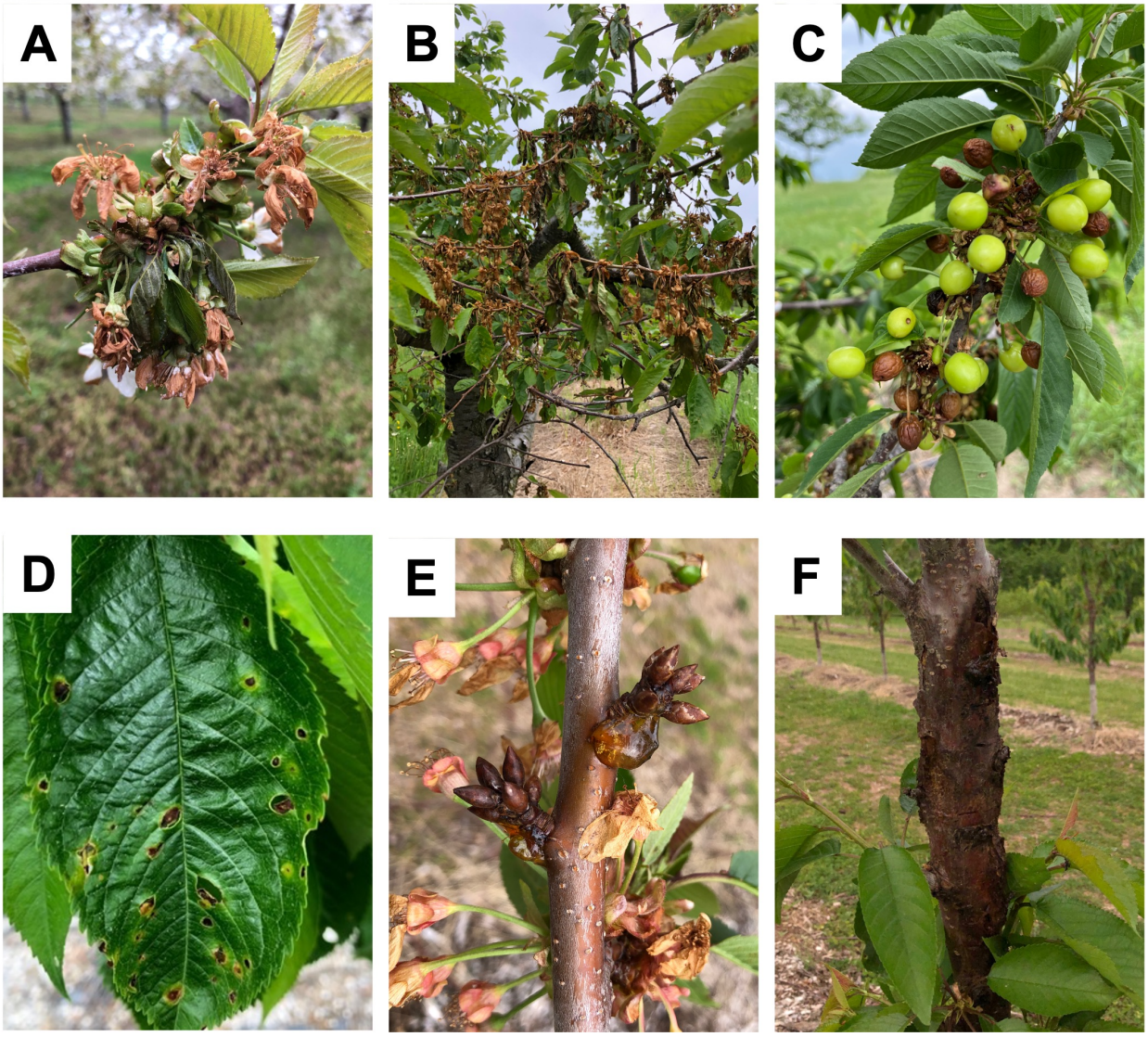

Figure 1. Symptoms of bacterial canker of sweet cherry trees cause by *Pseudomonas syringae* pv. syringae including A, blossom blast and collapse of fruiting spur; B, severe blossom blast in tree canopy following a spring freezing event; C, infected fruit; D, leaf spot with characteristic "shot-hole" appearance; E, dead-bud in spring with gummosis; sunken trunk canker beginning to girdle young tree.

CHAPTER 2

IN VITRO AND *IN VIVO* STUDIES ON BACTERIOPHAGE: A POTENTIAL BIOCONTROL TARGETING *PSEUDOMONAS SYRINGAE* PV. SYRINGAE

2.1 Abstract

Pseudomonas syringae pathovar *syringae* (PSS) is the most virulent pathogen causing bacterial canker of sweet cherry (*Prunus avium*). Historically, copper compounds were the only registered chemical control for the disease. The efficacy of copper is limited by widespread resistance of *P. syringae* populations in Michigan and elsewhere, and there are also problems with phytotoxicity from copper use on sweet cherry trees when disease control is most needed i.e., during bloom. In 2020 the antibiotic Kasumin was labelled for use on cherries in the United States. Bacteriophage (phage) are of interest as a biological control; however, all phages are highly sensitive to degradation by UV radiation. To improve the viability of phage, kaolin clay was investigated as a UV protectant. Kaolin clay at 4.8 mg ml^{-1}, 2.4 mg ml^{-1}, and 1.2 mg ml^{-1} protected the phage from 156 J m^{-2} UVC radiation by hundred-fold. Kasumin and a commercially available cocktail of *Pseudomona*s phage were applied to PSS-inoculated flowers during bloom in central and northern Michigan orchards in 2021 and 2022. In one field trial, the phage cocktail reduced PSS populations by ten-fold by 48 hrs after treatment. However, in other field trials phage treatments exhibited no control. Kasumin decreased floral populations of PSS in all field experiments but caused phytotoxicity and efficacy was inconsistent.

2.2 Introduction

Bacterial canker of stone fruit, caused by *Pseudomonas syringae* pvs. syringae (PSS) and morsprunorum (PSM), results in major economic losses in Michigan cherry orchards (Jones 1971; Renick et al. 2008). While both sweet cherry (*Prunus avium*) and tart cherry (*Prunus cerasus*) are susceptible, severe disease infection is more common in sweet cherry trees. The *P. syringae* pathovar syringae is more virulent on sweet cherry and pathovar morsprunorum on tart cherry (Jones 1971; Latorre and Jones 1979; Kennelly et al. 2007).

Typical disease symptoms of bacterial canker include blossom blast and spur dieback, necrotic lesions on leaves and fruit, and cankers on woody tissue that are commonly associated with gummosis. The spread of cankers in woody tissue over time can result in girdling of scaffold limbs or trunks and subsequently whole tree death. This can occur rapidly in young trees. Infection by PSS can occur at any point of the growing season and the pathogen exhibits both epiphytic and endophytic lifestyles during its disease cycle. PSS survives epiphytically on healthy plant tissue in the spring and colonizes flowers. Populations increase during cool and wet weather, reaching 10^4 to 10^6 CFU g^{-1} per flower (Kennelly et al. 2007).

Since bacterial canker was first reported in Michigan by Jones (1971), there have been various epidemic years of infection on sweet cherry, most recently in 2021 (Jones 1971; Renick et al. 2008). These disease outbreaks are associated with frost damage of flowers during bloom. Trees are weakened and predisposed to infection following freeze injury (Sobiczewski and Jones 1992). Many isolates of PSS exhibit ice nucleation activity (INA), in which the bacterial cells nucleate ice formation that damages plant tissue to occur at warmer temperatures only slightly below freezing (Lindow 1983). Blossom blast results in large yield losses in the current season

and higher incidence of canker initiation, making bloom one of the most critical timings for disease control (Kennelly et al. 2007).

There is no effective chemical control for bacterial canker disease. Copper compounds can be used for disease management; however, copper resistance is widespread in Michigan (Sundin et al. 1989; Renick et al. 2008). Also, copper is phytotoxic to sweet cherries, especially during bloom, so adequate rates for efficacy are unusable when control is needed most without risking tree damage (Kennelly et al. 2007). The antibiotic Kasumin (kasugamycin) was labelled for control of bacterial canker on sweet cherries in 2020. Development of resistance to the antibiotic in populations of PSS and subsequent horizontal gene transfer to other plant pathogens, such as *Erwinia amylovora* causing fire blight of apples, is a major concern (McGhee and Sundin 2011). For these reasons, alternative means of control are needed.

Bacteriophage (phage) have been recently investigated as biological controls for various bacterial plant diseases, including bacterial canker of stone fruit (Akbaba and Ozaktan 2021; Rabiey et al. 2020). Phages are viruses that infect bacterial cells and require the host bacterial cell to replicate. In this process the host cell is killed (lysed) and more phage virions are released into the environment (Kering et al. 2019; Svircev et al. 2018). In a recent growth chamber study of five *Pseudomonas* phages isolated in sweet cherry orchards in Turkey, four of the five phages and a cocktail of the phages reduced disease incidence on micropropagated cherry plantlets inoculated with a highly virulent PSS isolate (Akbaba and Ozaktan 2021). Rabiey et al. (2020) tracked PSS populations in controlled conditions on bean leaves, cherry leaves, and cherry twigs treated with four different phages and two phage cocktails and observed population reductions in every assay type. Studies on phage as biological controls for other pathovars of *Pseudomonas syringae* have also been conducted including pathovars tomato, actinidiae, and porri (Pinheiro et

al. 2020; Prior et al. 2007; Rombouts et al. 2016). While there have been many studies on the isolation and characterization of lytic phage, comparatively few have involved field studies. Commercial phage products have been developed, including a line called AgriPhage by the company OmniLytics Inc. (Sandy, UT) for controlling certain diseases of tomato, pepper, apple, and citrus (Buttimer et al. 2017).

A major challenge for the effective deployment of phage as biocontrols in plant agriculture is that phages are highly sensitive to UV degradation (Fuhrman 1999). Methods of decreasing degradation through protective formulations have been studied including kaolin clay, vegetable extracts, peptone, pregelatinized corn flour, casein, sucrose, and skim milk (Balogh et al. 2003; Born et al. 2015; Vettori et al. 2000). Kaolin clay was found to protect phage from UV radiation, with clay-treated phage titer after 30 min radiation was comparable to untreated phage after 10 min. radiation exposure (Vettori et al. 2000).

Though there have been studies on the isolation, characterization, *in vitro*, and *in vivo* efficacy of phage for controlling PSS pathogenic on sweet cherry, there have been no studies conducted in the field. In addition, the efficacy of phage on controlling populations of PSS on flowers, an important avenue of infection in the disease cycle of bacterial canker has not been documented. We hypothesized that a multi-year field study on the population dynamics of PSS on phage-treated sweet cherry flowers would greatly improve our understanding of phage biological controls in agricultural environments and would inform future refinement of such products or applications to improve efficacy. We also hypothesized that adding an Organic Materials Review Institute listed kaolin clay product called Surround, already registered for use on sweet cherry trees, to the phage treatment would protect the phage from UV degradation, thereby increasing the number of viable phages on treated flowers and improving efficacy. The

company OmniLytics has developed an AgriPhage product including three lytic *Pseudomonas* phage. In this study we investigated PSS populations of inoculated flowers in central and northwest Michigan orchards during bloom 2021 and 2022 treated with the Agriphage cocktail of *Pseudomonas* phage, the Agriphage cocktail with kaolin clay added, and the antibiotic Kasumin. We also assessed protection of phage from UVC radiation with different rates of kaolin clay the pH and temperature stability of the Agriphage cocktail.

2.3 Materials and Methods

Bacterial strains, bacteriophage, and culture conditions. We used two pathogenic strains of *Pseudomonas syringae* pv. syringae, BY25 and BP38, that were isolated from sweet cherry orchards in northwest Michigan in 2019. Spontaneous rifampicin- and nalidixic acid-resistant mutants of these strains were selected by spreading approximately 10^8 bacterial cells on King's medium B (KB) (King et al. 1954) amended with 100 μg ml^{-1} of the respective antibiotic. Each of the PSS strains and their respective spontaneous antibiotic-resistant mutants were stored in 15% glycerol at -80°C.

Wild-type bacteria were grown on KB agar medium, and antibiotic-resistant strains were grown on KB agar medium amended with nalidixic acid or rifampicin to concentration of 100 μg ml^{-1}. We also used KB agar medium for field isolations; this medium was amended with 50 μg ml^{-1} cycloheximide to inhibit fungal growth. Bacteria were incubated at 28°C or at room temperature (≈ 21°C) on a laboratory bench. Light-sensitive rifampicin-amended medium were covered during incubation. For broth cultures, we used KB liquid medium, and cells were grown at 28°C in an incubator shaker set to 220 rpm. Antibiotic-resistant strains were grown in liquid KB amended with the appropriate antibiotic.

Three phages, φSHL2, φCOT4, and φREC1, that were included in a commercial AgriPhage preparation for use in the treatment of bacterial diseases of stone fruit trees, were obtained from OmniLytics Inc. (Sandy, UT). The phages were maintained in phage buffer (10 ml 1 M Tris HCl; 10 ml 1 M $MgSO_4$; 4 g NaCl; 1 ml 0.1 M $CaCl_2$) at 4°C. The kaolin clay used in this study is the agricultural crop product Surround WP (95% active ingredient kaolin) (Novasource, Phoenix AZ). Surround will be referred to as kaolin clay for the remainder of this chapter.

***In vitro* ultraviolet radiation sensitivity determination of φSHL2, φCOT4, and φREC1 bacteriophage.** To assess the sensitivity of individual phage isolates to ultraviolet radiation (UVR), phage preparations were irradiated with UVC (254 nm) radiation, and survival was assessed using a soft agar petri dish overlay. To prepare a bacterial host suspension, a single bacterial colony of PSS BY38 was incubated in KB broth overnight at 28°C, after which 1 ml of cells was washed twice with 1 ml of sterile phosphate buffer. After washing, the suspension was adjusted turbidimetrically (optical density at 600 nm) to $1x10^9$ CFU μl^{-1} using a Tecan Spark plate reader (Männedorf, Switzerland).

Sixty-mm glass petri dishes were filled with 4.95 ml phage buffer. Phage stock solutions ($\approx 2x10^7$-10^9 PFU ml^{-1}) were vortexed, and 50 µl was added to the phage buffer in petri dish and gently swirled to mix. Two replicate dishes were prepared for each phage tested. A100 µl sample was removed from each petri dish prior to UVR exposure, and serially diluted in 900 µl phage buffer. The two replicate dishes containing each individual phage were then exposed to UV-C radiation from an UV-C Sterilaire Lamp (UVP Inc., Upland, CA) placed horizontally 1.2 m above the phage suspensions. To ensure stabilization of the UV output, the lamp was turned

on 15 min prior to use. The energy output of the lamp was measured with a UVX Digital Radiometer (UVP Inc., San Gabriel, CA) fitted with a UV-25 sensor and determined to be 1.3 J m^{-2} s^{-1}. Petri dishes were placed towards center of UV light box and lids removed. One dish was covered with an opaque covering and the other left exposed. The UV doses utilized for all UVR experiments were 117 and 156 J m^{-2}. Samples of 100 µl were taken after each dose and serially diluted in phage buffer.

Falcon tubes of molten soft KB agar (1:1 KB agar and diH_2O) were removed from a water bath and allowed to cool until just warm to the touch but still liquid before adding 100 µl bacterial cell suspension and 100 µl of the phage dilution. The cap was then replaced, and the tube inverted to gently combine. The mixture was then poured over the top of KB agar in a petri dish and dried in a laminar flow hood. Two soft agar petri dishes were prepared for each treatment. When solidified, the petri dishes were incubated for 24 hours at 28°C, after which plaques counted. The experiment was repeated three times for each of the three phages.

Effect of kaolin clay on *in vitro* UVR sensitivity of phages φSHL2, φCOT4, and φREC1. To investigate a kaolin clay product as a protectant for bacteriophage against UVR degradation, a soft agar overlay for counting plaque forming units was conducted as above. The bacterial suspension was prepared in the same manner. When preparing the glass petri dishes, a stock solution of kaolin clay and phage buffer was vortexed well and added for a final concentration of 4.8 mg ml^{-1}. A control of phage in buffer without clay was prepped. The phage solutions were then exposed to UVR as described above. Sampling, soft agar overlay, incubation, and plaque counting were conducted in the same way as previously described. The experiment was repeated three times for each of the three phages.

We further investigated the effect of lower rates of kaolin clay product as a protectant against UVR degradation using the φCOT4 phage and the UVR irradiation soft agar overlay technique described above. The different treatments tested were phage buffer alone as a control and phage buffer with 4.8 mg ml^{-1}, 2.4 mg ml^{-1}, and 1.2 mg ml^{-1} kaolin clay. The stock solution of kaolin clay and phage buffer was diluted with phage buffer to achieve these final concentrations. Bacterial suspension prep, sampling, UVR exposure, soft agar overlay, incubation, and plaque counting were conducted in the same way as above. The experiment was repeated three times.

Bacteriophage stability at differing temperature and pH. The temperature stability of a blend of bacteriophage developed by OmniLytics Inc. for controlling *Pseudomonas syringae* on Nut & Stone Fruit (NSF-041001) was evaluated. Phage stability was examined at three temperatures: 4°C, 21°C, and 37°C. The phage stock was diluted in phage buffer to a concentration of $1x10^6$ PFU ml^{-1}, and three 1.5-ml replicates of the diluted stock were incubated at the different temperatures in covered 1.7-ml centrifuge tubes. Samples of 200 µl were taken at day 0, day 14, and day 28 of the experiment, and 100 µl was serially diluted. A soft agar overlay with only bacterial cell suspension of PSS strain BY38 added to the molten agar was conducted as previously described. After the soft agar cooled, three droplets of 25 µl from each serial dilution were pipetted onto the medium and dried in a biosafety cabinet. Petri dishes were incubated for 12 hours at 28°C, after which plaques were counted. This experiment was repeated three times.

The pH stability of the same bacteriophage cocktail was also tested. The stability was evaluated at pH 4, 7, and 10. Phage stock was diluted in phage buffer to a concentration of $1x10^6$ PFU ml^{-1}. The solution was then adjusted to the pH 4.0, 7.0, or 10.0 with either sodium

hydroxide (NaOH) or hydrochloric acid (HCl). Three 1.5-ml replicates of each treatment were incubated at room temperature (≈ 21°C) in 1.7-ml centrifuge tubes. Samples of 200 µl were removed at 0, 24, and 48 hrs and serially diluted. Phage titer of the treatments was calculated at each sampling time following the soft agar overlay and drop plating method described above for testing bacteriophage temperature stability.

Population dynamics of *P. syringae* pv. syringae in field experiments on sweet cherry flowers treated with bacteriophage and antibiotics. All experiments were conducted on the sweet cherry cultivars 'Benton', 'Black Gold', and 'Ulster'. Experiments were conducted in the spring of 2021 and 2022. The trees used for the field experiments in 2021 were located at the Northwest Michigan Horticultural Research Center (NWMHRC) (44.881996, -85.675251) in Traverse City, Michigan and in a commercial orchard in Suttons Bay, Michigan. The trees used for the field experiments in 2022 were located at the Michigan State University Plant Pathology Research Farm (42.689167, -84.485278) and at the NWMHRC. Branches of trees were flagged at first bloom. At least four branches were flagged for each treatment across multiple trees. Multiple branches per tree were flagged for different treatments on larger trees. When trees reached full bloom, any unopened flowers were removed, and open flowers were inoculated with antibiotic-resistant PSS strains BP25 and BY38.

Prior to field inoculation, strains BP25 and BY38 were cultured in antibiotic-amended KB broth overnight at 28°C, and populations were adjusted turbidimetrically ($OD_{600\ nm}$) to $1x10^9$ CFU μl^{-1} using a Tecan Spark plate reader (Männedorf, Switzerland). Cells were washed and resuspended in PB (Phosphate buffer) solution, and the prepared inoculum stock was maintained on ice. The inoculum stocks were diluted with water, and a mixture of strains BP25 and BY38

was prepared in a 15.12 L backpack sprayer with a final concentration of $1x10^{6-7}$ CFU ml^{-1}. Immediately after dilution and mixing, all open flowers on flagged branches were sprayed with the inoculum. Inoculations were conducted in the evening between 6:30 and 8:00 pm.

Twelve to fourteen hours after inoculation, the treatments were applied using 15.12 L backpack sprayers. Treatments at the NWMHRC in 2021 included a water control, phage cocktail, phage cocktail amended with 8% Surround WP (4.8 g L^{-1} kaolin clay), and 8% Surround WP (4.8 g L^{-1} kaolin clay) control. Treatments at the commercial orchard in 2021 and MSU Plant Pathology Research Farm and NWMHRC in 2022 were the same but included the registered antibiotic Kasumin 2L (Arysta Corp.; Cary, NC).

The phage cocktail consisted of a mixture of φSHL2, φCOT4, and φREC1 phage stock. Each phage stock was diluted accordingly for a final concentration of 2.5×10^7 PFU ml^{-1} in the cocktail treatment. Kasumin antibiotic treatment consisted of 5 ml L^{-1} Kasumin 2L and 1.24 ml L^{-1} Li-700 surfactant (Loveland Products, Inc., Loveland, CO) diluted in water. The rate of 8% Surround WP included in certain treatments was 4.8 g L^{-1}. Immediately after mixing, all open flowers on flagged branches corresponding to each treatment were coated with the solution. Treatment application was conducted in the morning.

An initial flower sample was collected prior to treatment 12-14 hrs after inoculation. Subsequent samples were taken at approximately 24-hr intervals after treatment. At each sampling time point, four replicates of each treatment were collected. A replicate consisted of six flowers collected arbitrarily across different branches. Flowers were placed into plastic bags and kept in a cooler on ice until processed. Samples were brought back to the lab and then weighed. Flowers were removed from the bag using sterile forceps and placed into 60-ml glass culture tubes with 10 ml of PB solution and kept on ice. The tubes were then sonicated for 7 min

(model 250T; VWR Scientific, Houston, TX) and returned to ice. Samples (100 µl) from each tube were serially diluted in 900 µl of PB solution in 1.7-ml microcentrifuge tubes. Serial dilutions were drop-plated onto KB agar medium amended with 100 µg ml^{-1} nalidixic acid and 50 µg ml^{-1} cycloheximide to isolate solely for nalidixic acid-resistant BP25 and BY38 PSS inoculated strains and reduce fungal contamination. In 2022, the KB agar plates used were amended with 75 µg ml^{-1} rifampicin and 50 µg ml^{-1} cycloheximide. Drop-plating consisted of pipetting three, 25 µl droplets of each dilution onto the amended agar and drying the petri dish in a laminar flow-hood. Once dry, petri dishes were stacked in plastic sleeves and incubated at room temperature (≈ 21°C) for at least 48 hrs before colonies were counted. Colony counts and sample weight were used to calculate the population size of PSS BP25/BY38 over the course of the experiment in CFU g^{-1}.

Weather data. Weather data were collected and analyzed for each year and location during the span of field experiments. Historical data for each location were also compiled for analysis. All weather data were accessed from the Michigan Automated Weather Network (http://enviroweather.msu.edu). The weather stations in closest proximity to each study site were used. The weather station used for the field experiment at the MSU Plant Pathology Research Farm was Station *msuhort* (42,6734, -84.4870) located 1.6 km from the study site. The weather station used for the NWMHRC and off-site location in northwest Michigan was the Station *nwmhrs* (44.8831, -85.6777) located 0.5 and 6 km respectively. Hourly data corresponding to the dates of each field experiment were downloaded. Data included the air temperature (C°), precipitation (cm), relative humidity, and total solar flux (kJ m^{-2}). Hourly data were averaged for each day of the experiment (Table 1). Total solar flux was converted to daily light integral

($mol \cdot m^{-2} \cdot d^{-1}$) as previously described (Faust and Logan 2018). The total solar flux in kJ m^{-2} was converted to W m^{-2} then divided by a total sun spectrum conversion factor of 0.51 and calculated for length of day to find daily light integral ($mol \cdot m^{-2} \cdot d^{-1}$). Historical data for daily total solar flux from May 1 to May 15 for years 2000-2020 were downloaded for each location and also converted to daily light integral (DLI) (Fig. 5). DLI measurements can be used to assess solar irradiance delivered to a crop (Faust and Logan 2018).

Data and statistical analyses. All statistical analyses were performed with RStudio for statistical computing (R 4.0.2, R Core Team 2013). Statistical differences in the phage titer for *in vitro* studies were calculated using a one-tailed t-test. For *in vitro* studies, duplicate plate events were averaged to create one daily replicate. Three daily replicates were averaged, and standard deviation calculated for each treatment and irradiance tested. For *in vivo* experiments, four replicates were averaged per treatment per time point and standard deviation calculated. Statistical differences between phage titer of various kaolin clay rates and relative growth rates for field experiments were calculated using a one-way analysis of variance (ANOVA), and mean separation was determined by Tukey's honestly significant difference test.

2.4 Results

***In vitro* ultraviolet radiation sensitivity determination of φSHL2, φCOT4, and φREC1 bacteriophage**. Three bacteriophages, φCOT4, φREC1, and φSHL2, were examined *in vitro* for sensitivity to UVR. All three bacteriophages were susceptible to UV degradation, as phage titers were significantly lower ($P < 0.01$) in the UVR-exposed treatments after both 117 and 156 J m^{-2} radiation compared to the covered, non-exposed treatment (Fig. 2).

Effect of kaolin clay on *in vitro* UV sensitivity of φSHL2, φCOT4, and φREC1 bacteriophage. Kaolin clay was added to the three bacteriophages and examined as a UV-protectant. Pairwise comparisons of the kaolin clay and untreated treatment were conducted at each dose of UVR. Kaolin clay at a rate of 4.8 mg ml^{-1} protected φCOT4 up to the highest dose of irradiance measured, 156 J m^{-2} ($P< 0.01$) (Fig. 2B). The untreated φCOT4 titer decreased by nearly three log units after the same dosage. Kaolin clay at the same rate also protected φREC1 ($P< 0.01$) and φSHL2 ($P< 0.01$) after 156 J m^{-2} irradiance (Fig. 2D, 2F). After the same dosage, the untreated φREC1 and untreated φSHL2 titers decreased by over 2 log units.

Different amounts of kaolin clay were then tested with the φCOT4 bacteriophage to find the lowest rate that would still provide UVR protection. The 2.4 mg ml^{-1}, and 1.2 mg ml^{-1} rates of kaolin clay were as effective as the previously tested rate of 4.8 mg ml^{-1} at protecting φCOT4 at the highest tested level of irradiance ($P< 0.01$). The lowest rate tested; 0.6 mg ml^{-1} kaolin clay, offered some protection ($P< 0.01$) compared to the untreated control, but was not as effective than the higher rates tested (Fig. 3).

Bacteriophage stability at differing temperature and pH. The stability of the AgriPhage cocktail under different pH or temperature conditions was evaluated. In the temperature study, AgriPhage was stable after 28 days at 4°C, 21°C, and 37°C (Fig. 4A). AgriPhage was stable after 48 hrs in phage buffer at pH 7 and pH 10 (Fig. 4A). The cocktail was not stable at pH 4 after 24 hrs, as observed by a decrease of 1.6 log units ($P<0.01$) (Fig. 4B).

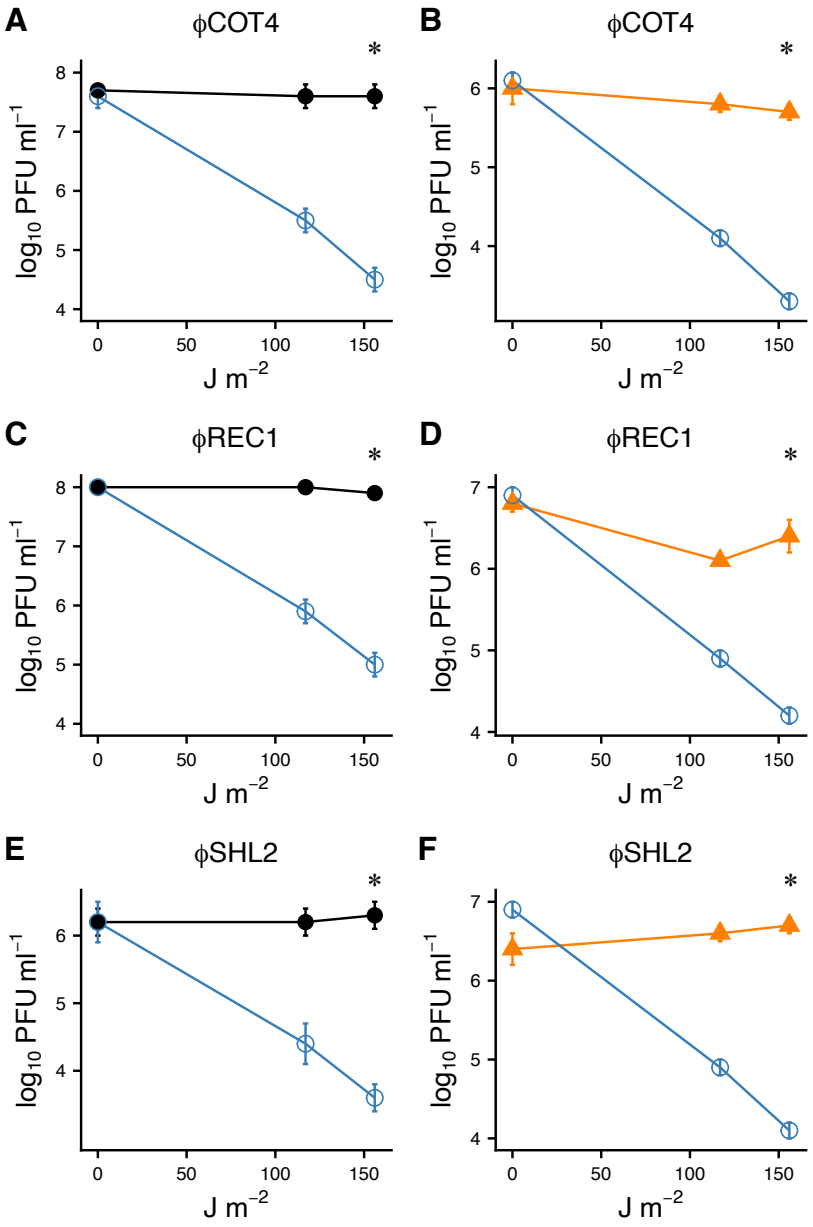

Figure 2. Effect of kaolin clay on UV sensitivity of φCOT4, φREC1, and φSHL2 bacteriophages. (A, C, E) Phage were exposed to UVC irradiation uncovered (blue circle) or covered (black filled circle). (B, D, F) Phage were exposed to UVC irradiation uncovered (blue circle) or with 4.8 mg ml^{-1} kaolin clay added (orange filled triangle). Points represent the mean of three experimental replicates and error bars are standard deviation. Asterisks represent $P<$ 0.01 from one-tailed t-test.

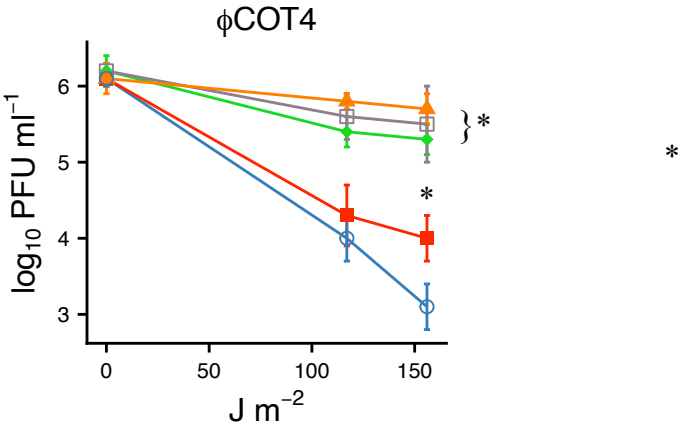

Figure 3. Effect of kaolin clay at different rates on UV sensitivity of φCOT4. Phage were exposed to UVC irradiation treated: uncovered (blue circle), 0.6 mg ml^{-1} kaolin clay (red filled square, 1.2 mg ml^{-1} kaolin clay (green filled diamond), 2.4 mg ml^{-1} kaolin clay (gray square), and 4.8 mg ml^{-1} kaolin clay (orange filled triangle). Points represent the mean of three experimental replicates and error bars are standard deviation. Asterisks represent $P< 0.01$ from Tukey's honestly significant difference test.

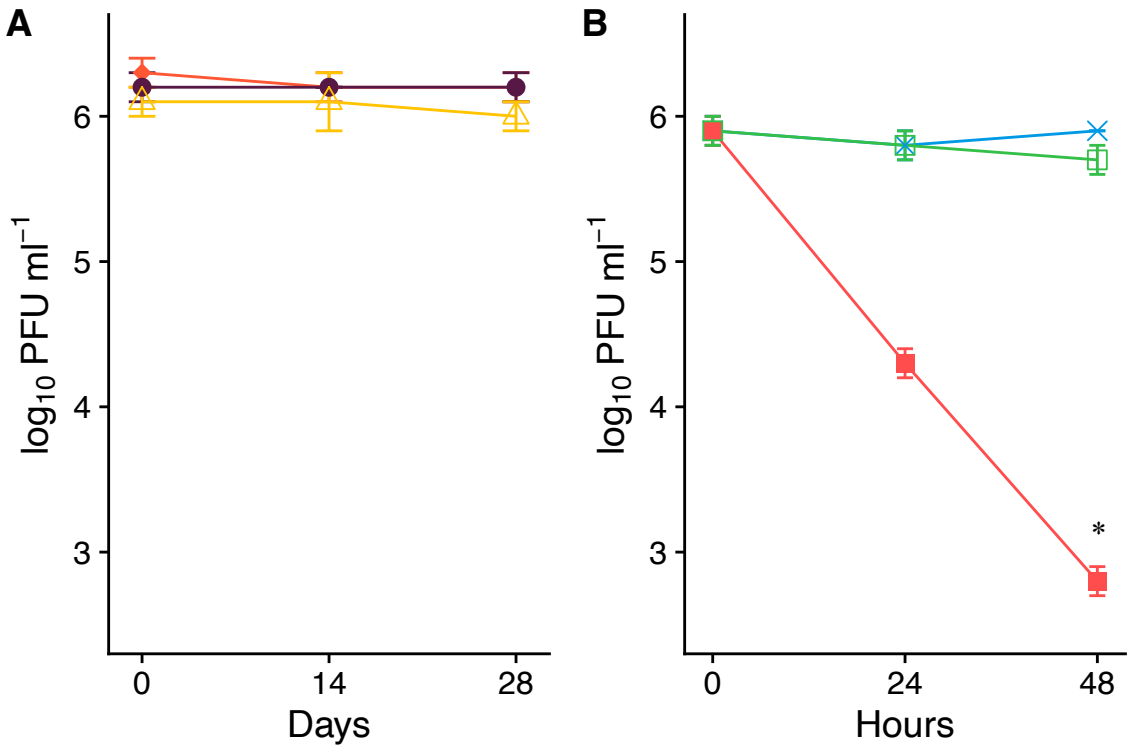

Figure 4. Temperature and pH stability of *Pseudomonas* AgriPhage cocktail. The AgriPhage cocktail developed for controlling bacterial canker of stone fruit caused by *Pseudomonas syringae* pv. syringae was incubated in phage buffer under different temperature and pH conditions: (A) 4°C (yellow triangle), 21°C (orange filled diamond), 37°C (burgundy filled circle) or (B) pH 4 (red filled square), pH 7 (green square), pH 10 (blue cross). Points are the mean and error bars the standard deviation of technical replicates. Results were similar for all three experimental replicates. Asterisks represent $P < 0.01$ from Tukey's honestly significant difference test.

Population dynamics of *P. syringae* pv. syringae in field experiments on sweet cherry flowers treated with bacteriophage and antibiotics. A lower population of PSS was detected with the phage cocktail treatment during one year in one location at one sampling point: 48 hrs after treatment at the NWMHRC in 2021. This field experiment was unique in other ways as well. For example, this was the only field replicate with measurable precipitation for more than one day of the experiment. The day of the 48 hrs after treatment sampling had the highest Total Daily Precipitation of 2.5 cm. The 2021 NWMHRC experiment also had a higher relative humidity, 77% on average, than any other replicate: 53-57% on average (Table 1). The average DLI for the course of the experiment (April 30-May 4) was 26 $mol \cdot m^{-2} \cdot d^{-1}$ which is lower than the average DLI at the same location for 19 of the last 20 years (Fig. 9). The only average DLI for the historical data at the NWMHRC that was lower was 24.9 $mol \cdot m^{-2} \cdot d^{-1}$ in 2014. Mean air temperature was lower over the course of the experiment as well, an average of 9.28°C compared to the next lowest of 11.15°C in the 2021 commercial orchard replicate (Table 1).

In 2021 at the NWMHRC, flower populations of a mixture of PSS strains BP25 and BY38 increased over one order of magnitude reaching $\log_{10}$ 5.9 CFU g^{-1} in the water-treated control from 14 hrs post inoculation to 72 hrs after treatment (Fig. 5). Flower populations were lower with the phage cocktail treatment 48 hrs after treatment ($P<0.05$) but this effect was lost by 72 hrs after treatment. In the commercial orchard in 2021, flower populations of the mixture of PSS strains BP25 and BY38 increased from 5.1 $\log_{10}$ CFU g^{-1} 12 hrs after inoculation to 6.8 $\log_{10}$ CFU g^{-1} after approximately 96 hrs (Fig. 6). No phage treatments were effective at reducing population of PSS compared to the water control except the 5 ml L^{-1} Kasumin at 94 hrs after treatment ($P<0.01$) (Fig. 6).

At the MSU Plant Pathology Farm in 2022, initial flower populations of the PSS mixture were 4.4 $\log_{10}$ CFU g^{-1}, which increased to 5.1. $\log_{10}$ CFU g^{-1} by 96 hrs after treatment. The flower populations were decreased by the Kasumin treatment 24 hrs after treatment (P<0.05), 48 hrs (P<0.01), and 72 hrs after treatment (P<0.01) (Fig 7). No treatments were effective at lowering PSS in flowers 96 hrs after the spray applications.

Flower populations for the NWMHRC field experiment in 2022 were initially 4.5 $\log_{10}$ CFU g^{-1} and increased to 6.3 $\log_{10}$ CFU g^{-1} over the course of the study (Fig. 8). Kasumin was the only treatment that reduced the PSS population 24, 48, and 72 hrs after treatment (P<0.01) (Fig. 8). Populations of PSS on flowers treated with Kasumin recovered to levels similar to the water control by 96 hrs after treatment (Fig. 8).

A summary of weather conditions for the duration of each field experiment is shown in Table 1. Measurable precipitation only occurred at the NWMHRC in 2021, and on one day during the MSU study in 2022 (Table 1). Mean air temperatures were lower during experiments in 2021 compared to 2022 (Table 1). Relative humidity values were similar across all experiments (average 57.3%, 53.8%, 57%) except for NWMHRC in 2021 which was higher (77%) (Table 1). The average DLIs of solar irradiance for the NWMHRC and commercial orchard experiments in 2021 were 26 and 45.7 $mol \cdot m^{-2} \cdot d^{-1}$, respectively and for MSU and NWMHRC experiments in 2022 were 40.7 and 47 $mol \cdot m^{-2} \cdot d^{-1}$, respectively (Table 1).

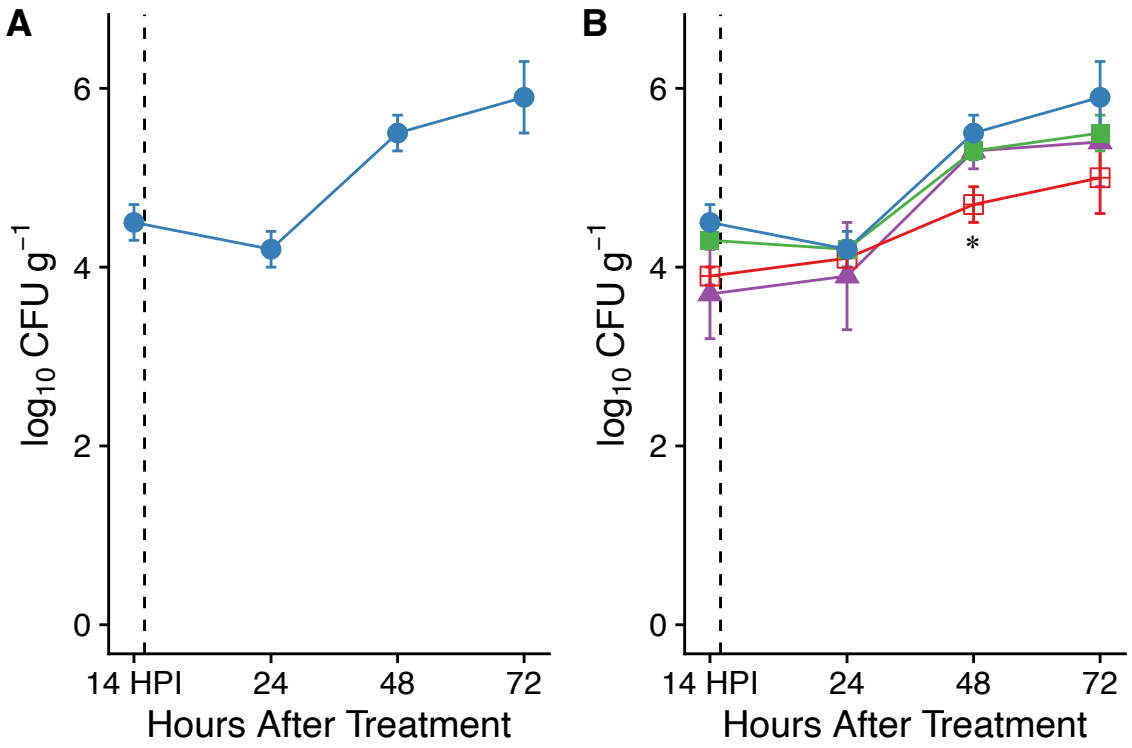

Figure 5. Population dynamics of combined *Pseudomonas syringae* pv. syringae strains BP25 and BY38 on 'Benton' sweet cherry flowers treated with AgriPhage cocktail or water sprays: NWMHRC 2021. HPI stands for hours post inoculation. Dashed line represents timing of treatment application. Labelling to the right of the dashed line correspond to number of hours after treatment. Points represent mean of four technical replicates and error bars represent standard deviation. Asterisk represents $P< 0.05$ from Tukey's honestly significant difference test. (A) Water treatment. (B) Treatments: Water (blue filled circle), 7.5 x 10^7 PFU ml^{-1} AgriPhage cocktail (red square), 7.5x10^7 PFU ml^{-1} AgriPhage cocktail with 4.8 g L^{-1} kaolin clay (green filled square), Water with 4.8 g L^{-1} kaolin clay (purple filled triangle).

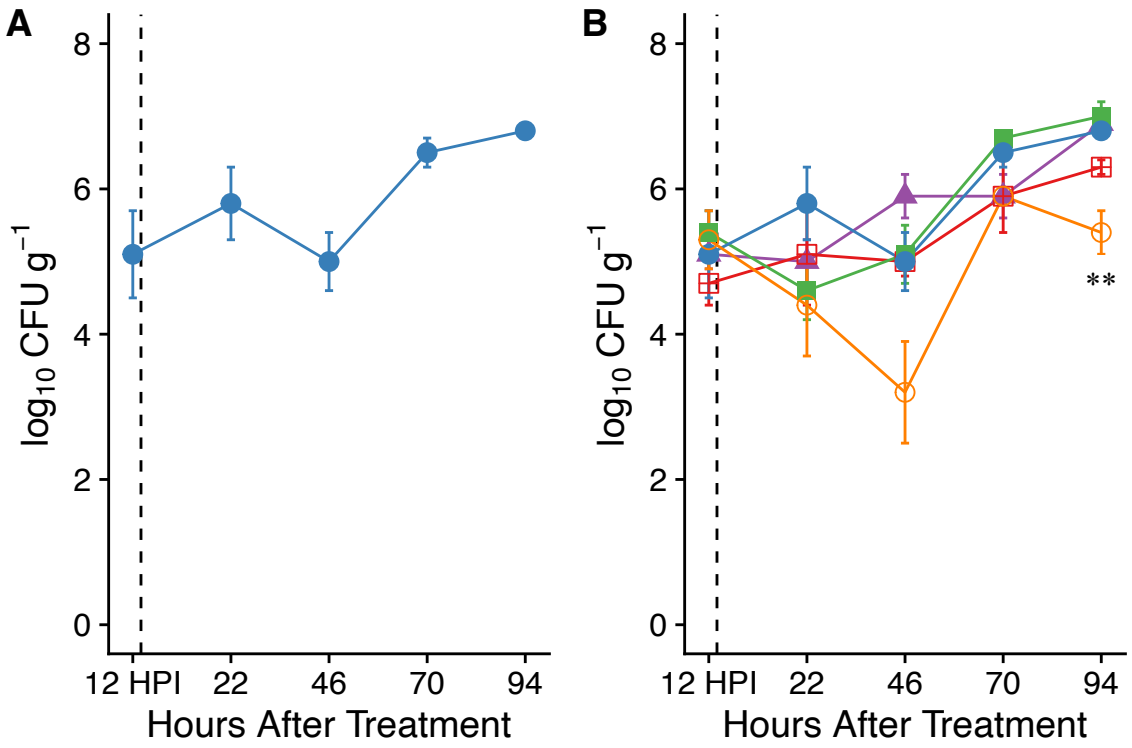

Figure 6. Population dynamics of combined *Pseudomonas syringae* pv. syringae strains BP25 and BY38 on 'Black Gold' sweet cherry flowers treated with AgriPhage cocktail, antibiotic, or water sprays: commercial orchard in Leelanau County 2021. HPI stands for hours post inoculation. Dashed line represents timing of treatment application. Labelling to the right of the dashed line correspond to number of hours after treatment. Points represent mean of four technical replicates and error bars represent standard deviation. Double asterisk represents $P<0.01$ from Tukey's honestly significant difference test. (A) Water treatment. (B) Treatments: Water (blue filled circle), $7.5x10^7$ PFU ml^{-1} AgriPhage cocktail (red square), $7.5x10^7$ PFU ml^{-1} AgriPhage cocktail with 4.8 g L^{-1} kaolin clay (green filled square), Water with 4.8 g L^{-1} kaolin clay (purple filled triangle) and 5 ml L^{-1} Kasumin (orange circle).

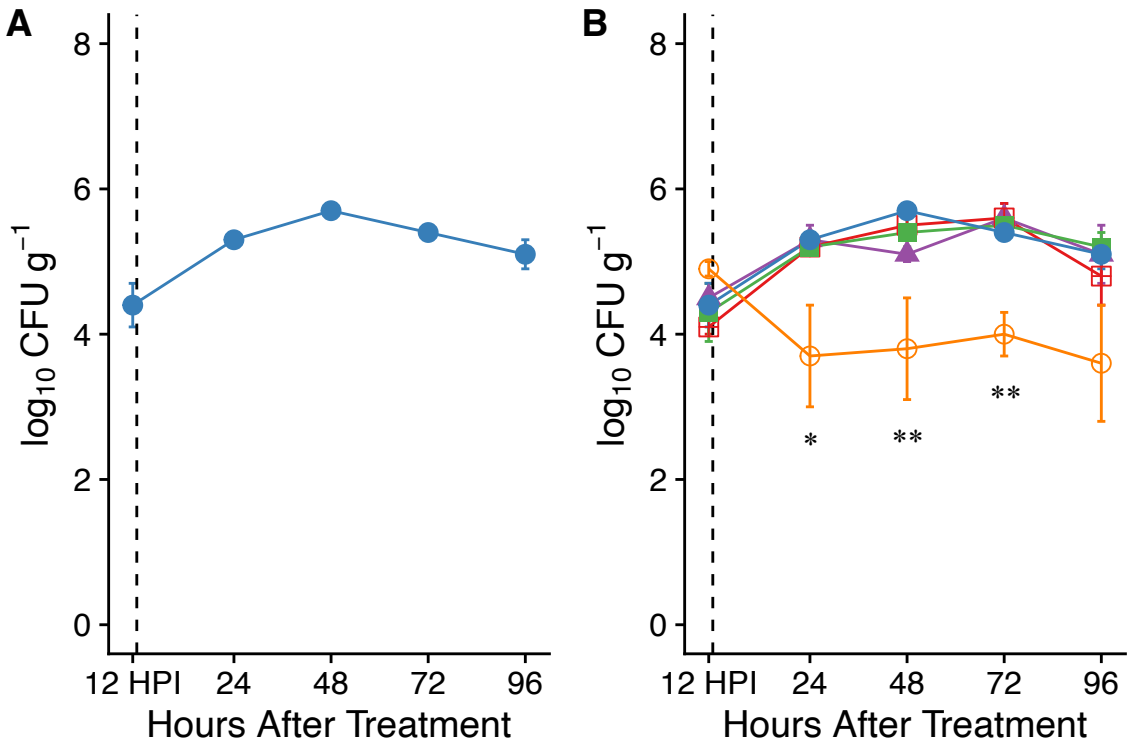

Figure 7. Population dynamics of combined *Pseudomonas syringae* pv. syringae strains BP25 and BY38 on 'Ulster' sweet cherry flowers treated with AgriPhage cocktail, antibiotic, or water sprays: MSU 2022. HPI stands for hours post inoculation. Dashed line represents timing of treatment application. Labelling to the right of the dashed line correspond to number of hours after treatment. Points represent mean of four technical replicates and error bars represent standard deviation. Single asterisk represents $P<0.05$ and double asterisk represents $P<0.01$ from Tukey's honestly significant difference test. (A) Water treatment. (B) Treatments: Water (blue filled circle), $7.5x10^7$ PFU ml^{-1} AgriPhage cocktail (red square), $7.5x10^7$ PFU ml^{-1} AgriPhage cocktail with 4.8 g L^{-1} kaolin clay (green filled square), Water with 4.8 g L^{-1} kaolin clay (purple filled triangle) and 5 ml L^{-1} Kasumin (orange circle).

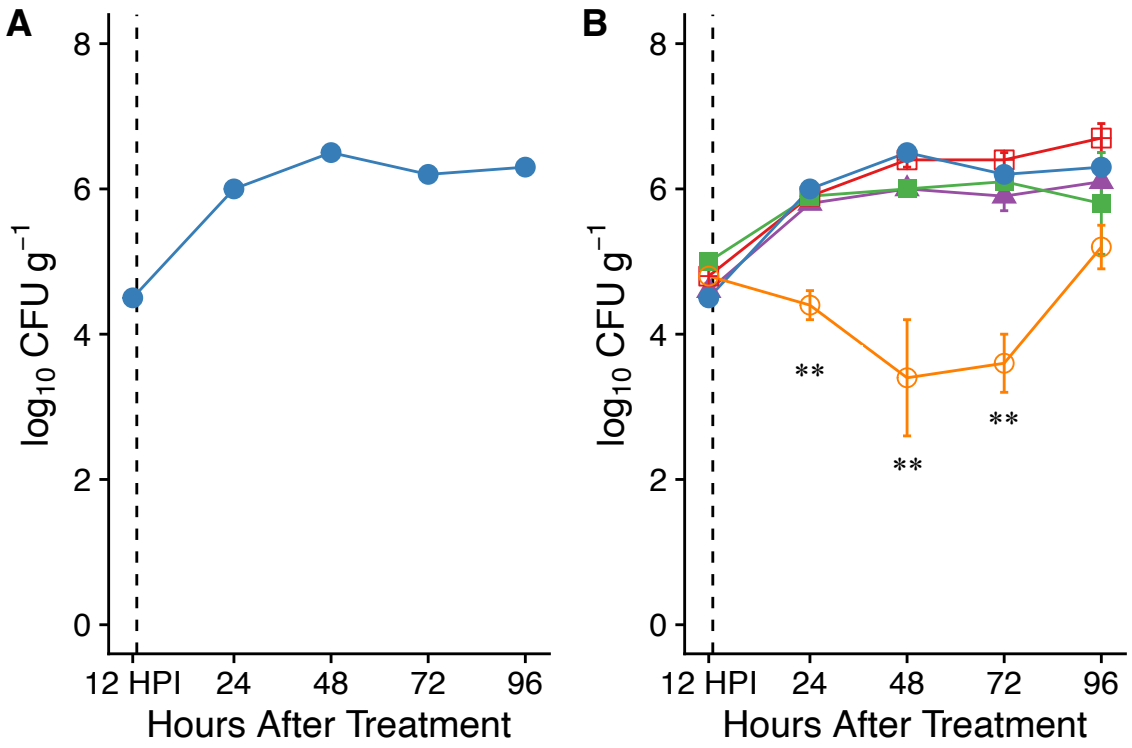

Figure 8. Population dynamics of combined *Pseudomonas syringae* pv. syringae strains BP25 and BY38 on 'Benton' sweet cherry flowers treated with AgriPhage cocktail, antibiotic, or water sprays: NWMHRC 2022. HPI stands for hours post inoculation. Dashed line represents timing of treatment application. Labelling to the right of the dashed line correspond to number of hours after treatment. Points represent mean of four technical replicates and error bars represent standard deviation. Double asterisk represents $P<0.01$ from Tukey's honestly significant difference test. (A) Water treatment. (B) Treatments: Water (blue filled circle), $7.5x10^7$ PFU ml^{-1} AgriPhage cocktail (red square), $7.5x10^7$ PFU ml^{-1} AgriPhage cocktail with 4.8 g L^{-1} kaolin clay (green filled square), Water with 4.8 g L^{-1} kaolin clay (purple filled triangle) and 5 ml L^{-1} Kasumin (orange circle).

Table 1. Summary of weather conditions each year during each experiment. All weather stations are maintained by the Michigan Automated Weather Network. The weather station was located 6 km from the commercial orchard study site and 0.5 km from the NWMHRC study site. The weather station for MSU location was 1.6 km from the study site (https://enviroweather.msu.edu). Daily light integral was converted from kJ•m-2.

Location	Year	Time point	Mean air temp. (°C)	Total daily precipitation(cm)	Average daily relative humidity (%)	Daily light integral ($mol \cdot m^{-2} \cdot d^{-1}$)
NWMHRC	2021	30-Apr	5.3	0.1	67.1	52
		1-May	11.1	0	51.4	37
		2-May	12.3	2.1	74.5	19
		3-May	11.3	2.5	97.8	6
		4-May	6.4	0.4	94.8	16
Commercial	2021	11-May	6	0	67.5	49
Orchard		12-May	8.3	0	58.7	57
		13-May	11.3	0	49.6	57
		14-May	14.3	0	41.6	51
		15-May	14.3	0	46	33
		16-May	12.7	0	80.6	27
MSU	2022	9-May	16.3	0	45.4	52
		10-May	20.9	0	51.2	31
		11-May	22.5	0	61.1	23
		12-May	22	0	39.1	50
		13-May	21.9	0	54.8	51
		14-May	20.9	0.5	71.1	37
NWMHRC	2022	12-May	23	0	61.2	38
		13-May	24	0	43.4	52
		14-May	19.1	0	63.4	39
		15-May	17	0	55.2	48
		16-May	13.9	0	54.2	48
		17-May	8.6	0	64.7	57

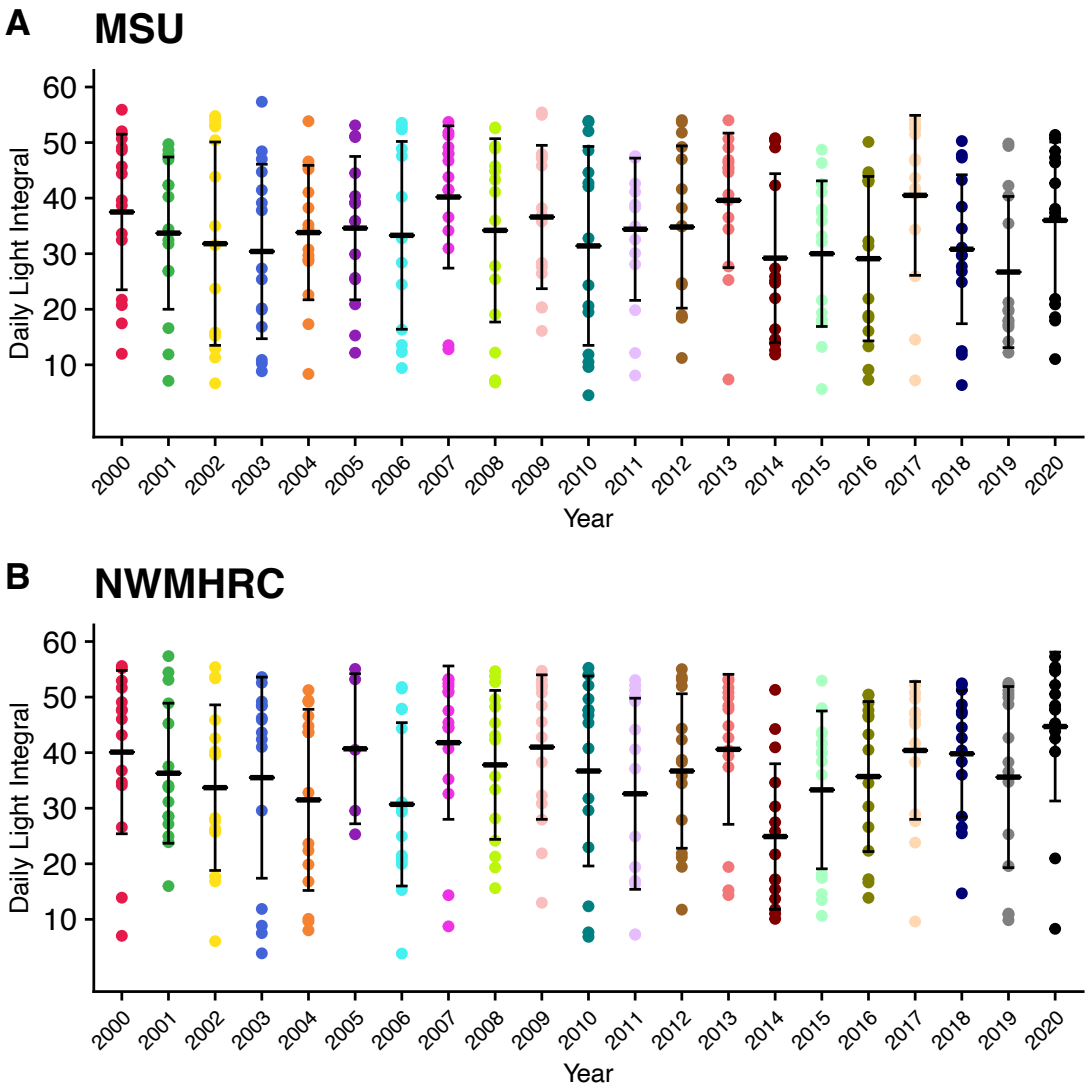

Figure 9. Historical Daily Light Integral (DLI) from 2000-2020. DLI for May 1 thru May 15 of the years 2000-2020 were acquired from the Michigan Automated Weather Network (https://enviroweather.msu.edu) and are represented by points. Mean DLI for each year is represented by the black horizonal bar. Error bars represent standard deviation. DLI was converted from kJ•m-2. (A) MSU weather station was 1.6 km from the study site (B) NWMHRC weather station was located 6 and 0.5 km from the study sites.

2.5 Discussion

Kasumin was the only other treatment able to reduce PSS flower populations during the experiments. However, control was inconsistent. Populations were only significantly lower at 94 hrs after treatment in the 2021 replicate. In both 2022 replicates, populations were lower at 24, 48, and 72 hrs after treatment, but the population recovered to the size of the control by 94 hrs. Since bloom is usually longer than 72 hrs, a second application of Kasumin may be necessary to control blossom blast. The greatest reduction of PSS observed across the experiments with Kasumin was 3.1 $\log_{10}$ CFU g^{-1} at 48 hrs after treatment at the NWMHRC in 2022 (Fig. 9). The average reduction across the replicates with significantly lower populations was 1.9 $\log_{10}$ CFU g^{-1}. The PSS population on Kasumin treated flowers never fell below 3 $\log_{10}$ CFU g^{-1}. This contrasts a study on control of *Erwinia amylovora* observed with Kasumin on apple flowers where populations were reduced by multiple logs during most experiments and the bacterial population recovered little over time (Slack et al. 2021). A higher rate of Kasumin may need to be applied to achieve similar control of PSS causing bacterial canker. We also observed phytotoxicity on Kasumin treated flowers. To ensure that the adjuvant LI-700 was not the cause, we applied Kasumin only to sweet cherry flowers in 2022 and still observed the phytotoxicity (Fig. 10). Applying a higher rate of Kasumin would exacerbate this issue and could have impacts on fruit set. Kasumin is also expensive, so applying more to achieve disease control may not be economical for sweet cherry growers.

In this study, we evaluated the UVR sensitivity of three bacteriophage that targeted the plant pathogen PSS, identified kaolin clay as a potential UVR protectant, and field-tested preparations of the phage with or without kaolin clay for effects on population reduction of PSS on sweet cherry flowers. The results presented here demonstrate that kaolin clay amendments

for *in vitro* experiments effectively protected the three bacteriophages tested, φCOT4, φREC1, and φSHL2, from UVR degradation. These results corroborate results of previous studies of kaolin clay as a protectant for bacteriophage that lyse the apple fire blight pathogen *Erwinia amylovora* (Dobbins 2020). In the current work, *Pseudomonas* phages were irradiated with UVC radiation (254 nm), which are the shortest and therefore most destructive wavelength of the UV spectrum (Jacobs et al. 2005). Although UVC wavelengths are effectively blocked from reaching the surface of the earth by atmospheric ozone, UVC can be used as a proxy for DNA-damaging UVB wavelengths. While UVA and UVB were not investigated in this study, Dobbins (2020) investigated UVA and UVB irradiation effects and found that UVA did not degrade bacteriophage and kaolin clay was effective at reducing degradation of φ21-4 and φ31-3 even up to 2000 J m^{-2} UVB irradiation.

Rates of kaolin clay lower than 4.8 mg ml^{-1} can protect bacteriophage from UVR degradation. φCOT4 was chosen for experiments to study the effect of kaolin clay rates but there is no indication that results would differ for the other phages present in the *Pseudomonas* AgriPhage blend as the phages all behaved similarly in the previous UVR experiments. One half and one quarter rates of the previously tested rate of kaolin clay demonstrated similar levels of protection. The lowest rate tested offered some protection compared to the untreated control. These results are significant when considering the economic feasibility of a fruit grower adding kaolin clay as a protectant when applying a commercial bacteriophage biological control product. Orchard management is already costly for producers, so determining the least amount of any product needed to have effective results is an important cost-saving measure. The 4.8 mg ml^{-1} rate of kaolin clay is already only 8% of the recommended 60 g L^{-1} field rate of Surround WP for its labelled use as a crop barrier. This lower amount is below the minimum specified on

the product label. A supplemental registration for the special use of Surround as a phage protectant will be necessary to use the product at these lower rates. Our results indicate that fruit growers could protect a phage product against UVR damage with an 8% (4.8 mg ml^{-1} kaolin clay), 4% (2.4 mg ml^{-1} kaolin clay) or 2% (1.2 mg ml^{-1} kaolin clay) rate of Surround WP. Utilizing a lower rate means lower input costs for the grower and less wear over time of spray equipment, as the fine clay particles are very abrasive.

Phage stability should be considered when developing a commercial product as the phage need to survive during production, distribution, and storage. Our results on the stability of the *Pseudomonas* AgriPhage cocktail of phages indicate that AgriPhage is stable at neutral and basic pH and was briefly stable at an acidic pH. If fruit growers apply AgriPhage in a spray tank mix, considerations must be made regarding the pH of the water source and any other products applied as acidic conditions could de-stabilize the phages and decrease efficacy. AgriPhage was stable at all temperatures studied; this indicates that temperature will not be a limiting factor for efficacy of bacteriophage as a biological control in plant agriculture. Temperatures tested were constant rather than fluctuating, nonetheless a lack of difference in titer even after 28 days across a spread of 33 degrees is promising. The stability of AgriPhage in terms of temperature is also advantageous since the storage of AgriPhage will not require climate-controlled conditions, making it practical and cost/price accessible for more growers to use. The temperature results are similar to a study on phages for controlling bacterial blight of leek which were stable between 4° and 37°C for 24 hrs. In contrast, those phages were stable from pH 4 to 12 for 24 hr (Rombouts et al. 2016).

We observed severe natural infection of bacterial canker in Michigan sweet cherry orchards in the spring of 2021 and did not observe any infection in 2022. Northwest and central

Michigan experienced a severe freezing event during the night of 21 April 2021. In 2022, temperatures reached over 29°C during the NWMHRC study. Even with these highly divergent weather conditions, bacterial populations reached similar levels on water-treated flowers in all field studies across the two years. Although these flowers were inoculated, we believe this demonstrates the real versatility of PSS; capable of growing to large populations on healthy and frost damaged plant tissue, in cold and warm weather and even with almost no measurable precipitation during three of the four studies. This also demonstrates the importance of freeze events in the disease cycle of bacterial canker. Even with similar population sizes on flowers across the two years, only severe blossom blast, spur infection and subsequent canker initiation was observed after the frost event in 2021.

We observed a reduction through phage in the PSS population under conditions with precipitation. We hypothesize this to contribute to increased efficacy of phage in two ways: through added cloud coverage and increased moisture in the flowers. Cloud coverage generally causes attenuation of UV radiation (Calbó et al. 2005). This corresponds to the lower DLI observed during the experiment. We hypothesize that the cloud coverage caused the phage to experience less UV radiation and therefore less degradation, resulting in a greater number of viable phages present to lyse the bacteria on the flowers, reducing the PSS flower population. This illustrates the value in developing strategies for protecting or otherwise reducing the UV degradability of phages to improve efficacy of phage-based biological controls in the field.

Precipitation also causes periods of time where the flowers are wet. It is well known that rain is key in the movement and dissemination of bacterial cells of many plant pathogens (McManus Jones 1994). It is also understood that plant pathogenic bacteria colonize different parts of flowers such as the stigma and hypanthium (Mansvelt and Hattingh 1988; Pusey 2000).

Phages are not actively motile and therefore can only interact with bacterium of which they come into contact (Simmons et al. 2018). We hypothesize that free moisture from precipitation increases the amount of contact between phage and bacterial host cells through the increased movement of the bacteria, phage particles, or both. It is possible that the spray coverage achieved through backpack sprayers like we used are not adequate for getting the phage to the niches of floral bacterial colonization, like the floral cup. The free moisture available during the 2021 NWMHRC trial, in particular the 48 hr sampling point with the highest observed Total Daily Precipitation, may have increased the number of host and virus interactions, and thus increased lysis of the PSS and reduced populations of phage treated flowers. Future work could be done to investigate means of improving spray coverage, perhaps with certain surfactants, of phage biological controls as a means of improving efficacy. The reduction in PSS populations by the phage cocktail treatment was only observed at 48 hrs after treatment. The population was similar again to the water treated control after another 24 hrs. This could indicate the necessity for multiple applications of a phage cocktail treatment during sweet cherry bloom for maintaining sufficient control of PSS.

The phage cocktail with kaolin clay did not demonstrate any efficacy in the field after it was shown to improve phage survival *in vitro*. We hypothesize that this may be caused by *in vitro* and *in vivo* differences in sorptive interactions between phage and clay particles. Various physico-chemical mechanisms such as ionic composition, pH, and electrical charges are involved in this interaction (Bitton 1975). These mechanisms are not controlled in a field setting and we believe may be confounding variables. Further work needs to be done to understand phage adsorption onto clay in the environmental conditions of flowers before kaolin clay can be optimized as a protectant for phage controlling bacterial canker disease.

Further research is required before bacteriophage are a viable biological control for bacterial canker of stone fruit. UVR, a well-known challenge, can be mitigated using a kaolin clay protectant *in vitro*, but acceptable reduction of *P. syringae* pv. syringae for disease prevention has yet to be seen in the field by either our phage cocktail alone or supplemented with kaolin clay. Our work contributes to the understanding of bacteriophage and phytopathogenic bacteria *in vivo*; an area of study that is currently lacking.

These environmental factors: higher humidity, greater precipitation, lower temperature, and lower DLI, could indicate conditions in which bacteriophage are able to be effective, demonstrated by the lower population of PSS at the 48 hrs sampling point. These cold and wet conditions are also those known to favor infection of bacterial canker (Kennelly et al. 2007). We believe this shows promise for bacteriophage as a biological control for bacterial canker when conditions are optimal and indicates how phage may be further optimized to this purpose.

Figure 10. Phytotoxicity on sweet cherry ‘Coral Champagne’ after treatment with Kasumin 4L antibiotic.

CHAPTER 3

PHENOTYPIC AND GENETIC ANALYSIS OF *PSEUDOMONAS SYRINGAE* PV. SYRINGAE POPULATIONS IN NORTHWEST MICHIGAN SWEET CHERRY ORCHARDS

3.1 Abstract

To better understand the populations of *Pseudomonas syringae* pv. syringae (PSS) inhabiting sweet cherry orchards in northwest Michigan, bacterial isolates were collected in 13 orchards. From an initial collection of 395 isolates of which 237 were pathogenic on sweet cherry, a group of 65 pathogenic isolates of PSS were identified and screened for sensitivity to copper and to the antibiotic kasugamycin, and for ice nucleation activity: a critical phenotype contributing to disease incidence and severity in the field. All 65 isolates were ice nucleation active, seven were resistant to copper sulfate at 250 μg ml^{-1} and all isolates were sensitive to kasugamycin at 100 μg ml^{-1}. This demonstrates the prevalence of virulent phenotypes in the population, a further loss in efficacy of copper control, and establishes a foundation for future studies on kasugamycin sensitivity in the state. Multi-locus sequence typing with four housekeeping genes was conducted, and a phylogenetic tree was generated, and which shows a high level of diversity with isolates grouping in three clades of Group 2 of the *P. syringae* complex. This population study will aid in developing bacteriophage mixtures that are effective and specific as a biological control for PSS, causal agent of bacterial canker of stone fruit.

3.2 Introduction

Pseudomonas syringae is one of the most common bacterial plant pathogens (Mansfield et al. 2012). There are over 60 pathovars of the species with over 80 plant species considered hosts of pathovar syringae (Baltrus et al. 2017; Hirano and Upper 2000). Many phenotypes exhibit ice nucleation activity (INA) where the bacterium produces proteins that catalyze ice formation at warmer temperatures; bacterial INA and is recognized as a factor in frost injury in plants and subsequent invasion by the pathogen (Lindow 1983). Other virulence factors of *P. syringae* include the production and secretion of hypersensitive response and pathogenicity (Hrp) effector proteins, phytohormones, and various phytotoxins such as coronatine, syringolin A, syringomycin, syringopeptin, phaseolotoxin, and tabtoxin (Ichinose et al. 2013).

The first multi-locus sequence testing (MLST) analysis of a plant pathogenic bacterium was conducted on *P. syringae* by Sarkar and Guttman in 2004. This analysis was conducted using seven housekeeping genes of the core genome of *P. syringae* and demonstrated a highly clonal species of four major groups of strains with weak association to host of isolation (Sarkar and Guttman 2004). Hwang et al. (2005) later reduced the number of MLSY loci examined to four while still retaining phylogenetic resolution. The four housekeeping genes included *rpoD*, encoding sigma factor 70; *gyrB*, encoding DNA gyrase B; *gltA* (or *cts*), encoding citrate synthase; and *gapA*, encoding glyceraldehyde-3-phosphate dehydrogenase. This analysis included a larger number of strains and identified a fifth group (Hwang et al. 2005). Many other MLST analyses of *P. syringae* including some combination of these housekeeping genes have been reported (Ahmadi et al. 2017; Morris et al. 2008; Parkinson et al. 2011; Vasebi et al. 2020). In 2014, Berge et al. combined 216 strains from agricultural and environmental studies on *P.*

syringae and identified 13 total phylogroups with 23 clades, further expanding understanding of the classification and diversity of the *P. syringae* complex (Berge et al. 2014).

Pseudomonas syringae pv. syringae (PSS) is the primary causal agent of bacterial canker disease on sweet cherry trees (*Prunus avium*). Michigan is the fourth largest producer of sweet cherries in the US by weight (NASS 2017). Bacterial canker is an economically important disease of sweet cherry trees as it can infect all parts of the tree and can cause major yield losses and death of the tree over time (Jones and Sutton 1996; Kennelly et al. 2007). Options for effective disease control are very limited. Copper products are available; however, sweet cherry trees are highly susceptible to phytotoxicity, and resistance has been detected in Michigan since 1989 (Renick et al. 2008; Sundin et al. 1989).

The most recent analysis of *P. syringae* from sweet cherry in Michigan was conducted by Renick et al. (2008). Healthy flowers were collected in 2003 and 2004 across the major fruit-growing regions of Michigan, including Northwest Michigan, and from which *P. syringae* was isolated to track levels of copper resistance within populations. Copper resistant and copper sensitive *P. syringae* isolates were recovered from all 39 orchards. Copper resistance differed over the years of sampling but averaged 6.6% of the population in the northwest region. An average of 78.5% of the isolates characterized were pathogenic across the two sampling years and 94% were ice nucleation positive (Renick et al. 2008). Renick identified three groups: Group 1 with 33 isolates and Groups II and III with two isolates each. PSS reference strains B728a, originally isolated in Wisconsin from bean leaves (Loper and Lindow 1987) clustered in Group I and FF5 isolated in Oklahoma from ornamental pear clustered in Group II (Sundin and Bender 1993). It is likely that these groups correspond to some of the 13 Phylogroups of the *P.*

syringae complex identified by Berge et al. (2014). The clustering of Michigan isolates in the dendrogram was not dependent on geographical location or copper resistance.

Levels of copper resistance in Michigan PSS populations have not been investigated for almost 20 years, nor has the existence or prevalence of PSS isolates resistant to kasugamycin in Michigan sweet cherry orchards been documented. This work is the first genetic analysis of the PSS population in this region. We hypothesized that a phylogenetic study of PSS in Northwest Michigan orchards using MLST analysis would demonstrate a diverse population of isolates capable of causing bacterial canker symptoms on host *Prunus avium*. In this study, we characterized a subset of 65 PSS isolates collected from 13 sweet cherry orchards in Northwest Michigan, including pathogenicity, ice nucleation activity, copper sensitivity, and streptomycin sensitivity. Using the four housekeeping genes *gapA*, *gltA*, *gyrB* and *rpoD* we built a phylogenetic tree of Michigan isolates, other species of *Pseudomonas*, and strains of PSS pathogenic to stone fruit and other hosts from around the world.

3.3 Materials and Methods

Isolation and characterization of *Pseudomonas syringae* pathovar *syringae* from Michigan sweet cherry orchards. Flower samples were collected at 13 different sweet cherry orchards in Northwest Michigan during bloom in 2021 (Fig. 11). Six orchards each were sampled in Leelanau and Grand Traverse counties and one orchard was sampled in Benzie County. Five replicates were collected at each orchard. A replicate consisted of five healthy flowers sampled randomly from individual sweet cherry trees in a transect across the orchard. Flowers were placed in Ziploc sandwich bags and kept in a cooler on ice until processing. Samples were processed within 6 hours of collection.

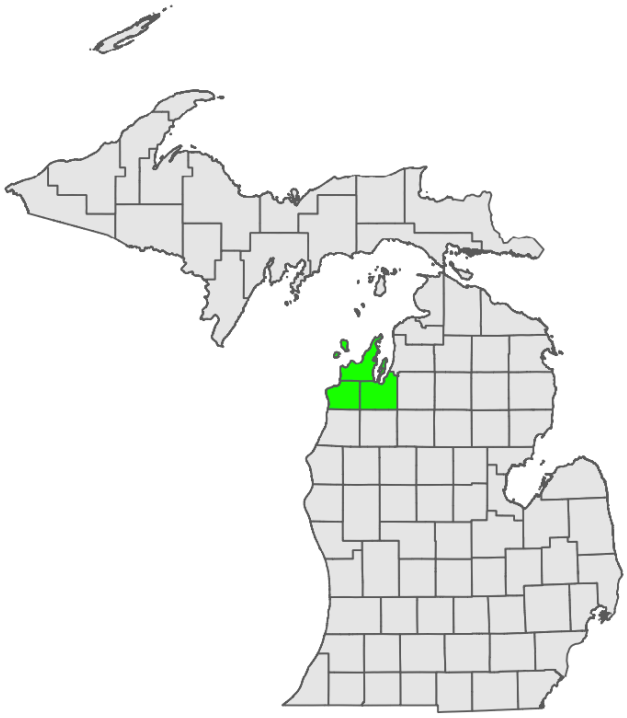

Figure 11. Map of counties sampled in Michigan. Flower samples for isolation of *P. syringae* pv. syringae were collected from sweet cherry orchards located in three counties in Northwest Michigan; Leelanau, Grand Traverse, and Benzie counties, shaded in green.

Samples were taken back to the lab. Flowers were removed from the bag using sterile forceps and placed into 60 ml glass culture tubes with 10 ml of PB solution and maintained on ice between all manipulations. Tubes were then sonicated for seven minutes and placed back on ice. Aliquots of 100 µl from each tube were serially diluted in 900 µl of PB solution in 1.7 ml microcentrifuge tubes. One hundred µl of the dilution was spread on KB agar medium amended with 50 $\mu g/ml^{-1}$ cycloheximide to inhibit fungal growth. Cultures were dried in a laminar flow hood, wrapped in plastic sleeve, and incubated at 21°C for at least 48 hours before colony selection.

Single colonies from every replicate were selected based on colony morphology matching *Pseudomonas*, including cream-color, convex, round with irregular/undulate margins and fluorescence on KB medium. Selected colonies were streaked to confirm morphology using sterile toothpicks onto fresh KB medium also amended with cycloheximide. To test for presence of cytochrome oxidase activity, a 1% (w/v) aqueous tetramethyl-p-phenylenediamine dihydrochloride solution was prepared fresh and used to moisten a filter paper. A sterile toothpick was then used to rub bacterial cells onto the saturated filter paper. *Pseudomonas* isolates that developed a blue color within ten seconds of spreading were considered oxidase positive. The cytochrome oxidase biochemical test is rapid and unlike many species of *Pseudomonas*, *P. syringae* is negative (lacking blue color) making this a valuable test for differentiation (Schaad et al. 2001). Oxidase negative isolates were also streaked onto aesculin medium to test for aesculin hydrolysis (Sneath 1956). Isolates that were able to hydrolyze aesculin were grown on KB agar medium and isolates were stored long-term in 15% glycerol at -80°C.

To continue characterization, isolates were assessed for biochemical activity by GATTa tests (Latorre and Jones, 1979). Single colonies were streaked onto gelatin medium (Lelliott et al. 1966), tyrosine medium (Lelliott et al. 1966), and tartrate medium (Holding and Collee 1971) for testing for gelatin liquefaction, tyrosinase activity, and utilization of tartrate, respectively. PSS is positive for gelatin liquefaction and aesculin hydrolysis and negative for tyrosinase activity and utilization of tartrate (Latorre and Jones 1979; Janse 2010).

Pathogenicity testing. Unripe, green sweet cherries were picked stem-on for pathogenicity testing of the putative PSS isolates. To surface sterilize, cherries were soaked in a 70% bleach solution for 1 minute then soaked for 1 minute in sterile diH_2O, rinsed with fresh sterile diH_2O, and air dried in a laminar flow hood. Sterile sharp-pointed surgical scissors were used to make a hole in caps of 5 ml Polystyrene round-bottom tubes (Corning, Glendale, AX). Tubes were filled with sterile diH_2O, and the cherry stem inserted through cap so that the end of the stem was submerged in water and cherry fruit was sitting on top of tube. Two replicates for pathogenicity of each isolate were conducted by poking two holes into each cherry, one on each cheek, with a 16-gauge sterile needle. Cells from a single colony were swabbed over each puncture wound using a sterile toothpick. Negative (sterile water) and positive (pathogenic PSS strain BY38) controls were included in each test group. The inoculated cherries were placed in a humidity chamber and incubated at 27°C for 72 hours. Isolates that developed a brown, sunken lesion around the wound were considered pathogenic (Fig. 12).

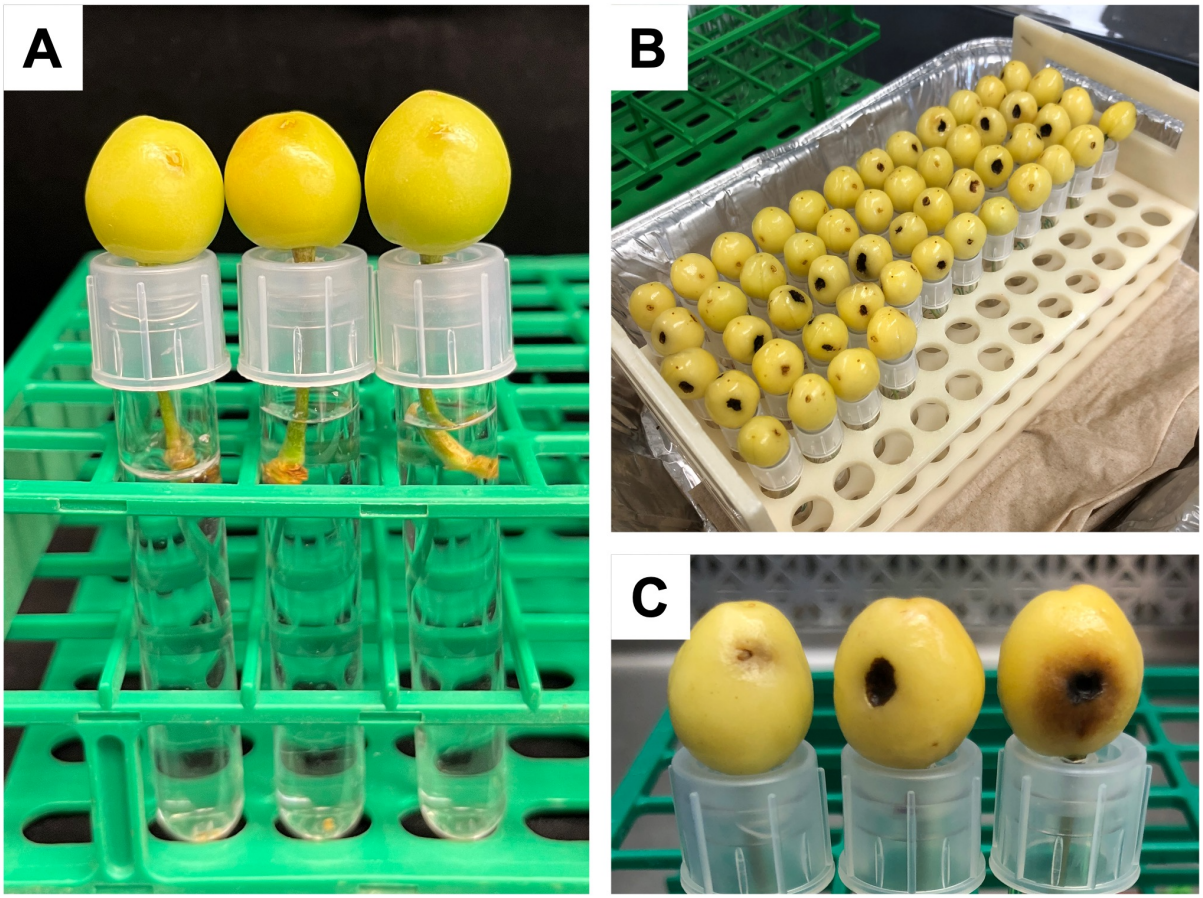

Figure 12. Pathogenicity test of *Pseudomonas syringae* isolates on green sweet cherries. (A) Experiment setup with stems of cherries inserted through hole in cap of 5 ml tube and submerged in water. Cherries were wounded on either cheek with a syringe. (B) Cherries after incubation in a humidity chamber. (C) On left, cherry 72 hrs after inoculation with non-pathogenic isolate. Middle and right cherries 72 hrs after inoculation exhibiting lesions indicative of pathogenic isolates.

Polymerase chain reaction for *syrB* gene. Isolates were tested for presence of the *syrB* gene which encodes for the phytotoxin syringomycin and is specific to *Pseudomonas syringae* (Mo and Gross 1991). Putative PSS isolates were grown from -80°C storage on KB at 27°C. Cells from these cultures were added to 50 µl sterile diH_2O and lysed by heating at 95°C for 10 minutes. Amplification was completed with GoTaq Master Mix (Promega Co., Madison, WI). Forward and reverse primers B1 and B2 for *syrB* gene were used as previously described (Vasebi et al. 2020) (Table 2). The thermocycler model used was a MiniAmp Plus Thermocycler (Applied Biosystems, Waltham, MA). Thermocycler settings are listed in Table 2.

Copper and kasugamycin resistance screening. Kasugamycin (100 µg ml^{-1}) or copper/cupric sulfate (250 µg ml^{-1}) was added to mannitol-glutamate medium supplemented with yeast extract at 1 g L^{-1} (MGY medium) (Keane et al. 1970). PSS isolates were streaked onto each type of amended media (MGY+ksg or MGY+Cu) and incubated at 27°C. Petri dishes were checked for the presence of colonies after 24, 48, 72, and 96 hrs. Isolates that showed any growth were streaked onto the corresponding medium to confirm if the isolate could tolerate the presence of the antibiotic or copper. Isolates that exhibited robust growth on the amended medium were considered resistant.

Ice nucleation activity assessment. PSS isolates were grown from -80°C storage on KB agar medium. Petri dishes were incubated at 21°C for 96 hours prior to testing to optimize ice nucleation frequency of cells (Lindow et al. 1982). Cellular suspensions of each isolate were prepared by adding a 10-ml loop of bacterial cells to 900 µl of PB in 1.7 ml centrifuge tube and vortexing. Ten replicate droplets of 10 µl of the suspension was pipetted onto an aluminum foil

boat coated with paraffin wax. Each isolate was tested twice, independently. Sterile PB solution was included as a control. The foil boat was floated in a refrigerating circulating bath (LAUDA-Brinkmann, Delran, NJ) filled with a 1:1 ethylene glycol and diH_2O mixture. The ice bath was set to an initial temperature of -3°C. Development of ice formation was noted after 15 minutes. Then the temperature was lowered by 0.5°C and droplets checked after an additional 15 minutes. The half-degree temperature reduction and incubation were repeated three additional times. Isolates with droplets frozen after -5°C were considered positive for ice nucleation activity.

Multi-locus sequence typing analyses. A subset of 65 PSS isolates were sequenced for MLST analysis using the *gap1*, *gltA*, *gyrB*, and *rpoD* housekeeping genes. Forward and reverse primers were as previously described (Vasebi et al. 2020) (Table 2). Thermocycler settings are listed in Table 2. PCR products were purified with ExoSAP-IT Express following manufacturer's recommended reaction conditions (Applied Biosystems, Waltham, MA). After clean-up, 9 µl of product was combined in 96-well plate with 3 µl forward or reverse sequencing primer of corresponding gene. Each gene was sequenced in the forward and reverse directions using the primers in Table 2. Purified PCR products were sequenced on the ABI 3730xl platform at MSU's Research Technology Support Facility Genomics Core (Michigan State University, East Lansing, MI). Reads were checked visually for quality using, low quality leading and trailing bases were removed using 4Peaks application (Nucleobytes, Amsterdam, NL). Forward and reverse sequences of each gene were aligned and terminally trimmed to include only base pairs that were present in both reads.

Sequences of the four housekeeping genes from *Pseudomonas* reference strains were acquired from NCBI GenBank or the Plant Associated and Environmental Microbes Database

(PAMDB). Reference strains include PSS9097 isolated from cherry in the UK, PSS isolated from other hosts including B728a, PsyCit7, PsyFF5, and 508, *P. syringae* pv. tomato strain DC3000, *Pseudomonas savastanoi* pv. glycinea strain Pf-95 and *Pseudomonas fluorescens* strain Pf95 as an outgroup. Ahmadi et al. (2017) identified 12 isolates of PSS from stone fruit in Iran and are included as reference strains. Strain information is listed in Table 3. Each of Pseudomonas phylogroup 2 clades (2a, 2b, 2c, and 2d) previously characterized by Berge et al. (2014) were represented by at least one reference strain included in the analysis (Table 3).

Sequences of reference strains and field isolates of each housekeeping gene were aligned using the ClustalX feature in MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar et al. 2018). Global trimming of gene alignments resulted in alignment lengths of 439 bp, 500 bp, 483 bp, 500 bp for *gap1, gltA, gyrB, rpoD*, respectively. Alignments of the four housekeeping genes were concatenated, and these were used to construct a Maximum-Likelihood Tree (Tamura-Nei model) with 1000 bootstraps in MEGA X. The tree was rooted using the outgroup strain of *Pseudomonas fluorescens* Pf95. Branches with internal bootstrap values lower than 50% were collapsed.

Table 2. Primers and reaction conditions for syrB and multi-locus sequence typing (MLST) used in thsis study. Al l primers are from Vasebi et al. 2020.

Target gene	Primer[a]	Reaction conditions
gap1		95ºC - 10:00
	gap1+264p	94ºC - 2:00 67ºC - 1:00
	gap1+312s	72ºC - 1:00 72ºC - 5:00
	gap1-874ps	
gyrB	gyrB+271ps	As above
	gyrB-1022ps	
rpoD	rpoD+174p	As above
	rpoD+364s	
	rpoD-1222ps	
gltA	gltA+174p	95ºC - 10:00 94ºC - 2:00
	gltA+513s	63ºC - 1:00 72ºC - 1:00
	gltA-1130s	72ºC - 5:00
	gltA-1192p	
*syr*B	B1	95ºC - 2:00 95ºC - 0:30
	B2	48ºC - 0:30 72ºC - 1:00

[a]Forward primers denoted by "+' and reverse by "-". MLST primers are denoted with "p" for PCR primer and "s" for sequencing primer.

Table 3. Reference strains for multi locus sequence typing used in this study.

Strain	Host of isolation	Origin of isolation	Accession No.[ab]	MLST subgroup[c]	Reference
P. fluorescens					
Pf95	Peach	Iran	KX160024, KX160044, KX160064, KX160084		Ahmadi et al. 2018
P. savastanoi pv. glycinea					
Psg86-3	Soybean	USA	MF666696, MF666697, KJ719502, MF666695		Dutta et al. 2018
P. syringae					
508	Apple	USA	PAMDB	2c	Burr et al. 1996
PsyCit7	Orange	USA	CP073636	2a	Lindow 1985
P. syringae pv. tomato					
DC3000	Tomato	UK	AE016853	1a	Cuppells 1986
P. syringae pv. syringae					
B728a	Bean	USA	CP000075	2d	Loper and Lindow 1987
Pss17	Peach	Iran	KX160009, KX160029, KX160049, KX160069	2d	Ahmadi et al. 2018
Pss18	Peach	Iran	KX160010, KX160030, KX160050, KX160070	2d	Ahmadi et al. 2018
Pss20	Nectarine	Iran	KX160011, KX160031, KX160051, KX160071	2d	Ahmadi et al. 2018
Pss22	Peach	Iran	KX160012, KX160032, KX160052, KX160072	2d	Ahmadi et al. 2018
Pss72	Almond	Iran	KX160017, KX160037, KX160057, KX160077	2b	Ahmadi et al. 2018
Pss75	Plum	Iran	KX160018, KX160038, KX160058, KX160078	2d	Ahmadi et al. 2018
Pss77	Nectarine	Iran	KX160020, KX160040, KX160060, KX160080	2d	Ahmadi et al. 2018
Pss92	Plum	Iran	KX160023, KX160043, KX160063, KX160083	2b	Ahmadi et al. 2018
Pss102	Almond	Iran	KX160025, KX160045, KX160065, KX160085	2b	Ahmadi et al. 2018
Pss103	Peach	Iran	KX160026, KX160046, KX160066, KX160086	2d	Ahmadi et al. 2018
Pss107	Peach	Iran	KX160027, KX160047, KX160067, KX160087	2d	Ahmadi et al. 2018

Table 3 (cont'd.)

Pss108	Peach	Iran	KX160028, KX160048, KX160068, KX160088	2d	Ahmadi et al. 2018
Pss9097	Cherry	UK	CP026568	2d	Hulin et al. 2018
PsyFF5	Ornamental Pear	USA	PAMDB	2b	Sundin and Bender 1993

[a]Four accession numbers correspond to each housekeeping gene: *gap1*, *gltA*, *gyrB*, *rpoD*.

[b]PAMDB = Plant Associated and Environmental Microbes Database (https://pamdb.org)

[c]Described in Berge et al. 2014. Strain either included in Berge et al. 2014 or described as such in reference.

3.4 Results

Isolation and characterization of *Pseudomonas syringae* pathovar *syringae* from Michigan sweet cherry orchards. A total of 395 isolates of bacteria displaying cream-colored, convex, round with irregular/undulate margined colonies that were oxidase negative and aesculin positive were isolated from flowers and stored from 13 sweet cherry orchards across the Northwest region of Michigan (Fig. 11). Of these isolates, 237 were pathogenic on green sweet cherries. The 237 pathogenic strains were subjected to GATTa biochemical tests and 232 were confirmed *Pseudomonas syringae* pv. syringae (PSS).

Sixty-five of these isolates, five from each of the 13 orchards, were selected randomly and confirmed as PSS through detection of the *syrB* gene with PCR (Table 4). All 65 exhibited ice nucleation by -5°C and were considered positive for ice nucleation activity. Copper resistance was observed in four of the thirteen orchards. Nine of the 65 isolates (13.8%) were resistant to 250 μg/ml $CuSO_4$ and zero showed resistance to kasugamycin antibiotic at rate of 100 μg/ml (Table 4).

Table 4. *Pseudomonas syringae* pv. syringae isolates from Michigan sweet cherry orchards used in this study.

Strain[a]	County[b]	*syrB*[c]	Pathog.[d]	GATTa[e]	INA[f]	Copper (growth at 250 µg ml$^{-1)}$	Kasugamycin (growth at 100 µg ml-1)	Clade
1-7	Leelanau	+	+	+ + - -	+	+	-	2a
1-9	Leelanau	+	+	+ + - -	+	-	-	2d
1-11	Leelanau	+	+	+ + - -	+	+	-	2a
1-12	Leelanau	+	+	+ + - -	+	+	-	2a
1-13	Leelanau	+	+	+ + - -	+	+	-	2a
2-4	Leelanau	+	+	+ + - -	+	-	-	2d
2-10	Leelanau	+	+	+ + - -	+	-	-	2d
2-20	Leelanau	+	+	+ + - -	+	-	-	2d
2-26	Leelanau	+	+	+ + - -	+	-	-	2d
2-30	Leelanau	+	+	+ + - -	+	-	-	2d
3-1	Leelanau	+	+	+ + - -	+	-	-	2d
3-6	Leelanau	+	+	+ + - -	+	-	-	2d
3-10	Leelanau	+	+	+ + - -	+	-	-	2d
3-20	Leelanau	+	+	+ + - -	+	+	-	2a
3-27	Leelanau	+	+	+ + - -	+	+	-	2a
4-2	Leelanau	+	+	+ + - -	+	+	-	2a
4-8	Leelanau	+	+	+ + - -	+	-	-	2d
4-14	Leelanau	+	+	+ + - -	+	-	-	2d
4-20	Leelanau	+	+	+ + - -	+	-	-	2d
4-31	Leelanau	+	+	+ + - -	+	+	-	2d
5-3	Leelanau	+	+	+ + - -	+	-	-	2d
5-9	Leelanau	+	+	+ + - -	+	-	-	2d
5-14	Leelanau	+	+	+ + - -	+	-	-	2d
5-25	Leelanau	+	+	+ + - -	+	-	-	2d
5-41	Leelanau	+	+	+ + - -	+	-	-	2d
6-15	Leelanau	+	+	+ + - -	+	-	-	2d
6-18	Leelanau	+	+	+ + - -	+	-	-	2d
6-20	Leelanau	+	+	+ + - -	+	-	-	2d
6-21	Leelanau	+	+	+ + - -	+	-	-	2d
6-26	Leelanau	+	+	+ + - -	+	-	-	2d
7-1	GT	+	+	+ + - -	+	-	-	2d
7-5	GT	+	+	+ + - -	+	-	-	2d
7-8	GT	+	+	+ + - -	+	-	-	2d

Table 4 (cont'd.)

7-15	GT	+	+	+ + - -	+	-	-	2d
7-20	GT	+	+	+ + - -	+	-	-	2d
8-3	GT	+	+	+ + - -	+	-	-	2d
8-7	GT	+	+	+ + - -	+	-	-	2d
8-12	GT	+	+	+ + - -	+	-	-	2d
8-16	GT	+	+	+ + - -	+	-	-	2d
8-19	GT	+	+	+ + - -	+	-	-	2d
9-2	GT	+	+	+ + - -	+	-	-	2d
9-10	GT	+	+	+ + - -	+	-	-	2d
9-17	GT	+	+	+ + - -	+	-	-	2d
9-25	GT	+	+	+ + - -	+	+	-	2d
9-34	GT	+	+	+ + - -	+	-	-	2d
10-1	GT	+	+	+ + - -	+	-	-	2d
10-8	GT	+	+	+ + - -	+	-	-	2d
10-13	GT	+	+	+ + - -	+	-	-	2d
10-28	GT	+	+	+ + - -	+	-	-	2b
10-51	GT	+	+	+ + - -	+	-	-	2d
11-8	GT	+	+	+ + - -	+	-	-	2d
11-15	GT	+	+	+ + - -	+	-	-	2d
11-21	GT	+	+	+ + - -	+	-	-	2d
11-34	GT	+	+	+ + - -	+	-	-	2d
11-43	GT	+	+	+ + - -	+	-	-	2d
12-1	GT	+	+	+ + - -	+	-	-	2b
12-7	GT	+	+	+ + - -	+	-	-	2d
12-16	GT	+	+	+ + - -	+	-	-	2b
12-20	GT	+	+	+ + - -	+	-	-	2d
12-34	GT	+	+	+ + - -	+	-	-	2d
13-2	Benzie	+	+	+ + - -	+	-	-	2d
13-15	Benzie	+	+	+ + - -	+	-	-	2d
13-23	Benzie	+	+	+ + - -	+	-	-	2d
13-37	Benzie	+	+	+ + - -	+	-	-	2d
13-50	Benzie	+	+	+ + - -	+	-	-	2d

[a]First number corresponds to the orchard number and second is isolate number collected from within the orchard

[b]GT = Grand Traverse

[c]Detection of syrB gene by PCR

[d]Pathogenicity tested on green cherry

[e]G, gelatin liquefaction; A, aesculin hydrolysis; T, tyrosinase activity; Ta, tartrate utilization.

[f]Ice nucleation activity

Multi-locus sequence typing analyses. Branching of the tree occurred by Pseudomonas phylogroup 2 clades (Fig. 13). Clade 2a included reference strain PsyCit7 and seven Michigan isolates from three different orchards. Clade 2b included reference strain PsyFF5, three strains isolated from stone fruit in Iran, and three Michigan isolates. Michigan isolate 10-28 had the greatest similarity to PsyFF5 within the clade. No Michigan isolates fell into clade 2c, the only strain in clade 2c was reference strain 508, a non-pathogenic isolate (Burr et al 1995). The largest clade represented in the tree was 2d with 55 Michigan isolates, nine reference isolates from Iran, Pss9097, and B728a. Strains not identified as *Pseudomonas syringae* pv. syringae: Psg86-3 (*P. savastano*i pv. *glycinea*) and DC3000 (*P. syringae* pv. *tomato*), did not cluster with any of these clades. Strain DC3000 is associated with Group 1 of the *Pseudomonas syringae* complex (Berge et al. 2014). Eight of the thirteen orchards included isolates only from clade 2d. Three orchards included isolates from clades 2a in addition to 2d. Two orchards included isolates from clades 2b and 2d (Fig. 14).

A subset of the Michigan orchard isolates had identical sequences for the four housekeeping genes. The largest difference within sequences, if any, across any of the collapsed branches was one base pair. These polytomy groups were collapsed and represented in the tree as branches ending in triangles (Fig. 13). Five of these identical groups consisted of isolates from the same orchard and four consisted of isolates from different orchards. All isolates from Orchard 6 were identical.

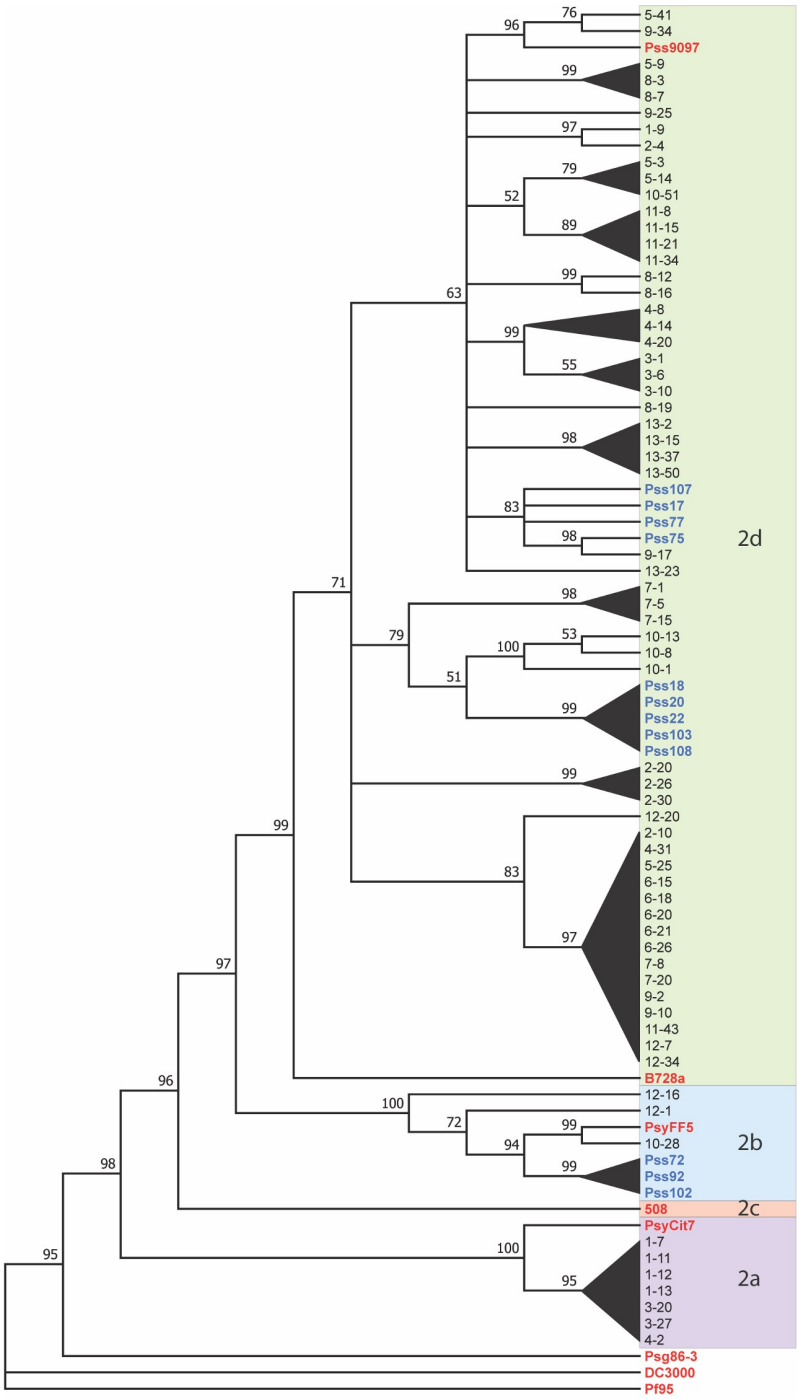

Figure 13. Phylogenetic tree of *Pseudomonas syringae* pv. *syringae* isolated from Michigan sweet cherry orchards and other *Pseudomonas* strains based on the MLST analysis of concatenated sequences of *gap1*, *gltA*, *gyrB*, and *rpoD* genes. Dendrogram was generated by Maximum-likelihood, Tamura-Nei model. Bootstrap scores above 50 based on 1000 replicates are shown above the nodes. Polytomy groups of strains were collapsed and clade names of phylogroup 2 are indicated as reported in Berge et al. (2014). Font color for isolates from this study are in black and reference strains in color. Names with blue color are from Ahmadi et al. 2018. The tree was rooted on *P. fluorescens* Pf95.

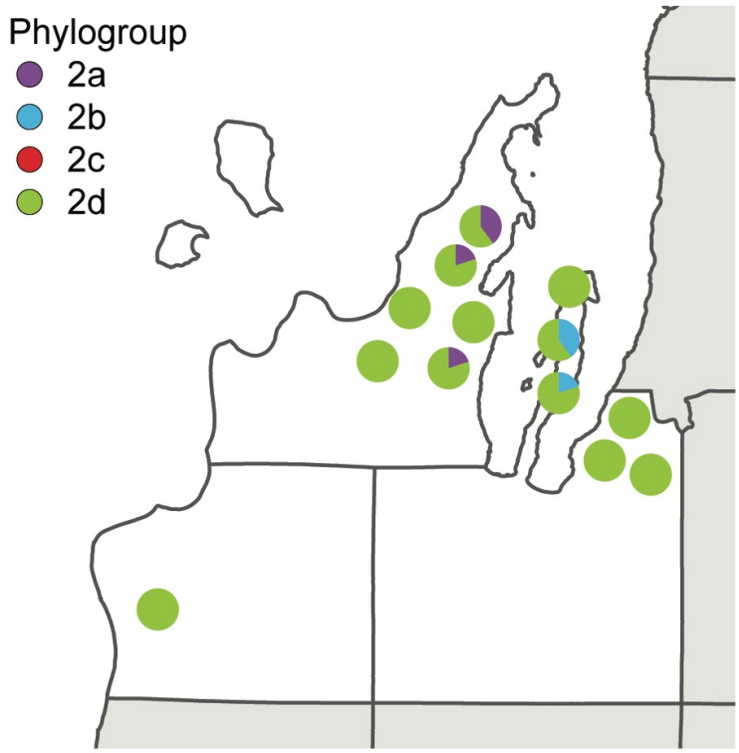

Figure 14. Northwest Michigan county map of *Pseudomonas syringae* phylogroup clades identified within orchards through multi locus sequence typing of isolates of *P.s.* pv. syringae. Each pie chart represents one sweet cherry orchard and consists of five isolates. Clades are described in Berge et al. 2014.

3.5 Discussion

The population of *P. syringae* pv. syringae capable of causing bacterial canker disease on sweet cherries in Michigan is diverse. The diversity detected in this work was expected given the current literature regarding the broad host range of Pseudomonas syringae as a species and particularly of pathovar *syringae* (Hirano and Upper 1990) (Sarkar and Guttman 2004). A subset of 65 isolates collected in this study from Northwest Michigan orchards represent three clades of Phylogroup 2 of the *Pseudomonas syringae* complex. This expands our previous knowledge of the genetics of this population.

Renick et al. (2008) identified three groups from the collection of *Pseudomonas syring*ae isolates in 2003 and 2004 with genetic fingerprinting. PSS reference strains B728a clustered in Group I and FF5 clustered in Group II. These reference strains have been reported as part of Phylogroup 2 of the *P. syringae* complex with FF5 in subgroup 2b and B728a in subgroup 2d (Ahmadi et al. 2018) (Berge et al. 2014) (Sarkar and Guttman 2004). With this understanding, it is reasonable to infer Renick Group I belongs in clade 2d and Group II in clade 2b. These were also the most common clades 2b and 2d detected in the current population study. Group III of the Renick study included two isolates but no reference strains for linking these isolates to a specific clade of the *P. syringae* complex (Berge et al. 2014). Based on grouping patterns in the current work, this Renick Group III likely belongs to clade 2a, the only other clade we identified in Michigan orchards through MLST. Grouping of the isolates in the present study did not correspond to geographical location, nor was copper resistance restricted by group or orchard, similar to Renick et al. (2008). We observed that seven of the nine isolates resistant to copper clustered in clade 2a, however it is uncertain if this observation is significant.

The average frequency of copper resistance in isolates of the present study (13.8%) is higher than the average resistance frequency observed among the total epiphytic populations collected in the northwest region in 2003-2004 (Renick et al. 2008). This increase may demonstrate a further loss of bacterial canker disease control with copper compounds that has occurred in the last 18 years. Given that we only tested a subset of pathogenic PSS isolates, the current frequency of copper resistance in the total epiphytic population of *P. syringae* could actually be much greater or lower.

The antibiotic kasugamycin was recently registered for controlling bacterial canker disease and our results indicate no pre-existing resistance in the population. This is promising in terms of longevity of the products efficacy. Resistance should continue to be monitored in this population considering it is also used in the region to control fire blight on apple and horizontal gene transfer between the species is a major concern (McGhee and Sundin 2011).

This population study exemplifies some of the challenges when considering successful management of bacterial canker disease. Our analyses and culture collection can aid in the development of phage biological controls for controlling bacterial canker in Michigan. By understanding the diversity of pathogenic isolates, we can ensure that a cocktail of phage can lyse the spectrum of PSS present in Michigan sweet cherry orchards, thereby more effectively managing bacterial canker disease. Future work should be done on host specificity with these Michigan isolates and φCOT4, φREC1, and φSHL2 of the *Pseudomonas* AgriPhage product. If these phages are incapable of lysing the full diversity represented by the present study, our PSS isolates can be used to isolate new lysogenic phage to include in the cocktail.

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