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The Development of Control Methods for *Xylella fastidiosa* by Manipulating the Grapevine Microbiome, Host Defense System, and its Abiotic Environment

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Biagio DiSalvo

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Dissertation Committee:
Dr. Caroline Roper, Chairperson
Dr. Hailing Jin
Dr. Patrick H. Degnan

The Dissertation of Biagio DiSalvo is approved:

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

The Development of Control Methods for *Xylella fastidiosa* by Manipulating the Grapevine Microbiome, Host Defense System, and its Abiotic Environment

by

Biagio DiSalvo

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Xylella fastidiosa is a xylem-limited, Gram-negative, gammaproteobacterium, and the causal agent of Pierce's disease (PD) of grapevine and several other diseases in many economically important crops. In the PD patho-system in California, it is natively vectored by the blue green sharpshooter (*Graphocephala atropunctata*) and vectored by the invasive glassy-winged sharpshooter (*Homalodisca vitripennis*). When transmitted into a healthy vine, *X. fastidiosa* colonizes the xylem leading to blockages both directly through biofilm formation and EPS build up and indirectly through the induction of tyloses and pectin gels. These blockages are believed to contribute to symptom development which includes marginal leaf necrosis, abscission of the leaf blade from the petiole, green islands in the periderm, desiccation of the berries, and eventually vine death. While there is no current cure, common management practices include severe pruning of diseased tissue and insecticide sprays to control vector populations. However,

new management strategies are needed to create cost effective and sustainable solutions to protect these culturally valuable crops.

As of late, biological control agents, the enhancement of existing treatments, and the development of transgenic disease-resistant or disease-tolerant plants have been implemented in an attempt to cope with phytopathogenic microbes. First, the concept of a biological control agent or biocontrol can be defined as the use of a third organism to act antagonistically against a pathogen and lessening disease symptoms in the host. This antagonism can occur via antibiotic production, direct predation, or nutrient competition and exclusion. We have identified two endophytic bacterial isolates that impart varying levels of protection to the grapevine from *X. fastidiosa* and have speculated on the mechanisms by which they do so. Second, the transgenesis of plants has been proven to be effective at controlling pathogens. Transcriptomic data can be used to identify genes that are upregulated in a successful defense response against a pathogen and these genes can be transgenically engineered to be overexpressed and confer resistance or tolerance. Here, we have overexpressed a class III peroxidase and found that it conferred tolerance to the grapevines against *X. fastidiosa*. Third, metal ions have recently been found to break down the resistance of some pathogenic bacteria to certain antibiotics. We investigated the *in vitro* activities of copper, a common metal used in vineyards, on *X. fastidiosa*. We found that it affects several characteristics such as cell surface hydrophobicity and cell attachment to a surface. We found that it also increases the susceptibility of *X. fastidiosa* cells in a biofilms to antibiotics. Collectively, this research has set the framework for the development of novel PD management strategies and can

potentially serve as a model for other *X. fastidiosa* patho-systems affecting other economically important crops.

Table of Contents

List of Figures

Chapter II

Chapter III

Chapter IV

Appendix A

Chapter I

Introduction

1.0.1 Historical Context

Initially known as California vine disease or Anaheim disease, Pierce's disease of grapevine (PD) was first described in the late 1800s by Newton B. Pierce, a special agent of the United States Department of Agriculture (Pierce 1892). Tasked with the challenge to cure the disease, Pierce attempted to discover its causal agent which he ultimately failed to do. He believed the infection to be caused by a virus, since he could not isolate a disease-causing microbe and like other viruses, the disease was graft transmissible, or transmitted from cutting to rootstock. Unfortunately, he also could not find a cure and tried several chemical treatments including the Bordeaux Mixture, a copper-based fungicide developed in the 1880s (Pierce 1892; Lamichhane et al. 2018). It was not until 1973 that micrographs of PD-infected grapevine tissue showed *Rickettsia*-like bacteria present in the xylem, the water conducting tissue of the grapevine (Hopkins and Mollenhauer 1973). Finally, in 1978, pure cultures of this bacterium were isolated and inoculated into healthy grapevines. These vines developed symptoms and the same bacterium was re-isolated and found to be indistinguishable from the original inoculum. Thus, Koch's postulates were completed and the bacterium, *Xylella fastidiosa,* was determined to be the causal agent of PD (Davis et al. 1978). Since this discovery, *X. fastidiosa* has also been shown to be the causal agent of several other diseases in economically important crops.

1.0.2 Taxonomic and Microbiological Traits

X. fastidiosa is a Gram-negative, gammaproteobacterium in the *Xanthomonadaceae* family (Wells et al. 1987). It is a rod-shaped bacterium ranging from

0.25-0.35 by 0.9-3.5 microns in size (Rapicavoli et al. 2018). While previously thought to be the only species in the genus *Xylella*, a recent finding shows a closely related second species, *X. taiwanensis* (Su et al. 2016). *X. fastidiosa* is non-flagellate and mediates motility via a Type IV pilus (Meng et al. 2005). *X. fastidiosa* is a fastidious prokaryote and is difficult to culture on artificial media, which explains Pierce's initial conclusion that a virus was responsible for the disease he studied. However, now several complex media such as Periwinkle wilt and PD3 and defined, minimal media such as XFM exist to facilitate its growth *in vitro* (Davis et al. 1978; Almeida et al. 2004).

1.0.3 Host Range

X. fastidiosa is a xylem-limited bacterium and has a broad host range including ornamental, ecological, and agricultural monocots and dicots (Rapicavoli et al. 2018). To date, it has been found in 87 plant families, covering almost 300 genera and spanning over 600 plant species (European Food Safety Authority (EFSA) 2021). While known to be pathogenic in about 100 plant species, *X. fastidiosa* can also reside in the xylem of many plant species as a non-pathogenic endophyte (Purcell and Saunders 1999). Often in these cases, *X. fastidiosa* does not multiply well or migrate systemically. However, asymptomatic plants can still serve as important reservoirs for susceptible plant hosts (Hill and Purcell 1995).

Strains of *X. fastidiosa* have been grouped into a single species, as they have shown greater than 70% similarity in early DNA-DNA hybridization studies (Kamper et al. 1985). More recently, taxonomists have used multilocus sequence typing systems to further group strains together into three major subspecies in North America (Scally et al.

2005; Schunzel at el. 2005). Genetic recombination is considered to be a major driver of this development of subspecies allowing certain strains of *X. fastidiosa* to adapt to different hosts (Kung et al. 2011). For example, *X. fastidiosa* ssp. *fastidiosa* is found in grapes, almond, and alfalfa. *X. fastidiosa* ssp. *multiplex* is found in almond, peach, plum, and oak. *X. fastidiosa* ssp. *sandyi* has been found in oleander (Scally et al. 2005; Schuenzel et al. 2005). In South America, *X. fastidiosa* ssp. *pauca* which infects citrus and coffee is the predominant subspecies and historically was the first plant pathogenic bacterium to have its genome sequenced (Simpson et al. 2000). Now in Europe, *X. fastidiosa* is an emerging pathogen in olives in the Apulia region of Italy, where it is the causal agent of Olive Quick Decline Syndrome (OQDS)(Loconsole et al. 2014). The subspecies responsible for OQDS has been found to be most similar to the *pauca* subspecies of South America (Jacques et al. 2016). Additionally, subspecies *fastidiosa* and *multiplex* have also been detected in Europe (Marcelletti et al. 2016; Schneider et al 2020).

1.0.4 PD in California

In California, *X. fastidiosa* affects the state's table grape and wine industry, valued at nearly $5 billion USD. PD costs CA grape growers over $100 million USD annually, which does not include the millions spent on preventative measures and PD research (Alston et al. 2015). Major symptoms of PD include marginal leaf necrosis, abscission of the leaf blades leaving matchstick-like petioles attached to the cane, desiccation of the berries, green islands in the periderm, cane die back, and eventually vine death (Guilhabert and Kirkpatrick 2005). In grapes, most symptoms are similar to,

but not exactly like those caused by drought stress due to blockages in the xylem, the vine's water conducting vessels. These blockages are caused by the build up of *X. fastidiosa* biofilm and EPS production and the induction of plant host responses such as tyloses and pectin gels (da Silva et al. 2001; Perez-Donoso et al. 2007).

1.0.5 Vector Biology (Previously published in *PLOS Pathogens*)

X. fastidiosa is obligately vectored by xylem-feeding hemipteran insects primarily belonging to the sharpshooter leafhopper (*Cicadellidae*) and spittlebug (*Cercopidae*) families (Nunney et al. 2013; Hill and Purcell 1995; Almeida et al. 2005). These insects are polyphagous (*i.e.* they feed on many plant species) and are present in temperate and tropical regions across the globe (Almeida et al. 2005). *X. fastidiosa* is acquired when the insect feeds on the xylem sap of the plant. The bacteria colonize and multiply in the insect foregut (mouthparts) in a persistent, but non-circulative manner (Hill and Purcell 1995; Krugner et al. 2019). This type of pathogen-vector relationship is unique among insect-vectored plant pathogens because the bacterial cells propagate within the insect mouthparts, but do not circulate throughout the hemolymph of the insect, whereas most propagative pathogens circulate within the insect (Backus and Morgan 2011). When sharpshooters feed on the xylem of infected vines, *X. fastidiosa* attaches to and colonizes the insect foregut where it forms adhesive biofilms. *X. fastidiosa* experiences extreme shear stress during the xylem sap ingestion and egestion processes that occur during insect feeding. During transmission into a healthy vine, bacterial cells dislodge from the insect foregut, presumably as a result of the high shear stress created during feeding, and are deposited directly into the xylem of healthy vines (Purcell and Finlay 1979). There is

no apparent specificity between a particular *X. fastidiosa* subspecies and insect vector species. In fact, the glassy-winged sharpshooter (GWSS) (*Homalodisca vitripennis*) can acquire more than one *X. fastidiosa* subspecies in its foregut and can potentially transmit these strains to a variety of plants where the bacterium can behave as pathogen or a commensal endophyte (Chatterjee et al. 2008; Backus et al. 2015).

In the context of Pierce's disease of grapevine caused by *X. fastidiosa* subsp. *fastidiosa*, the pathosystem with the broadest literature base, the two xylem-feeding insects transmit *X. fastidiosa* that have received the most research focus are the blue-green sharpshooter (BGSS) (*Graphocephala atropunctata*) and the GWSS. The BGSS is native to riparian areas in California and feeds on new plant growth that emerges in the spring (Nunney et al. 2013; Hill and Purcell 1995). The GWSS is invasive to California and is thought to have been introduced from the southeastern United States into Southern California on nursery stock in the 1990s. The introduction of this invasive pest drastically changed the epidemiology of PD in the southern part of California because GWSS can feed on both green and dormant woody tissues meaning that transmission can occur even in winter. In addition, GWSS can fly longer distances than native sharpshooter species, which could explain how PD incidence was elevated to epidemic proportions in Southern California. Subsequently, there has been a concerted effort among growers and the California Department of Food and Agriculture to control populations and prevent the spread of GWSS.

1.0.6 Plant Responses to *X. fastidiosa*

Plants can respond to a bacterial pathogen invasion in a number of different ways. They can produce phytoalexins, oxidative bursts, or induce structural changes to fight or prevent infection. Phytoalexins are low molecular weight secondary metabolites which exhibit antimicrobial properties and include phenolic compounds such as flavonoids like anthocyanins. Since they are metabolically expensive to produce, they accumulate in large quantities only when the plant perceives a pathogenic threat (Nicholson and Hammerschmidt 1992). In PD-infected vines, phenolic production increases during early infection which may serve to delay disease progression but is not sufficient to repress it. (Wallis and Chen 2012).

Plant host structural changes can include callose or suberin deposition or the formation of tyloses and pectin gels (Rapicavoli et al. 2018). Tyloses are outgrowths of parenchymal cells adjacent to the xylem into the lumen of the xylem. They can fill the entire vessel lumen, thereby blocking ingress of microbial pathogens in the xylem, but this can also consequently disrupt the flow of water and solutes (Evert 2006). Tyloses, along with pectin gels, are the predominant cause of xylem occlusions in PD-infected vines in the stem and petioles (Fry and Milholland 1990; Stevenson et al. 2004). Tylose production in grapevine is linked to production of the phytohormone, ethylene (Sun et al. 2007; Diaz et al. 2002). Elevated levels of ethylene during *X. fastidiosa* infection are linked to prolific tylose production (Perez-Donoso et al. 2007; Sun et al. 2013). Ethylene is a phytohormone that can both promote and inhibit plant growth and senescence depending upon its concentration, timing of release, and plant species (Iqbal et al. 2017).

In terms of plant pathogen defense, ethylene can accelerate senescence, bringing about a hypersensitive response or localized death of infected tissue, in addition to sealing off vessels which can be conduits for pathogens (Diaz et al. 2002; Sun et al. 2013).

Plants can recognize invading pathogens through highly conserved pathogen-associated molecular patterns (PAMPs). Lipopolysaccharides (LPS) are PAMPs found embedded in *X. fastidiosa*'s cell envelope (Caroff and Karibian 2003). They are composed of highly conserved lipid A, a core oligosaccharide, and a variable O-antigen chain (Lerouge and Vanderleyden 2002). LPS induces PAMP-triggered immunity (PTI); however, *X. fastidiosa*'s O-antigen seems to mask this plant immune response long enough to allow *X. fastidiosa* to successfully colonize the grapevine's xylem. *X. fastidiosa*'s LPS is synthesized partially by the Wzy polymerase which creates a long chain of individual O-subunits (Raetz and Whitfield 2002). In early stages of infection, the vine perceives wild type *X. fastidiosa* as an abiotic stress. In contrast, when the Wzy polymerase is deleted in a mutant strain of *X. fastidiosa*, a shorter chain of O-subunits is synthesized and the vine is able to recognize this strain as a biotic stress. The vine launches a different defense response which reduces the symptoms (Rapicavoli et al. 2018a).

In vines inoculated with wild type *X. fastidiosa,* genes associated with jasmonic acid (JA)-mediated defense pathways are enriched both locally and systemically. Additionally, as early as 8 hours post inoculation, ethylene-mediated signaling begins to occur which initiates the production of tyloses (Rapicavoli et al. 2018a). These swellings form occlusions in the vasculature in an attempt to stop the spread of *X. fastidiosa*, but in

turn, increase the severity of PD symptoms (Sun et al. 2013). Conversely, in vines inoculated with the *Δwzy* mutant strain of *X. fastidiosa*, genes associated with salicylic acid (SA)-mediated defense pathways are upregulated. Genes, including pathogenesis-related (PR) genes and enhanced disease susceptibility 1 (*EDS1*) genes, are enriched locally and systemically and are maintained over time. Class III peroxidase genes are also upregulated, which is phenotypically reflected by an increase in reactive oxygen species (ROS) production in the xylem of *Δwzy* mutant-inoculated vines (Rapicavoli et al. 2018a). Although the infection was not cleared, disease symptoms were significantly reduced in vines inoculated with *Δwzy* mutant compared to vines inoculated with wild type *X. fastidiosa*. Therefore, overexpressing genes associated with reduced disease symptom severity in transgenic grapevines may be a promising avenue to mitigate PD development in vineyards.

1.0.7 Grapevine Abiotic Environment

The vasculature of vines serves several key biological functions for a plant. The xylem and phloem provide structure and allow for the transport of water and nutrients throughout the plant. Due to environmental and temporal factors, the chemical composition of the sap of the internal vasculature of plants depends on geographic location and time of day (Andersen et al. 1995). For example, in the spring, the xylem of grapevines is rich in sugars such as glucose and fructose and amino acids such as glutamine as the vine remobilizes nutrient reserves for bud break and new growth (Keller 2020).

In grapevine xylem sap, concentrations of essential nutrients such as phosphorus, calcium, potassium, zinc and iron, and cofactors are in the micromolar (μM) range (Campbell and Strother 1996). In particular, the availability of iron is very limited. Plants acquire iron through roots in the rhizosphere and the amount of uptake is dictated by environmental factors such as soil redox potential and pH (Morrissey and Guerinot 2009). In the phloem, iron is commonly transported as ferric nicotianamine (Fe-NA). In the xylem, however, it is commonly transported as ferric citrate (Fe-citrate) and is generally found at less than 10 μM concentration in the xylem sap (Briat et al. 2007; Lopez-Millan et al. 2000).

Copper is also present in grapevine xylem sap. Copper is a transition metal and is required as a micronutrient by plants for photosynthesis, respiration, and other important cellular activities (Pilon et al. 2006). Since the late 1800s and the development of the Bordeaux Mixture, a copper-based treatment, copper has been applied heavily in vineyards to treat fungal and fungal-like pathogens (Lamichhane et al. 2018; Schneider et al. 2019; Behlau et al. 2017). While copper has been shown to be taken through into the vasculature of model organisms and agricultural crops for disease management of other microbes, no mitigation of PD has been observed (Pierce 1892; Ge et al. 2020; Ballabio et al. 2018; Cha and Cooksey 1991).

1.0.8 Grapevine Microbiome

A plant's microbiome is defined as the consortia of microbes within the rhizosphere (soil-derived microbes associated with the roots), phyllosphere (microbes associated with the aerial surfaces), and endosphere (microbes associated with the

internal tissues) (Turner et al. 2013). Endophytic organisms, particularly bacteria, are generally considered to be non-pathogenic and reside in symptom-less plants, but sometimes endophytes can be latent pathogens depending on host genotype and other environmental factors (James et al. 1998; Monteiro et al. 2012). There are a number of ways a bacterium can enter the internal tissues of the plant. Many enter through the rhizosphere through junctions in the roots or naturally occurring cracks, while some enter through insect feeding (Reinhold-Hurek and Hurek 2011; Monteiro et al. 2012; Lopez-Fernandez et al. 2017). Once colonized, endophytic bacteria can have a myriad of microbe-host interactions and can lead to mutual benefits for the plant host and microbe (Pacifico et al. 2019).

Investigators have targeted plant microbiomes to identify community members that may work as biocontrol agents against invading pathogens. For example, *Pseudomonas* sp. from the rhizosphere can induce systemic resistance to pathogens by sensitizing their hosts by defense priming them against pathogen challenges (Bolwerk et al. 2003). In grapevines, the microbiome composition is determined by geographic location and time, and more diverse in wild *V. vinifera* compared to cultivated varieties (Bokulich et al. 2019; Campisano et al. 2015). A few recent studies have started looking at potential biocontrol agents against grapevine pathogens including *X. fastidiosa* (Perrazzolli et al. 2014; Deyett et al. 2017; Baccari et al. 2019).

Furthermore, the grapevine microbiome has been linked to the “escape vines” phenotype where vines appear healthy in an otherwise heavily *X. fastidiosa* infected vineyard (Jones and Oliveras 2004; Deyett et al. 2017). Because all vines are clonally

propagated, this suggests that a non-genetic component such as the microbiome is the cause of this "escape" phenomenon. Characterization of the bacterial and fungal communities in diseased and escape vines identified the bacterial *Pseudomonodales* and fungal *Pleosporales* orders were the most abundant in "escape vine" endophytic communities (Deyett et al. 2017). Among the differing taxa, two bacterial genera, *Pseudomonas* and *Achromobacter,* had a strong negative correlation with disease severity suggesting their possible role in vine "escape" (Deyett et al. 2017). Furthermore, these microbes are now potential candidates for *X. fastidiosa* biocontrol agents.

Biocontrol agents are defined as living organisms that suppress disease, by suppressing the pathogen through a number of modes of action (Kohl et al. 2019). Biocontrol agents can directly antagonize the pathogen via production of antimicrobial compounds, parasitism, and competition via niche occupation, or indirectly by activating plant defense responses or plant growth promoting compounds (Sunar et al. 2015; Turner et al. 2013; Zarraonaindia et al. 2015). There are many examples of the success of biocontrol agents in agriculture such as the application of *Agrobacterium radiobacter* as a preventative treatment to protect against *Agrobacterium tumefaciens*, the causal agent of crown gall (Kerr 1972). Thus, we hypothesize that the aforementioned endophytes specific to the "escape" vine microbiome may have efficacy as biocontrol agents against *X. fastidiosa* infection of grapevines.

1.0.9 Current Management Practices:

There are several strategies currently implemented to manage PD including, but not limited to: controlling insect vectors and cropping practices such as severe pruning

(Kyrkou et al. 2018). While these show some effectiveness, none are completely sufficient in controlling the disease. Soil and foliar applications of insecticides such as imidacloprid, have proven effective against sharpshooters to suppress the spread of PD. However, unintended environmental effects such as developing insecticide resistance and environmental contamination have led to its recent ban in the European Union and drive the search for safer solutions (Daugherty et al. 2015; EFSA 2018). Weeding around and pruning of vines have also shown some effectiveness in reducing *X. fastidiosa* reservoirs (Hopkins and Purcell 2006). However, with the invasion of GWSS which feeds on the permanent structures of the vines, more drastic techniques have been implemented such as severe pruning (Daugherty et al. 2018).

In addition, breeding or genetic engineering techniques are being examined to identify a genetic solution within the grapevine hosts. Breeding programs have been established to cross resistant wild grape varieties with commercially grown *V. vinifera* and have identified at least 15 hybrids which are resistant to PD in greenhouse trials (Kyrkou et al. 2018). Resistance genes such as those in the locus PdR1 have also been identified and ongoing studies are showing promising results on PD resistance, while maintaining the economically valuable characteristics of the berries (Walker and Tenscher 2006).

Researchers have aimed to harness plants' immune systems' ability to recognize pathogen invaders and effectors to develop transgenic, disease resistant plants (Van Esse et al. 2020). Most current studies use transgenic root stocks to produce enzyme inhibiting proteins, antimicrobial peptides, or signaling molecules to interfere with *X. fastidiosa*'s

normal behavior (Agüero et al. 2005; Dandekar et al. 2012; Lindow et al. 2014). However, an area ripe for exploration is the overexpression of innate grapevine genes found to be involved in the successful suppression of PD.

This research aims to contribute to the development of PD management strategies by understanding how *X. fastidiosa* interacts with its microbial neighbors, abiotic environment, and plant host. First, we examine the potential of native grapevine bacteria, which have been found to negatively correlate with PD disease severity, to act as biological controls against *X. fastidiosa*. Second, we developed transgenic vines that constitutively overexpress genes that have been found to be upregulated early on in the infection process to successfully suppress disease. Third, we examined how copper interacts with *X. fastidiosa* in order to set the foundation to understand how biofilms may behave *in planta*. Together, this research will lead to the development of management strategies for PD and can serve as a baseline to be applied to diseases affecting other economically important crops.

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Chapter II

Grapevine Bacterial Endophytes Reduce Disease Severity of Pierce's Disease of Grapevine.

Abstract

Xylella fastidiosa, the causal agent of Pierce's disease (PD) of grapevine (*Vitis vinifera*), is a xylem-limited bacterium for which effective management strategies are limited to vector control, severe vine pruning or removal, and the production of resistant varieties through established breeding programs. Novel approaches, using biocontrol agents of *X. fastidiosa,* if effective, would lessen the environmental and economic impacts of chemical spraying and plant removal. *Pseudomonas* spp. and *Achromobacter* spp. are abundant in the endosphere of healthy grapevines and their abundance is negatively correlated with both *X. fastidiosa* titer and PD symptom severity. In this study, we evaluated two native endophytic microbes in these genera for their ability to mitigate *X. fastidiosa* titer and disease progression. We assessed the potential biocontrol efficacy of the two strains using *in vitro* antagonism assays and bioassays. We also assessed temporal colonization and spatial distribution patterns for these two strains. Our results indicate that the grapevine-associated *Pseudomonas* spp. and *Achromobacter* spp. significantly suppress PD symptoms and pathogen titer.

Introduction

Xylella fastidiosa has affected California vineyards since the 1880s. This Gram-negative, xylem-limited bacterium can colonize plants belonging to over 63 plant families, but is only known to cause disease in a smaller subset of plants, that includes grapevine, citrus, olive, alfalfa, peach, plum, almond, elm, and coffee among others

(Chatterjee et al., 2008; Rapicavoli et al., 2017). It is vectored mainly by xylem feeding insects in the sharpshooter (Cicadellidae) and spittlebug (Cercopidae) families. The introduction of the invasive glassy-winged sharpshooter (GWSS) (*Homalodisca vitripennis)* has been associated with outbreaks in California (Janse & Obradovic, 2010). Despite existing management strategies for Pierce's disease (PD) such as insecticide applications to control vector population, which are often expensive and off-target, and breeding programs to produce resistant varieties, *X. fastidiosa* still causes large economic losses (Pathak et al., 2017; Sarigiannis et al., 2013; Krivanek & Walker, 2005). It is estimated to cost the California grape industry alone, valued at 6.25 billion USD, an average of 104 million USD per year (Tumber et al., 2014). Plant-associated microbes can act as important disease antagonists and harnessing the potential of beneficial microbes can be important components of integrated disease management strategies (Kyrkou et al., 2018). Identification and application of beneficial grapevine associated microbes that can inhibit *X. fastidiosa* infections and limit disease progression could have immense benefits for the California grape industry, and potentially other crop systems devastated by *X. fastidiosa* globally.

A healthy plant's native microbiome is a potential pool of effective biocontrol candidates for the suppression of diseases and the complexity of plant-microbe-pathogen dynamics has yet to be fully understood (Compant et al. 2019). Microbes within the plant endosphere can trigger induced systemic resistance (ISR), a phenomenon known as priming, and as a result, the plant can be more resilient to abiotic and biotic stresses (Aziz et al., 2015; Santoyo et al., 2016). Furthermore, microbes may directly interact with each

other through a variety of mechanisms including antibiosis, nutrient/niche exclusion, or quorum quenching activities which indirectly protecting the host (Brader et al., 2014; Hartmann & Schikora, 2012; Sarrocco & Vannacci, 2017). While some effects may rely on an entire community of microbes, there are some studies suggest disease suppression can be mediated by individual taxa (Deyett et al. 2017).

Avirulent strains of *X. fastidiosa* have been used as biocontrol agents of PD (Hao et al., 2017). However, due to their variability in success, the identification of further biocontrol candidates are warranted. In a recent amplicon sequencing project of healthy-appearing grapevine endospheres in heavily PD-infected vineyards, the abundances of *Pseudomonas* spp. and *Achromobacter* spp. were negatively correlated with pathogen abundance and disease symptom severity (Deyett et al., 2017). Here, we test the potential efficacy of vine-derived isolates, a *Pseudomonas* spp. and an *Achromobacter* spp., for their ability to mitigate PD in both *in vitro* and *in planta* assays. We find that the *Pseudomonas* spp. and *Achromobacter* spp. perform as well as *Paraburkholderia phytofirmans* strain PsJN, an established biocontrol agent for PD. We hypothesize that the *Pseudomonas* spp. reduces disease symptom severity through direct antibiosis against *X. fastidiosa*, while the *Achromobacter* spp. acts indirectly by interacting with the plant host to reduce disease symptom severity

Materials and Methods

Identification of Biocontrol Agents. Two bacterial strains were assessed as biocontrol agents in this study: a *Pseudomonas* spp. and an *Achromobacter* spp. These strains were isolated from grapevine hosts in California and were chosen based on negative correlation to disease symptom severity and pathogen titer (Jones 2004; Deyett et al., 2017). *Pa. phytofirmans* strain PsJN is a biocontrol agent for PD of grapevine that was originally isolated from onion and was used as a positive control for biocontrol efficacy in our bioassays (Baccari et al. 2018). The *Pa. phytofirmans, Pseudomonas* spp., and the *Achromobacter* spp. were grown on Tryptic Soy Agar (TSA) for 24 h at 28°C. *X. fastidiosa subsp. fastidiosa* strain Temecula1 was grown on PD3 medium for 5 days at 28°C according to standard methods (Roper et al. 2007) .

To verify species identification, Sanger sequencing was performed. First, DNA was extracted from overnight grown cultures streaked for DNA extraction using the Qiagen Blood and Tissue kit per the manufacturer's protocol. DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen, Waltham, MA) according to the manufacturer's protocol. 16S rDNAs were PCR amplified using the universal bacterial primers (27F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R 5'-GGTTACCTTGTTACGACTT-3') (Miller et al., 2013). PCRs were performed in a 25µl volume with the following reagents: 12.5 µL of the APEX 2x Hot start master mix buffer I, 0.4 µM of each primer, and 2 µL of DNA template using the following thermocycling parameters: 94°C for 2 min; 35

cycles of 94°C for 60 s, 55°C 30 s, 72°C for 60 s; followed by a final elongation step for 5 min at 72°C.

Amplicons were visualized by electrophoresis at 110 V in 1% agarose gel amended with GelRed® (Biotium, Fremont, CA) in 1X TBE buffer. The PCR product was then purified using the Qiagen QIAquick PCR Purification kit (Qiagen, Germantown, MD) and quantified as previously described. PCR products were then Sanger sequenced using the same 16S primers as above at the Institute for Integrative Genome Biology, at the University of California, Riverside. Sequences were blasted against NCBI BLAST nucleotide database to obtain identifications.

Growth Curve Analysis. The *Pseudomonas* spp. and *Achromobacter* spp. were grown overnight in 2 ml of TSB medium at 180 rpm. Cultures were normalized to an OD_{600} =0.1 and 150 µL of this culture was placed into a 96-well plate and OD_{600} values were measured in 30 minutes intervals using the Synergy HTX multi-mode reader (Biotek Instruments, Winooski, VT). Each strain was done in 6 biological replicates and the plate was incubated at 28°C.

For *X. fastidiosa,* cells were plated onto solid PD3 medium and incubated at 28°C for 5 days and harvesting cells from plates using sterile 1X PBS buffer and adjusted to OD_{600} =0.25 (10^8 CFU/ml) and 0.5ml of this inoculum was used to inoculate 4.5 mL of PD3 bringing the final OD_{600} = 0.025. Cultures were grown in a shaker incubator at 28°C at 180 rpm for 7 days. OD_{600} measurements were taken every 24 hours for 6 days.

PD bioassays. For *in planta* assays, *Vitis vinifera* cv. Cabernet Sauvignon one-bud cuttings were rooted (generously provided by Foundation Plant Services, University of California, Davis) in vermiculite. Rooted cuttings were potted in UCR soil mix and amended with 5 g of controlled release fertilizer (Scotts Osmocote Classic, N-P-K: 14-14-14). Vines were propagated in the greenhouse and trained vertically as a single shoot onto a stake.

Inoculations were prepared as follows: cells were harvested from 5 day old plates and suspended in sterile 1X PBS and adjusted to an OD_{600} =0.25. For each trial, 10 plants were inoculated with one of 4 treatments: *Pa. phytofirmans, Pseudomonas* spp.*, Achromobacter* spp.*,* or 1X PBS. Plants were inoculated with 10 µL of biocontrol agent or 1X PBS solution between the first and second node of the plant on both sides by mechanical needle inoculation as previously described (Hill et al., 1995). Ten µL of *X. fastidiosa* suspension was needle-inoculated into the plant between the second and third node (above the biocontrol inoculant) at 1 day or 7 days post biocontrol agent inoculation. A randomized block design was utilized. Visual PD severity symptom ratings (0 = healthy vine, 1 = one or two leaves with scorching at the margins, 2 = two or three leaves with more developed scorching, 3 = all the leaves have some scorching with a few matchstick petioles, 4 = all leaves have heavy scorching and many matchstick petioles, and 5 = dead vine) (Deyett et al., 2019)) were taken through sample collection occurring when 1X PBS-inoculated control vines reached an average disease rating of 3.

Detection and quantification of *X. fastidiosa in planta*. Detection and quantification of *X. fastidiosa* was determined using the method described by Deyett *et al*., 2019. Briefly, three petioles were harvested from the point of inoculation (POI) and pooled. Petioles were frozen, lyophilized for 36 h with a FreeZone 2.5 L benchtop freeze dry system (Labconco, Kansas City, MO) and ground to a powder at room temperature using a MM300 grinder (Retsch, Bucks County, PA; 45 s 25 oscillations per second) in a 35 mL stainless-steel grinding jar (Retsch, Bucks County, PA) with 20 mm stainless steel balls. DNA was extracted from 15 mg of ground petiole tissue using the ZymoBIOMICS DNA miniprep kit per manufacturer's protocol (Zymo Research, Irvine, USA). DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen, Waltham, MA) according to the manufacturer's protocol.

X. fastidiosa ITS primers (XfITSF6: 5'-GAGTATGGTGAATATAATTGTC-3' and XfITSR6: 5'-CAACATAAACCCAAACCTAT-3') were utilized to quantify pathogen abundance in each sample using quantitative PCR (qPCR). qPCR was performed in a CFX96 Real-Time PCR cycler using CFX Maestro V. 1.1 software (Bio-Rad) using default settings for amplification curve analysis. Reactions occurred at a final volume of 25 µl of 12.5 µL of Quantitect SYBR Green Master Mix reagent (Qiagen, Germantown, MD), 0.4 µM of each primer, and 2 µL of DNA template. The cycling program consisted of 95 °C for 15 min; 40 cycles of 55 °C for 15 s, 72 °C for 45 s, 95 °C for 15 s; and a melt curve analysis of 65–95 °C (0.5 °C increments every 5 s). Each qPCR plate was accompanied with both sterile water as a negative control and a standard curve

consisting of four tenfold dilutions of 6 ng of *X. fastidiosa* DNA. Each sample was tested in triplicate.

Quantitative assessment of temporal and spatial distribution of biocontrol strains *in planta.* For *in planta* assessment of biocontrol presence and movement, vines and bacterial cultures were prepared as described for the PD bioassays. Plants were inoculated with 10 µL of an individual biocontrol agent suspension or 1X PBS solution between the first and second node of the plant on both sides by mechanical needle inoculation (Hill et al., 1995). Three plants were used for each treatment at each time point of 2, 4, 8, 12, 16 weeks. Thus for 4 treatments at five different time points 75 plants were utilized. Plants were grown under greenhouse conditions for 16 weeks.

At each time point (2, 4, 8, 12, 16 weeks), stem samples were destructively sampled at the point of inoculation (POI), 3 nodes above the POI, and 7 nodes above the POI. Each sample was surface sterilized in 70% ethanol for five minutes and rinsed three times in sterile distilled water. The samples were placed in individual mesh lined, sample bags (Agdia, Inc., Elkhart, IN) and homogenized with a hammer in 2 mL 1X PBS. 10 µL from each resulting slurry solution was plated on Tryptic Soy Agar and allowed to grow at 28°C for 48 hours. Colony counts were recorded and normalized to colony forming units per gram of tissue (cfu/g), and biocontrol identity was confirmed via Sanger sequencing with 16S universal primers (16S U1: 5'-CCAGCAGCCGCGGTAATACG-3' and 16S U2: 5'-ATCGGCTACCTTGTTACGACTTC-3').

***In vitro* inhibition assay**. To determine if the *Pa. phytofirmans, Pseudomonas* spp.*,* and *Achromobacter* spp. inhibited *X. fastidiosa* through an antibiosis mechanism, we utilized a dot inhibition assay. Briefly, inocula of the individual biocontrol agents, the *Pa. phytofirmans, Pseudomonas* spp.*,* and *Achromobacter* spp., of OD_{600}= 0.25 were prepared in sterile 1X PBS buffer. Inoculum of *X. fastidiosa* of OD_{600}=0.25 was also prepared in sterile 1X PBS buffer. A 10 µL drop of each inocula was plated on PD3 agar plates and spread across a length of 5 cm by gently tilting the plates. Single drops of 5 µL of *X. fastidiosa* inoculum were plated 1, 2, 3, 4, and 5 cm away from the endophyte drip line. The inocula were allowed to air dry in a biosafety cabinet and incubated at 28°C for 7 days and observed for growth. The distance to the first sign of growth was recorded.

Measurement of tylose production. Two centimeter stem pieces were collected 3 internodes above the point of inoculation of *X. fastidiosa* in vines that were pretreated with the *Pseudomonas* spp.*, Achromobacter* spp.*,* and *Pa. phytofirmans,* or 1X PBS. Stem segments were hand sectioned with a razor blade and rinsed in sterile water. Sections were then stained in 500 µL of Toluidine Blue O (0.05% in deionized water) for 5 minutes. In order to minimize background staining, stem sections were then rinsed in water. A Zeiss Axioskop 2 light microscope (20x objective/0.5/component number: 440341) was used to visualize stem sections that were mounted on a glass slide in 50% glycerol solution. Three sections per two cm stem piece were analyzed to obtain a representation of occluded vessels. Briefly, the total number of occluded vessels was divided by the total number of vessels to obtain a percentage of occlusion.

Assessment of 1-aminocyclopropane-1-carboxylic acid (ACC) catabolic activity. Dworkin and Foster (DF) salts medium was used to assess the ACC catabolic activity for each biocontrol agent (Dworkin and Foster, 1958). The medium was prepared with the following reagents per 1 L of distilled water: 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2.0 g glucose, 2.0 g gluconic acid, 2.0 g citric acid, 1 µg $FeSO_4.7H_2O$, 10 µg H_3BO_3, 11.19 µg $MnSO_4.H_2O$, 124.6 µg $ZnSO_4.7H_2O$, 78.22 µg $CuSO_4.5H_2O$, and 10 µg MoO_3, pH 7.2. The following solution was then amended with either 3 mM ACC or 3 mM $(NH_4)_2SO_4$, a nitrogen source used as a positive control and 4.5 mL of each media were inoculated with 0.5 mL of biocontrol agent inoculum at an OD_{600} =0.25, bringing the final OD_{600} of each tube to 0.025. Cultures were grown in a shaker incubator at 28 °C at 180 rpm. OD_{600} measurements were taken every 24 hours for 4 days.

Statistical Analyses. All statistical analyses and graph visualizations were done using R v. 4.6.1 (http:// www.R-project.org/). Differences in PD visual symptoms and pathogen quantities were determined through Kruskal-Wallis rank sum statistical rating and pairwise Wilcoxon test was also used to calculate the differences between both biocontrol agents and time periods. P-values were adjusted with a false discovery rate. Parametric survival model using log normal distributions with *post hoc* Tukey HSD test was conducted to determine the differences in survival between biocontrol agents and analysis was adapted from Schandry (2017).

Results

Pre-treatment with biocontrol agents reduces PD symptoms. PD symptom development, including scorched leaves, began 10 weeks after the plants were inoculated with the pathogen. Disease symptom severity was significantly reduced for all vines inoculated with each biocontrol agent compared to buffer control (Figure 1A; pairwise Wilcoxon: $P < 0.05$). By the end of the disease bioassay, the vines pre-treated with 1X PBS and challenged with *X. fastidiosa* 1 or 7 days later exhibited average disease ratings of 2.9 and 1.12, respectively based on the 0 to 5 disease progression scale mentioned above. Vines pre-treated with *Pa. phytofirmans* and challenged with *X. fastidiosa* 1 or 7 days later exhibited average disease ratings of 0.67 and 0.26, respectively. Vines pre-treated with the *Achromobacter* spp. and challenged with *X. fastidiosa* 1 or 7 days later exhibited average disease ratings of 0.85 and 0.32, respectively. Finally, vines pre-treated with the *Pseudomonas* spp. and challenged with *X. fastidiosa* 1 or 7 days later exhibited average disease ratings of 0.6 and 0.23, respectively.

In addition, we performed a vine survival assay. A visual rating of 2 was considered a critical point in terms of disease symptom development, because once this visual rating was achieved, the disease progression was considered fatal. By the end of the disease bioassay, 44 plants were given a fatal rating of 2 or greater across both time points of *X. fastidiosa* challenge. Thirty of these vines occurred in control vines pre-treated with 1X PBS buffer, 8 in the *Achromobacter* spp. pre-treated vines, 3 in *Pa. phytofirmans* pre-treated vines and 3 in the *Pseudomonas* spp. pre-treated vines.

Therefore, vines inoculated with any of the biocontrol agents had greater than 75% survival probability at the end of the bioassay compared to control vines which had less than a 10% survival at the end of the bioassay (Figure 1B; pairwise Wilcoxon : $P<0.05$). Pairwise log-rank tests confirmed that the *Pa. phytofirmans*, the *Achromobacter* spp., and the *Pseudomonas* spp. all had significantly higher survival rates compared to the 1X PBS negative buffer control, regardless of the timing of *X. fastidiosa* challenge (Figure 1B; pairwise Log-Rank test: $p<0.0001$). Between biocontrol agents, the *Achromobacter* spp. had a decreased survival rate compared to the other two biocontrols regardless of the timing of a *X. fastidiosa* challenge (Figure 1B; pairwise Log-Rank test: ($p<0.05$). Cox proportional hazard analysis, a survival analysis regression model, showed the *Pa. phytofirmans*, the *Achromobacter* spp., and *Pseudomonas* spp. each confer a significant reduction in risk of death when inoculated with *X. fastidiosa* regardless of the timing *X. fastidiosa* challenge (Figure 1C; Cox Proportional hazards; $p<0.001$).

Pre-treatment with biocontrol agents reduced *X. fastidiosa* titer. For vines challenged with *X. fastidiosa* 1 day after pre-treatment, all vines inoculated with the *Pa. phytofirmans*, the *Achromobacter* spp., and *Pseudomonas* spp. showed a significant reduction in average pathogen titer (7.25, 10.5, and 8.06 log *X. fastidiosa* DNA quantification per total DNA extracted, respectively) compared to the average pathogen titer found in the 1X PBS buffer pre-treated vines (11.44 log *X. fastidiosa* DNA quantification) (Figure 2; pairwise-Wilcoxon: $p < 0.05$). In addition, vines pre-treated with *Pa. phytofirmans* exhibited significantly lower pathogen titer compared to vines pre-

treated with the *Achromobacter* spp.*,* which had the highest average pathogen titer (Figure 2; pairwise-Wilcoxon: $p < 0.05$). For vines challenged with *X. fastidiosa* 7 days after pre-treatment, only vines pre-treated with *Pa. phytofirmans* and the *Achromobacter* spp. showed a significant reduction in average pathogen titer (5.66 and 2.74 log *X. fastidiosa* DNA quantification, respectively) compared to the average pathogen titer found in the sterile buffer pretreated vines (9.70 log *X. fastidiosa* DNA quantification) (Figure 2; pairwise-Wilcoxon: $p < 0.01$). At the 7 day *X. fastidiosa* challenge, *the Pseudomonas* spp. inoculated vines did not exhibit less pathogen titer (7.24 log *X. fastidiosa* DNA quantification) and showed the largest variation in *X. fastidiosa* titer, with some titers exceeding the largest titers associated with 1X PBS buffer pre-treated vines (Figure 2; pairwise-Wilcoxon). In contrast to the 1 day *X. fastidiosa* challenged vines, the *Achromobacter* spp. pre-treated vines exhibited significantly less average pathogen titer compared to both *Pa. phytofirmans* and the *Pseudomonas* spp. pre-treated vines (Figure 2; pairwise-Wilcoxon: $p < 0.05$).

Biocontrol agents persist in the vines over 16 weeks and remain largely localized to the point of inoculation. Each biocontrol agent persisted in the vines throughout the 16-week experiment and the vines showed no decline in health indicating the biocontrol agents do not have a detrimental effect on the plant. Population sizes of the *Pa. phytofirmans*, the *Achromobacter* spp., and *Pseudomonas* spp. were quantified at the point of inoculation, three internodes above the point of inoculation, and seven internodes above the point of inoculation on mature grape stems (Figure 3). Endophytes were not

detected seven internodes above the point of inoculation throughout the 16-week growth period, with the exception of one sample at the two-week time point inoculated with the *Achromobacter* spp. *Pa. phytofirmans* was recovered from the point of inoculation at the point of inoculation (10^6 CFU/g stem tissue) up until four weeks post inoculation. However, *Pa. phytofirmans* was only recovered at 3 internodes above the point of inoculation (10^5 CFU/g stem tissue) at two weeks post inoculation. Following this, it declined in titer to ~200 CFU/g stem tissue for all subsequent time points. The *Achromobacter* spp. was recovered from the point of inoculation at an average titer of 10^6 CFU/g stem tissue at the point of inoculation and 3 internodes above the point of inoculation at 10^5 CFU/g stem tissue up until four weeks post inoculation. Subsequent time points indicate that the *Achromobacter* spp. colonized the point of inoculation at 10^5 CFU/g stem tissue and three nodes above the point of inoculation at ~500 CFU/g stem tissue. The *Pseudomonas* spp. declined in titer over the 16-week assay at the point of inoculation and was only found three internodes above the point of inoculation (at an average concentration of < 300 CFU/g stem tissue) at the two-week time point post inoculation and not at subsequent time points.

Antibiosis activity of the biocontrol agents. One mechanism found in successful biocontrol agents is the production of antibiotics that inhibit or kill target pathogens. We assessed these novel biocontrol agents for their ability to inhibit the growth of *X. fastidiosa* on solid PD3 medium (Figure 4). Inhibition was assessed by calculating the percentage of growth of *X. fastidiosa* along a five cm distance. Of the endophytic

bacteria, *Pa. phytofirmans* and the *Pseudomonas* spp. exhibited the strongest inhibition of *X. fastidiosa* growth. No *X. fastidiosa* growth was observed within five cm of *Pa. phytofirmans* in every replicate and *X. fastidiosa* growth was only observed on average after 4.54 cm ± 0.72 from the *Pseudomonas* spp. per replicate. In contrast, the *Achromobacter* spp. exhibited the least amount of inhibition on the growth of *X. fastidiosa*, which was observed on average after 1.24 cm ± 0.24 from the *Achromobacter* spp. per replicate.

Grapevines pre-treated with biocontrol agents exhibited significantly fewer tylose-occluded xylem vessels. Tyloses are outgrowths of parenchymal cells lining the xylem vessels. They are induced as part of plant defense responses to invading microbes with the goal of acting as a physical barrier to stop the systemic spread of a pathogen. However, they can also block the flow of water and nutrients and prolific production of tyloses is a hallmark symptom of PD. Cross sections of stem segments three nodes above the point of inoculation of *X. fastidiosa* from the PD bioassay were assessed to calculate the percentage of occluded xylem vessels. Vines pre-treated with 1X PBS buffer and challenged with *X. fastidiosa* had on average 73% occluded vessels. The *Pseudomonas* spp. and *Achromobacter* spp. had 14% and 19% occluded vessels, respectively. *Pa. phytofirmans* had the largest reduction in occluded vessels with an average of 3.3% occlusion (Figure 6).

Biocontrol agents show catabolic ACC activity *in vitro*. Achromobacter spp. putatively possesses the genes to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Blaha et al. 2006). This enzyme is important because it breaks down the precursor to ethylene, a plant hormone that is linked to tylose formation (Sun et al., 2007). To assess the ability of each strain to catabolize ACC, the biocontrol agents were grown in Dworkin and Foster (DF) medium in which ACC is the only available nitrogen source. If a bacterium can grow in DF medium amended with ACC, it can catabolize ACC to achieve its nitrogen nutrient requirement. When grown in liquid DF medium without an ACC amendment or nitrogen source, the optical densities of the three biocontrol agents did not increase. However, when the DF medium was amended with ACC, the optical densities of each biocontrol agent did increase, demonstrating that the biocontrols were able to catabolize ACC to acquire nitrogen (Figure 5).

Discussion

We have assessed two novel grapevine endophytic microbes for their ability to mitigate PD of grapevine *in planta.* The *Pseudomonas* spp. and the *Achromobacter* spp. persist within grapevine microbiomes across years and tissue types in California (Deyett, 2020). They have also been found in the xylem, like *X. fastidiosa,* and to be negatively correlated with disease symptom severity and pathogen titer (Deyett *et al.*, 2017). Similarly to *Pa. phytofirmans*, an effective biocontrol agent of PD (Baccari et al. 2019), we have shown that the *Pseudomonas* spp. and the *Achromobacter* spp. have biocontrol qualities and confer protection to *V. vinifera* against *X. fastidiosa* under greenhouse

settings. Vines inoculated with the *Pa. phytofirmans*, the *Achromobacter* spp., and *Pseudomonas* spp. exhibited both lower visual disease ratings of PD and lower pathogen titer, regardless of the time at which the vines were challenged with *X. fastidiosa*.

Several species in the genus *Achromobacter* improve plant health by several different mechanisms including stimulating nitrate uptake (Bertrand et al., 2000). Additionally, it has been noted that some species may confer protection against *Fusarium* wilt of tomato through iron competition (Moretti et al., 2008). In our findings, vines pre-treated with the *Achromobacter* spp. had the highest average *X. fastidiosa* titer of the biocontrol agents tested. However, high *X. fastidiosa* titers did not appear to affect disease symptom severity as observed visual disease ratings were low and not significantly different from the observed visual disease ratings of vines pre-treated with the other biocontrol agents.

Although *Achromobacter* species are known to produce antibiotic compounds toward other microorganisms (Yan et al., 2004), little *in vitro* antagonism was observed against *X. fastidiosa* on PD3 medium. While the *Achromobacter* spp. tested here may produce antibiotics within the vine to have a greater inhibitory effect than observed in our *in vitro* assay, it may also interact with the plant host to confer protection to *V. vinifera*, rather than interacting directly with the pathogen. *Achromobacter* strains can putatively produce a 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Blaha et al. 2006). We determined that our *Achromobacter* spp., in addition to our other biocontrol agents, can catabolize ACC, suggesting that this deaminase may be functional. ACC is a precursor molecule in the ethylene production reaction and ACC catabolism prevents ethylene

synthesis (Gamalero and Glick 2015). Elevated levels of ethylene during *X. fastidiosa* infection have been suggested to be the cause of increased tylose production, increasing disease symptom severity during pathogenesis (Perez-Donoso et al. 2007; Sun et al. 2013). We propose that the *Achromobacter* spp. catabolism of ACC decreases the amount of ethylene within the plant to reduce tylose formation, consequently decreasing disease severity.

Similarly to the *Achromobacter* spp., several *Pseudomonas* species have plant growth promoting and pathogen suppression attributes including phosphate solubilization, indole acetic acid, hydrogen cyanide production, ACC deaminase, quorum sensing and siderophore (iron chelating metabolite) production (Samad et al., 2017). Thus *Pseudomonas* spp. could potentially mitigate pathogen titer through a combination of both direct and indirect mechanisms. Our data indicate that our *Pseudomonas* spp. can directly antagonize *X. fastidiosa in vitro*, and we speculate the *Pseudomonas* spp. can inhibit *X. fastidiosa in planta* by direct antibiosis. While the viable *Pseudomonas* spp. titer is the lowest of the biocontrols by end of the 16-week experiment, antibiotic titer may be sufficient to antagonize *X. fastidiosa* to slow disease progression during the disease bioassay. It is also possible that endophyte titer fluctuates over different phenological grapevine stages and may reach abundant titers in the field to confer protection against *X. fastidiosa* at the onset of an infection event, accounting for its previously observed negative correlation with pathogen abundance (Deyett et al. 2019; Deyett et al. 2017).

As an established biocontrol agent for PD, *Pa. phytofirmans* reduces pathogen titer and reduces disease symptom development (Baccari et al. 2019). Baccari *et al.* attribute this protective quality of *Pa. phytofirmans* to priming of the grapevine evidenced by the higher abundance of PR1 expression compared to control plants (Baccari et al. 2018). Our *in vitro* results indicate that the *Pa. phytofirmans* is also inhibitory to *X. fastidiosa* within a 5 cm radius, suggesting it can inhibit *X. fastidiosa* growth via antibiosis. The low *X. fastidiosa* titer recovered from the vines inoculated with *Pa. phytofirmans* and low percentage of tyloses found in these vines suggest that the titer of *X. fastidiosa* was reduced or eliminated before significant disease could occur. This, in combination with the fact that other studies in different plant systems have found that living PsJN cells are required for biocontrol activity (Timmermann et al. 2017), suggests that titer of metabolically active cells plays a role in biocontrol. Together, these findings suggest that it is likely a combination of both direct microbe-microbe interactions and indirect microbe-plant-pathogen interactions that account for *Pa. phytofirmans*'s successful biocontrol activity of PD.

Future functional genomics and transcriptomics experiments will help further decipher the role that these microbes play and enhance our understanding of their biocontrol capabilities. Together, our results demonstrate that all biocontrol agents tested have biocontrol properties and they expand the repertoire of biological control agents available for prophylactic therapy for plant diseases such as PD.

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Figures

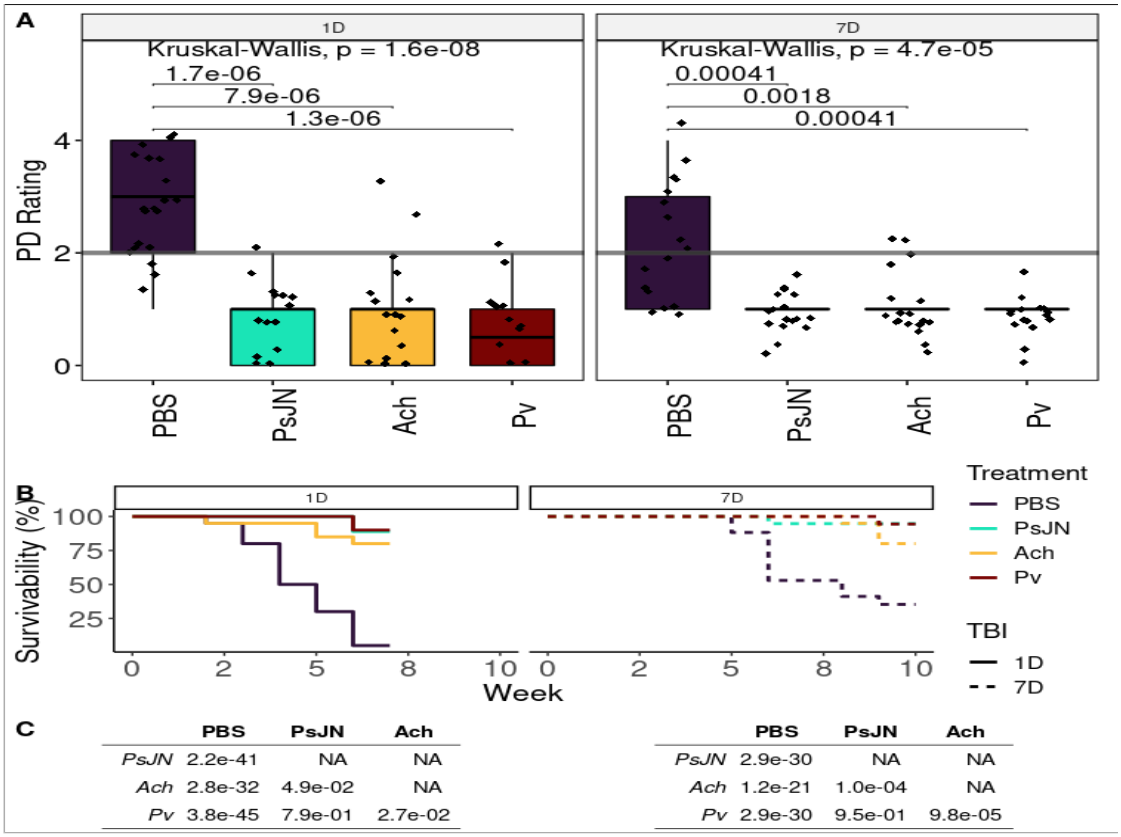


Figure 2.1. PD symptom severity is reduced in vines pretreated with endophytes compared to vines pretreated with 1X PBS. A. Severity of PD symptoms on Cabernet Sauvignon grapevines pretreated with *Pa. phytofirmans* (PsJN), *Achromobacter* spp. (ACH), or *Pseudomonas* spp. (PV) or 1X PBS and then challenged with *X. fastidiosa* either 1 or 7 days later. Boxplots represent the median and variation of the disease rating scores (two biological replicates of approximately 8-10 technical replicate plants) at 12 post inoculation for 1 day vines and 15 weeks for 7 day vines. Gray bars reflect the disease rating at which vines will no longer survive the season (Disease rating of 2 or more). B. The percentage of survivability of vines over time, where vines scoring a disease rating of 2 or higher will not survive the season. Solid lines represent vines challenged with *X. fastidiosa* 1 day after biocontrol inoculation, where data were collected over the course of 6 weeks. Dotted lines represent vines challenged with *X. fastidiosa* 7 days after biocontrol inoculation, where data were collected over the course of 9 weeks. C. Cox proportional hazard analysis, a survival analysis regression model, showed each endophyte confer a significant reduction in risk of death when inoculated with *X. fastidiosa* regardless of the timing *X. fastidiosa* challenge (Cox Proportional hazards; $p<0.001$).

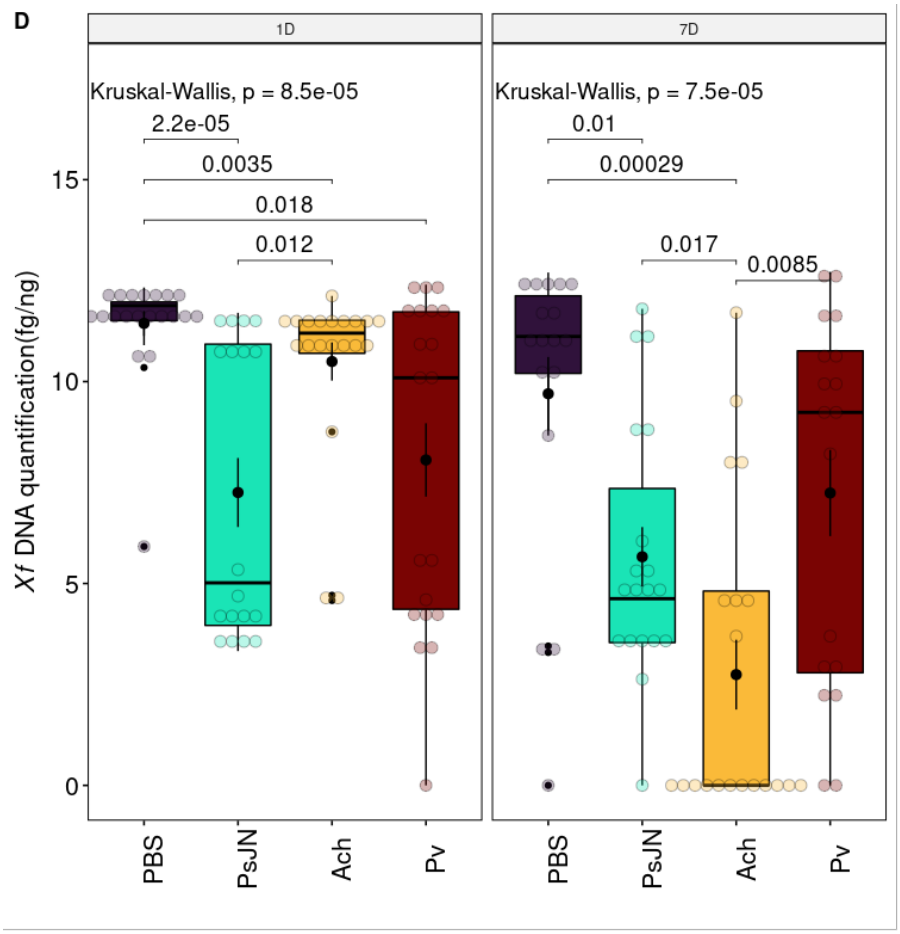


Figure 2.2. *X. fastidiosa* DNA recovered from the petioles of Cabernet Sauvignon grapevines pretreated with endophytes is reduced compared to vines pretreated with 1X PBS. Boxplots represent the median and variation of *X. fastidiosa* titer (fg *X. fastidiosa* DNA per ng of plant DNA extracted) from each of 18-20 replicate plants (two biological replicates of 8-10 technical replicate plants). *X. fastidiosa* titer was significantly reduced in vines pretreated with *Pa. phytofirmans* (PsJN), *Achromobacter* spp. (ACH), and *Pseudomonas* spp. (PV) compared to pretreatment with 1X PBS at the day 1 challenge. *X. fastidiosa* titer was significantly reduced in vines pretreated with *Pa. phytofirmans* and the *Achromobacter* spp. compared to pretreatment with 1X PBS at the day 7 challenge, but not the *Pseudomonas* spp.

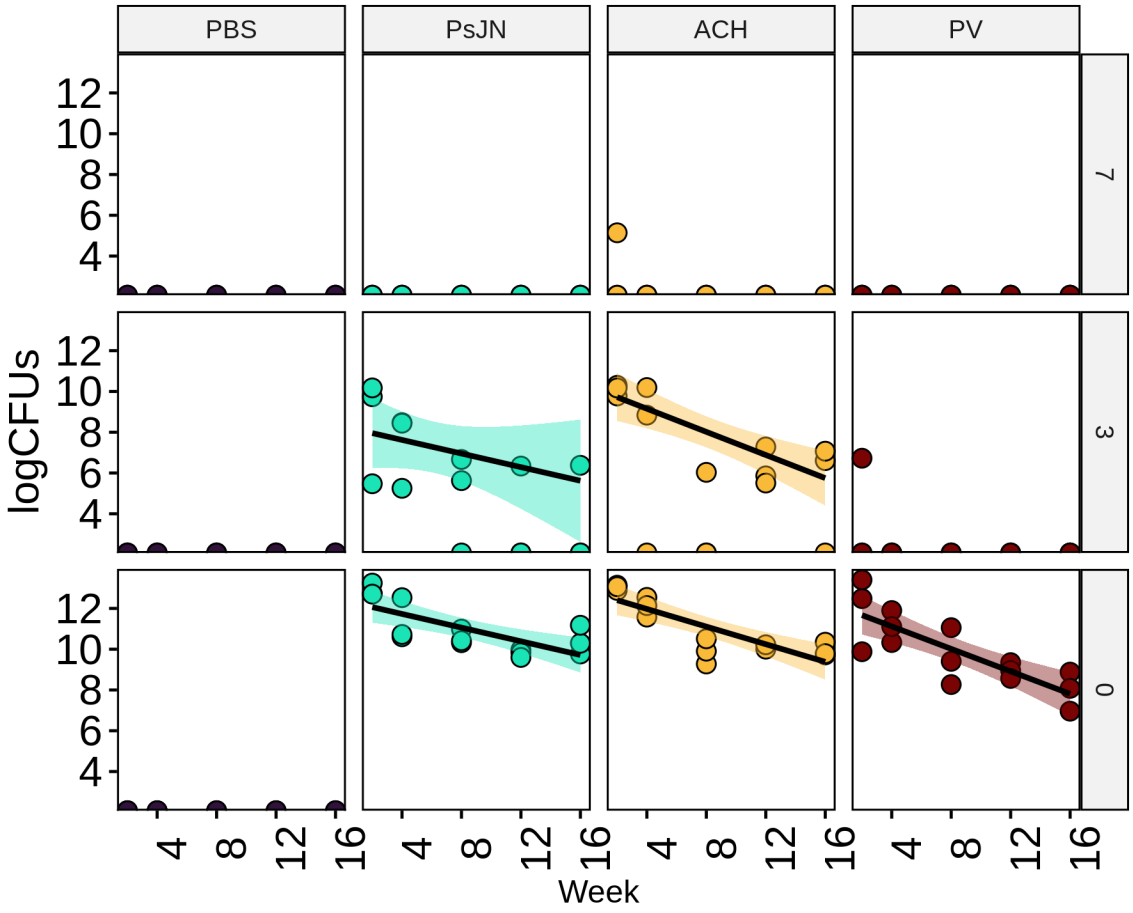
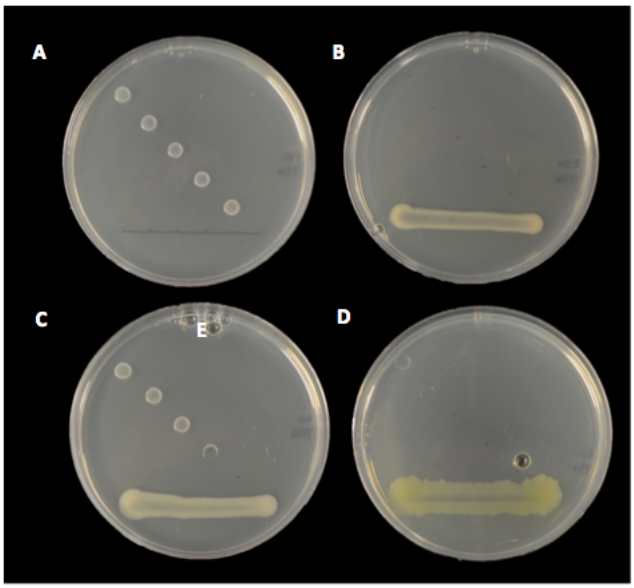


Figure 2.3. Viable populations of endophytes persist in Cabernet Sauvignon grape stems over time. Viable population sizes of *Pa. phytofirmans* (PsJN), *Achromobacter* spp. (ACH), and *Pseudomonas* spp. (PV) in Cabernet Sauvignon grape stems at the point of inoculation (POI), 3 internodes above the POI, and 7 nodes above the POI. Horizontal rows represent the internode sampled and vertical columns represent the endophyte. Circles represent bacterial population sizes and lines represent the regression line from three plants measured at 5 times points.


A
B
C
E
D

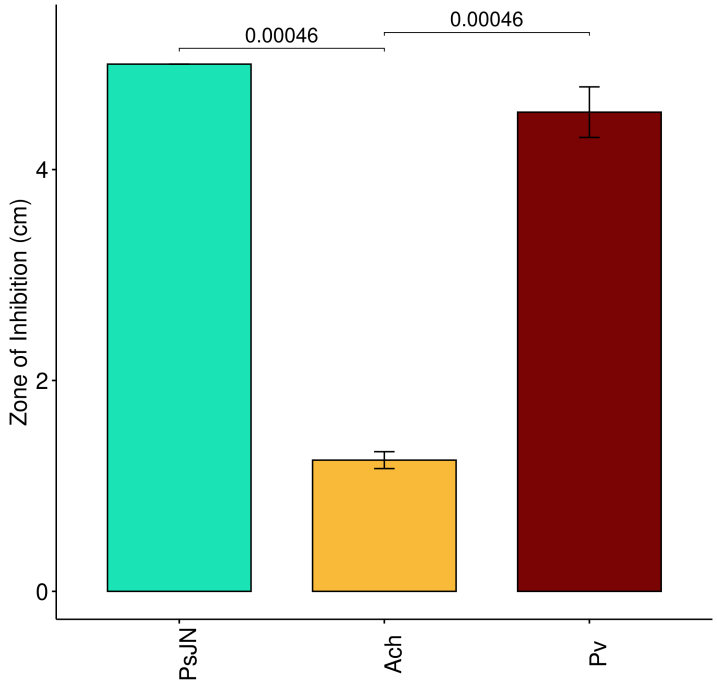

0.00046
0.00046
Zone of Inhibition (cm)
0
2
4
PsJN
Ach
Pv

Figure 2.4. Inhibition of growth of *X. fastidiosa* in the presence of endophytes. 1X PBS (A) *Pa. phytofirmans* PsJN (B), *Achromobacter* spp. (C), and *Pseudomonas* spp. (D) were plated against 5 μL droplets of *X. fastidiosa*. Bar plots represent average distance from from *Pa. phytofirmans* (PsJN), *Achromobacter* spp. (ACH), and *Pseudomonas* spp. (PV) to the first sign of *X. fastidiosa* growth on solid PD3 plates. Vertical bars represent the standard error of the average distance of inhibition from 9 replicate plates.

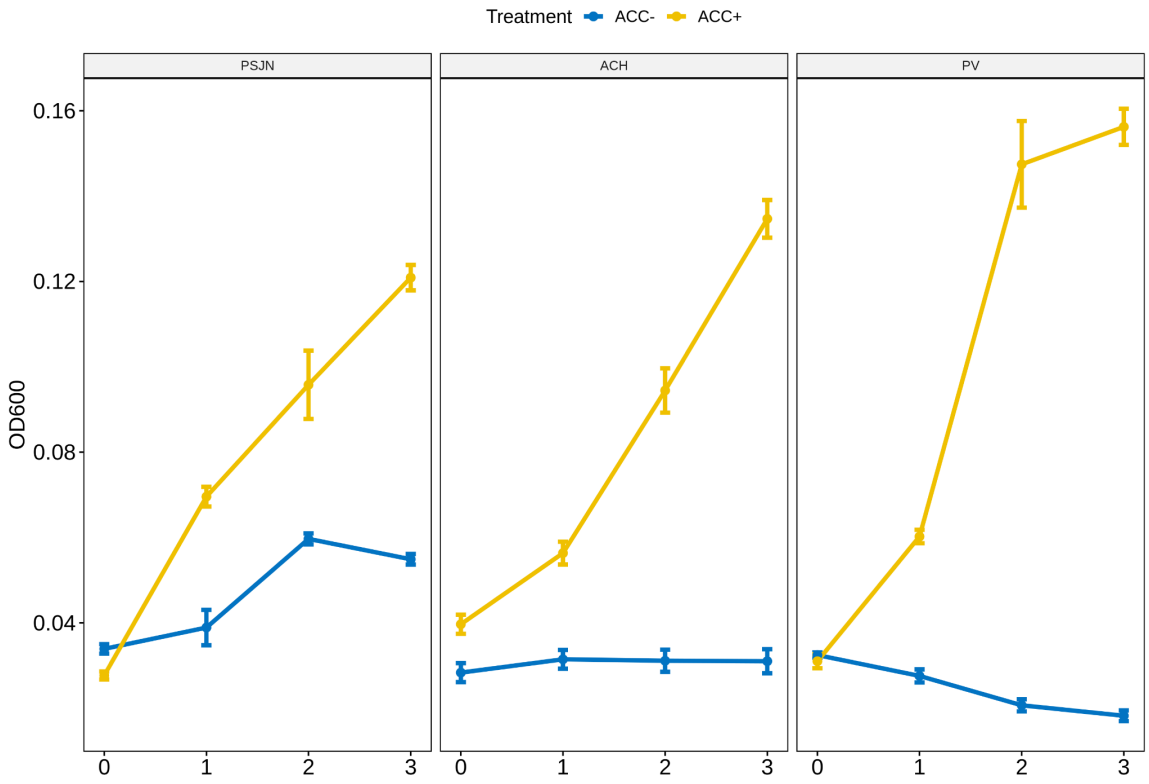


Figure 2.5. Functional assessment of endophytes' abilities to catabolize 1-aminocyclopropane-1-carboxylate (ACC). *Pa. phytofirmans* (PsJN), *Achromobacter* spp. (ACH), and *Pseudomonas* spp. (PV) were grown in Dworkin and Foster medium containing either $(NH_4)_2SO_4$ as a positive control, ACC, or no nitrogen source as a negative control. Each endophyte was able to catabolize ACC.

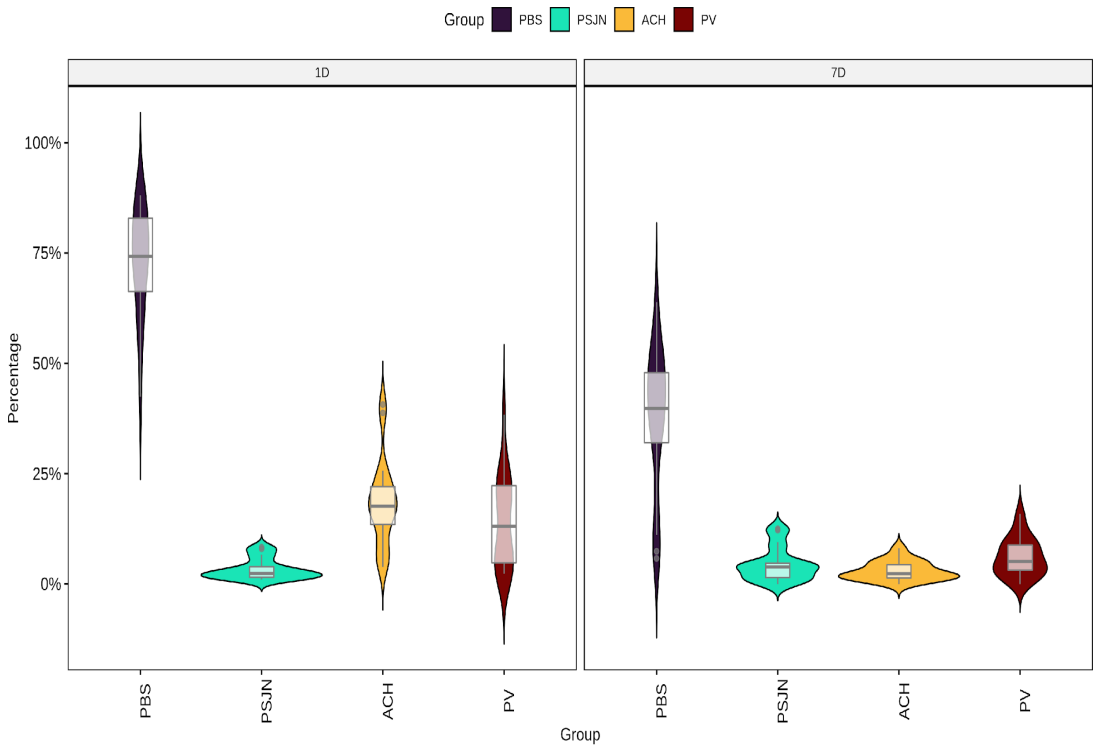


Figure 2.6. Endophyte pretreated vines have significantly fewer tylose-occluded xylem vessels. Cross sections of grapevine stem tissue were examined 3 nodes above the point of inoculation of *X. fastidiosa*, challenged either 1 or 7 days after treatment with *Pa. phytofirmans* (PsJN), *Achromobacter* spp. (ACH), and *Pseudomonas* spp. (PV), or 1X PBS. Samples were collected at the same time as the samples for *X. fastidiosa* titer quantification mentioned above. Percentages were calculated by dividing the number of occluded vessels by the total number of vessels. Violin plots represent the distribution of the percentage of occluded xylems from three cross sections from each of 6 replicate plants per treatment.

Chapter III

Transgenic Overexpression of a Class III Peroxidase Gene Confers Tolerance to Pierce's Disease in *Vitis vinifera*

Abstract

Plant immune systems rely on pathogen recognition receptors (PRRs) to identify invading pathogens and activate early and then later stage defenses. The lipopolysaccharide (LPS) produced by *Xylella fastidiosa*, a plant pathogenic bacterium and the causal agent of Pierce's disease (PD) of grapevine, elicits a strong immune response in grapevines. This early elicitation of basal defense responses leads to the activation of systemic and prolonged defense pathways against *X. fastidiosa* infection of grapevines. We previously identified a gene encoding a class III peroxidase (*CP1-like*), an enzyme responsible for the production of reactive oxygen species (ROS), which is upregulated during an effective immune response against *X. fastidiosa*. Here we developed a transgenic line constitutively overexpressing the *CP1-like* gene (OE-*CP1-like* vines) and assessed it for its ability to confer protection against *X. fastidiosa*. In a greenhouse trial, OE-*CP1-like* vines exhibited a slower rate of disease progression and an overall reduction of pathogen titer, suggesting that the *CP1-like* gene contributes to PD resistance. This work illuminates a way forward for creating tolerant transgenic lines and rootstocks for use in commercial vineyards.

Introduction

Unlike animals, plants lack an adaptive immune system and must rely on innate immunity strategies to fend off pathogens. Conserved pathogen- or microbe-associated molecular patterns (PAMPS or MAMPS, respectively) such as flagellin and lipopolysaccharides (LPS) can trigger an immune response in the host (Jones and Dangl.

2006). LPS are a major component of the cell envelope of Gram-negative bacteria, including *Xylella fastidiosa*, a bacterium limited to the xylem *in planta* and the causal agent of Pierce's disease (PD) of grapevine and several other diseases affecting economically important crops around the world (Castro et al. 2021). The LPS of *X. fastidiosa* includes the canonical lipid A anchor, a core oligosaccharide, and a terminal, surface-exposed O-antigen (Caroff and Karibian. 2003). Critically, *X. fastidiosa's* high molecular weight, rhamnose rich O-antigen aids in masking the presence of *X. fastidiosa* from its plant host and delays host immune response allowing colonization (Clifford et al. 2013; Rapicavoli et al. 2018b). However, purified wild type *X. fastidiosa* LPS and whole mutant *X. fastidiosa* cells with a truncated O-antigen elicit robust immune reactions leading to a systemic and prolonged immune response to *X. fastidiosa* in grapevine. Transcriptome analysis identified a subset of genes that are significantly upregulated during a successful immune response against *X. fastidiosa* including three class III peroxidases (Rapicavoli et al. 2018b). Transgenic overexpression of these genes may be an effective means to confer grapevines with protection against *X. fastidiosa*.

Class III peroxidases are heme-containing glycoprotein enzymes that accumulate in the xylem sap of plants during pathogen colonization (Hiraga et al. 2001; Yadeta and Thomme. 2013). They have several roles including contributing to the production of reactive oxygen species (ROS) during a host defense response which is congruent with what is seen in the xylem of *X. fastidiosa* LPS primed grapevines (Almagro et al. 2009; Rapicavoli et al. 2018b). Class III peroxidases are also known to confer resistance to

other pathogens that are taxonomically relatively closely related to *X. fastidiosa* species, such as *Xanthomonas citri* subspecies *citri* in sweet orange (Li et al. 2020).

To determine the role of class III peroxidases in grapevines during infection with *X. fastidiosa*, we generated transgenic grapevines to constitutively overexpress a class III peroxidase gene (*VvCabSauv08_P0003F.ver1.0g290450.m01*; VIT_18s0001g06850; NCBI LOC100257440*;* herein referred to as *CP1-like* gene) in Thompson Seedless *Vitis vinifera* vines (the transgenic vines are herein referred to as OE-*CP1-like* vines). Here we explored the ability of these transgenic vines to cope with infection and hypothesized that overexpression of this *CP1-like* gene would confer protection by increasing ROS to reduce *X. fastidiosa* titer and PD symptom severity.

Materials and Methods

Construction of *35S:CP1-like* construct. The *CP1-like* coding region (0.97-kb*)* was amplified from a Cabernet Sauvignon petiole cDNA sample using Q5® High-Fidelity DNA Polymerase (NEB). Amplification was as follows: denaturation at 98°C for 30s min, followed by 30 cycles of 98°C for 10s, 60°C for 30s, and 72°C for 1 min, followed by 1 cycle of final extension at 72°C for 15 min, using primers forward-NheI : (5′-AAGCTAGCATGGCTTCCCTATCCTTG-3′) and reverse-BstE II (5′-TTGGTCACCCTAAGATCCATTGATTTTCTTGCA-3′) primers contained introduced restriction sites to facilitate cloning. The amplified fragment was cloned into the pCR-Blunt vector (according to the protocol Zero Blunt™ PCR Cloning Kit, Invitrogen),

sequenced to verify its integrity, and subcloned subsequently into binary vector pCAMBIA1302 (digested with *SpeI* and *BstEII* restriction enzymes) to replace mgfp5-6xHis tag gene with *CP1-like* coding region to crate p*35S: CP1-like* plasmid. This construct was introduced into somatic embryos of Thompson Seedless Grape (*Vitis vinifera* L.) plants.

Grapevine Transgenesis. For generating transgenic grape, somatic embryos were transferred to 5 mL of liquid Lloyd and McCown WPM supplemented with 20 g/L sucrose, 1g/L casein, 1 mM MES, 500 mg/L activated charcoal, 0.5 mg/l BAP, 0.1 mg/L NAA, 200 uM acetosyringone and 12.5 uL pluronic F68. The embryos were heat shocked by placing the tube in a 45°C water bath for 10 minutes. Transformed *Agrobacterium tumefaciens* was added to the solution to an OD_{600nm} of 0.1 to 0.2. After 5 minutes, the supernatant was removed and saved in a 60 x 15 mm petri dish. The embryos were transferred to an empty 100 x 20 mm petri dish containing a 7 mm whatman filter paper disk with a sterile spoonula. The petri dish was wrapped with parafilm and incubated in the dark at 23°C. After 2-3 days, the embryos were harvested off the filter paper disk and transferred to selection medium consisting of WPM supplemented with 20 g/l sucrose, 1g/L casein, 1mM MES, 500 mg/L activated charcoal, 0.5 mg/L BAP, 0.1 mg/L NAA, 400 mg/L carbenicillin, 150 mg/L timentin, 200 mg/L kanamycin, (or 25 mg/l hygromycin), 50 g/L sorbitol, 14 g/L agar and 4 mL PPM. They were then incubated in the dark at 26°C. After seven days, the embryos were subcultured to fresh medium of the same formulation. After 14 days, the embryos were subcultured again to fresh medium. The subculture was repeated once more. After an additional 14 days, the embryos were

then subcultured to WPM supplemented with 20 g/L sucrose, 1g/L casein, 1mM MES, 500 mg/L activated charcoal, 0.5 mg/L BAP, 0.1 mg/L NAA, 400 mg/L carbenicillin, 150 mg/L timentin, 200 mg/L kanamycin (or 25 mg/L hygromycin) without sorbitol and 8 g/L agar. The embryos were divided on the plate into five independent clusters. Incubate under 16 hours light 26 degrees. The embryos were subcultured every two weeks. As individual clusters of embryos expand and before they merge into one another, each cluster was subcultured to its own plate. The embryos were subcultured every 14-21 days until plants formed. Germinating plants were harvested and the root was excised and the shoot was placed on WPM minimal organics medium supplemented with 30 g/L sucrose, 0.01 mg/L IBA, 150 mg/L timentin, 400 mg/L carbenicillin and 100 mg/L kanamycin (or 25mg/L hygromycin). Once shoots root, they were transferred to a 2-inch pot containing moistened soil (1 part sunshine mix 2 part vermiculite). The pot was placed in a Ziploc bag and sealed for 7 days. The bag was incubated at 26°C under 16 hours of 100 um light covering the flat with a plastic dome. After one week, the Ziploc bag was opened to reduce the humidity. Once new growth was evident, the plant was removed from the Ziploc bag and placed under the dome for an additional 7 days to complete acclimatization.

RNA isolation and semi-quantitative RT-PCR analysis of transcript levels in OE-*CPI-like* vines. Total RNA from petiole tissue was extracted using PureLink™ Plant RNA Reagent according to the recommendations of the manufacturer (Invitrogen, Waltham, MA). First-strand cDNA was synthesized from 2µg DNase (Promega) treated

total RNA using Superscript IV First-Strand Synthesis system (Invitrogen, Waltham, MA) with oligo(dT)15 primer. The cDNA was used as the template for RT-PCR. RT-PCR was performed with denaturing, annealing, and extension conditions of 95°C for 1 min, 56°C for 30 sec and 72°C for 1 min, for 30 or 33 cycles, respectively. The primers for *CP1-like* (forward: 5- ACATGGACCCTGCAATTAGGCAG -3′; reverse: 5′-GCATTGGCCACATCCGTAAA -3′) were used to detect the expression levels of total *CP1-like* in the transgenic grapevines. *ACTIN* forward primer: 5′-CTATGAGCTGCCTGATGGGC -3′; reverse primer: 5′-GCAGCTTCCATCCCAATGAG -3′) was used as a reference gene for data normalization. The amplification products were electrophoresed on 2% agarose gel. Gels were stained with ethidium bromide and photographed.

Propagation of Transgenic Grapevines. Parental genotype and transgenic Thompson Seedless grapevines were shipped from the UC Davis Plant Transformation Facility to the Roper Lab at UC Riverside and grown in a growth chamber at 28°C with 16 hours of light and 8 hours of darkness. In the growth chamber, each individual line was vegetatively propagated to generate more plant material. One mother plant was kept for each line. Stem cuttings with two nodes were hand sectioned from the mother plants and rooted in vermiculite. These plants were grown in the growth chamber and transferred to the greenhouse in 1 gallon pots containing PP UC Mix Soil for greenhouse trials.

Disease bioassay. Five parental genotype Thompson Seedless vines and 15 clonal OE-*CPI-like* vines were used in biological replicates of 5 technical replicates each. *X. fastidiosa* subsp. fastidiosa (Temecula 1) inoculum was prepared in sterile 1X PBS to OD_{600nm}= 0.25 (approx. 10^8 CFU/mL). Briefly, two 20 µL volumes of inoculum were mechanically inoculated using a 20-gauge needle, one on either side of the stem of the vine between the third and fourth nodes (Hill and Purcell 1995). Within each biological replicate, one vine served as a negative control and was inoculated with sterile 1X PBS in the same manner. All plants were randomized in the greenhouse and each vine was given a weekly PD disease rating based on a scale from 0 to 5 as described by Guilhabert and Kirkpatrick 2005. Briefly, 0 = healthy vine, 1 = one or two leaves with scorching at the margins, 2 = two or three leaves with more developed scorching, 3 = all the leaves have some scorching with a few matchstick petioles, 4 = all leaves have heavy scorching and many matchstick petioles, and 5 = dead vine. The plants were rated weekly over a 9 week period. The mean PD disease rating scores per week were calculated and a comparison between the disease progression of the vine types was made by calculating the area under the disease progression curves throughout the 9 weeks.

Quantification of *X. fastidiosa* titer *in planta*. *X. fastidiosa* titer per gram of tissue was quantified by isolating *X. fastidiosa* from the intact petiole closest to the point of inoculation. Petioles were collected from each vine, surface sterilized for 5 minutes in 70% ethanol and ground in 2 mL of sterile 1X PBS. The tissue suspension was then serial diluted and the series was plated on solid PD3 medium according to standard methods

(Roper et al. 2007). The titers from each vine were normalized by dividing the colony forming units by the weight of their corresponding petiole. The titers of the parental genotype Thompson seedless vines were compared with the titers of the transgenic vines.

Quantification of ROS in grapevine leaves. ROS was quantified in grapevine leaves according to Alexou (2013) with some modifications. Specifically, leaf samples were cut into rectangles weighing 60 mg. The tissue was frozen in liquid nitrogen and ground in 500 µL of distilled water at 4°C and placed in a 1.5 mL tube. The tubes were then centrifuged for 20 minutes at 4°C and 100 µL of supernatant was transferred to a clean Eppendorf tube. 20 µL of 3 mM luminol was added to the 100 µL of supernatant and the optical density was measured at 347 nm. The concentration of ROS was calculated using the following equation:

$$A = \varepsilon * d * c$$

A is the absorbance. ε is the molar extinction coefficient, d is the path length in cm, and c is the Molar concentration. At 347 nm, ε is approximately 7650 for luminol.

ROS Survivability Assay. *X. fastidiosa*'s survival in the presence of H_2O_2, a ROS generator, was evaluated using a modified version of an established protocol (Wang et al. 2017). In brief, *X. fastidiosa* was harvested from PD3 solid medium after 5 days of incubation at 28°C and suspended in sterile 1X PBS to OD_{600nm}= 0.25 (approx. 10^8

CFU/mL). 40 µL of inoculum was inoculated into 960 µL of sterile 1X PBS in a 1.5 mL tube. Tubes were then treated with H_2O_2 to create concentrations of 0 µM, 200 µM, 250 µM and 2.0 mM H_2O_2. Tubes were shaken at 180 rpm at 28°C for 30 minutes and then incubated on ice. Cell cultures were serially diluted using sterile 1X PBS. 10 µL of each dilution was drip plated onto PD3 solid medium and incubated at 28°C for 10 days. The number of colony forming units (CFUs) were quantified and the survival percentage was calculated by dividing the total number of CFUs by the number of CFUs in the untreated cultures.

Anthocyanin Extraction and Quantification. Systemic leaves, defined as 10 or more nodes above the point of inoculation, were chosen at approximately the same stage of development at 0, 4, and 9 weeks post inoculation. A leaf punch was created at random and the tissue weight was recorded. The leaf punches were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle to which 2 mL of methanol with 2 µL of HCl was added. This solution was incubated overnight in 4°C in the dark overnight. To separate anthocyanin from the solution, 500 µL chloroform was added to 500 µL of the aforementioned solution and centrifuged at 16,000 x g for 10 minutes. 500 µL of the supernatant was added into 1 mL 60% methanol 1% HCl and 40% deionized water. The absorbance was measured at 530 nm for anthocyanin and 657 nm for chlorophyll. The following equation was used to determine the concentration of anthocyanin:

$$A530nm - (0.25*A657nm)/g \text{ fresh weight}$$

Qualitative and quantitative assessment of tylose production. Stem segments were sectioned by hand with a razor blade and rinsed in water briefly. Sections were then stained with 500 μL of Toluidine Blue O (0.05% in water) for 5 minutes. Sections were rinsed thoroughly again with water to minimize background staining. Sections were mounted on a glass slide in 50% glycerol solution and examined under a light microscope. The number of occluded vessels were counted. The percentage of occluded vessels was calculated by dividing the number of occluded vessels by the total number of vessels per cross section. Three sections per node were assessed to visualize the occluded vessels in multiple planes. The averages of the three sections were taken to represent the number of occluded vessels per node. Images were taken on a Leica DFC295 camera (Serial #642284412). The cane segments can be stored in 80% ethanol at 4°C for later use.

RESULTS

Transgenic overexpression of the *CP1-like* gene confers resistance to PD. Vines were monitored for PD symptoms once a week over the course of 9 weeks and observed disease rating scores were used to generate disease progression curves (Figure 3A). By week 9, the parental genotype Thompson Seedless vines had an average disease rating score of 3.25 out of 5 and the OE-*CP1-like* vines had an average disease rating score of 1.36 out of 5. In addition, the areas under the disease progression curves for the OE-*CP1-like* vines were significantly lower than areas corresponding to the disease progression

curves for the parental genotype Thompson Seedless vines (Student's *t*-test, p = 0.006629)(Figure 3B).

Transgenic overexpressing *CP1-like* vines harbor less *X. fastidiosa.* *X. fastidiosa* titer was also measured at the closest intact petiole above the point of inoculation on each vine. The average bacterial titer in the OE-*CP1-like* vines (4.38 x 10^7 CFU/g tissue) was significantly lower than the average bacterial titer in the parental genotype Thompson Seedless vines (1.67 x 10^8 CFU/g tissue) (Student's *t*-test, p = 0.0124)(Figure 4).

Transgenic overexpressing *CP1-like* vines contain fewer tyloses. The percentage of tyloses, protrusions of the parenchymal cells into the lumen of the xylem to occlude the vessels, were calculated by dividing the total number of tylose-occluded vessels by the total number of xylem vessels in the cross section. Cross sections of the stem of the vines were stained with Toluidine Blue O and assessed under light microscopy (Figure 5A). At 9 weeks post-inoculation, the OE-*CP1-like* vines had fewer occluded vessels (21% occlusion) than the parental genotype Thompson Seedless vines (52% occlusion) (Student's *t*-test, p < 0.0001)(Figure 5B).

ROS and Anthocyanin Production. ROS in the parental genotype and the OE-*CP1-like* vines were quantified in leaf tissue prior to inoculation to understand basal levels of ROS in each genotype. The OE-*CP1-like* vines had a higher basal level of ROS than the parental genotype vines (One way ANOVA, p = 0.001056)(Figure 6A). Furthermore, *X.*

fastidiosa was significantly more susceptible to the level of ROS quantified from in the OE-*CP1-like* vines than the ROS concentration found in the wild type vines (Student's *t*-test, $p < 0.0001$)(Figure 6B).

We quantified anthocyanin production because anthocyanins are produced to scavenge ROS to protect the plant from self-harm (Xu et al., 2018.) We quantified anthocyanin just prior to inoculation (0 weeks), 4 weeks, and 9 weeks post inoculation (Figure 7). At time 0, the OE-*CP1-like* vines produced significantly more anthocyanins than the parental genotype Thompson seedless vines (Student's *t*-test, $p < 0.001$). Anthocyanin levels were similar throughout the course of the investigation with no significant difference between concentrations at 0, 4, and 9 weeks post inoculation. In contrast, there is a significant increase in anthocyanin concentration in the parental genotype Thompson Seedless vines from week 0 to week 4 (Student's *t*-test, $p < 0.001$) and another significant increase in concentration from week 4 to week 9 (Student's *t*-test, $p < 0.001$). At 4 and 9 weeks, there was no significant difference between anthocyanin concentration in the OE-*CP1-like* vines and the parental genotype vines.

Discussion

Peroxidases are involved in many processes in plants including lignification, auxin catabolism, wound healing, and defense against pathogen infection (Hiraga et al. 2001). They are secreted into the extracellular space where they can oxidize various substrates in the presence of hydrogen peroxide (Jovanovic et al. 2018). In regards to plant defense against invading pathogens, peroxidases can increase ROS production,

induce phytoalexin production, and reinforce cell wall physical barriers (Reimers et al. 1992; Vance et al. 1980; Kristensen et al. 1999). Here we successfully transformed *Vitis vinifera* cv. Thompson Seedless vines to constitutively overexpress the *CP1-like* gene and further assessed their ability to cope with PD and found that overexpression of *CP1-like* conferred protection.

We challenged both parental genotype and transgenic vines with inoculum of wild type *X. fastidiosa* in a controlled greenhouse environment. By the end of the trial, the OE-*CP1-like* vines exhibited less scorching and chlorosis and fewer symptomatic leaves than parental genotype vines. In addition, the average rate of disease progression for the OE-*CP1-like* vines was significantly lower than the average rate of disease progression for the parental genotype vines. Furthermore, *X. fastidiosa* titer near the point of inoculation of the OE-*CP1-like* vines was approximately tenfold lower than the titer of the parental genotype vines suggesting that overexpression of *CP1-like* greatly reduces symptom development with only a modest decrease in *X. fastidiosa* colonization.

The occlusion of the xylem vessels is caused by tyloses, a plant defense caused by outgrowths of the neighboring parenchyma cells into the lumen of the xylem. While tyloses can block the movement of xylem-limited pathogens, they can also potentially disrupt the flow of water and nutrients (Evert 2006). Tyloses are one of the predominant vascular occlusions in PD-infected vines. Grapevines produce prolific amounts of tyloses in response to *X. fastidiosa* infection which causes a drastic reduction in hydraulic conductivity and correlates with an exacerbation of external symptom development (Fry and Milholland 1990; Stevenson et al. 2004). OE-*CP1-like* vines developed fewer

occluded vessels than the parental genotype and had less severe symptoms supporting our hypothesis that reducing overproduction of tyloses can result in PD resistance.

During the invasion of a pathogen, a plant's first response is to produce a burst of ROS to damage and kill the cells of the invading microbe (Adam et al. 1989). This phenomenon is observed in several *X. fastidiosa* patho-systems as plant hosts often induce a ROS burst upon recognition of *X. fastidiosa* (Rapicavoli et al. 2018b; Novelli et al. 2019). Interestingly, in the olive - *X. fastidiosa* pathosystem, higher expression levels of transcripts of ROS-inducing genes were found in *X. fastidiosa* -resistant plants compared to susceptible plants (De Pascali et al. 2019). Because the OE-*CP1-like* vines had a higher basal level concentration of ROS we speculate that the transgenic plants more effectively inhibited *X. fastidiosa* during the early infection process. At the end of the disease bioassay, the transgenic plants showed fewer symptoms which suggests elevated levels of ROS in the OE-*CP1-like* vines are linked to the disruption of pathways that are linked to the manifestation of internal and external symtpoms. ROS can also cause oxidative stress to host cells by damaging membranes and interfering with crucial reactions such as photosynthesis (Mittler 2002). To mitigate self-damage, plants can produce antioxidants including anthocyanins (Xu et al. 2017). Anthocyanins are polyphenolic pigments found throughout plant tissue types (seeds, flowers, leaves). They are water-soluble derivatives of flavonoids made through the Shikimic Acid pathway and serve several functions including environmental stress tolerance and attracting pollinators to flowers (Chalker-Scott. 1999; Saito and Harborne. 1992). Anthocyanins have the ability to scavenge free radicals such as ROS to prevent cellular damage (Nimse et al.

2015). Not surprisingly, the OE-*CP1-like* vines had a significantly higher basal level concentration of anthocyanins per gram of tissue than the parental genotype. In *Arabidopsis thaliana*, ROS production positively correlates with anthocyanin accumulation (Xu et al. 2017). Interestingly, the concentrations of anthocyanins in the OE-*CP1-like* vines are not significantly different throughout the course of infection, in contrast to the parental genotype vines which show a significant increase in anthocyanin accumulation over time. We speculate that the initial higher concentration of ROS in the OE-*CP1-like* vines reduces *X. fastidiosa* populations within the vine and slows disease development. A similar phenomenon was observed in the olive - *X. fastidiosa* pathosystem, where higher expression levels of transcripts of ROS-inducing genes were found in *X. fastidiosa* -resistant plants compared to susceptible plants (De Pascali et al. 2019).

This study serves as a baseline for further investigation into how class III peroxidases contribute to the production of ROS and can further elucidate which genes are activated to tolerate the presence of *X. fastidiosa* populations. Furthermore, the development of OE-*CP1-like* rootstocks and investigation into their ability to impart protection to scion plant material could facilitate their incorporation into commercial viticulture.

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Figures

A.

B.

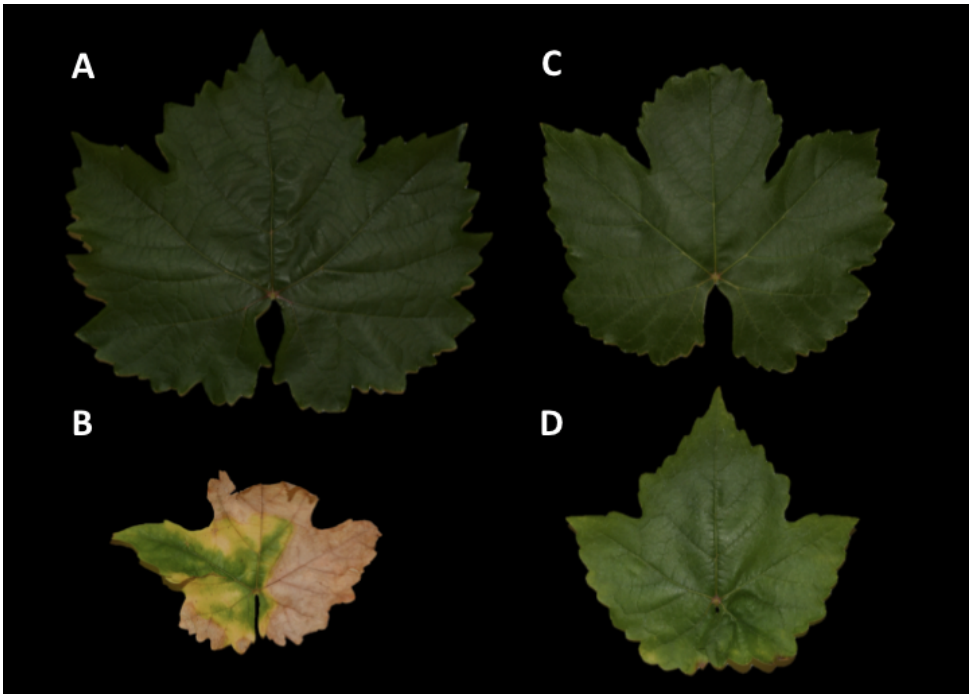


Figure 3.1. Wild type *Vitis vinifera* "Thompson Seedless" and OE-*CP1-like* vines inoculated with wild type *X. fastidiosa*. Panel A depicts a representative vine from each treatment 9 weeks after inoculation. A. Parental genotype Thompson Seedless inoculated with 1X PBS. B. Parental genotype Thompson Seedless inoculated with *X. fastidiosa*. C. OE-*CP1-like* vines inoculated with 1X PBS. D. OE-*CP1-like* vines inoculated with *X. fastidiosa*. Panel B depicts a representative leaf blade from each treatment 9 weeks after inoculation. A. Parental genotype Thompson Seedless inoculated with 1X PBS. B. Parental genotype Thompson Seedless inoculated with *X. fastidiosa*. C. OE-*CP1-like* vines inoculated with 1X PBS. D. OE-*CP1-like* vines inoculated with *X. fastidiosa*.

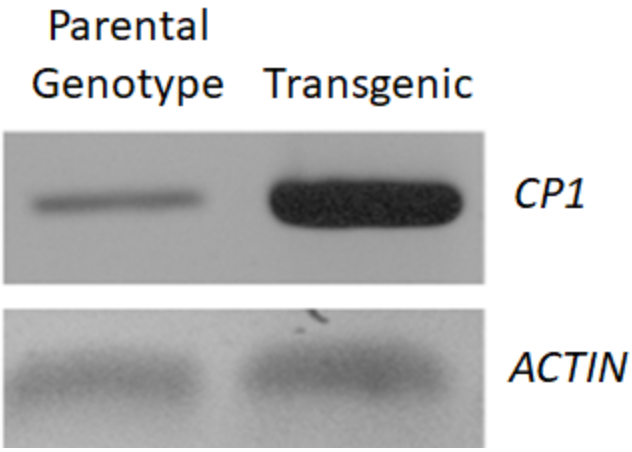


Figure 3.2. *CP1-like* transcript accumulation is higher in OE-*CP1-like* vines compared to wild type vines. RT-PCR products were detected by RT-PCR with gene-specific primers 35 cycles of amplification. *ACTIN* was used as a control, with 30 cycles of amplification.

A. B.

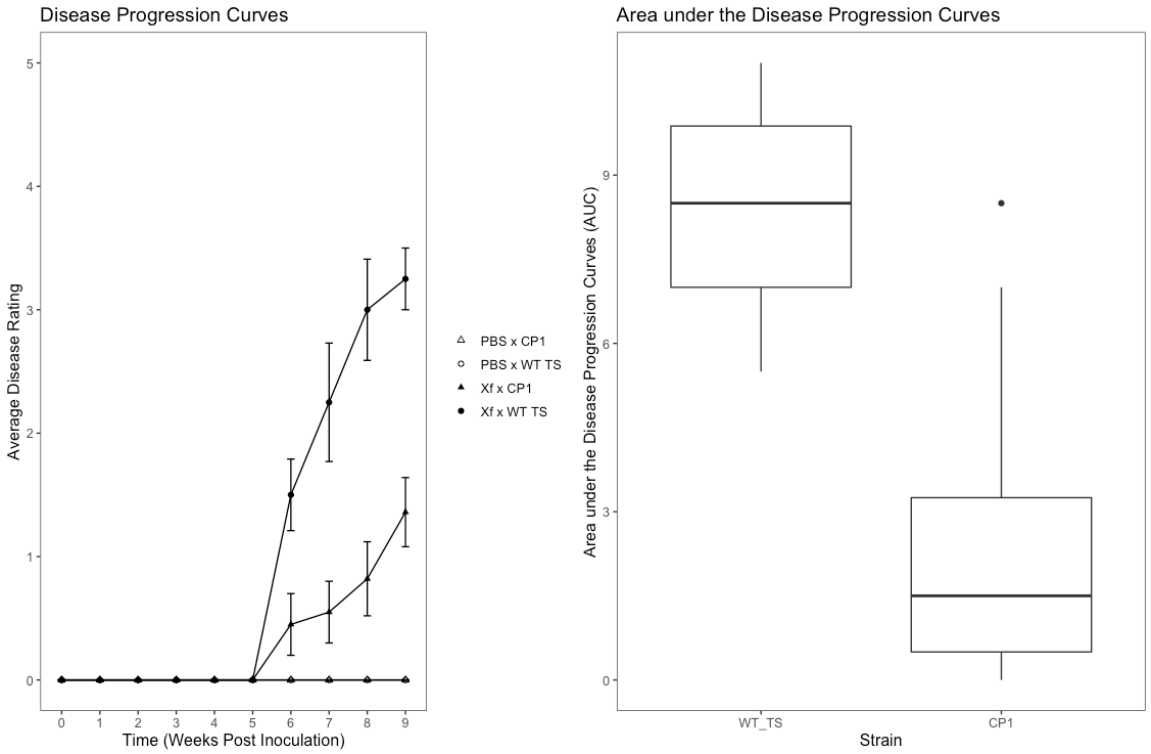


Figure 3.3. Disease symptom progression was significantly reduced in OE-*CP1-like* compared to wild type vines. A. Disease symptom progression in parental genotype Thompson Seedless (solid circle) and in OE-*CP1-like* (solid triangle) vines. PBS-inoculated parental genotype Thompson Seedless (open circle) and OE-*CP1-like* (open triangle) vines showed no symptom development. Vertical lines represent the standard error of the mean of disease severity at a given time point. B. Area under the disease progression curve reveals a significantly lower disease progression for OE-*CP1-like* vines compared to parental genotype Thompson Seedless vines (Student's *t*-test, p = 0.006629).

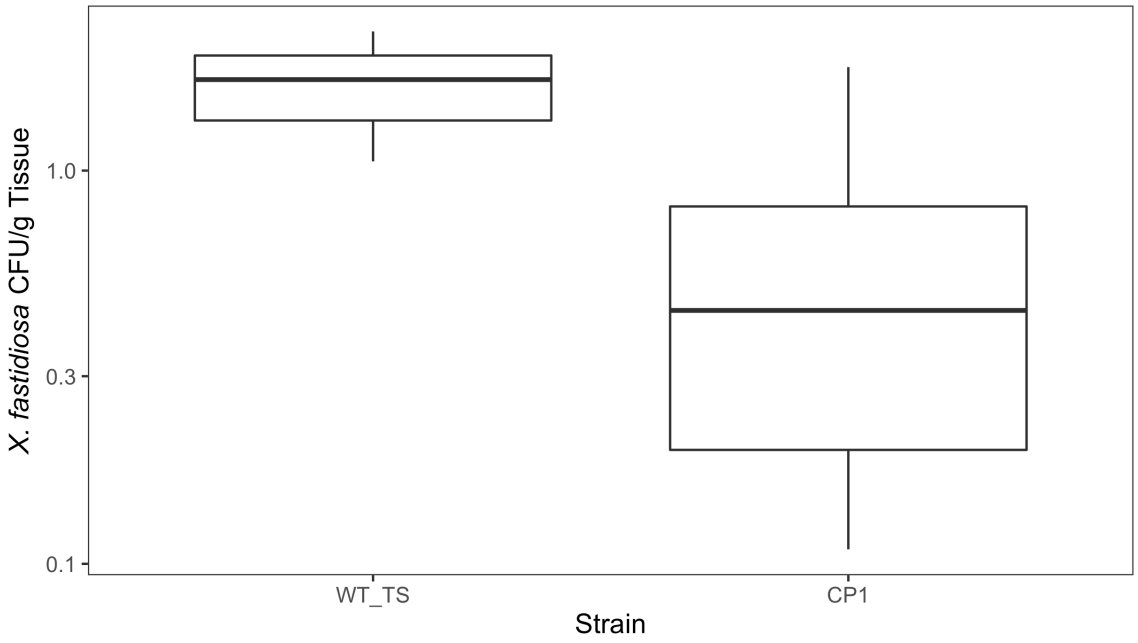
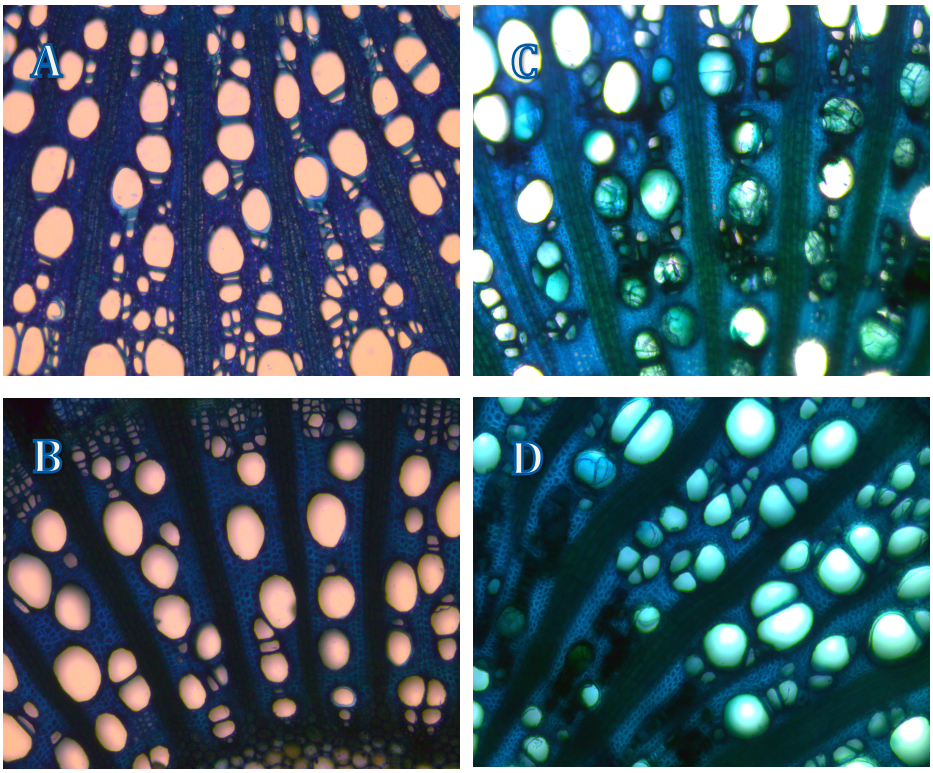


Figure 3.4. Viable *X. fastidiosa* titer was significantly lower in OE-*CP1-like* vines compared to wild type vines. *X. fastidiosa* populations were recovered from the first intact petiole above the point of inoculation 9 weeks after inoculation. Four replicates were sampled from parental genotype Thompson Seedless vines and 12 replicates were sampled from OE-*CP1-like* vines. *X. fastidiosa* titer was significantly lower in the OE-*CP1-like* vines (Student's *t*-test, p = 0.0124).

A.


A
C
B
D

B.

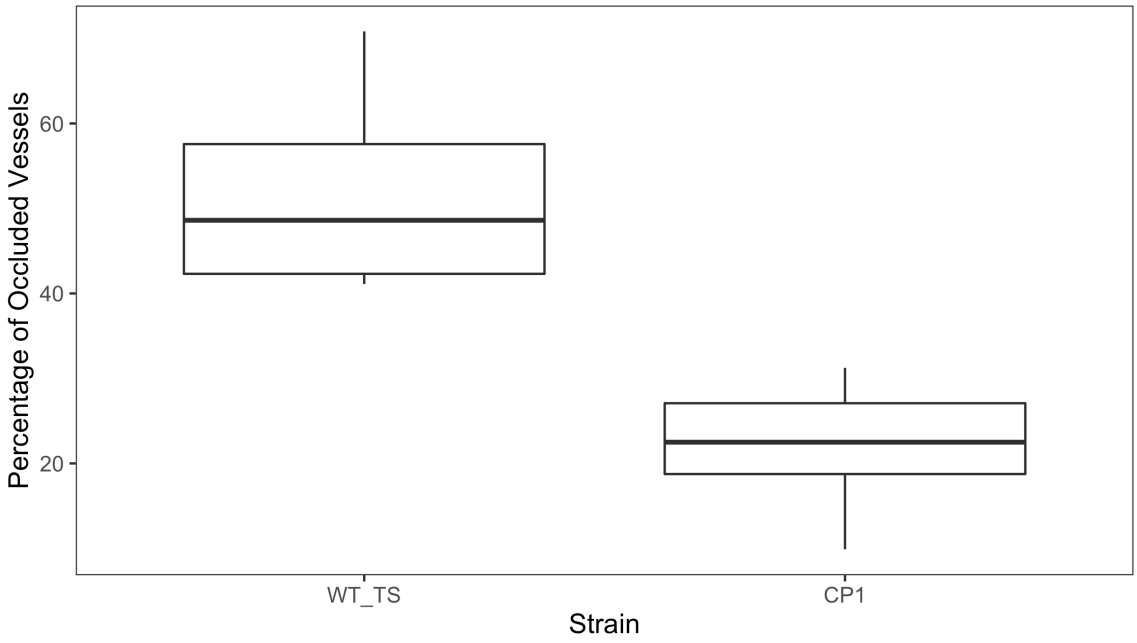
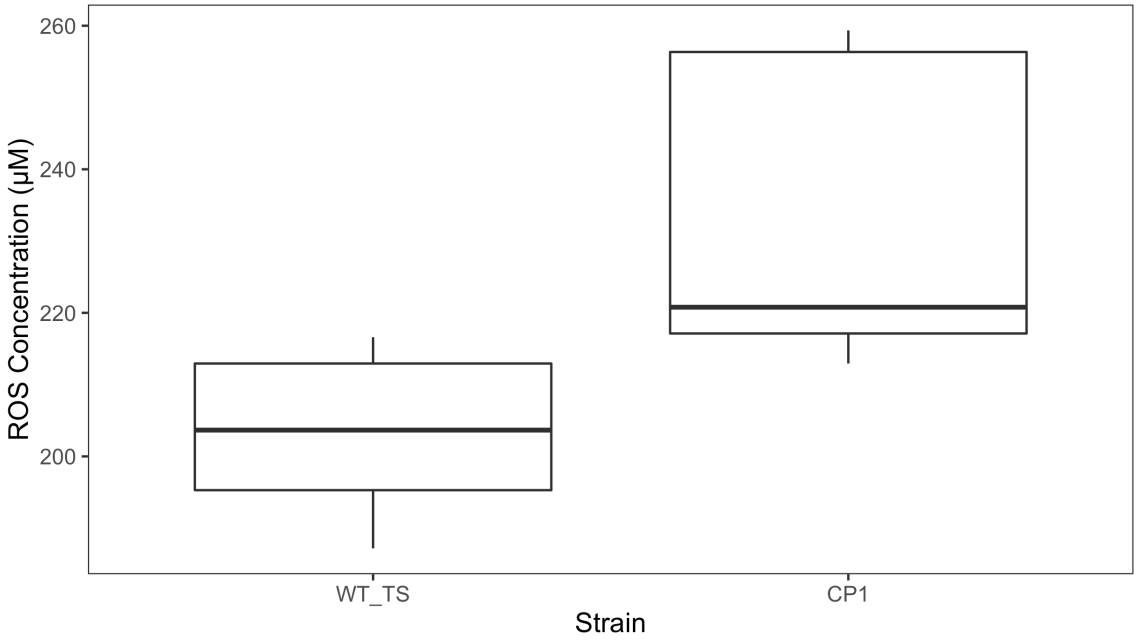

Percentage of Occluded Vessels
60
40
20
WT_TS
CP1
Strain

Figure 3.5. The number of occluded xylem vessels are reduced in *X. fastidiosa* inoculated OE-*CP1-like* vines compared to wild type vines. A. Cross sections of canes were examined 3 nodes above the point of inoculation 9 weeks after inoculation. A. Parental genotype Thompson Seedless inoculated with 1X PBS. B. Parental genotype Thompson Seedless inoculated with *X. fastidiosa*. C. OE-*CP1-like* vines inoculated with 1X PBS. D. OE-*CP1-like* vines inoculated with *X. fastidiosa*. B. The percentages of occluded vessels per vine were calculated. The OE-*CP1-like* vines showed significantly lower percentages of occluded vessels compared to the parental genotype Thompson Seedless vines (One Way Anova, $p < 0.00001$).

A.


260
240
220
200
ROS Concentration (μM)
WT_TS
CP1
Strain

B.

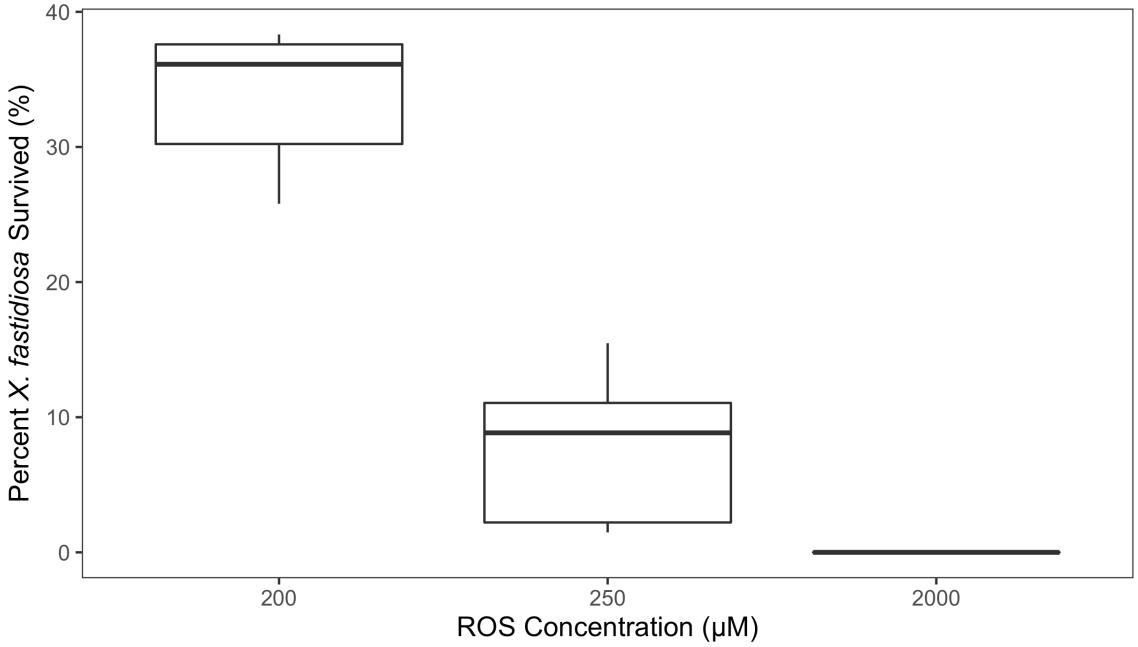

40
30
20
10
0
Percent X. fastidiosa Survived (%)
200
250
2000
ROS Concentration (μM)

Figure 3.6. ROS concentrations prior to *X. fastidiosa* challenge are significantly higher in OE-*CP1-like* vines. A. ROS concentrations were assessed at baseline prior to inoculation. OE-*CP1-like* vines produced significantly more ROS at baseline than parental genotype Thompson Seedless vines (One Way Anova, $p = 0.001056$). B. An approximate ROS concentration (250 μM) found in the OE-*CP1-like* vines was found to kill significantly more *X. fastidiosa* cells than the ROS concentration found in the parental genotype vines (200 μM)(Student's *t*-test, $p < 0.0001$). A ROS concentration of 2000 μM was used as a positive control to show no surviving *X. fastidiosa* cells.

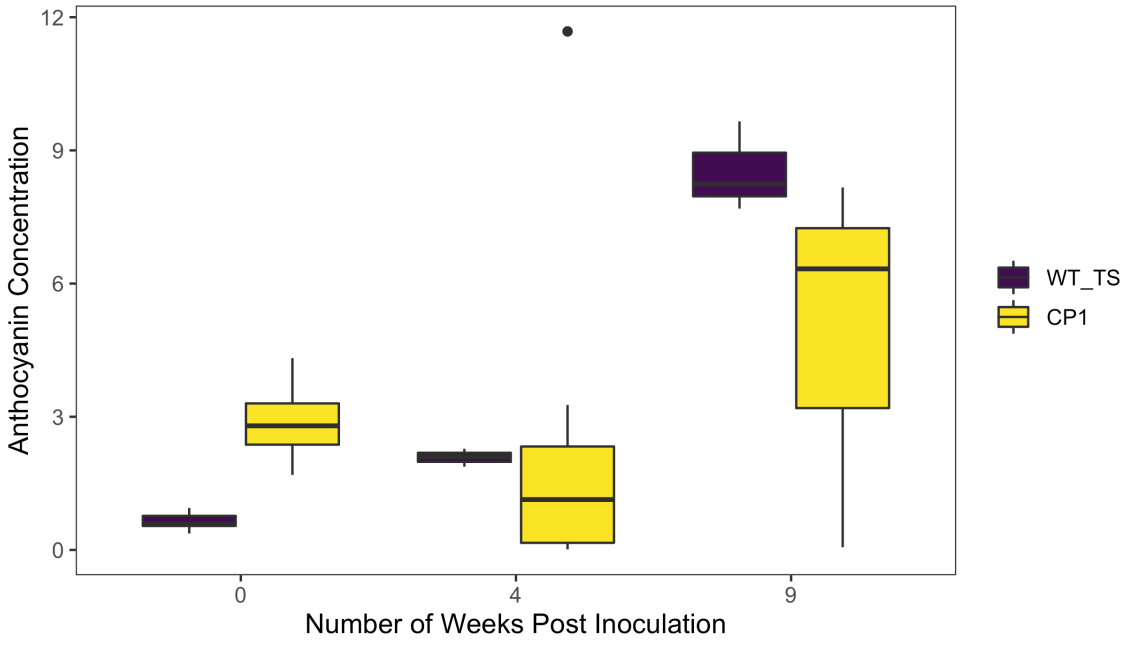


Figure 3.7. Anthocyanins significantly increased in wild type vines over time, but not in OE-*CP1-like* vines. Anthocyanin concentration was measured in vines at 0, 4, and 9 weeks post inoculation. Vines inoculated with 1X PBS experienced no difference in anthocyanin concentration over time (data not shown). OE-*CP1-like* vines produced more anthocyanin at baseline than parental genotype Thompson seedless vines (Student's *t*-test, $p < 0.001$). Parental genotype Thompson Seedless vines experienced a significant increase in anthocyanin concentration from week 0 to week 4 (Student's *t*-test, $p < 0.001$) and another significant increase in concentration from week 4 to week 9 (Student's *t*-test, $p < 0.001$). OE-*CP1-like* vines experienced no significant increase in concentration over time.

Chapter IV

In Vitro Activities of Copper Ions and Antibiotics on *Xylella fastidiosa*, the Causal Agent of Pierce's Disease of Grapevine

Abstract

Xylella fastidiosa is a Gram-negative, plant pathogenic bacterium capable of producing biofilms. *X. fastidiosa* biofilms play a role in occluding the xylem vasculature of grapevines which leads, in part, to impaired water transport and symptom development in Pierce's disease (PD) of grapevine. Copper treatments are commonly used for fungal and oomycete pathogens in vineyards, but they appear to have no effect on inhibiting the pathogenesis of *X. fastidio*sa *in planta* at concentrations below phytotoxic levels. Understanding how *X. fastidiosa* interacts with elements such as copper in their environment may elucidate how they behave and affect pathogenesis *in planta*. Based on recent findings showing that copper ions weaken antibiotic resistance in *Bacillus subtilis* NCIB 3610, we hypothesized that copper ions also weaken the resistance of *X. fastidiosa*'s biofilm to antibiotics. The objectives of this study were to evaluate the *in vitro* effects of copper ions and antibiotics on *X. fastidiosa*. We found a low concentration of copper significantly increases cell surface hydrophobicity, increases biofilm surface attachment, and reduces viable cells associated with biofilms in the presence antibiotics. A *Δwzy* mutant of *X. fastidiosa* with truncated lipopolysaccharide (LPS) structure and an *ΔengXCA2* mutant with a deregulation of exopolysaccharide (EPS) production, both of which have structurally altered biofilms, did not show a reduction in viable cells associated with biofilm.

Introduction

Xylella fastidiosa is a Gram-negative, xylem-limited bacterium that causes Pierce's disease (PD) of grapevine and several other diseases in economically important crops (Rapicavoli et al. 2018). PD symptoms (e.g. marginal leaf necrosis (leaf scorching)) are attributed in part to disruptions in the xylem vasculature (Guilhabert and Kirkpatrick. 2005). These disruptions are a result of both plant immune responses, such as tylose formation and pectin gel secretions, and the formation of biofilms by *X. fastidiosa* obstructing xylem vessels (Fry and Milholland 1990; Stevenson et al. 2004). Microbial biofilms provide microbes protection from host immune responses and antimicrobial compounds such as antibiotics (Sharma et al. 2019). As such, breaking down the protection provided by *X. fastidiosa* biofilms could be an effective means of reducing PD symptom severity.

Biofilms form as planktonic cells attach to a surface and mature into a community within complex structure of secreted components such as proteins, nucleic acids, and exopolysaccharides (EPS) (Muhammad et al. 2020; Sutherland 2001). Recently, it has been demonstrated that copper ions weaken the antibiotic resistance conferred by biofilms of *Bacillus subtilis* NCIB 3610 by making the biofilm structure smoother and hydrophilic (Garcia et al. 2020). While required in trace amounts by many microbes as a cofactor for redox-related enzymes, copper in higher amounts can be toxic to most microbes. As a result copper has been used as an antimicrobial agent over the course of

human history against diverse pathogens including fungi, oomycetes, and bacteria (Grass et al. 2011; Lamichhane et al. 2018; Schneider et al. 2019; Behlau et al. 2017).

Greenhouse applications of copper in model organisms such as *Nicotiana benthamiana* results in systemic distribution of copper including the xylem where concentrations reach 0.1 mM, before the plants begin to experience phytotoxicity. However, this low concentration is insufficient at reducing PD symptom severity (Ge et al. 2020). In fact, low concentrations of copper have been shown to induce *X. fastidiosa* biofilms. For example, in the Citrus Variegated Chlorosis-causing strain of *X. fastidiosa* subspecies *pauca*, subinhibitory concentrations of copper (<3 mM) induce genes that encode cell-cell adhesion proteins and repress genes that encode Type IV pilus proteins related genes, suggesting that *X. fastidiosa* cells stop moving and build biofilm in part of a resistance mechanism to copper (Muranaka et al. 2012). Similarly, it has been reported that the PD-causing strain *X. fastidiosa* subspecies *fastidiosa* also exhibits an increase in biofilm production at copper concentrations below 0.2 mM (Ge et al. 2020). However, it is unclear what secondary sensitivity *X. fastidiosa* may have to other compounds in the presence of copper.

The ability of copper to both induce biofilm formation in *X. fastidiosa* as well as potentially weaken the protection provided by biofilms as shown in *B. subtilis* (Garcia et al. 2020) raises questions about how copper interacts and changes *X. fastidiosa* biofilms. Since *X. fastidiosa* is a biofilm producer, we hypothesized that copper ions also weaken *X. fastidiosa*'s biofilm resistance to antibiotics. In this study, *in vitro* effects of three antibiotics, tetracycline, polymyxin B, and ampicillin, were observed in the presence and

absence of a sublethal concentration of copper. In order to begin to understand the role of the structural integrity of the biofilm plays in antibiotic resistance, two *X. fastidiosa* mutants with structurally altered biofilms, *Δwzy* mutant with a truncated O-antigen and *ΔengXCA2* EPS-overproducing mutant, were also examined.

Materials and Methods

Strain used in this study. *X. fastidiosa* subsp. *fastidiosa* strain Temecula1, *Δwzy* mutant, *Δwzy/+* complement, and *ΔengXCA2* were used in this study (Clifford et al., 2013; Ingel et al., 2019). The Wzy polymerase is responsible for polymerizing the O-subunit to produce the O-antigen chain of *X. fastidiosa*'s lipopolysaccharide (LPS) which functions as a barrier to antimicrobial compounds (Clifford et al. 2013). A mutation in the *wzy* gene results in a significantly shortened O-antigen, drastically changing an integral component of biofilm structure. β-1,4- endoglucanases are cell wall-degrading enzymes that hydrolyze β-1,4 glycosidic linkages. When one of these, *engXCA2*, is knocked out, there is an over-production of exopolysaccharide (EPS), causing a compromised biofilm structure (Ingel et al. 2019; Castro 2021). These strains were grown in PD3 liquid medium, PD3 solid medium, and PD3 liquid amended with varying concentrations of $CuSO_4$. Stocks of *X. fastidiosa* were stored in liquid PD3 medium plus 15% glycerol at -80°C.

Growth curves in the presence of copper. Wild type *X. fastidiosa* was grown on PD3 solid medium for 5 days at 28°C. Following this incubation, colonies were gently scraped from plates to prepare inoculum in PD3 liquid medium. The resulting cell suspension was collected in one 50 mL sterile conical tube and inoculum was prepared to OD_{600nm}= 0.25 (approx. 10^8 CFU/mL). Briefly, 4.5 mL of PD3 liquid medium in 30 x 10 mm glass culture tubes were inoculated with 0.5 mL of inoculum. Culture tubes were amended with 0.1 mM $CuSO_4$ to compare to no copper supplementation. The tubes were grown in a shaker incubator at 28°C for 7 days at 180 rpm. Optical density measurements were taken every 24 hours. The mean OD_{600nm} per time point was calculated and a comparison between treatments was made by calculating the area under the growth curves.

Cell-surface hydrophobicity assay in the presence of copper. Cell-surface hydrophobicity was assessed under copper free and 0.1 mM $CuSO_4$ conditions as previously described, with minor modifications (Rosenberg et al. 1980). Briefly, *X. fastidiosa* inoculum was prepared in the same manner as the growth curve assay and 0.5 mL of inoculum was inoculated into 4.5 mL of PD3 liquid medium in 30 mm glass culture tubes. Culture tubes were amended with 0.1 mM $CuSO_4$ to compare to no copper supplementation. The tubes were grown in a shaker incubator at 28°C for 7 days at 180 rpm. The absorbance at day 7 was measured to calculate the initial OD_{600nm} and 4.0 mL of this suspension were added to 1 mL dodecane and vortexed for 2 minutes. The suspensions were allowed to rest for 45 minutes. The absorbance of 200 µL of the

suspension from the very bottom of the tubes was measured to calculate the final OD_{600nm}. Percent hydrophobicity was calculated by the following equation:

$$((\text{Initial } OD_{600nm} - \text{Final } OD_{600nm})/ \text{Initial } OD_{600nm}) * 100$$

Surface attachment assay in liquid media in the presence of copper and antibiotics. Wild type *X. fastidiosa* was evaluated for its ability to attach to a glass surface under different concentrations of $CuSO_4$, antibiotics, or both, as previously described with some modifications (Espinosa-Urgel et al. 2000). Concentrations of antibiotics were chosen based on minimum inhibitory concentrations found in previously published work (Kuzina et al. 2006). The concentrations used for tetracycline, polymyxin B, and ampicillin were 4 µg/mL, 32 µg/mL, and 4 µg/mL, respectively. Briefly, *X. fastidiosa* inoculum was prepared as described in the growth curve assay and 0.5 mL of inoculum was added into 4.0 mL of PD3 liquid medium in 30 mm glass culture tubes. The tubes were grown in a shaker incubator at 28°C at 180 rpm for 2 days to initiate biofilm formation. Following this, culture tubes were amended with either $CuSO_4$, antibiotic, or both and incubated for 5 more days. To quantify biofilm attachment, 500 µL of filtered 1% crystal violet stain was added to the tubes and incubated at room temperature for 20 minutes before removing the crystal violet suspension and rinsing three times with deionized water. The stained, attached biofilm was detached in 30% acetic acid and OD_{600nm} of the eluent was measured. Values were normalized by dividing OD_{600nm} measurements by the mean of OD_{600nm} of the eluent of untreated samples.

Quantification of viable biofilm-associated *X. fastidiosa* cells in the presence of copper and tetracycline. *X. fastidiosa* inoculum was prepared as described in the growth curve assay and 0.5 mL of inoculum was added into 4.0 mL of PD3 liquid medium in 30 mm glass culture tubes. The tubes were grown in a shaker incubator at 28°C at 180 rpm for 2 days to initiate biofilm formation. Following this, culture tubes were amended with either 0.1 mM $CuSO_4$, tetracycline, or both and incubated for 5 more days. Liquid culture was removed and culture tubes were rinsed three times with deionized water. Attached biofilm cells were gently scraped into 1 mL of sterile 1X PBS. The suspension was then serially diluted and the series was plated on solid PD3 medium according to standard methods (Roper et al. 2007). Colonies were counted to quantify viable cells per treatment. The *Δwzy* mutant, *Δwzy/+* complement, and *ΔengXCA2* mutant were also analyzed in this manner.

Results

Low concentrations of $CuSO_4$ reduce growth, but are not completely lethal. The inhibitory capacity of a low concentration of copper was assessed to determine if it would not be completely lethal against wild type *X. fastidiosa*. Liquid PD3 medium was amended with 0 and 0.1 mM $CuSO_4$ concentrations, based on xylem concentrations of copper associated with non-phytotoxic symptoms (Ge et al. 2020). Growth was observed at both concentrations (Figure 1). The areas under the growth curve of cells grown in PD3 amended with 0.1 mM $CuSO_4$ (0.448 OD_{600nm} x Time) were significantly lower than cells grown in PD3 with no$CuSO_4$ (0.383 OD_{600nm} x Time), (Student's *t*-test, $p = 0.0404$).

Because 0.1 mM $CuSO_4$ was not completely lethal, it was chosen as the concentration for the proceeding experiments.

$CuSO_4$ increases the percentage of cell surface hydrophobicity and biofilm attachment. 0.1 mM $CuSO_4$ in liquid PD3 medium significantly increased the cell surface hydrophobicity, a factor associated with adhesion to abiotic and biotic surfaces, of planktonic *X. fastidiosa* cells (Figure 2). The percentage of cell surface hydrophobicity in liquid PD3 medium without copper was 14.6%, whereas the percentage of cell surface hydrophobicity in liquid PD3 medium amended with $CuSO_4$ was 23.2%. Cell-surface attachment was also significantly increased, qualitatively evidenced by a wider purple ring in glass culture tubes containing liquid PD3 medium amended with 0.1 mM $CuSO_4$ (Figure 3A and 3B). Quantitatively, the OD_{600nm} of crystal violet eluent of attached biofilm grown in 0.1 mM $CuSO_4$ was significantly increased compared to the OD_{600nm} of the crystal violet eluent of attached biofilm grown in absence of $CuSO_4$ (Figure 3C)(Student's *t*-test, $p < 0.00005$).

Activities of sublethal concentrations of $CuSO_4$ and antibiotics on *X. fastidiosa*. Liquid cultures of *X. fastidiosa* were treated with an antibiotic (tetracycline, polymyxin B, ampicillin), with and without 0.1 mM $CuSO_4$. In combination with each antibiotic, the sublethal concentration of $CuSO_4$ significantly reduced biofilm attachment to a glass surface compared to a treatment with antibiotic alone (Figure 4). For tetracycline, the average normalized OD_{600nm} of treatment with antibiotic alone was significantly higher at

0.88 compared to a treatment of both antibiotic and copper at 0.65 (Student's *t*-test, p = 0.00108). For polymyxin B, the average OD_{600nm} of a treatment with antibiotic alone was significantly higher at 1.13 compared to a treatment of both antibiotic and copper at 1.01 (Student's *t*-test, p = 0.00682). For ampicillin, the average OD_{600nm} of a treatment with antibiotic alone was significantly higher at 0.93 compared to a treatment of both antibiotic and copper at 0.79 (Student's *t*-test, p = 0.0253).

Finally, the ability of copper to reduce the number of biofilm-associated *X. fastidiosa* cells in combination with tetracycline was also assessed. The number of colony forming units (CFUs) for wild type *X. fastidiosa* cells treated with tetracycline alone (2.98×10^5 CFUs) was significantly higher than a treatment of antibiotic and copper (3.4×10^4 CFUs) (Student's *t*-test, $p < 0.0001$). The number of CFUs for the *Δwzy* mutant treated with tetracycline alone (3.0×10^4 CFUs) was not significantly reduced with the addition of 0.1 mM $CuSO_4$ to the antibiotic treatment (2.94×10^4 CFUs). Similarly, the number of CFUs for the *ΔengXCA2* mutant treated with tetracycline alone (3.8×10^4 CFUs) was not significantly reduced with the addition of 0.1 mM $CuSO_4$ to the antibiotic treatment (4.3×10^4 CFUs). However, the *Δwzy*/+ complement did show a reduction in CFUs from cells treated with tetracycline alone (2.12×10^4 CFUs) to cells treated with both (9.2×10^3 CFUs)(Student's *t*-test, $p < 0.001$) (Figure 5).

Discussion

Understanding *X. fastidiosa* biofilms, their development, and the potential changes they can undergo based on their environment *in vitro* may elucidate how they

behave and interact with their environment *in planta*. Here, we have demonstrated that a sublethal concentration of $CuSO_4$ in combination with antibiotics significantly reduces biofilm surface attachment. During five days of growth, there is less biofilm development and with less biofilm development, there is a decrease in the number of biofilm-associated viable cells.

A concentration of 0.1 mM $CuSO_4$ significantly increased the cell surface hydrophobicity of the cells, which is often correlated with surface attachment and has been shown to typically yield more biofilm formation across several different bacterial species such as *Pseudomonas putida* and *Staphylococcus* species (Krasowska et al. 2014; De la Pinta et al. 2019; Krepsky et al. 2003). Indeed, we found a significant increase in biofilm formation at 0.1 mM $CuSO_4$, suggesting that this increase in cell surface hydrophobicity may contribute to an increase in biofilm formation.

However, in combination with the application of the antibiotics tetracycline, polymyxin B, and ampicillin, the addition of 0.1 mM $CuSO_4$ significantly reduced biofilm surface attachment of *X. fastidiosa* when compared to treatment with the antibiotic alone. For tetracycline in particular, the number of viable *X. fastidiosa* cells associated with the biofilm was significantly reduced. Interestingly, there is not a reduction in viable *Δwzy* and the *ΔengXCA2* mutant cells, both of which are known to have structurally compromised biofilms (Clifford et al. 2013; Castro 2021). A reduction in viable cells is observed in the *Δwzy/+* complement, suggesting that copper may be interacting with a structural component such as the LPS, EPS, or both to decrease cell viability.

Δwzy mutant cells have a truncated O-antigen and form thinner biofilms (Clifford et al. 2013). We speculate that the truncated O-antigen may leave the cells more vulnerable to antibiotics because there is less of a physical barrier between the environment and the cells. Interestingly the *ΔengXCA2* also has a structurally impaired biofilm. The *ΔengXCA2* overproduces EPS which we speculate leads to an unstable and loose biofilm (Castro 2021), which may increase exposure of the biofilm-associated cells to antibiotics as well. In wild type *X. fastidiosa* biofilms, copper ions might interact with these structural components to increase the exposure of cells to the antibiotics.

Further studies are needed to assess whether or not cells locked in a biofilm state are more susceptible to antibiotics in the presence of copper. It is possible that the reductions observed in these assays are due to different rates of recruitment of planktonic cells to the biofilm because they are being killed by the antibiotics or their behavior is being altered by the antibiotics. In order to assess a differential susceptibility of cells within biofilms, one could grow the biofilm with no amendments, remove the planktonic cells, and replace with sterile media containing treatments of antibiotics or a combination of antibiotics and copper.

Copper homeostasis genes can also play a role in sensitivity to copper, which itself is antimicrobial against *X. fastidiosa* at high concentrations (Rodrigues et al. 2008). When *X. fastidiosa*'s copper homeostatic genes, *copA* and *copB,* are knocked out; both mutants show an increase in susceptibility to copper ions at concentrations above 0.15 mM $CuSO_4$. However, below 0.140 mM $CuSO_4$, these mutants act similarly to wild type

X. fastidiosa, in that they also exhibit an increase in biofilm formation compared to a no copper treatment (Ge et al. 2021).

In this study, we report that a sublethal concentration of copper increases cell-surface hydrophobicity. We found that on its own, a concentration of 0.1 mM $CuSO_4$ increases biofilm formation, however significantly reduces biofilm surface attachment in the combination with tetracycline, polymyxin B, and ampicillin compared to antibiotic treatment alone. Furthermore, this sublethal concentration of copper significantly reduces the number of viable wild type *X. fastidiosa* cells associated with the biofilm to tetracycline, but not to two mutants of *X. fastidiosa* with compromised biofilm structures. Future studies will elucidate whether or not the addition of copper enhances the susceptibility of cells locked in a biofilm state to antibiotics.

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Figures

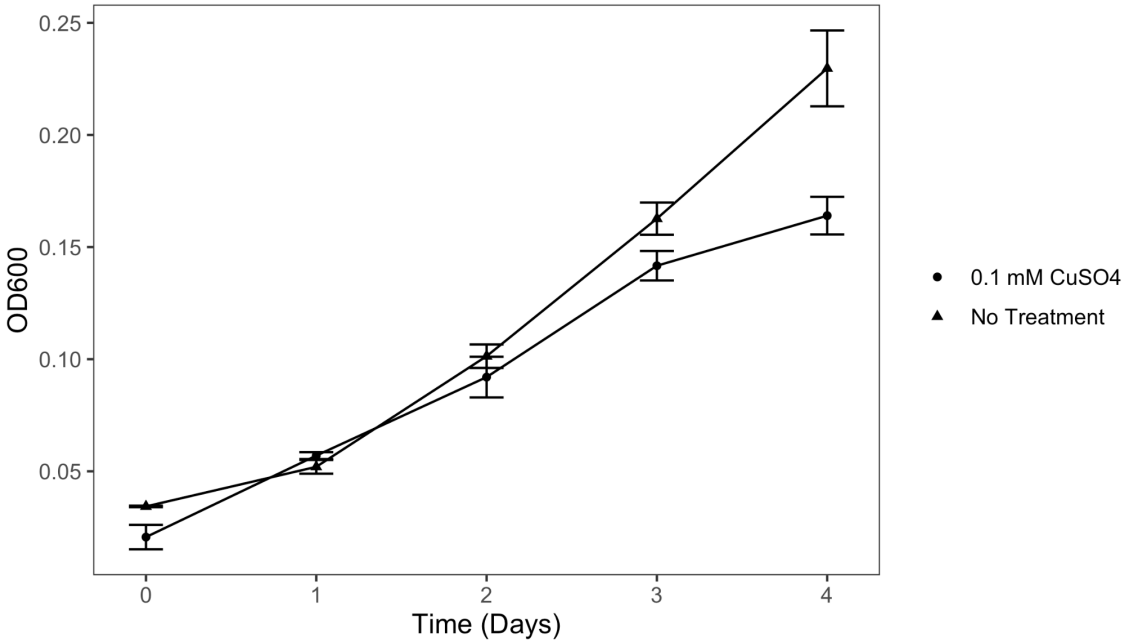

Figure 4.1. Growth of *X. fastidiosa* in PD3 liquid medium amended with $CuSO_4$ is reduced compared to growth in PD3 liquid medium in the absence of $CuSO_4$. The addition of 0.1 mM $CuSO_4$ in the liquid PD3 reduced growth, but it was not completely lethal to *X. fastidiosa* in liquid culture. Area under the growth curve analysis indicated a significantly reduced growth rate (Student's *t* test, $p < 0.5$).

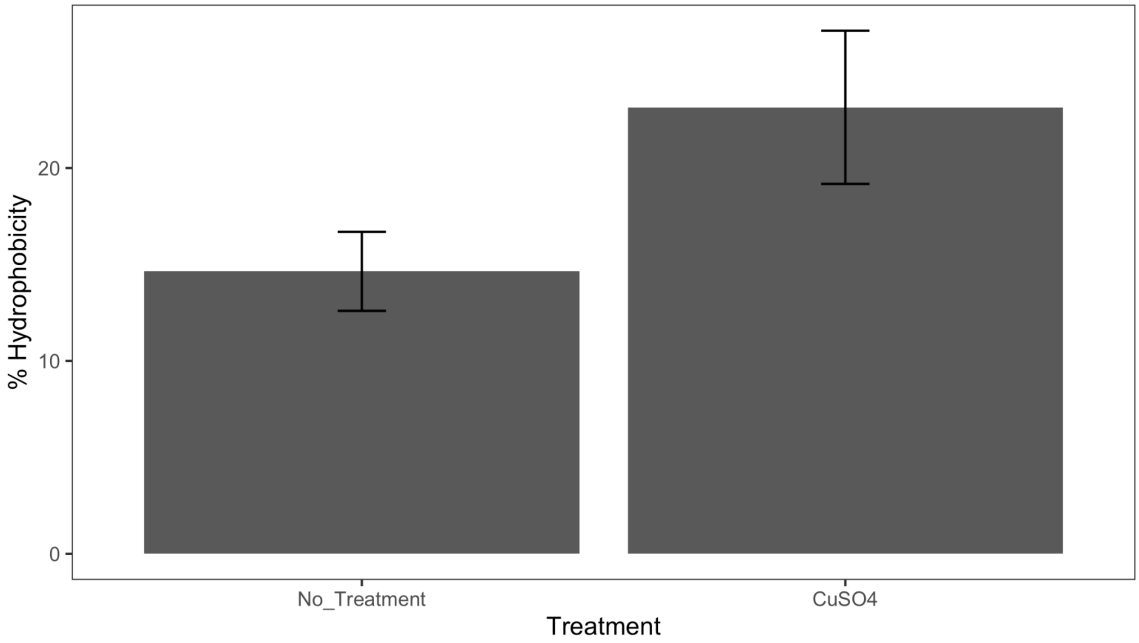

Figure 4.2. $CuSO_4$ increased *X. fastidiosa* cell surface hydrophobicity. The addition of 0.1 mM $CuSO_4$ in the liquid PD3 significantly increased the hydrophobicity of the planktonic cells compared to cells grown in liquid PD3 without $CuSO_4$ (Student's *t*-test, $p < 0.05$).

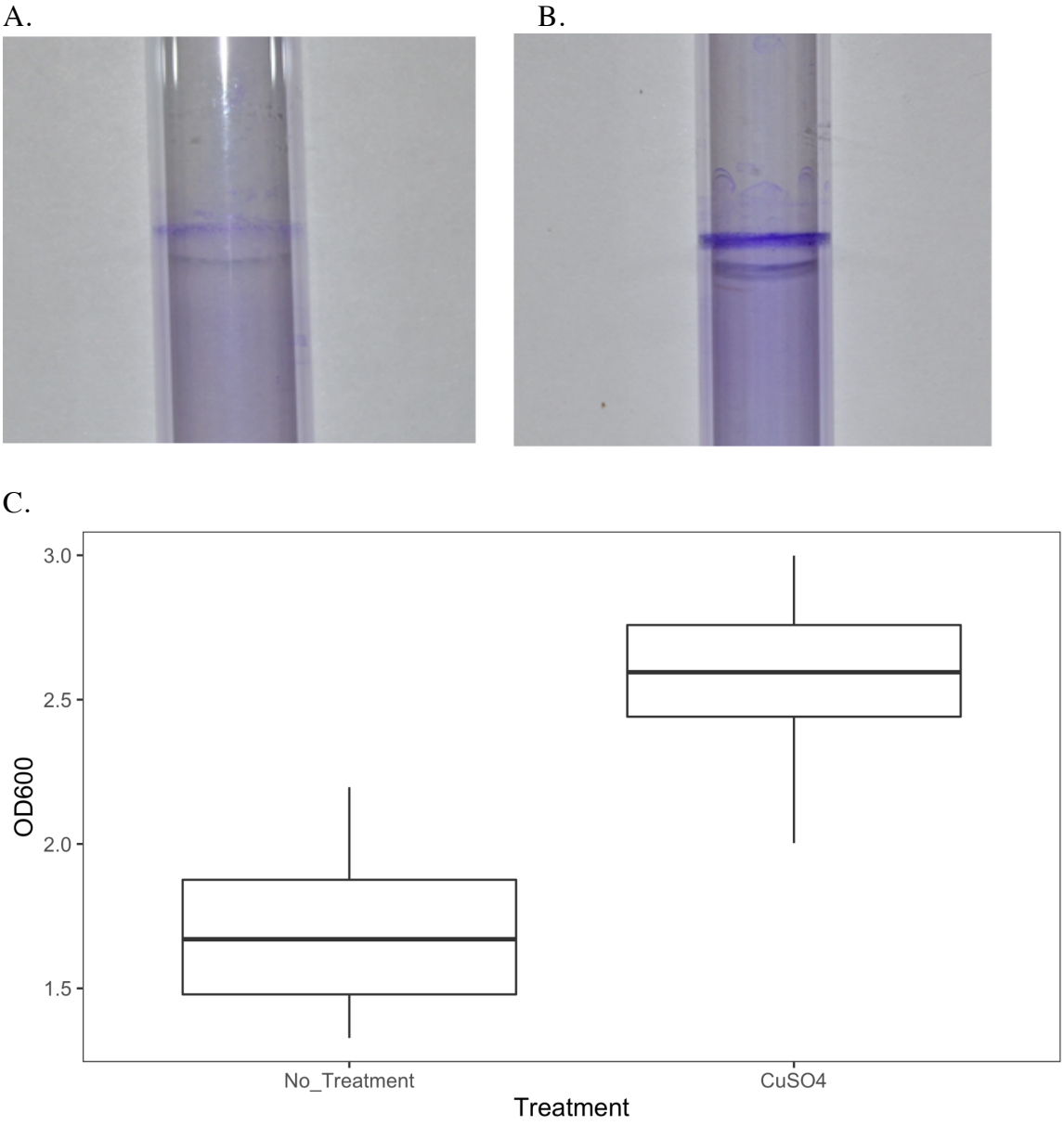

Figure 4.3. $CuSO_4$ enhances attachment of wild type *X. fastidiosa* to a glass surface. A. Attached cells grown in 0 mM $CuSO_4$ stained with 1% crystal violet. B. Attached cells grown in 0.1 mM $CuSO_4$ stained with 1% crystal violet. C. The quantification of crystal violet eluted from the attached cells grown under both copper conditions. The attachment of wild type *X. fastidiosa* cells significantly increased in liquid PD3 amended with 0.1 mM $CuSO_4$ compared to 0 mM $CuSO_4$ (Student's *t*-test, $p < 0.00005$).

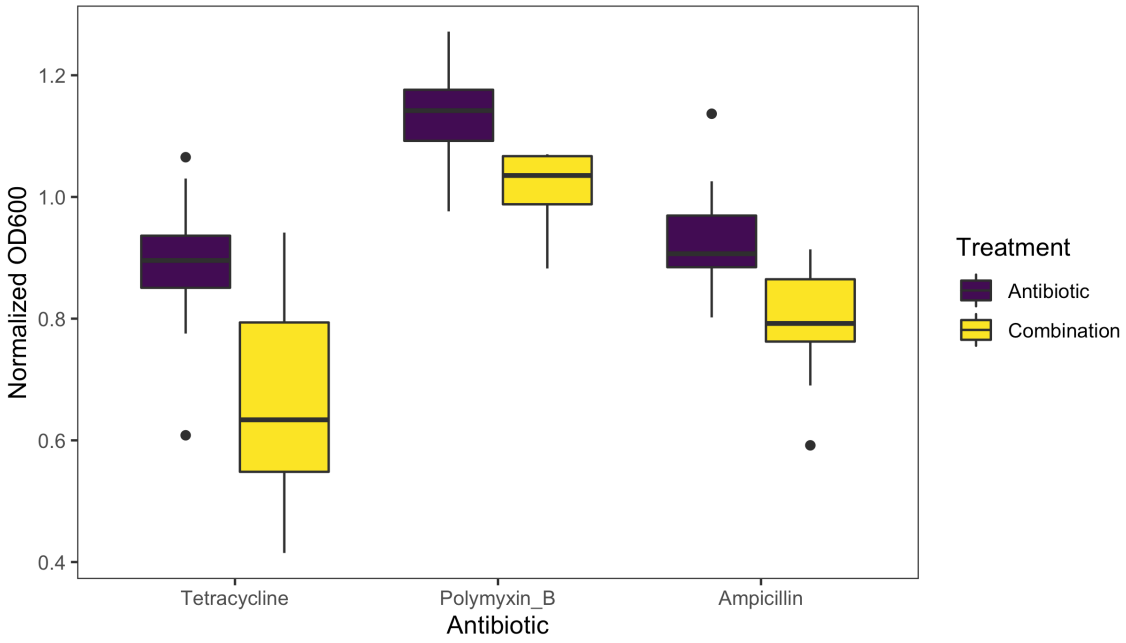

Figure 4.4. $CuSO_4$ in conjunction with antibiotics significantly decreased attachment to a glass surface compared to antibiotic treatment alone. Cultures of *X. fastidiosa* were treated with antibiotic or antibiotic and 0.1 mM $CuSO_4$. Biofilms were stained with 1% crystal violet for visualization and subsequent optical densities were measured for biofilm quantification and were normalized to no treatment samples. For each antibiotic (tetracycline, polymyxin B, ampicillin), the sublethal concentration of copper significantly reduced cell surface attachment in combination with the antibiotic compared to antibiotic treatment alone (For Tetracycline: Student's *t*-test, p = 0.00108; For Polymyxin B: Student's *t*-test, p = 0.00682; For Ampicillin: Student's *t*-test, p = 0.0253).

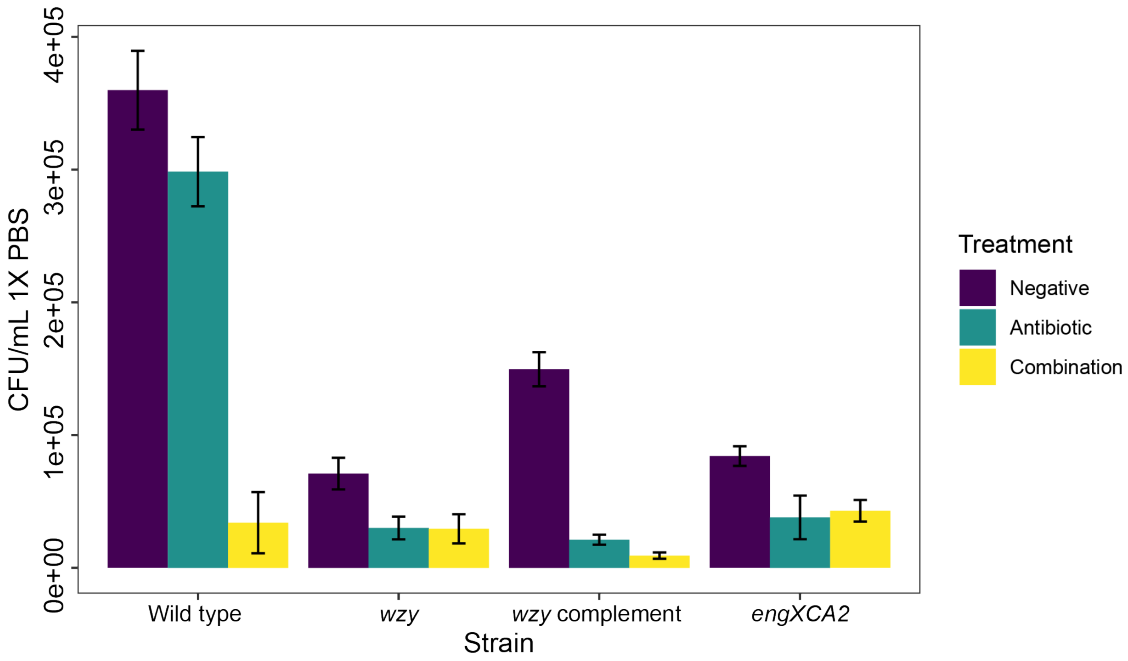

Figure 4.5. $CuSO_4$ in combination with tetracycline reduces the number of viable wild type *X. fastidiosa* cells compared to treatment with tetracycline alone, but does not for mutants with structurally compromised biofilms. Cultures of each strain were grown and treated with antibiotic or antibiotic and 0.1 mM $CuSO_4$. No significant difference was found between treatments of antibiotic or antibiotic and 0.1 mM $CuSO_4$ with biofilm structural mutants *Δwzy* and *ΔengXCA2*. However, a significant reduction in the number of cells was observed between treatments of antibiotic or antibiotic and 0.1 mM $CuSO_4$ in *Δwzy/+* complement strain (Student's *t* test, $p < 0.001$).

Appendix

Xylella fastidiosa: A Re-emerging Plant Pathogen that Threatens Crops Globally

Castro C, DiSalvo B, Roper MC. Xylella fastidiosa: A reemerging plant pathogen that threatens crops globally. PLoS Pathog. 2021;17: e1009813.

Xylella fastidiosa is a fastidious, gram-negative bacterium in the family *Xanthomonadaceae* and is a major threat to agricultural crops and ecological and ornamental landscapes in the world. This bacterium is quite remarkable in regards to its very broad host range that includes over 600 plant species belonging to 63 diverse plant families. It is specifically limited to the xylem tissue of its plant hosts [1]. In some of these hosts it causes severe and devastating disease. However, in the vast majority of its hosts it is considered a benign commensal.

X. fastidiosa is endemic to the Americas. Historically, Europe was considered to be free of *X. fastidiosa*, but the bacterium was recently detected in Italy. In 2013, olive trees in the Apulia region of southern Italy began exhibiting leaf scorch symptoms that were later confirmed to be caused by *X. fastidiosa*. Since then, thousands of olive trees have died and *X. fastidiosa* has been detected in plants in France, Spain, and Portugal [1–3]. *X. fastidiosa* is responsible for significant economic losses in regions like the US, Italy, and Brazil. For example, *X. fastidiosa* subsp. *fastidiosa*, the causal agent of Pierce's disease of grapevine, leads to crop losses of approximately $104 M and costs growers approximately $50 M in preventative strategies each year for the California viticulture industry [4,5]. In the Apulia region, *X. fastidiosa* subsp. *pauca* infection in olive orchards is projected to cost Italy up to €5.2 billion over the next 50 years if trees are not replaced [6]. Current management strategies to minimize *X. fastidiosa* spread in the field include removal of infected plants, severe pruning, and control of insect vectors with insecticides. The development of resistant plant lines is an active area of research with the goal of devising a sustainable and long-term solution to help control the spread of *X. fastidiosa*.

Recently, five new PD resistant grape varieties were commercially released to the grape industry [7].

The *X. fastidiosa* species is subdivided into multiple subspecies that include subsp. *fastidiosa*, *multiplex*, *pauca*, and *sandyi* [8]. The subspecies designations are loosely associated with host range. In general, disease symptoms associated with these *X. fastidiosa* strains are characterized by marginal leaf necrosis or leaf scorching like those observed in grapevines infected with *X. fastidiosa* subsp. *fastidiosa*. However, symptoms caused by *X. fastidiosa* subsp. *pauca* can be characterized by foliar wilt and interveinal chlorosis and symptoms caused by *X. fastidiosa* subsp. *multiplex* can exhibit dense canopies and reduced fruit size [1]. *X. fastidiosa* has no free-living component of its lifestyle and has only been found associated with its plant and insect hosts.

Xylella fastidiosa has a unique association with its xylem sap-feeding insect vectors.

X. fastidiosa is obligately vectored by xylem-feeding hemipteran insects primarily belonging to the sharpshooter leafhopper (*Cicadellidae*) and spittlebug (*Cercopidae*) families (Figure 1) [9–11]. These insects are polyphagous (i.e., they feed on many plant species) and are present in warm regions across the globe [11]. *X. fastidiosa* is acquired when the insect feeds on the xylem sap of an infected plant. The bacteria colonize and multiply in the insect foregut (mouthparts) in a persistent, but non-circulative manner [10,12]. This type of pathogen-vector relationship is unique among insect-vectored plant pathogens because the bacterial cells propagate within the insect mouthparts but do not circulate throughout the body of the insect, whereas most propagative pathogens circulate

within the insect. When sharpshooters feed on the xylem of infected vines, *X. fastidiosa* attaches to and colonizes the insect foregut where it forms adhesive biofilms (Figure 1). *X. fastidiosa* experiences extreme shear stress during the xylem sap ingestion and egestion processes that occur during insect feeding. During transmission into a healthy vine, bacterial cells dislodge from the insect foregut, presumably as a result of the high shear stress created during feeding, and are deposited directly into the xylem of healthy vines [13]. There is no apparent specificity between a particular *X. fastidiosa* subspecies and insect vector species. In fact, the glassy-winged sharpshooter (GWSS) (*Homalodisca vitripennis*) can acquire more than one *X. fastidiosa* subspecies in its foregut and can potentially transmit these strains to a variety of plants where the bacterium can behave as pathogen or a commensal endophyte [2,14].

In the context of Pierce's disease of grapevine caused by *X. fastidiosa* subsp. *fastidiosa*, the pathosystem with the broadest literature base, the two xylem-feeding insects transmit *X. fastidiosa* that have received the most research focus are the blue-green sharpshooter (BGSS) (*Graphocephala atropunctata*) and the GWSS. The BGSS is native to riparian areas in California and feeds on new plant growth that emerges in the spring [9,10]. The GWSS is invasive to California and is thought to have been introduced from the southeastern United States into Southern California on infested nursery stock in the late 1990s. The introduction of this invasive pest drastically changed the epidemiology of PD in the southern part of California because GWSS can feed on both green and dormant woody tissues meaning that transmission can occur even in winter. In addition, GWSS can fly longer distances than native sharpshooter species, which could

explain how PD incidence was elevated to epidemic proportions in Southern California. Subsequently, there has been a concerted effort among growers and the California Department of Food and Agriculture to control populations and prevent the spread of GWSS. The predominant vector linked to Olive Quick Decline Syndrome in Italy is the meadow spittlebug, *Philaenus spumarius* [15].

Xylella fastidiosa **colonizes host compartments that are primarily non-living.**

As far is presently known, *X. fastidiosa* interacts primarily with non-living tissues in both its insect and plant hosts. These include the cuticular surface of the insect foregut and the plant xylem, which is non-living at maturity (Figure 1). The xylem consists of a network of vessels that are connected by pit membranes. These are thin, porous structures composed of primary plant cell wall, which allow for the passage of water but prevent the movement of pathogens and air embolisms. *X. fastidiosa* produces plant cell wall degrading enzymes, a polygalacturonase and several endoglucanases, that act in concert to degrade pit membranes allowing *X. fastidiosa* to move from vessel to vessel to achieve systemic colonization [16–18]. Interestingly, *X. fastidiosa* does not possess a Type III secretion system (T3SS) typical of other pathogenic bacteria that that injects cognate Type III effectors into living host cells, likely because the bacterium interacts primarily with non-living cells. Instead of relying on T3SS effectors to bypass host immunity, *X. fastidiosa* delays early plant recognition in grapevines by camouflaging itself with a rhamnose-rich O-antigen, the most external portion of its lipopolysaccharide layer as one mechanism that allows it to skirt initial triggering of the grape immune system to

establish itself in the plant [19]. It is not known which living plant tissues are primarily responsible for initiating and propagating a response to *X. fastidiosa*, but it is likely the living xylem parenchyma cells adjacent to the xylem vessels.

One of the remarkable internal symptom phenotypes of infected grapevines is the prolific production of tyloses in response to *X. fastidiosa* colonization of the xylem (Figure 1). Tyloses are outgrowths of the living xylem parenchyma cells that protrude into the xylem and are part of the plant defense response. Their role, in part, is to slow or prevent pathogen movement within the xylem. However, overproduction of tyloses can cause a reduction in hydraulic conductivity within the xylem that is detrimental to the plant [20,21]. In PD-infected vines, tyloses become the dominant form of xylem occlusion during the early stages of disease and as a consequence, infected vines have a significant loss in hydraulic conductivity. Tyloses exacerbate PD symptoms and it is thought that this uncontrolled production of tyloses is what ultimately leads to the quick demise of the plant [22].

Another notable feature of *X. fastidiosa's* behavior *in planta* is the manner in which it regulates its own biofilm formation as it colonizes the xylem. In general, entering into and maintaining robust biofilms is linked to promoting virulence for many bacterial pathogens [23]. On the contrary, mutant strains of *X. fastidiosa* that are impaired in biofilm formation and effectively locked in a planktonic phase have a hypervirulent phenotype in grapevines [24–27]. Thus, it is speculated that *X. fastidiosa* enters the surface adhesive biofilm state as a means to attenuate its own virulence by controlling its movement *in planta* by adhering to the xylem wall. This self-limiting behavior during

parasitism in symptomatic/susceptible hosts may be a remnant from its lifestyle as a commensal in non-symptomatic hosts where tightly regulating and limiting rapid movement in the plant would promote a commensal interaction rather than a parasitic interaction.

X. fastidiosa acts as both a commensal and a pathogen depending on its host environment.

The bulk of the research on *X. fastidiosa* is biased towards isolates that are pathogenic in economically important hosts. The mechanism by which *X. fastidiosa* causes disease only in certain hosts, but not others has not been fully elucidated and its interactions with commensal hosts is largely understudied. However, it is speculated that compatibility between xylem pit membrane carbohydrate composition and *X. fastidiosa*-secreted cell wall-degrading enzymes mediate disease onset and progression [18,28]. In addition, the O-antigen is a critical component in evading initial immune recognition in the susceptible grapevine immune system and it is tempting to speculate that O-antigen composition dictates the type of symbiotic association with the plant-commensalism vs. parasitism [19]. Understanding the mechanisms that underlie how different *Xylella*-plant host interactions skew towards parasitism or commensalism is an area of research that is ripe for exploration.

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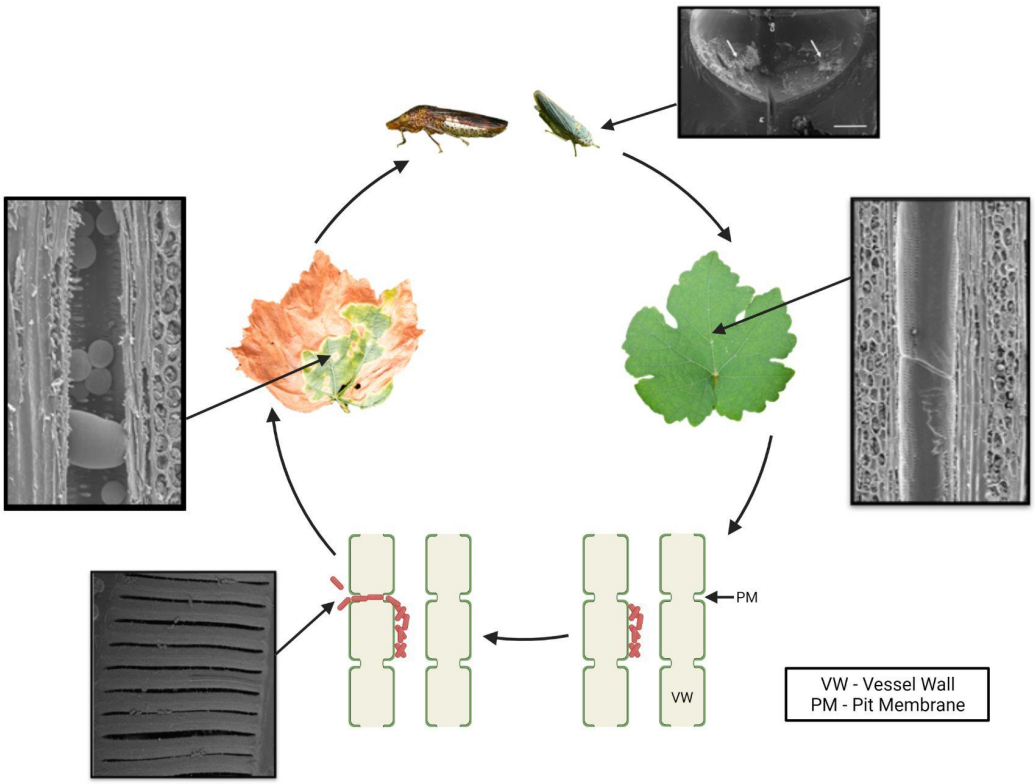

Figure 1. Pierce's disease of grapevine cycle. *Xylella fastidiosa* is acquired by its xylem-feeding insect vectors, such as the glassy-winged sharpshooter and the blue green sharpshooter, during the feeding process. Once acquired it colonizes the insect's foregut and forms robust biofilms (indicated by white arrows). *X. fastidiosa* is transmitted to a new host plant when the insect vector feeds on a new plant and deposits *X. fastidiosa* cells directly into the plant xylem. *X. fastidiosa* achieves systemic colonization of the xylem by enzymatic degradation of the xylem pit membranes that connect adjacent xylem vessels. *X. fastidiosa* colonization induces prolific production of balloon-shaped defense-related protrusions called tyloses in the xylem. Systemic colonization and vessel occlusion by bacterial biofilms and excess tylose production lead to PD symptom

development. Photo credit for the blue green sharpshooter: Rodrigo Krugner. Photo credit for the xylem longitudinal sections: Qiang Sun. Pit membrane photo reprinted from Ingel et al., 2019, Molecular Plant-Microbe Interactions Vol. 32, No. 10: 1402-1414. Insect foregut image reprinted from Rapicavoli et al., 2015, Applied and Environmental Microbiology Vol 81, No. 23: 8145-8154.