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Mining the Endophytic Microbiome and Leveraging the Pedigrees of *Vitis vinifera* for Integrated Management Strategies of Pierce's Disease

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Dedication

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ABSTRACT OF THE DISSERTATION

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Agricultural chemicals have been an invaluable tool to fertilize crops and manage diseases, but as consumer demand for sustainable and safer products continue to rise, its becoming imperative to find alternative management strategies that are environmentally sustainable and safe to humans. Grapevine (*Vitis vinifera L.*) is a staple crop to California agriculture and is valued at $6.25 billion. This perennial plant is plagued by Pierce's Disease (PD), caused by the xylem limited bacterium, *Xylella fastidiosa*. Under field conditions, grapevine varieties can exhibit a spectrum of disease phenotypes and some vines appear to escape the disease (i.e., no to little symptoms) . We hypothesize that the endophytic microbiome as well as the variety of the host may impact disease outcome. Harnessing this knowledge can lead to integrated and sustainable approaches for agriculture.

We present the first comprehensive culture-independent microbial study of an agricultural crop, including previously unexplored niches: sap and wood. Our results indicate that the majority of endophytes are derived from the rhizosphere. Disease, season, year, and biocompartment all play crucial roles in microbial assemblage. However, a core microbiome

shapes the backbone of the grapevine endosphere. Our results showed that two endophytes native to California grapevines, *Pseudomonas* and *Achromobacter* negatively correlated with *Xylella*. We developed novel pathogen-specific qPCR primers to assess *X. fastidiosa* population *in planta*. *In planta* bioassays indicated that 'Thompson Seedless'exhibited the highest pathogen titre, disease ratings and lowest stem hydraulic conductivity; suggesting a cultivar's pedigree and underlining xylem anatomy shape disease susceptibility. *In planta*, *P. viridiflava* and *A. xylosidans* showed protective qualities against *X. fastidiosa*. *In vitro* assays were conducted to elucidate potential microbe-pathogen interactions and indicated that the mechanism of protection is not through direct antagonism but possibly via alteration of plant-pathogen interaction. In conclusion, this research elucidates the endophytic grapevine microbiome and deepens our understanding of grapevine-*Xylella*-microbe relationship, producing two potential biocontrols and the foundation to new management strategies fo Pierce's Disease.

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Chapter 1

Introduction

1.0.1 The Current State of Pesticide for Agricultural Systems

Bordeaux mixture (copper-based formulation) discovered in the 19th century in France by Dr. Millardet to control grapevine downy mildew, was the first commercial fungicide used in agriculture. Since then, a countless number of synthetic agrochemicals have been developed and commercialized. It has been estimated that in 2016 the four largest agricultural producers, USA, EU, China and Brazil use 827 million, 831 million 1.2 billion and 3.9 billion pounds of pesticides respectively (Donley, 2019). Those resources are largely deployed to manage plant diseases and pests that account for 20-30% in crop losses annually(Savary *et al.*, 2019). The intensification of agriculture coupled with the broad adoption of conventional chemicals by Ag industries has had a measurable environmental impact including erosion of biodiversity, increase of greenhouse gas emissions (Syed Ab Rahman *et al.*, 2018), water contamination and impact on human health (Pathak *et al.*, 2017), (UNEP, n.d.), (Pimentel & Burgess, 2014). In addition, the research showed that

25-95% of chemical pesticides are off-target (Sarigiannis *et al.*, 2013; Zadoks & Waibel, 2000) and overuse application on crops has selected for resistance among many pathogen populations (Defoirdt *et al.*, 2010)(Langdon *et al.*, 2016)(REX Consortium, 2013).

Public health and environmental pollution, off target effects, high productivity costs and rise in pathogen resistance has exacerbated the need for an alternative approach. To sustain our current population growth, agriculture must double its output in the next 30 years while coping with the challenge of climate change, water scarcity and land availability (Balasubramanian, 2017). Moreover, the growing consumer demand for organic food/food products and safer agricultural practices has been driving the market demand towards alternative management strategies with lower environmental impact. Alternative and integrative methods such as probiotics and prebiotics are becoming increasingly necessary to combat pathogens both for human health, food security and environmental stability as we transition into a different farming philosophy.

1.0.2 Integrated Pest and Disease Management

Disease development is often thought of as the interaction of four major variables: (i) a susceptible host, (ii) a favorable environment, (iii) a viable/prevalent pathogen and (iv) appropriate time (Scholthof, 2007). Manipulating or altering any one of these variables could result in a less severe epidemic or avoidance of disease entirely. Pathogens and their reservoirs/vectors can be directly targeted with pesticide applications to reduce viability and inoculum levels(Kyrkou *et al.* 2018; Hopkins and Purcell, 2002). However, as previously discussed, this tactic may be expensive, inefficient and harmful. In the 1970s, a holistic approach, known as integrated pest and disease management (IPDM) was intro-

duced which looked to assimilate multiple approaches that targets several or all disease variables in a way which minimized risk for people and the environment. UC ANR defines this term as an ecosystem-based strategy that focuses on long-term prevention of pests or their damage through a combination of techniques such as biological control, habitat manipulation, modification of cultural practices, and use of resistant varieties (UC ANR Statewide IPM Program, 2020). In this tactic, cultural practices may include crop rotation, irrigation alterations, and adjusting harvest/planting times (Altieri *et al.*, 1996).

Microbes and their host have a deep bond forged by years of coevolving; molding mutualistic or antagonist relationships between any number of interconnected partners. Human intervention by means of selective breeding, geographical distribution of plant material, neglect of soil nutrients, or misunderstanding microbial needs has unintentionally severed this bond and can often result in dysbiosis of the plants; resulting in a myriad of negative effects(Singh and Trivedi, 2017). Unsurprisingly, management strategies have been shown to significantly alter the microbiome(Campisano *et al.* 2014). Due to the microbiomes capability to assist in plant growth, yield, pathogen mitigation, disease suppression and to change with management strategy and individual crop varieties; the plant native microbiome is now recognized as a valuable part of IPDM.

Harnessing these interactions can lead to microbiome manipulation and increase/restore symbiosis; ultimately leading to higher quality/quantity of product, control of pests and pathogens , and improve crop fitness (Singh and Trivedi 2017). Likewise, understanding crop susceptibility to a pathogenic members of the microbiome can lead to better breeding programs and more resistant/tolerant varieties can be utilized in a variety of geographical

locations (Riaz *et al.* 2018). Unveiling the dynamic crop-pathogen-microbiome interactions as it pertains to disease development over time and understanding the unique microbial fingerprint through geographical space and crop varieties is a holistic approach to finding competitive and effective biological control agents for new and integrated disease management strategies. Mining the innate microbiome and leveraging host pedigree for agriculture manipulation provides a sustainable and integrated approach to prevent or to keep diseases below the point of economic injury .

1.0.3 Mechanisms of Microbial Symbiosis and Plant Protection

There is compelling evidence that microbes influence plant health and productivity much like they do with humans. Unraveling the function and inhabitants of the native plant microbiome could lead to the discovery of new plant pro-and prebiotics. Potential plant pro- and prebiotics may act antagonistically towards the pathogen and/or symbiotically with the host. Antagonism may occur through antibiosis, parasitism, activation of plant defenses, competition for nutrients and niche exclusion of pathogens (Sunar *et al.*, 2015; Turner *et al.*, 2013; Zarraonaindia *et al.*, 2015)(Sarrocco & Vannacci, 2017). Symbiotically, microbes can stimulate plant growth through production of plant hormones, priming the host immune systems, solubilization of phosphate, nitrogen fixation, increase root architecture, soil fertility, carbon and nutrient cycling (Bonito *et al.*, 2014; Pieterse *et al.*, 2016; Sunar *et al.*, 2015). Many of these interactions are controlled by 1+ of over 20,000 known microbial secondary metabolites (Gaiero *et al.*, 2013). The output of metabolites from microbes is highly situational and changes with the environment, due to this, many metabolites and their functions are still left undiscovered. However, there are some metabolites such

as 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, cycloheximide, actinomycin A, novobiocin, among others which have been reported to function antagonistically towards both microbes and insect pests (Brader *et al.*, 2014; Gaiero *et al.*, 2013; Pallavi *et al.*, 2017; Kim & Anderson, 2018; Pathak *et al.*, 2017).

Probiotics could also supply the plant with growth promoting attributes, an ability not seen with pesticides. Plant growth promoting bacterial endophytes (PGPBE) have adapted several ways to benefit their hosts, both directly and indirectly. Microbes directly supply the plant with essential nutrients such as fixed nitrogen, iron, potassium, zinc and phosphate or can modify essential plant hormones such as auxin, ethylene or cytokinin (Pallavi *et al.*, 2017; Srinivasarao & Manjunath, 2017). Indole-3 acetic acid, another hormone that can be produced by both plants and microbes, regulates plant growth and development. It is reported about 80% of rhizospheric microbes can synthesize this hormone (Srinivasarao & Manjunath, 2017). Hormone production and nutrient acquisition by either microbial inhabitants or host plays an important role in their interaction and can influence mineral/nutrient uptake, gene expression, signalling molecule production, stress tolerance, plant growth and immune responses (Kaul *et al.*, 2017).

Induced systemic resistance (ISR), often called priming, is a plant immune response similar to the immune cell training that occurs in the human gut (Bron *et al.*, 2011). Priming the plant increases the hosts sensitivity to hormonal changes, allowing for a quicker response when challenged with a pathogen(Compant *et al.*, 2005). In ISR, beneficial microbes trigger plant defenses through microbe associated molecular patterns (MAMPS). Lipopolysaccharides, siderophores, cyclic acetoin, 2,3 butanediol and benzaldehyde can all

act as triggers for ISR (Pallavi *et al.*, 2017). This leads to the host activating defense transcription factors and could lead to systemic activation, triggered by jasmonic acid and ethylene (Gruau *et al.*, 2015; Pineda *et al.*, 2010). Ethylene levels have also been shown to be lowered in the presence of bacteria that contain ACC deaminase. They can hydrolyze ACC, promoting shoot and root growth and lowering ethylene levels ultimately priming the plant (Shaharoona *et al.*, 2006)(Penrose & Glick, 2003). For example, Gruau *et al.* primed grapevines with *Pseudomonas fluorescens* PTA-CT2 and found plant defenses were upregulated throughout the vine. They also discovered that when plants were challenged with *Botrytis cinerea*, vines that were induced by *P. fluorescens* had reduced colonization of the pathogen (Gruau *et al.*, 2015). Primed plants are not only more capable to handle biotic stressors, but have also been shown to increase protection against abiotic stresses including high salt and drought (Santoyo *et al.*, 2016).

Through nutrient/niche exclusion, plant immune activation, antimicrobials and phytohormone production, microbes are a source of untapped potential in crop management. With further research into these fields, we can understand and take advantage of this natural and intimate interaction between plant and microbe. To promote symbiosis, the plant will release 5-20% of its photosynthetic products into the rhizosphere, becoming major fuel sources (Berendsen *et al.*, 2012; Turner *et al.*, 2013). Root exudates are sloughed off into the rhizosphere, adding organic acids, sugars, amino acids, vitamins, enzymes, hormones, proteins, flavonoids antimicrobials, and other secondary metabolites (Berendsen *et al.*, 2012; Kenny & Balskus, 2017; Velmourougane *et al.*, 2017; Berendsen *et al.*, 2012; Dennis *et al.*, 2010; Turner *et al.*, 2013). These molecules may attract or discourage microbes

from approaching the root. In addition, plant-microbe interactions may be bio compartment specific and understanding holistic endospheres of a plant is an important attribute to selecting microbial biostimulants, which will adequately infect the host (Deyett & Rolshausen, 2019). All these factors play into selecting which microbes are within the rhizosphere, but environmental factors like temperature, UV, radiation, nutrient availability (Berg *et al.*, 2014; Zarraonaindia *et al.*, 2015), pesticides (Pinto *et al.*, 2014), soil type, pH(Berg & Smalla, 2009), the species of plant among others factors (Doornbos *et al.*, 2011) also have a significant contribution to the microbial composition and community metabolite function. Discovery of plant probiotics can be quite challenging due the overwhelming diversity of microbes, but understanding the plant-microbe interactions and utilizing high-throughput modern techniques can make this impossible task a manageable enterprise.

1.0.4 Microbiome Technologies for the Discovery of Agricultural Probiotic Candidates

Classically, culture-dependent approaches were used to screen for microbes that had specific abilities to inhibit a pathogen either through direct antagonism or parasitism. In the former, biocontrol agents could be easily identified because of their ability to produce bioactive antimicrobial compounds, which inhibited pathogen growth. In the later, biocontrol agents completely parasitized the pathogen. Culture dependent methods can be long and arduous, requiring large quantities of microbes to be screened through laboratory and greenhouse bioassays with limited ability to pre-screen candidates. They are also limited in their ability to decipher the mechanism of antagonism. For instance, if the assay screens for antimicrobial production, microbes that are antagonistic towards the pathogen due to

nutrient or niche exclusion will be screened out, providing a limited scope of mechanistic discoveries and discarding potentially viable candidates. However, recent advances in microbial techniques and Omic tools have broadened the understanding of the host-microbe interaction and microbial diversity, supplying an efficient and cost effective manner to identify microbial inhabitants. Although new and difficult to analyze, these techniques are high throughput, cost effective, efficient and will aid in the discovery of novel probiotics (Papadimitriou *et al.*, 2015).

The plant microbiome is composed of microorganisms living either on the surface (i.e, rhizosphere, phylloplane, carposphere) or inside (a.k.a. the endosphere) plants organs. Epiphytic microbiome composition and function has been relatively well documented especially for a few plant model systems. In contrast, endophytes remain vastly underexplored although they possess many characteristics that make them prime candidates for probiotic/biocontrol agents, such as naturally self-replicating and the ability to colonize the plant tissue, supplying a very intimate association between host and microbe (Spadaro & Gullino, 2005). There are two main ways of finding beneficial microbes through microbiome analysis, targeted sequencing and whole-metagenomics. Both techniques sequence the microbiome from healthy and diseased hosts with the goal of finding candidate taxa that are more abundant or only found in the healthy host.

In targeted metagenomics, a small fragment of DNA is sequenced from each microbe. For bacteria the most common targeted gene for sequencing is the 16S rDNA (Bonito *et al.*, 2014; Chakravorty *et al.*, 2007; Prober *et al.*, 2015), particularly the V4 region (Caporaso *et al.*, 2011; Prober *et al.*, 2015; arth Microbiome Project, n.d.). Chloroplast and

mitochondrias nearly identical V4 rDNA region adds another level of difficulty to plant microbiome studies and can lead to >80% non-target sequences (Lundberg *et al.*, 2013). Plastid and mitochondria peptide nucleic acid (PNA) PCR clamps (Lundberg *et al.*, 2013; von Wintzingerode *et al.*, 2000) as well as sequencing the V5 region as opposed to the V4 region have both been successfully utilized to limit amplification of plastid and mitochondrial sequences (Hanshew *et al.*, 2013). In fungal, 18S, 5.8S and 28S rDNA genes as well as the internal transcribed spacer regions (ITS1 and ITS2) are commonly used to amplify and identify species (Huffnagle & Noverr, 2013). To trace back sequences to individual samples, a unique barcodes is assigned to each sample and is usually attached to one of the primer pairs (Kumar *et al.*, 2014). Pipeline technologies such as QIIME2 and DADA2 have been developed to handle much of the bioinformatic analysis in microbiome studies (Bolyen *et al.*, 2019; Callahan *et al.*, 2016). The taxonomy of both fungal and bacterial sequences can be determined by aligning to a database of sequences with known taxonomic identities. In contrast, whole metagenomics, sequences the total community of DNA, resulting in much larger quantities of genomic data of both host and microbes, requiring stronger bioinformatic expertise to be properly analyzed. This approach is useful in determining what genes and genetic pathways are within a particular sample (Rebollar *et al.*, 2016; Bhargava *et al.*, 2019). Having gene functional information can easily eliminate candidate microbes that may pose a risk to humans due to toxin production (Papadimitriou *et al.*, 2015).

Finding potential probiotic/biocontrol candidates using 'Omic'techniques often requires correlation between sick and healthy individuals. Correlation tests can reveal candidates which are negatively or positively correlated to the microbe of interest (the pathogen)

or the plant phenotypic condition. These methods include classic techniques like Pearson and Spearman correlations and Wilcox to determine statistical difference. Microbiome surveys often produce data which is over-dispersed, extremely zero-inflated and more recently incorporates longitudinal surveys as well. Thus the data does not meet many assumptions for the above classical statistics. In recent years, more robust techniques designed for microbiome analysis such as MENA (Microbial Ecological Network Analysis), CoNet (Co-occurrence Network Analysis), MIC (Maximal Information Coefficient) and SparcCC (Sparse Correlation for Compositional Data), CCLasso (Correlation inference for Compositional data through LASSO), BAnOCC (Bayesian Analysis Of Compositional Covariance), MPLasso (Microbial Prior LASSO), and SPIEC-EASI(SParse Inverse Covariance Estimation for Ecological Association Inference) have been designed to interpret microbiome interactions each with their unique strengths and weaknesses (Dohlman & Shen, 2019). DESeq2, adapted from RNASeq analysis, can also be utilized to determine statistically significant differential abundance of microbes between groups (Rebollar *et al.*, 2016). Together these tools supply insight into microbial composition among groups as well as supply specific genes that might be attributing to a 'healthy' vs 'diseased'phenotype.

Despite the great benefit and ease of this approach, it comes with several limitations and biases. Sequencing short fragments can often lead to erroneous taxonomic identifiers and may not reach the genus/species level with confidence. For example, Singh *et al.* 2016, found nearly 50% of sequences were unknown at the genus level and 82% were unknown at the species level (Singh *et al.*, 2016). This problem is more pronounced in fungi where current fungal databases often produce misclassification and high percentages of un-

classified taxa (Bonito *et al.*, 2014). Targeted metagenomics also results in a loss of DNA sequences throughout the process as well as an inability to determine the quantity of specific microbes due to primer binding and elongation efficiency in the PCR (Hughes *et al.*, 2001). Most importantly, this approach does not confer functionality of the microbiome (Prosser, 2015). Plants also contain a high quantity of phenolics and other various compounds which can inhibit PCR. CTAB during DNA extraction and TaqMan Environmental Master Mix, or including trehalose and BSA into PCR reactions can limit PCR inhibitors(Azmat *et al.*, 2012; Jane *et al.*, 2015; Samarakoon *et al.*, 2013). Uncovering the phytobiomes of a plant system is not only difficult but includes many unexplored limitations. However, as research into phytobiomes continues, better methods and more robust techniques will be developed to cope with both plant specific and global microbiome limitations.

1.0.5 The Susceptible host: *Vitis vinifera*

To explore the microbiome as it relates to disease development in agriculture, this thesis focuses on research of Pierce's Disease of Grapevine and the underlying endophytic microbiome. *V. vinifera* (grapevine) is a woody perennial crop best known for the oenological attributes but are also prized for their nutrition. It can be consumed in a variety of ways including, juice, wine, jams, and as table grapes. *V. vinifera* is one of the most cultivated crops in the world with 7.5 million hectares devoted to the creation of 27 million tons of wine per year (Pinto & Gomes, 2016). In California, the grape industry is the second top grossing agricultural industry at a value of $6.25 billion as of 2018 (California Department of Food & Agriculture, n.d.).

The domestication of grapes occurred during the neolithic period more than 6,000 year ago. Making grapes not only a current economic and nutrient driver but an important aspect of human history inspiring cuisines, medicines, cults and economies throughout the centuries. Through this domestication and selective breeding it is believed there are over 10,000 varieties of grapevines in existence today, each chosen for their distinct characteristics to provide a product of desired quality (McGovern *et al.*, 2017). Today these varieties are grouped into three pedigrees *Vitis vinifera*; *occidentalis, orientalis* and *pontica*. The first lineages consists of small-berried wine grapes of western Europe while *orientalis* are the large-berried table grapes of West Asia; and the final lineage is made up of intermediate type from the basin of the Black Sea and eastern Europe (Aradhya *et al.*, 2003). Vineyards are often clonal exhibiting one species chosen for flavor grafted onto a rootstock often selected for its ability to thrive under specific edaphic factors and display a range of tolerance/susceptibility to a variety of biotic or abiotic stressors (Terral *et al.*, 2009). Commercial grapes are exposed to a number of fungal, bacterial, viral and phytoplasma diseases, so much so that it is estimated that without chemical applications such as fungicides, California would lose 97% of their wine production (Cantu *et al.*, 2016). Due to *V. vinifera*'s economic worth, high susceptibility and clonal nature, this crop makes an excellent model to study disease development particularly through a microbial lense.

1.0.6 The Viable Pathogen: *Xylella fastidiosa*

One particularly devastating global pathogen is *Xylella fastidiosa*; an aerobic, Gram-negative bacteria transmitted by xylem feeding insects causing a number of diseases in a variety of plants including alfalfa, coffee, citrus, almonds, peaches, olives and grapes

throughout the Americas and Europe (Hao *et al.*, 2016). However, it is not a strict pathogen, as it has been found in a number of plant host without causing any symptoms. It is estimated that *Xylella* has been found in over 350 plants species from 75 different plant families (Baldi & La Porta, 2017). Due to its significance as a pathogen, *X. fastidiosa* was the first plant-pathogen to have its whole genome sequenced (Rapicavoli *et al.*, 2017). In grapevine, *X. fastidiosa* causes Pierce's Disease and plays a significant economic role as it is estimated to cost the industry $104 million annually (Tumber *et al.*, 2014).

X.fastidiosa is vectored most often by xylem sap feeding insects such as sharp-shooters and spittlebugs. The major vector in California grapevines are the glassy-winged and blue-green sharpshooters (Chatterjee *et al.*, 2008). This pathogen thus has a unique life cycle as it requires the appropriate mechanisms to thrive and survive in both the mouth-parts of the insect and the nutrient poor environment of the xylem. Transmission of the pathogen occurs upon feeding events of the vector of an infected vine. The pathogen is capable of forming a biofilm within the foregut of the insect as well as within the xylem environment. *X. fastidiosa* can be transmitted to the xylem vessels of a new susceptible host during the next feeding event.

The xylem environment is nutrient poor and consists of interconnected vessels of variable lengths which are separated by pit membranes (PMs). The PMs'pores are large enough for water and small solutes to pass through but too small for *Xylella fastidiosa* and other members of the microbial communities to pass. Thus, it is thought, *X. fastidiosa* utilizes cell wall degrading enzymes for systemic colonization of the grapevine (Ingel *et al.*, 2019). In an attempt to stop pathogen colonization, the plant will produce tyloses, an

expansion of the neighboring parenchyma cells wall. These balloon like structures have the capability of blocking off vessels, thus limiting the spread of the pathogen and subsequently limiting the vines ability to transport water (Sun *et al.*, 2013). Pierce's Disease symptoms, are thus similar to symptoms of water stress, and include berry desiccation, abscission of petioles and uneven maturation of canes (Rapicavoli *et al.*, 2017). Overtime, vines impacted by Pierce's Disease will show stunted canopies and poor quality of fruits eventually leading to the vine's death.

1.0.7 IPM for PD and Pesticide Alternatives

There is currently no cure for Pierce's Disease and management strategies rely heavily on suppressing vector populations through insecticide applications. In addition wild North American cultivars such as *V. arizonica/candicans* and *Muscadinia rotundifolia* have shown resistance to the disease but lack the oenological qualities possessed by V. *vinifera* varieties (Riaz *et al.*, 2018). A PD resistance gene, PDR1, has been identified from these cultivars and introgressed in V. *vinifera* lines (Krivanek *et al.*, 2006; Walker & Tenscher, 2012) and PD-resistant commercial grapes were recently released. Environmental conditions also affect disease incidence and severity. For instance, vineyards near citrus orchards (a XF-reservoir and common habitat for sharpshooters) are at higher risks of contracting Pierce's Disease. Furthermore, soil PH, latitude, temperature nutrient concentration and water stress have also been linked to PD incidence(Costello *et al.*, 2017). These strategies all incorporate scientific knowledge and host-microbe interactions to better manage this devastating disease.

Interestingly, many of the above factors are also known to alter the microbial fingerprint of an area. Furthermore, It has been observed that not all vines exhibit universal disease progression or symptom severity. Even clonal vines within the same vineyard, under high Pierce's Disease pressure, do not equally contract the disease. Some vines will be severely impacted by the disease while the adjacent vines shows no symptoms; they have 'escaped'. In this scenario, the environment is virtually the same, the host is the same, and the pathogen load is the same; it thus was our hypothesis that the microbial inhabitants living within the vine might play a role in disease progression and could potentially be manipulated for pathogen population suppression and novel IPM strategies.

This research aims to (i) define the grapevine microbiome in a variety of biocompartments, geographical locations, disease severity and through temporal shifts (ii) highlight potential negative correlates within the microbiome to mitigate Pierce's Disease, (iii) to validate and assess a novel *X. fastidiosa* qPCR detection method by assessing Pierce's disease susceptibility in *V. vinifera* (iv) develop and assess a greenhouse assay for biocontrols against Pierce's Disease. Taken together this research reveals the endophytic microbiome from symbiotic to pathogenic within the grapevine, deepens our understanding the grapevine susceptibility to an endophytic pathogen and mines the microbiome for novel approaches for Pierce's Disease management.

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Chapter 2

Endophytic Microbial Assemblage in Grapevine

2.0.1 Abstract

Grapevine has become a model system for crop microbiome research. The host vascular system has remained an under-explored niche despite its potential for hosting beneficial microbes. The aim of this work was to determine the origin of the microbial endophytes inhabiting grapevine. We focused on a single commercial vineyard in California over a two-year period and used an amplicon metagenomics approach to profile the bacterial (16S -V4) and fungal (ITS) communities of the microbiome across a continuum of six grapevine compartments; bulk soil, rhizosphere, root, cordon, cane and sap. Our data supported that roots are a bottleneck to microbial richness and that they are mostly colonized with soilborne microbes, including plant growth promoting bacteria recruited by the host, but also saprohytic and pathogenic fungal invaders. A core group of taxa was identified throughout the vine, however there were clear partitioning of the microbiome based on hosts niche. Above and below ground plant tissues displayed distinct microbial fingerprints and were intermixed in a limited capacity mostly by way of the plant sap. We

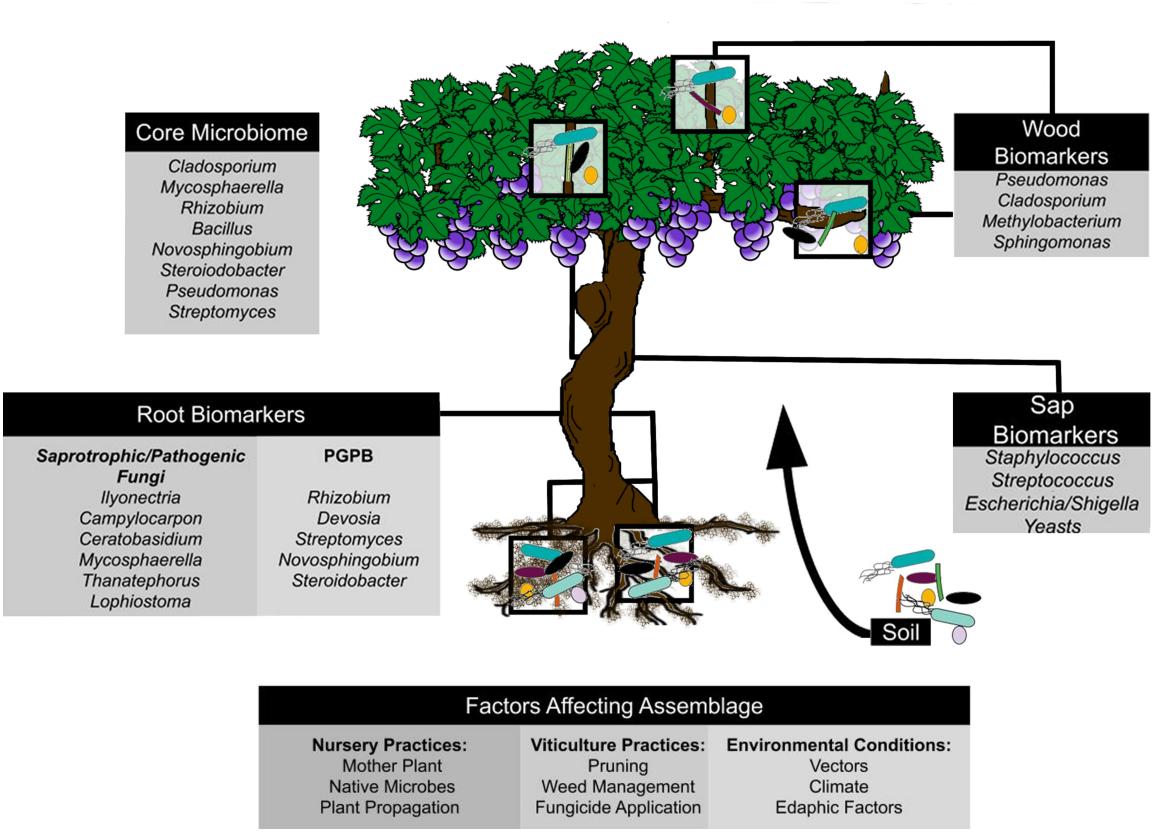

Figure 2.1: Graphical abstract depicting grapevine microbiome including core microbiome and biomarkers for wood, root and sap biocompartments.Factors affecting assemblage are also listed.

discuss how cultural practices and human contact may shape the endosphere microbiome and identify potential channels for transmission of its residents.

2.0.2 Introduction

The holobiont involves intricate interactions between the plant host and its residing microbial communities. Plant-associated microbes can be viewed as an extension of the plant genome facilitating its environmental fitness and supporting its health (Turner *et al.*, 2013, Berg *et al.*, 2016). Those attributes are especially valuable for agriculture. Characterizing healthy microbiomes for crop production systems has become the new frontier in plant science with the hope to identify beneficial microorganisms that could be further marketed into novel agricultural bioproducts. To date the field of plant microbiome research has focused mostly at the soil/rhizosphere/root interface. One major reason is the fundamental role that roots play in nutrition and its analogy to the human gut. Plants must actively and selectively recruit organisms from their surrounding environment through chemical signaling. Plants provide food substrate in the form of photosynthetically fixed carbon through rhizodeposits in exchange for increased nutrients assimilation and improved tolerance against abiotic and biotic stresses (Compant *et al.*, 2010, Bulgarelli *et al.*, 2013). Bulgarelli *et al.* (2013) described the root microbiota assembly as a dynamic two-step selection process that involves an initial acquisition of microbes from the soil to the rhizosphere and a sorting step that involves a host-driven mechanism of subsetting specific microbes into the root. As a result microbial diversity is reduced spatially along the soil-endorhiza continuum and microbial profiles are markedly differentiated between rhizocompartments (Bulgarelli *et al.*, 2012, Edwards *et al.*, 2015, Fitzpatrick *et al.*, 2018). Endophytic microbes are required to be highly specialized organisms that can migrate to the root zone, attach to the rhizoplane, move pass the endodermis and pericycle and colonize the central cylin-

der (Compant *et al.*, 2010). In addition they must be fitted to survive under the internal conditions of the plant roots that are vastly different than the external conditions of the rhizosphere. Subsequently, root endophytic microbes can move systemically and colonize other plant organs, using the vascular system as a freeway (Compant *et al.*, 2010, Deyett & Rolshausen, 2019). Not all microbes inhabiting the endosphere originate from the root as a fraction of them can also gain access from the aerial parts using natural openings (stomatas) or wounds (pruning) as entry points, while others are directly introduced by feeding insect vectors and pests (Munkvold & Marois, 1993, Compant *et al.*, 2011, Lopez-Fernandez *et al.*, 2017). It has been hypothesized that the plant endosphere is mainly composed of rhizospheric organisms that systemically colonize the host. However, most of the research has focused on either the below- or aboveground compartments of the plant but few studies have adopted a wholistic approach looking at the continuum between the two niches (Cregger *et al.*, 2018). The plant vascular system plays a key role of connecting all the plant organs and internally transport water, nutrients and signals both acropetally and basipetally across biocompartments. Characterizing plant endophytic microbes has gained traction because they are more likely to have bioactive functions including plant-growth promoting capabilities and protection against phytopathogens (Lugtenberg *et al.*, 2016, Santoyo *et al.*, 2016, Ek-Ramos *et al.*, 2019). This enticing research axis has been actively pursued in plant model systems and annual crops, but it has been limited in perennial cropping systems due in part to the lignified nature of the hosts vascular system. Grapevine (*Vitis*) is an excellent plant model system to study the microbiome of woody perennial crops. It is one of the most cultivated fruit plants in the world. The International Organization of Wine and

Vine (OIV) estimated the wine growing surface area at about 7.5 million hectares in 2016 (http://www.oiv.int/en/statistiques/). Metagenomics tools have been actively used to map the microbiome of the rhizocompartments, phyllosphere, carposphere and anthosphere of grapevine. Studies have clearly established that microbial communities are an integrated part of the identity of a viticulture region that define the terroir and showed that it is influenced by several factors including the host genotype and viticulture practices as well as climatic and edaphic factors (Bokulich *et al.*, 2014, Zarraonaindia *et al.*, 2015, Marasco *et al.*, 2018, Gupta *et al.*, 2019). The endophytic microbes of the grapevine vascular system have been mostly studied in the context of diseases, including crown gall (Faist *et al.*, 2016), Pierces Disease (Deyett *et al.*, 2017) and esca (Del Frari *et al.*, 2019). Deyett & Rolshausen (2019) showed that the sap microbiome is a dynamic entity that was enriched with specific bacteria depending on the phenology or disease condition of the grapevine. In addition, grapevine sap displayed strong imprint of microbes of rhizospheric origin, but because microbial communities of the rhizosphere and endorhiza were not spatially profiled, answers about microbial origin could not be addressed. The goal of this study was to address this gap in the knowledge with regards to the origin of endophytic microbes residing in the plant vascular system. We provide a microbial map of six distinct compartments (bulk soil, rhizosphere, root endosphere, cordon, cane and sap) of vines in a single commercial vineyard over a two-year period to determine the proportion of rhizospheric microbes capable of colonizing the host vasculature. This study enables a better understanding of the assemblage of microbes in the grapevine endosphere and dynamics along the below- and aboveground continuum, and identify potential channels for transmission of microbes.

2.0.3 Materials and Methods

Plant Sampling and Processing

The experiment was conducted in a cordon-pruned conventional commercial vineyard cv. 'Syrah'on 1103P rootstock in Temecula, California. The vineyard was planted in 2010 and all samples were collected after harvest (Fall) in 2017 and 2018. Cane, cordon, root and rhizosphere samples were collected from 30 grapevines in the vineyard. Plant sap was extracted from a subset of ten of the thirty vines. Five soil samples were also collected in 2018 from the four corners and the middle of the vineyard. Cordon pieces (10 cm in length) were removed from the vine with two-hand pruners. Root samples were dug-out with a shovel at a 10-30 cm soil depth. Four canes (two from each side of the vines cordon) were mechanically removed from the vine using pruners. Samples were placed in a chilled cooler on ice and transported back to the laboratory. All samples were processed within 24 h as followed; sap was extracted from two canes of ten vines using a Scholander pressure bomb as previously described (Deyett & Rolshausen, 2019). For all 30 vines, two canes were selected and petioles and leaves were stripped away. One internode per cane was cut off (two total per vine) and bark peeled-off using a sterile scalpel. The internodes were surface sterilized by flaming, cut into smaller pieces (about 5 mm ^{3}) and stored in a 15 ml conical tube at -70C for further processing. Root and rhizosphere samples were processed as described by Lundberg *et al.* (2012). Briefly, roots were placed in sterile 50 ml conical tube with 25 ml of PBS buffer with 200μl L$^-$1 Silwet L-77 surfactant. Samples were vortexed for 15 s. Roots were then transferred to a clean 50 ml conical tube with 25 ml of PBS buffer. The first tube was centrifuged at 3200 g for 15 min and the aqueous layer was

removed. The pellet was retained as the rhizosphere fraction. The roots continued to be vortexed and moved to a clean PBS tube until PBS remained clear after vortexing. Roots were then sonicated using a Branson Sonifier 450 at a low frequency for 5 min (five 30 s bursts followed by 30 s breaks). Roots were then stored at -70°C for further processing. Cane, cordon and roots were then lyophilized in the FreeZone 2.5 L benchtop freeze dry system (Labconco) for 48-72 h. Samples were then ground to a powder using the MM300 grinder (Retsch) in a 35 mL stainless-steel grinding jar (Retsch) with 20 mm stainless steel balls at 25 oscillations per second in 45 s increments until sample was fully pulverized.

Microbiome Library Prep

DNA was extracted from all samples using the ZymoBIOMICS DNA miniprep kit per manufacturers protocol, using 250 l of sap, 100 mg of dried tissue or 250 mg of wet rhizosphere (Zymo Research). DNA quantities were assessed using the Synergy HTX multi-mode reader (Biotek Instruments, Winooski, VT). Both bacterial 16S and fungal ITS rRNA region was amplified from all samples using the Earth Microbiome protocol and primers (http://www.earthmicrobiome.org/). Briefly, primers 515F and 806R were used for bacterial microbiomes and ITS1f-ITS2 for fungal ITS amplification (Caporaso *et al.*, 2010). PCR reactions of 25 μl were performed in triplicate and pooled for each sample using 10 l of Phusion hot start flex 2x master mix, 0.5 l of primer (10 m), 2 l of DNA. In bacterial woody tissue, universal pPNA and mPNA clamps were added at a starting concentration of 1.25l (25 m). These clamps were designed to reduce the amplification of host chloroplasts and mitochondria while having no effect on bacterial amplification (Fitzpatrick *et al.*, 2018). Every PCR was also accompanied by a negative control to ensure barcodes and master mix

were not contaminated. Successful amplification was verified on a 1% agarose gel and DNA quantification was checked using the Synergy HTX multi-mode reader (Bioteck Instruments, Winooki, VT). Equal quantities of each sample in a library were combined into an eppendorf tube and cleaned using the AMPure XP PCR purification system (Beckman Coulter) per manufacturers protocol. Final concentration of libraries were determined using both qPCR and bioanalyzer before being sequenced on the MiSeq instrument (Illumina, San Diego, USA) using 600-cycle run (2X300 paired end) for fungal reads and 500-cycle run (2x250 paired-end) for bacterial microbiome at the UC Riverside Genomics Core facility.

Computational Analysis

Trimmomatic (Bolger *et al.*, 2014) was used as an initial quality filtering with a sliding window 5:20. Primers and PhiX reads were removed from sequences and demultiplexed using QIIME v 1.9.1 (Caporaso *et al.*, 2010). Most processing for the reads were done in DADA2 v 1.14.0 (Callahan *et al.*, 2016) including further quality control (no ambiguous base calling, no more than 2 errors), dereplicating, sample inference using learned error rate algorithm, merging of paired reads, removal of chimeric sequences and construction of sequence tables. Taxonomy identification was assigned using the SILVA SSU r132 reference database and Unite database v 10.10.2017 for fungal taxa. With DADA2's learn error rate algorithm, authors claim sequences as ASVs (Amplicon Sequence Variants) and has the capacity to resolve sequences difference to a single nucleotide, allowing for more robust identification and capability of identifying taxa, in some cases to the species level (Callahan *et al.*, 2016). Phyloseq v 1.30.3 (McMurdie & Holmes, 2013) was used for much of the graphical and statistical analysis of the data. Unidentified microbes at the kingdom

or phylum level, or microbes that occurred less than three times were removed from the full data set. Taxa which were identified as chloroplast or mitochondria were also removed. Samples harboring less than 500 ASVs were also pruned out of the data set. Shannon diversity index was used as a metric of taxa diversity within the communities. Kruskall-Wallis and pairwise Wilcoxon tests were run to verify statistical differences among groups. Phylum pie charts were constructed by aggregating taxa at the Phylum level and samples by tissue compartments and transforming to relative abundance. Phyla occurring at less than 1% were removed before creating pie charts for clarity purpose. Bray Curtis dissimilarity was used to calculate the compositional similarities between samples and was visualized with PCoA plots using the Vegan package v 2.5-6. To determine statistical significance of beta-diversity, Adonis test were run. Classical Venn diagrams were created by transforming to relative abundance and filtering taxa to those which occur greater than 0.1% and prevalent in at least two samples of that tissue type. Graphs were created using VennDiagram v 1.6.20. For prevalent Venn diagrams, data was aggregated by genus and transformed to relative abundance. Taxa were denoted as prevalent in each biocompartment if they occurred in at least 50% of the samples of that biocompartment. Taxa which occurred less than 1% in the dataset were removed before visualizing Venn diagrams using UpSetR v 1.4.0. For concentric pie charts representing core microbiome, data was aggregated to the ASV or genus level and transformed to relative abundance. ASVs/genera were filtered based on core microbiome as previously defined. To find microbes associated with a biocompartment and above and belowground sections DeSeq2 v 1.26.0 was utilized and visualized using complexheatmap v 2.2.0. Taxa were filtered by p value and log 2 fold change, keeping only taxa

with p <0.01 and having a log 2 fold change greater than 5 or less than -5. Taxa were also filtered based on relative abundance >0.1%. Heat maps represent the relative abundance of the data and the log2 fold change as determined through DeSeq2.

2.0.4 Results

The bacterial dataset totaled 126 samples (21 cane, 23 cordon, 33 rhizosphere, 31 root, 13 sap and 5 soil samples) and the fungal dataset totaled 119 samples (24 cane, 26 cordon, 27 rhizosphere, 24 root, 13 sap and 5 soil samples) after filtering out poor quality reads, chloroplast, mitochondria, taxa with unidentified phyla and samples with too few ASVs. After removal of singletons and doubletons, the total ASVs were of 18305 (soil=3050; rhizosphere=12825; root=3320; cordon=2900; cane=1908; sap=995) and 3991 (soil=432; rhizosphere=2924; root=830; cordon=290; cane=198; sap=277) for the bacterial and fungal datasets, respectively.

Shannon diversity index was used on the entire datasets prior to removing singletons and doubletons (Figure 2.2). The plant bacteriome was overall more diverse than the plant mycobiome. The soil mycobiome was significantly richer than all other biocompartments, including rhizosphere (p <0.05 [pairwise-Wilcox]), whereas the soil bacteriome was only significantly different from the cane (p <0.01 [pairwise-Wilcox]). In contrast, the rhizosphere bacteriome displayed significant higher Shannon diversity index as compared to all endophytic tissues (p <0.0001 [pairwise-Wilcox]). Within the plant endosphere, Shannon index was significantly higher in the root vs. the cane for the bacterial dataset (p <0.001 [pairwise-Wilcox]) and in the cordon vs. the cane for both datasets (p <0.01 [pairwise-Wilcox]). Of all the biocompartments, the cane microbiome had the lowest average Shannon

diversity index although not significantly different from the sap.

Proteobacteria and *Ascomycota* were the most abundant phyla within the entire dataset representing on average 54.2% and 79.3% of all taxa, respectively (Figure 2.3). Phyla *Basidiomycota*, *Actinobacteria*, and *Firmicutes* were also important phyla as they occurred in greater than 10% on average across the entire datasets. *Proteobacteria* ranged from about 80% in relative abundance of the cane bacteriome to less than 40% in the sap and soil. It was the most abundant phylum in all tissue types except for the sap, which harbored a higher relative abundance of *Firmicutes* (39.7%) vs. *Proteobacteria* (37%). Likewise, *Ascomycota* ranged from over 80% in relative abundance of the endosphere mycobiome to below 50% in the rhizosphere. In contrast, several phyla with abundance above 1% were limited to a few compartments. Hence, *Mortierellomycota*, *Calcarisporiellomycota*, *Olpidiomycota*, *Gemmatimonadetes* and *Acidobacteria* were mainly found in the rhizosphere and soil.

Bray-Curtis beta diversity metrics with PCoA were used to visualize how biocompartments impacted fungal and bacterial community composition (Figure 2.4). Our data showed distinct clustering between above- (cane and cordon) and belowground (root, soil, and rhizosphere) microbiomes with a significant segregation of the two habitats, while plant sap microbiome clustered in between the two habitats ($p < 0.01$ [Adonis]). Year also had a significant effect ($p < 0.01$ [Adonis]) in the clustering pattern, most noticeably in the bacterial cane and cordon datasets.

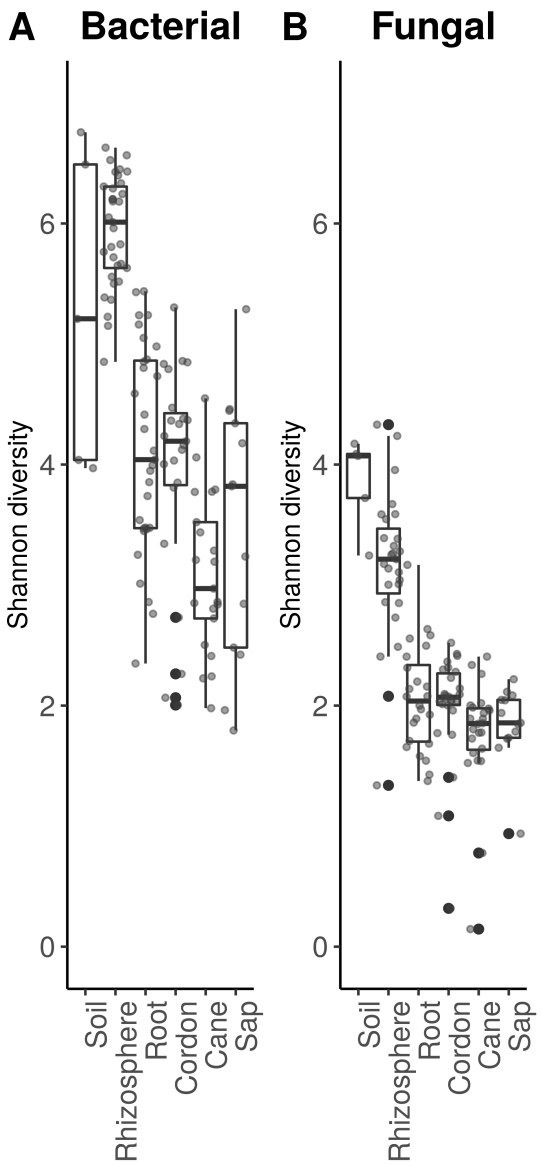

Figure 2.2: Shannon alpha diversity plots for (A) bacteria (B) fungi within six different grapevine biocompartments (soil, rhizosphere, root, cordon, cane, and sap).

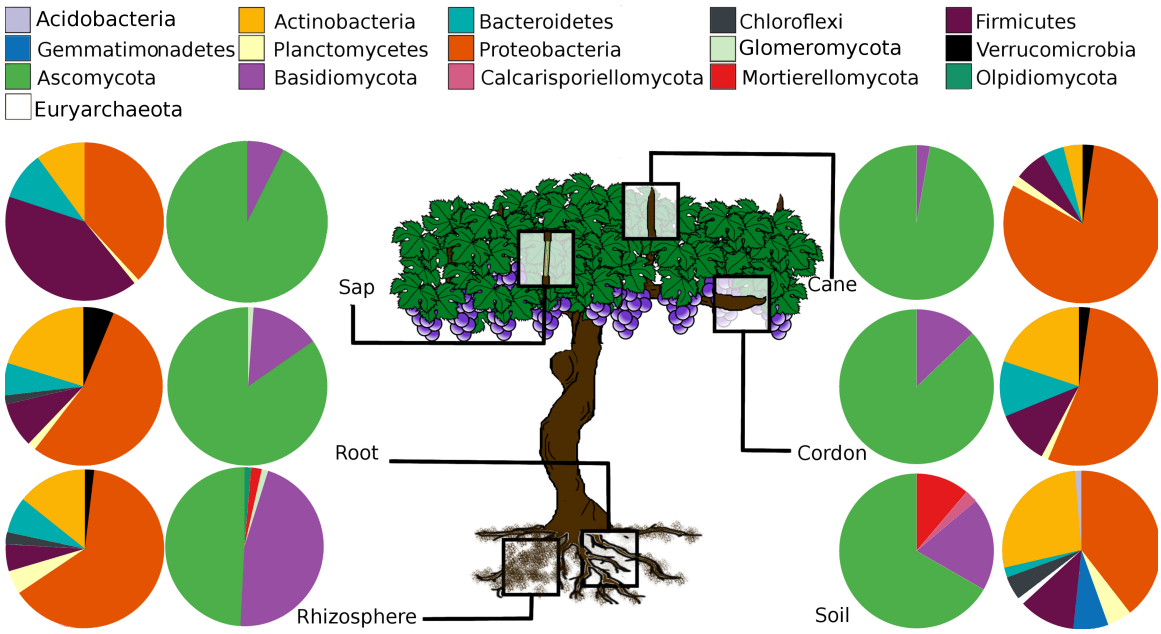

Figure 2.3: Relative abundance of bacterial and fungal phyla within individual grapevine biocompartement (soil, rhizosphere, root, cordon, cane, and sap). Only phyla occurring at $\geq$ 1% relative abundance are displayed.

We used classical Venn diagrams with a filtering of the bacterial and fungal datasets to determine the proportion and name of the most common and abundant ASVs shared amongst compartments (Figure 2.5). The filtering consisted of ASVs present in two or more samples and with a relative abundance >0.1% within each biocompartment (not across the entire dataset). The majority of all belowground ASVs (root, rhizosphere, soil) were found in the soil/rhizosphere (342 ASVs =68%), and 21% (102 ASVs) of the rhizosphere ASVs were able to colonize the root endosphere, whereas 11% (57 ASVs) of the remaining root ASVs were not soilborne (Figure 2.5A). When looking at the root microbiome, 64% of the filtered endophytic ASVs were of soil/rhizosphere origin (Figure 2.5A). However, when looking at the totality of the plant microbiome, 34% of the filtered endophytic ASVs were of soil/rhizosphere origin (Figure 2.5B). In addition, we found that

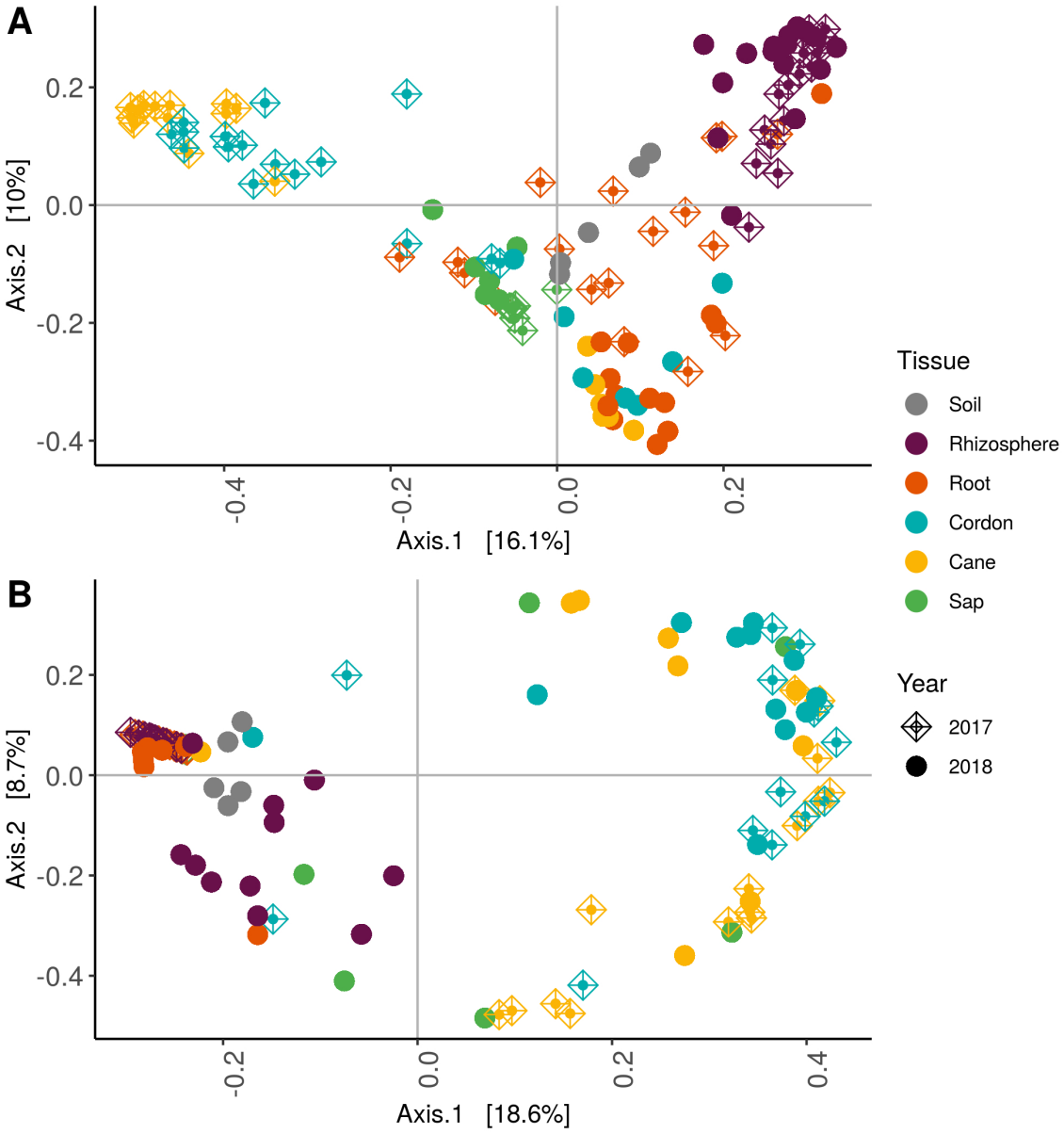

Figure 2.4: Bray Curtis beta diversity for (A) bacteria and (B) fungi. Points represent individual sample communities for one biocomparment from one vine at one year. Points are colored by biocomparment and shaped by year collected.

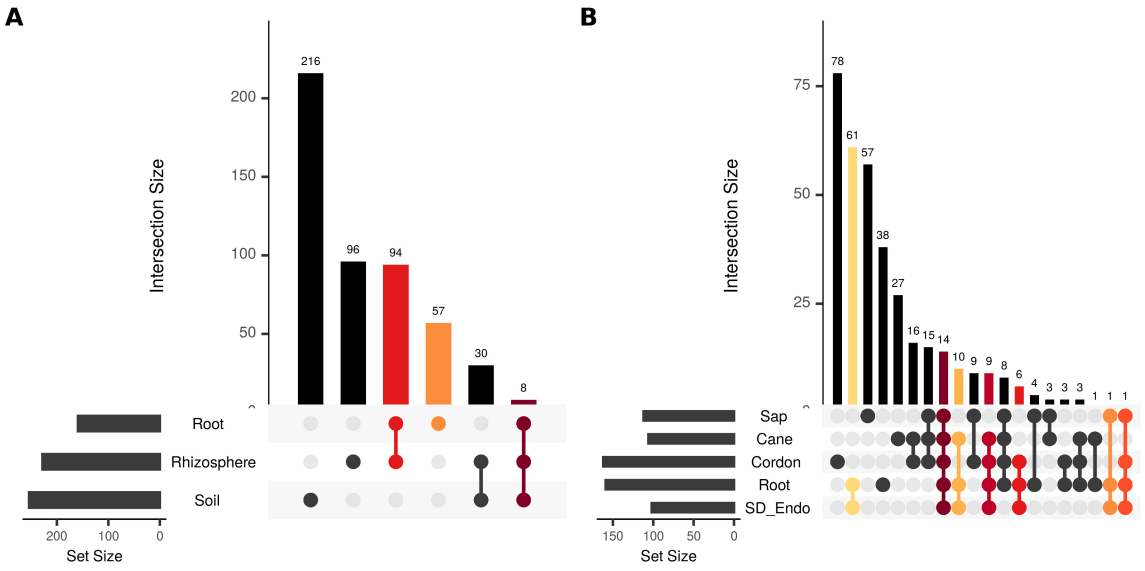

Figure 2.5: ASV Venn diagrams depicting overlapping taxa. (A) below ground; soil, rhizosphere and root and (B) endosphere; woody tissues (cane and cordon), sap and root. In B, the root category was divided into two sections based on below ground Venn diagram; soil derived endosphere (root taxa overlapping with rhizosphere and soil) and non-soil derived endosphere (root taxa not overlapping with rhizosphere and soil). Taxa were filtered based on a sample prevalence >1 and relative abundance of >0.1% for each biocompartment.

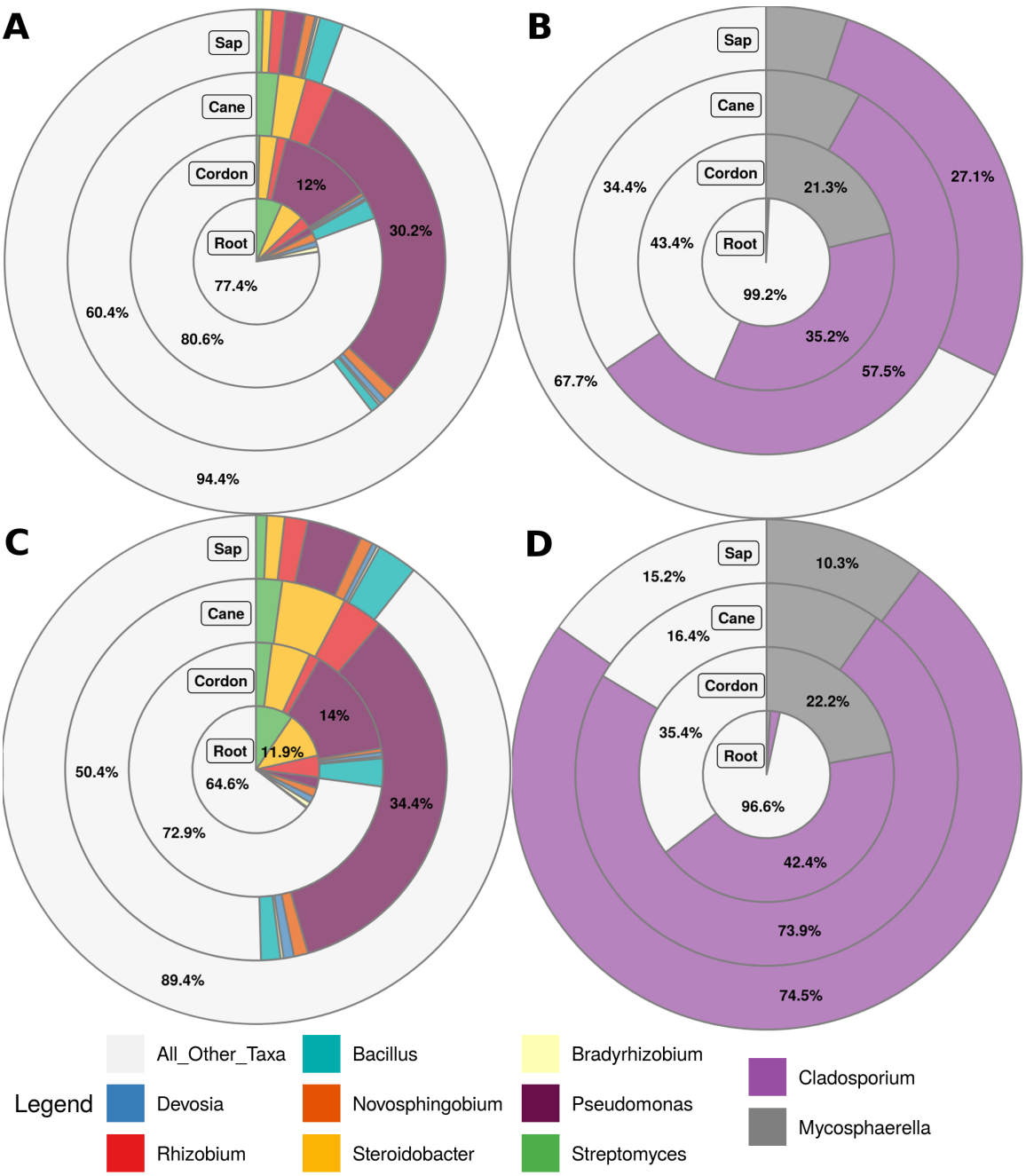

Figure 2.6: Core microbiome relative abundance. Each concentric circle represents the bacterial (A and C) or fungal (B and D) core microbiome communities of a tissue type as defined in Figure 4. A and B represent the 15 core ASVs identified in Figure 4 that are shared across all biocompartments, and labeled by their genera identifier. C and D represent the abundance of the ten genera identified as core microbiome.

only 5% of the filtered endophytic ASVs was shared among all biocompartments belonging to ten genera with two fungal (*Cladosporium* and *Mycosphaerella*) and eight bacterial (*Devosia*, *Rhizobium*, *Bacillus*, *Novosphingobium*, *Steroidobacter*, Brady*Rhizobium*, *Pseudomonas* and *Streptomyces*) taxa (Figure 2.5 and Figure 2.6). In contrast, 54% of filtered endophytic ASVs were specific to each compartment (root, sap or cordon/cane). A prevalence Venn diagrams with a 50% cut-off was also used to identify genera often associated with a specific tissue type as well as genera overlapping between biocompartments (Figure 2.7). Eight genera emerged as dominant taxa as they inhabited all the biocompartments of the grapevine endosphere and included one fungal genus (*Cladosporium*) and seven bacterial genera (*Escherichia/Shigella*,*Novosphingobium*, *Pseudomonas*, *Rhizobium*, *Sphingomonas*, *Bacillus* and *Steroidobacter*). *Streptomyces* was also abundant taxa commonly found in all the vine lignified tissues (root, cordon and cane), and could also be found frequently in the sap (although in less than 50% of our samples). *Mycosphaerella* was also found in all of the above ground tissues (sap, cordon, cane) and was also found frequently in the roots (although in less than 50% of our samples). *Devosia* and *Bradyrhizobium*, the remaining core microbes as defined by ASV Venn Diagrams, did not pass prevalence and abundance filters. Based on the combined results from the Venn diagrams (Figures 2.5 and 2.7), we identified two fungal (*Cladosporium* and *Mycosphaerella*) and six bacterial (*Rhizobium*, *Bacillus*, *Novosphingobium*, *Steroidobacter*, *Pseudomonas* and *Streptomyces*) taxa as members of the grapevine core microbiome. Our results also highlighted the shifts in microbial abundance for core taxa organisms across biocompartments between the two years of sampling (Figure 2.8), supporting beta diversity metrics data (Figure 2.4).

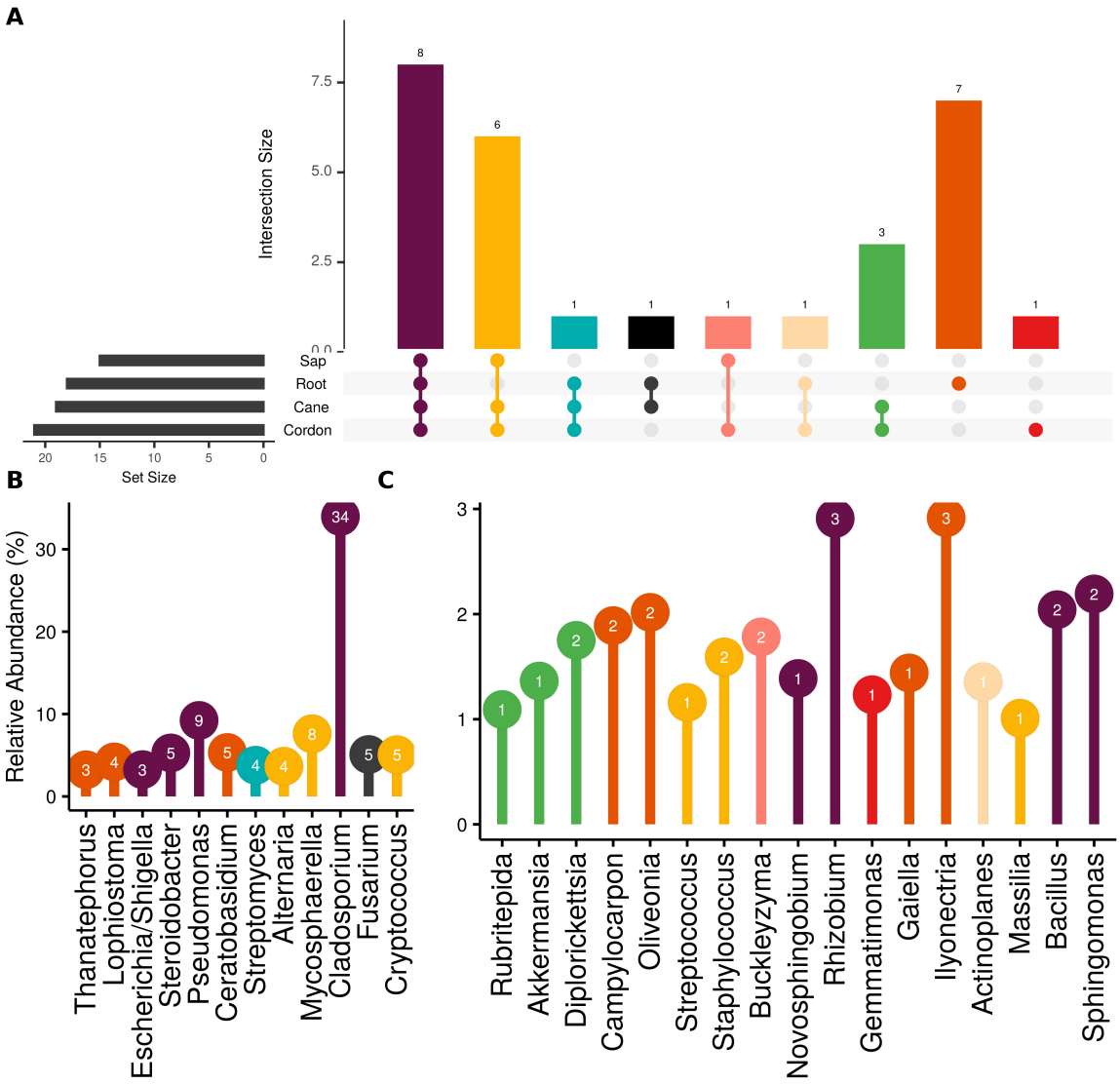

Figure 2.7: Prevalence Venn diagrams: genera that occur in $\geq 50\%$ of all samples from each biocompartment. (A) Intersections of genera associated with endospheric biocompartment (sap, cordon, cane and root) combination. (B) High and (C) low relative abundant for the genera colored by intersections in (A).

We used DeSeq2 analyses to indicate enrichment/rarefaction patterns of individual taxa across the different biocompartments (Figure 2.9). Results clearly indicated above and belowground microbial signature profiles. We measured an enrichment pattern for plant growth promoting bacteria (*Rhizobium*, *Devosia*, *Novosphingobium*, *Steroidobacter*, and *Streptomyces*), and pathogenic and saprophytic fungi (*Campylocarpon*, *Ilyonectria*, Ceratobasidium, Thanatephorus and Lophiostoma) in the root zone. In contrast, core (*Cladosporium*, *Mycosphaerella* and *Pseudomonas*) and non-core (*Methylobacterium*, *Alternaria*) microbiome genera were differentially abundant in above ground tissues, with specific enrichment of human-related bacteria in the sap (*Staphylococcus*, *Streptococcus*, and *Escherichia/Shigella*).

2.0.5 Discussion

This study lays the foundation to understanding endophytic microbial community assembly and functions in perennial cropping systems. We focused on a single commercial vineyard in California and spatially profiled the grapevine microbiome across a continuum of six biocompartments from the host ectospshere to its endosphere. Soil properties, climate, farming practices, disease pressure, host genotype and phenology are all selection drivers that shape the microbial identity of a vineyard and are an integrated part of its terroir (Bokulich *et al.*, 2014, Zarraonaindia *et al.*, 2015, Marasco *et al.*, 2018, Deyett & Rolshausen, 2019, Gupta *et al.*, 2019). Our data is in line with other grapevine studies that measured greater microbial diversity in below- vs. aboveground (Zarraonaindia *et al.*, 2015, Morrison-Whittle *et al.*, 2017) and that bacterial richness was overall superior to fungal richness (Gupta *et al.*, 2019) including inside the host vascular system. Fungal communities are influenced by tree species and host diversity (Urbanova *et al.*, 2015). Vineyards are monoculture cropping systems and the lack of host genotypic diversity across viticulture areas may be a bottleneck to diversity, particularly for fungi. It is also plausible that the cumulative inputs of synthetic and organic fungicides deployed for managing grapevine diseases have a negative impact on fungal biodiversity in the soil because of chemical residue run-offs but also inside the grapevine vasculature due to the systemic properties of some of those fungicides (Pancher *et al.*, 2012, Morrison-Whittle *et al.*, 2017, Del Frari *et al.*, 2019).

Plants invest resources into actively recruiting bioactive microbes by releasing photoassimilates into the rhizosphere (e.g., carbohydrates, amino and organic acids). Our data supports Bulgarelli *et al.* (2013) hypothesis of a two-step selection model for root bacteria

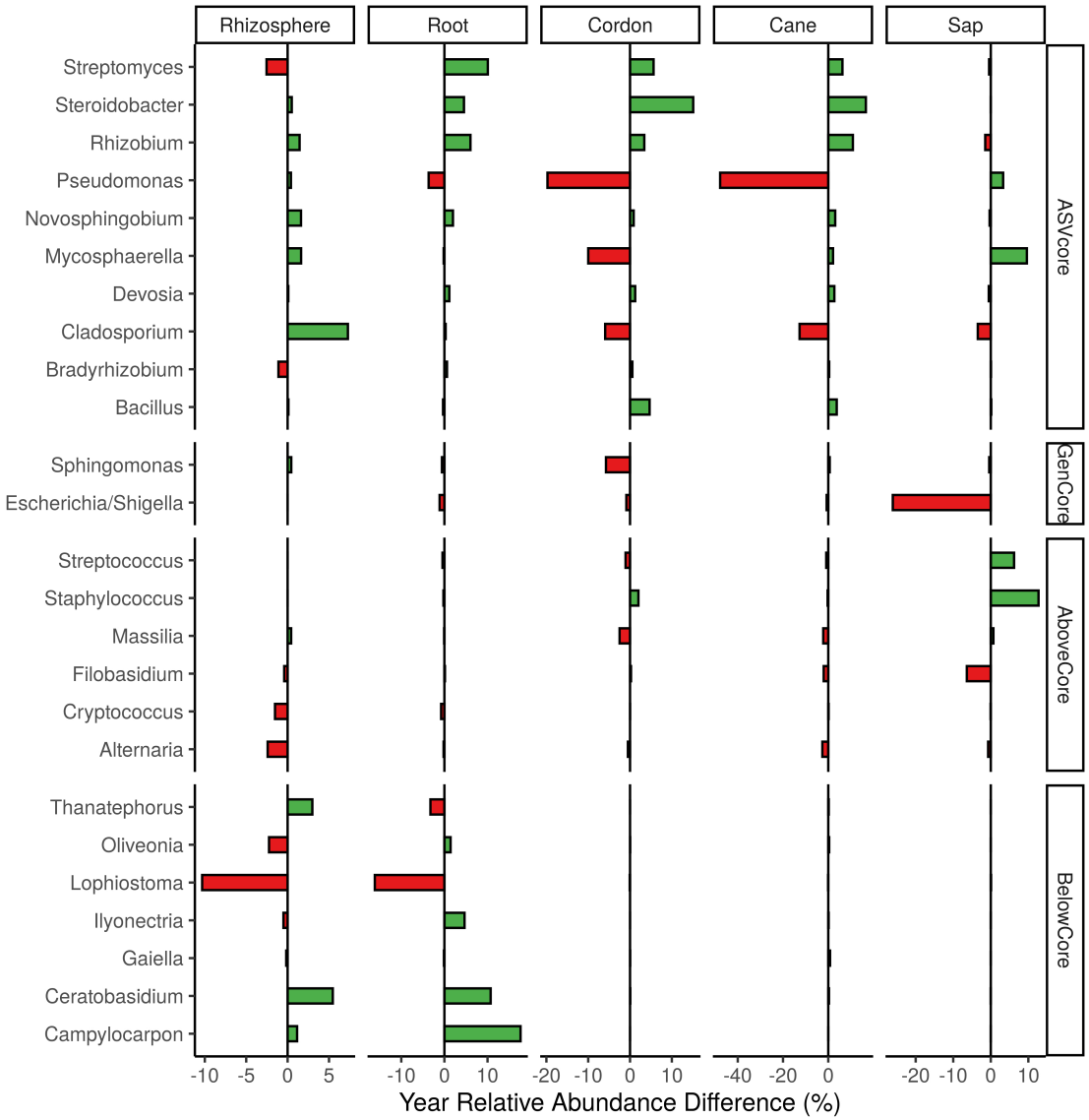

Figure 2.8: Relative abundance differences between years of prevalent microbes as described in Figure 4 and Figure 5. Red bars show a decrease in abundance from 2017 to 2018 while green bars represent an increase in mean abundance from 2017 to 2018 sampling times.

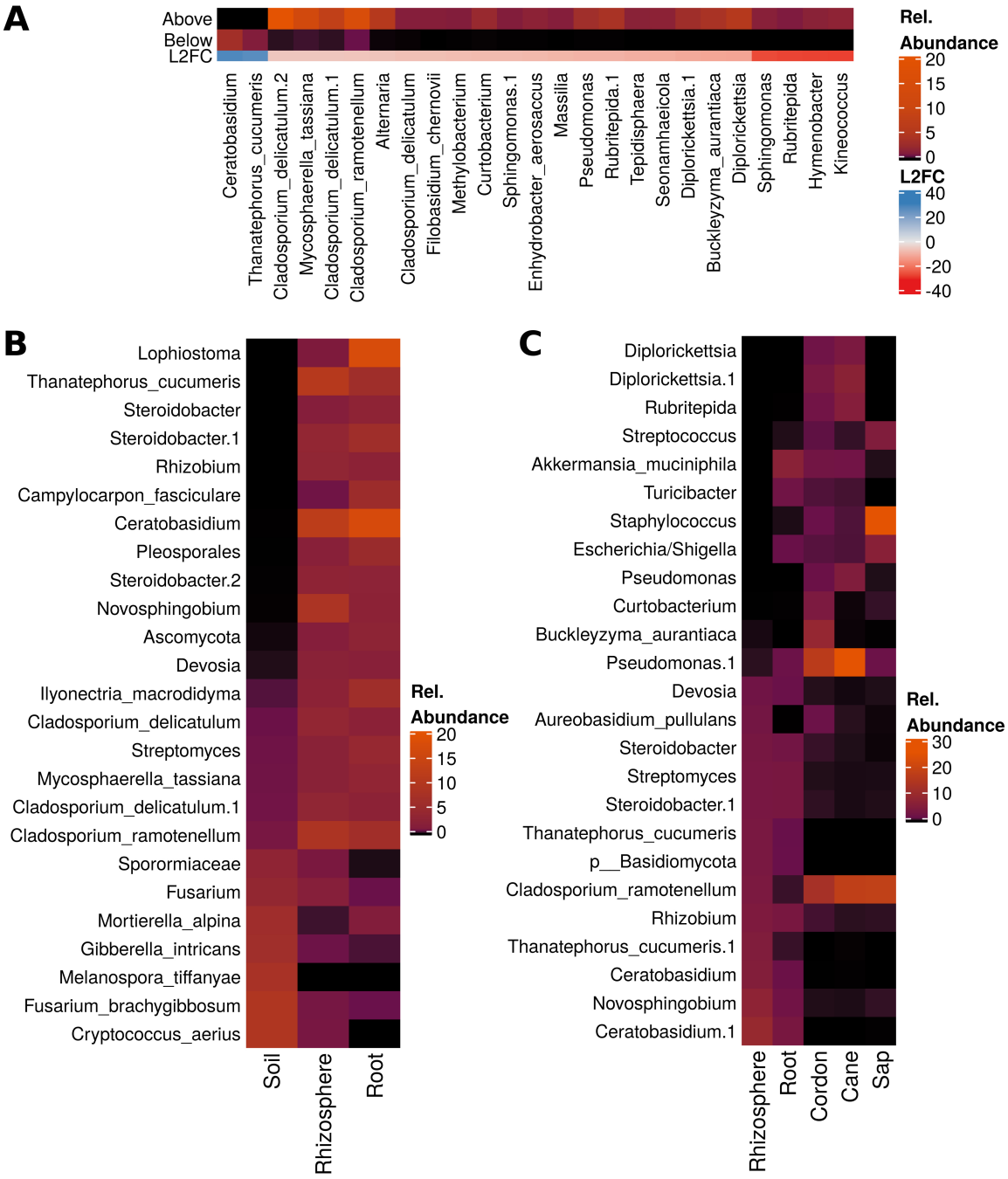

Figure 2.9: Relative abundance heatmaps of significant taxa as determined through DESeq2 analyses. (A) Data is grouped by above- (cane, cordon) and belowground (root) habitats and log2 fold change (L2FC) as determined through DESeq2 is displayed in a colorimetric bar. Tissue specific DESeq2 results for (B) belowground compartments and (C) endosphere biocompartments. Data was filtered to a p <0.01 cut-off and log2 fold change of >5 or <-5. The top 25 most abundant taxa are displayed. Black squares represent absence of taxa.

differentiation. Hence, the rhizosphere bacteriome profile was distinct from the soil profile with evidence of enrichment of plant growth promoting bacteria (*Rhizobium*, *Devosia*, *Streptomyces*, *Pseudomonas*). Zarraonaindia *et al.* (2015) also measured an enrichment of Rhizobiales, *Proteobacteria* and *Actinobacteria*, while Bona *et al.* (2019) showed that *Streptomyces*, *Pseudomonas* and Rhizobia were the most active bacteria involved in phosphorus and nitrogen metabolism in the grapevine rhizosphere. In addition, we measured a noticeable enrichment of *Novosphingobium* and *Steroidobacter* in rhizocompartments. These bacteria may play a role in bioremediation as they are known to metabolize aromatic compounds (Fahrbach *et al.*, 2008, Gan *et al.*, 2013). *Novosphingobium* was also found across viticulture areas and has been proposed to promote, by way of enhancing the quorum-sensing signal, the spread of pTi plasmid among Agrobacterium/Allo*Rhizobium* the causal agents of crown gall (Gan *et al.*, 2019). We should point out that there was no sign of crown gall in our vineyard, but Allo*Rhizobium* was found in our dataset. In contrast, we measured a drop in fungal diversity from soil to rhizosphere combined with a colonization of root pathogens (*Ilyonectria*, *Campylocarpon*, and *Thanatephorus/Rhizoctonia*) and saprobes (Ceratobasidium, *Mycosphaerella*, and Lophiostoma) suggesting that the acquisition model is not well supported with fungi, at least in a vineyard setting. Saprophytic fungi likely colonize the root zone in an opportunistic manner and decay products of the root exudates, while necrotrophic pathogens could also break-down root cell walls to gain access to the endorhiza. Martinez-Diz *et al.* (2019) made similar observations with both saprophytic and pathotrophic fungi colonizing the rhizocompartments of wine grapes in Spain. Surprisingly, we measured very low abundance of mycorrhizae (*Rhizophagus*, *Glomus*) in our

dataset. In agricultural tree and vine cropping systems, mycorrhizae play an essential role in supporting tree health by way of a mutualistic interaction with the host roots (Cheng & Baumgartner, 2004, Mercado-Blanco *et al.*, 2018). Those interactions are sensitive to soil management practices and the in-between rows mechanical weeding couples with the lack of cover cropping as implemented in our commercial vineyard are likely determinants for the low mycorrhizae incidence (Trouvelot *et al.*, 2015, Mercado-Blanco *et al.*, 2018).

As expected, we measured a significant decrease in both fungal and bacterial richness from the rhizosphere to the endorhiza, and 64% of the root endophytes were derived from the rhizosphere. The conditions encountered in those vastly different habitats select for a narrow group of microbes with plastic traits capable of adapting to environmental changes. It has been reported that culturable bacteria decrease from 107-109 CFU g-1 of soil in the rhizosphere to 105-107 CFU g-1 of fresh weight in the root (Compant *et al.*, 2010), which is in line with data from culture-independent studies conducted for several cropping systems (Edwards *et al.*, 2015, Cregger *et al.*, 2018, Fitzpatrick *et al.*, 2018) including grapevine (Zarraonaindia *et al.*, 2015, Marasco *et al.*, 2018, Martinez-Diz *et al.*, 2019). The vine endosphere collective microbiome was dominated by *Proteobacteria* and *Ascomycota*, but also displayed substantial abundance of *Basidiomycota*, *Actinobacteria*, *Bacteroides*, *Verrucomicrobia*, and *Firmicutes* in the root that were in the range of previous reports (Zarraonaindia *et al.*, 2015, Marasco *et al.*, 2018, Martinez-Diz *et al.*, 2019). A subgroup representing about 5% of the filtered ASVs residing in the vine endosphere and originating from the soil/rhizosphere was able to systemically colonize the grapevine vasculature and shape its core microbiome backbone including two fungal (*Cladosporium*, and

Mycosphaerella) and eight bacterial (*Streptomyces*, *Bacillus*, *Devosia*, *Novosphingobium*, *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, and *Steroidobacter*) taxa. Most of these taxa have been identified on or in grapevine organs both above and belowground, supporting the theory that rhizospheric microbes may use the host vascular system as a transportation pathway (Compant *et al.*, 2010, Compant *et al.*, 2011, Zarraonaindia *et al.*, 2015). We propose that only *Streptomyces*, *Bacillus*, *Pseudomonas*, *Novosphingobium* and *Rhizobium* should be viewed as keystone taxa due to their confirmed cosmopolitan distribution across viticulture areas combined with their high incidence in our dataset and across tissue habitats. In grapevine, *Streptomyces*, *Bacillus*, *Pseudomonas* and *Rhizobium* are well described plant growth promoters and have been shown to maintain environmental fitness against biotic and abiotic stressors (Barka *et al.*, 2002, Compant *et al.*, 2011, Baldan *et al.*, 2015, Rolli *et al.*, 2015, Andreolli *et al.*, 2016, Jiao *et al.*, 2016, Zhao *et al.*, 2016, Alvarez-Perez *et al.*, 2017, Deyett *et al.*, 2017, Ma *et al.*, 2017, Nigris *et al.*, 2018), while *Novosphingobium* may also play a role in abiotic stress tolerance through bioremediation of aromatic compounds (Gan *et al.*, 2013). The two nitrogen-fixing *Devosia* and Brady*Rhizobium* (Rivas *et al.*, 2002, Bona *et al.*, 2019), although present throughout the vine endosphere, were niche specific as they displayed higher prevalence in roots, and also were only detected in a small subset of samples and thus were not considered as part of the core microbiome. Moreover, the biological function of the two fungal members, *Cladosporium* and *Mycosphaerella*, is more ambiguous. Both have been found on the surface and inside grapevine (Zhang *et al.*, 2017, Dissanayake *et al.*, 2018, Singh *et al.*, 2018, Deyett & Rolshausen, 2019). Species of *Cladosporium* have also been reported as pathogenic of grape berries or act as a bio-

control agent on pruning wound surface against a causal agent of grapevine wood disease (Munkvold & Marois, 1993, Briceno & Latorre, 2008, Iasur-Kruh *et al.*, 2015, Zhang *et al.*, 2017), while Li *et al.* (2019) showed that *Cladosporium* sphaerospermum could promote plant growth. Additional research will need to determine if these taxa have a functional role in grapevine.

Our results also supported that within the host and across its different habitats there was clear partitioning of the microbiome with niche adaptation of distinct taxonomic groups (Zarraonaindia *et al.*, 2015, Cregger *et al.*, 2018). The microbiome of both below and above ground habitats intermixed in a limited capacity and mostly by way of plant sap flow. The sap microbial fingerprint was unique because it was colonized with yeasts (*Aureobasidium*, *Sporobolomyces*, *Cryptococcus/Filobasidium*) commonly found on grape berry surface (Barata *et al.*, 2012) and bacteria (*Staphylococcus*, *Escherichia/Shigella*, and *Streptococcus*) commonly associated with human (Zoetendal *et al.*, 2012, Lloyd-Price *et al.*, 2016), and plant (VanderZaag *et al.*, 2010, Barata *et al.*, 2012, Yousaf *et al.*, 2014, Zarraonaindia *et al.*, 2015) systems. Grapevine constantly interface with humans throughout its lifespan, during the propagation phase in nursery and the production phase within vineyard, which may be the source of cross inhabitation. Deploying metagenomics tools could further unfold the traceability and reveal the degree of host specificity of these bacterial populations. We found that only 34% of the total taxa residing in the host vascular system stems from the rhizosphere and those remained localized within the endorhiza. In fact the majority of the host endosphere microbiome likely originates either from above ground introduction or from native microbes that were already present in grapevine at the time of planting. The

communities residing in the aboveground compartments were predominantly colonized with *Pseudomonas* and *Cladosporium* and were structured around single ASVs capable of colonizing all the biocomparments and with a complex of strains/species adapted to a specific habitat. They both especially thrived in the young cane tissue and were previously detected as early as bloom (Deyett & Rolshausen, 2019), suggesting that they may be pioneers of young and developing green organs. In addition to *Pseudomonas*, *Methylobacterium* and *Sphingomonas* were signature taxa of the above ground vascular system as previously recognized, and could play a role in phytohormone production and defense against vascular pathogens occupying the same niche (Zarraonaindia *et al.*, 2015, Lai *et al.*, 2016, Asaf *et al.*, 2017, Deyett *et al.*, 2017). Interestingly, we also found that grapevine sap, cordon and cane were colonized by a wide range of bacteria found in grape berry and involved in malolactic fermentation (*Lactobacillus*), wine spoilage (*Gluconobacter*), or of unknown effect to wine making (*Acinetobacter*, *Enterobacter*), but could shape to some degree the identity of a wine region (Barata *et al.*, 2012, Belda *et al.*, 2017).

Several routes of horizontal transmission of microbes have been identified. Grapevines are pruned to a specific training system in order to optimize fruit quality and yield, and the exposed vascular system becomes the entry point for airborne microbes. This is a major doorway for vascular wood pathogens and study on timing of pruning wound susceptibility and protection with fungicides has been extensively studied for practical reasons to manage disease in vineyards (Munkvold & Marois, 1995, Rolshausen *et al.*, 2010, Diaz & Latorre, 2013, Pouzoulet *et al.*, 2019). In contrast, knowledge about the microbial epiphytes dynamic colonizing wound surface and their ability to adapt to an endophytic lifestyle has

been limited in scope. Munkvold & Marois (1993) found by culture-dependent approach that pruning wounds were colonized with ubiquitous bacteria (*Pseudomonas*, *Bacillus*) and fungi (*Aureobasidium*, *Penicillium*, *Fusarium*, *Cladosporium*, *Alternaria*, *Epicoccum*), but mostly yeasts were able to further colonize the host vascular system. Epiphytes of the phyllosphere, carposphere and anthosphere microbiome (*Bacillus*, *Pseudomonas*) have also been shown to enter the host via natural openings such as stomatas (Compant *et al.*, 2011). Finally, vector-assisted transmission of grapevine endophytic microbes has been clearly established (Lopez-Fernandez *et al.*, 2017). Several insects (e.g., mealybug, leafhopper, sharpshooter) commonly feed on the host xylem and phloem and could have transmitted some of the bacteria and fungi detected in our analyses (Hail *et al.*, 2011, Iasur-Kruh *et al.*, 2015, Lopez-Fernandez *et al.*, 2017). For example, we found evidence of *Xylella*, the causal agent of Pierce's Disease, in several of our samples likely because of introduction events from the glassy-winged sharpshooter, an important vector of the pathogen in southern California (Redak *et al.*, 2004). Another alternative route for microbial introduction is prior to vineyard planting, during the propagation phase in nurseries. In perennial cropping systems, plants are not grown from seeds but are propagated from wood cuttings and grapevines are a combination of a fruiting cultivar (scion) grafted on a rootstock. Plant propagation practices are known to spread many fungal vascular pathogens (Gimenez-Jaime *et al.*, 2006) and one can suspect that it also spreads other microbial endophytes (Waite *et al.*, 2013). Finally, we also anticipate that a fraction of the host microbiome is inherited from the mother vine in nursery and passed on to commercial plants. Further experiments will help determine the fraction of the native communities that persist in the vine endosphere

following planting and characterize which microbes are introduced during the propagation phase at the nursery and the production phase in the vineyard.

The endophytes residing in plants are major contributors to host health and productivity. In order to succeed in developing sustainable agricultural practices, one must better understand the factors that govern microbial assembly in the host and shed light on their biological functions. This study provides insightful information about the origin of the microbes in the vine endosphere and a perspective with regards to potential transmission from mother vines in nurseries and horizontal acquisition during the propagation and production phases of the hosts life. The profiling of microbial communities highlights target organisms with plant growth promoting capabilities. Whole genome metagenomics will aid in refining the population structure for some of the key organisms identified here and address the missing gaps about their source and habitat range. Metaproteomics and metabolomics will also aid in moving the scientific field from a descriptive phase to assigning functions to members of the microbiota. This fundamental knowledge may fuel the engineering of novel technological bioproducts with commercial applications or help with the implementation of cultural practices that support presence and abundance of key beneficial microbes associated with the host plant.

2.0.6 Referecnes

Alvarez-Perez JM, Gonzalez-Garcia S, Cobos R, Olego MA, Ibanez A, Diez-Galan A, Garzon-Jimeno E & Coque JJR (2017) Use of Endophytic and Rhizosphere *Actinobacteria* from Grapevine Plants To Reduce Nursery Fungal Graft Infections That Lead to Young Grapevine Decline. Appl Environ Microb 83.

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Chapter 3

Temporal Dynamics of the Sap Microbiome of Grapevine Under High Pierce's Disease Pressure

3.0.1 Abstract

Grapevine is a pillar of the California state economy and agricultural identity. The grapevine vascular system plays a key role by transporting nutrient, water and signals throughout the plant. The negative pressure in the xylem conduits, and low oxygen and nutrient content of its sap make it a unique and unexplored microbial environment. We hypothesized that grapevine hosts in its sap, microbes that have a beneficial impact on plant health by protecting against pathogen attack and supporting key biological processes. To address this hypothesis, we chose a vineyard under high Pierce's Disease (PD). PD is caused by the xylem-dwelling pathogenic bacterium *Xylella fastidiosa*. We selected ten grapevines within this vineyard with a range of phenotypes, and monitored them over 2 growing seasons. We sampled each vines at key phenological stages (bloom, veraison, and post-harvest) and used an amplicon metagenomics approach to profile the bacterial (16S -V4) and fungal (ITS) communities of the sap. This study is, to the knowledge of the

authors, the first comprehensive culture-independent microbiome analysis from the sap of a plant. We identified a core microbiome of the sap composed of seven bacterial (*Streptococcus*, *Micrococcus*, *Pseudomonas*, *Bacteroides*, *Massilia*, *Acinetobacter* and *Bacillus*) and five fungal (*Cladosporium*, *Mycosphaerella*, *Alternaria*, *Aureobasidium*, and *Filobasidium*) taxa that were present throughout the growing season. Overall, the sap microbial makeup collected from canes was more similar to the root microbial profile. Alpha diversity metrics indicated a microbial enrichment at bloom and in vines with moderate PD severity suggesting a host-driven microbial response to environmental cues. Beta diversity metrics demonstrated that disease condition and plant phenology impacted microbial community profiles. Our study identified several potential taxonomic targets with antimicrobial and plant growth promoting capabilities that inhabit the grapevine sap and that should be further tested as potential biological control or biofertilizer agents.

3.0.2 Introduction

The vascular system ensures several key biological functions in plants. The xylem and phloem elements are major vehicles for water and nutrient transportation and signaling routes between the below (i.e., root) and above (i.e., leaf, fruit) ground plant parts. The xylem and phloem saps differ in chemical composition and their profile is also influenced by the plant phenological stages and environmental factors (Andersen *et al.* 1995; Lucas *et al.* 2013; Savage *et al.* 2016). In grapevine, the xylem sap is initially rich in sugars (especially glucose and fructose) and amino acids (especially glutamine) in the spring time right after the dormant season, because of the remobilization of nutrient reserves (e.g. starch) stored in the main permanent structure of the vine (Keller 2010). This mobilization of nutrients is essential to support vegetative growth at bud-break until leaf becomes energy independent and transition from a sink to a source and export photosynthates and assimilates to the fruit via the phloem sap. To that end, research has focused on how irrigation and fertilization changed sap composition in order to maintain plant hydraulic function at maximum capacity and a balance between vegetative and reproductive growth (El-Razek *et al.* 2011; Peuke 2000).

Several biotic and abiotic factors such as freeze damage, drought, and pathogen infection compromise the integrity of the grapevine vascular system and impair plant sap flux (Hochberg *et al.* 2017; Pouzoulet *et al.* 2014; Todaro and Dami 2017). As a result the affected host shows a decrease in vigor and subsequently crop productivity and fruit marketability. One devastating vascular pathogen that plagues the California grapevine industry is Pierce's Disease (PD), caused by the gram-negative bacterium *Xylella fastidiosa*

(Wells *et al.* 1987). Insect sharpshooters transmit *X. fastidiosa* to the host upon feeding events (Redak *et al.* 2004). Following infection, the bacterium systemically colonizes susceptible host and multiplies to high titer causing vascular occlusions and a loss of xylem conductivity (Deyett *et al.* 2019). As a result, grapevine decline rapidly displaying symptoms of canopy dwarfing and thinning as well as leaf scorching and berry raisining (Varela *et al.* 2001). *In planta*, *X. fastidiosa* resides exclusively in the lumen of the xylem vessel where the negative pressure, low oxygen and nutrient content selects for adapted microorganisms. However, very little is known about the microbial taxa that inhabit the grapevine sap, their population dynamic over a growing season and their interaction with *X. fastidiosa*. Wallis *et al.* (2013) showed that PD affected the grapevine sap chemical composition but no study has looked at microbial community shifts in response to pathogen infection and colonization of the host and during disease symptoms development.

Grapevine microbiome research has mainly focused on plant organ epiphytes (i.e., berry, leaf and root) because of its importance with grape production and specifically with regards to fruit and foliar diseases management as well as the biological significance of indigenous microbes with the regional signature of a wine (Bokulich *et al.* 2014; Perazzolli *et al.* 2014; Zarraonaindia *et al.* 2015). Identification of the microbial communities inhabiting the grapevine endosphere was achieved using standard culture-dependent microbial techniques (Baldan *et al.* 2014; Compant *et al.* 2011; Dissanayake *et al.* 2018; Kraus *et al.* 2018; West *et al.* 2010). Culture-independent amplicon metagenomic approaches have recently been deployed to improve the microbial profiling of the grapevine woody organs including trunk and cane (Deyett *et al.* 2017; Dissanayake *et al.* 2018; Faist *et al.* 2016).

Figure 3.1: Pierce's disease (PD) rating scale; (A) No symptoms or mild PD symptoms with only a few canes (<10% of the canopy) showing marginal leaf scorching; (B) Moderate PD symptoms with visual dwarfing and thinning on 10-50% of the canopy and/or canes displaying leaf scorching. The affected canes can also show green island and match-stick petioles and berry raisining late in the season. (C) Severe PD symptoms with 50-100% of the vine canopy displaying dwarfing, thinning, leaf scorching and wood dieback. The affected canes showed green island and match-stick petioles and berry raisining late in the season.

Unraveling the microbiome of the grapevine sap would provide a comprehensive view of the microbes that systemically move throughout the plant and would establish a framework for targeting biological control agents (BCAs) of vascular pathogens or plant growth promoting agents. Identifying BCAs that can control *X. fastidiosa* through direct inhibition or niche displacement and/or commensal and symbiotic organisms that can support the host immune system could become an integrated alternative approach to the current insect vector management strategy. Our research was designed to address this gap in the knowledge by characterizing the bacterial and fungal taxa that shape the xylem sap microbial communities and determining how the host phenology and PD severity impact microbial profile.

3.0.3 Materials and Methods

Sap Sample Collection

Shoot samples were collected from a commercial vineyard in Temecula, California, subjected to high insect sharpshooter (the vector of *X. fastidiosa*) pressure. Ten grapevines were selected in 2016 on the basis on PD symptoms, with five healthy vines showing no or mild symptoms and five vines showing intermediate to severe symptoms (Figure 3.1). Canes were later sampled in 2017 and 2018 at bloom (spring), veraison (summer) and post-harvest (fall) from the same grapevines. At each time point, three canes were pruned-off randomly along the grapevine cordons. In addition, grapevines were visually rated for PD symptoms at post-harvest using the same disease rating scale (no/mild, intermediate, or severe PD-symptoms; Figure 3.1). Canes were placed in a cooler with ice packs and brought back to the laboratory. On the same day of the sampling, canes were cut to 40 cm long, inserted in a Scholander pressure chamber (PMS Instruments, Albany OR) and pressurized to 400-550 PSI. Liquid sap was harvested from the end of the cane with a pipette and stored at 4°C until further processing. To obtain culturable sap microbiome, 20 μl of sap was plated onto each of the following media: lysogeny broth (LB), trypticase soy agar (TSA), and potato dextrose agar (PDA). TSA and LB plates were stored at 28°C for 3-5 days and PDA plates at room temperature for 7 days. The bacteria and fungi recovered were sub-cultured to obtain single colonies and pure strains, respectively.

DNA Extraction, PCR Condition, Library Preparation and Sequencing

The DNA was extracted from 250 μl of sap using the ZymoBIOMICS DNA miniprep kit per manufacturer's protocol (Zymo Research). The DNA was quantified using a Synergy HTX multi-mode reader (Biotek Instruments, Winooski, VT) for nucleic acid quantification. In addition, the DNA was extracted from pure bacterial colonies and fungal strains using Qiagen blood and tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions for cultured cells with an initial extended incubation time of 30 min instead of 10 min.

The bacterial 16S and fungal ITS rRNA region was amplified for each DNA sample using the earth microbiome protocol and primers (http://www.earthmicrobiome.org/). Briefly, primers 515F and 806R with Caporaso barcodes were used for 16S amplification and ITS1f-ITS2 was used for fungal ITS amplification (Caporaso *et al.* 2010). Each sample was made in a 25 μl PCR reaction using 10 μl of Phusion hot start flex 2x master mix, 0.5 μl of primer (10 μm) and 2 μl of DNA. Thermal cycling parameters for ITS were 94°C for 1 min; 35 cycles of 94°C for 30 s, 52°C for 30 s, 68°C for 30 s; followed by 68°C for 10 min. Thermal cycling parameters for 16S amplification were 94°C for 3 min; 35 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 s; followed by 72°C for 10 min. Each PCR was accompanied with a negative control to ensure that the mastermix and barcodes were not contaminated. Samples were checked against a 1% agarose gel for visualization of appropriate band size. PCR products were quantified using a Synergy HTX multi-mode reader for nucleic acid quantification. Libraries were prepared by combining equal quantities of DNA from each sample. Libraries were cleaned using AMPure XP PCR purification system (Beckman Coulter) per

manufacturer's protocol. Final concentration of libraries was quantified using qPCR and bioanalyzer before being sequenced on the MiSeq instrument (Illumina, San Diego, USA) using 600-cycle (2X300 paired-end) V3 sequencing kit for fungal reads and the 500-cycle (2x250 paired-end) V2 sequencing kit for 16S at the UCR Genomics Core. Fungal and bacterial sequences were deposited in NCBI under the accession number PRJNA548584.

Culturable microbes were Sanger sequenced using the 27F/1392R primer pair of the 16S bacterial gene (Turner *et al.* 1999) and ITS1/ITS4 primer pair for fungal ITS region (White *et al.* 1990). Each sample was made in a 25 μl PCR reaction using 10 μl of Phusion hot start flex 2x master mix, 0.5 μl of each primer (10 uM) and 2 μl of DNA template. Thermal cycling parameters were 94°C for 2 min; 35 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min; followed by 72°C for 5 min. PCR product was visualized on a 1% agarose gel for appropriate band size, cleaned using Qiagen PCR Clean Up kit (Qiagen, Valencia, CA), quantified using Synergy HTX quantifier and submitted for Sanger sequencing at the UCR Genomics Core. Sequences were check for quality before using the NCBI BLAST database for identification.

Computational Analyses

Raw reads were first filtered for quality using sliding window 5:20 with trimmomatic (Bolger *et al.* 2014). Reads shorter than 125 bp were removed and low quality reads were filtered out. PhiX reads were then removed from the sequences. Due to the varying lengths of the fungal ITS reads, primers were removed using cutadapt version 1.18 and matching to primer sequences. Libraries were then demultiplexed using QIIME version 1.9.1 (Caporaso *et al.* 2010). DADA2 version 1.6.0 (Callahan *et al.* 2016) in R version

3.4.4 was used for the remainder of the processing. Within DADA2, sequences were filtered to contain no ambiguous base calling, and no more than 2 errors. 16S reads were fixed at lengths for 240 bp. Reads were then de-replicated, and further filtered using the sample inference algorithm and learned error rates within the DADA2 pipeline. Paired end reads were merged using standard arguments. Chimeric sequences were also filtered out. Taxonomy was assigned using the Unite database version 10.10.2017. DADA2 method creates ASV (amplicon sequence variant, similar to operational taxonomic units or OTUs, but is capable of resolving amplicons to a single nucleotide) and thus may make species level identification when 100% of sequences match to reference (Callahan *et al.* 2016). Bacteria identification was realized using the IDTAXA classification against the SILVA SSU r132 reference database. ClustalW and maximum likelihood parameters were used to create a bacterial tree for UniFrac metrics.

Data were then inputted into the phyloseq package version 1.22.3 (McMurdie and Holmes 2013). Microbes unidentified at the kingdom and phylum level or classified as plant or other contaminants were removed for both datasets. Phyla that did not occur in at least 5% of samples were removed as well as singletons, doubletons and taxa that only occurred in one vine. Samples that had too few reads were also removed. For analysis, comparing disease severity, only the post-harvest time points were used. For taxonomy charts, libraries were aggregated to the genus level and transformed to relative abundance. Fungal and bacterial genera were filtered to the 0.1% level. SunburstR version 2.1.0 was utilized to visualize the taxonomy pie charts. Statistical differences between genera in the *Xanthomonadaceae* were computed using Kruskal-Wallis and pairwise comparisons where

done with Wilcox pairwise test. Prevalence Venn diagrams was used to identify unique taxa that were more likely associated with the specific grapevine phenological stages at the different sampling time point and with specific disease condition. We set stringent filters with taxa needed to occur in at least 50% of the samples in a given category. UpSetR version 1.3.3 was used to create graphical representation of prevalence Venn diagrams.

Using the number of unique families as a proxy for alpha diversity, mean and standard error were calculated for each time point grouping. Error bars represent standard error of the mean. Statistics on count of family were determined by generalized linear model using Poisson regression and statistical significance on pairwise comparison were conducted through Tukeys test using the multcomp package version 1.4-8. To look at the difference between groupings, beta-diversity plots were created using weighted UniFrac and PCoA metrics for bacteria and Bray-Curtis dissimilarity matrix and NMDS ordination matrix for fungi using the vegan package version 2.4-5. Adonis test with 999 permutations were run to determine statistical differences among centroids. Pairwise comparisons were calculated with the RVAideMemoire package version 0.9-71. To find differentially abundant microbe associated with season or condition, DeSeq2 was used. Microbes that occurred in more then one sample and in more than one year were used in the analysis. Random Forests supervised machine learning algorithm was applied to the dataset to learn a function that relates predictors (ASVs) to classification, here phonological stage and disease condition (Breiman 2001). Random Forest creates a function using part of the dataset to train and validates that function by classifying the remaining subset of samples. ASVs are assigned with an importance score, which indicates the increase in the functions error rate if the

taxa would be removed. In this way, microbes with a high importance score are valuable predictors for a classification. Random forest was carried out using package randomForest version 4.6-14.

3.0.4 Results

DADA2 analysis revealed that there were 2,875 bacterial amplicon sequence variants (ASVs) and 2,694 fungal ASVs in 68 sap samples (two samples were removed based on poor quality sequences). After removal of contaminants, singletons, doubletons and taxa that did not occur in at least 5% of the samples, there were 168 fungal ASVs and 390 bacterial ASVs. Sap samples that did not have at least 500 reads were removed from the analyses. After filtering, we used a total of 61 samples from the bacterial dataset including 26 from post-harvest, 15 from bloom and 20 from veraison and a total of 65 samples from the fungal dataset including 29 from post-harvest, 16 from bloom, and 20 from veraison.

Phylogeny and relative abundance of fungal and bacterial taxa were represented in a Sunburst plot (http://rpubs.com/edeye001/487241). Results showed that fungal communities were dominated by the *Ascomycota* phylum (92%) and the remaining was composed with the phylum *Basidiomycota* (8%). *Cladosporium* represented over half (56%) of the total fungal genera followed by *Mycosphaerella* (16%) and *Alternaria* (9%). *Filobasidium* (6%) was the major genus among the *Basidiomycota*. The sap culturable mycobiome indicated that *Cladosporium* was also the main fungus recovered and also included the taxa *Filobasidium* and *Mycosphaerella*. The bacterial community composition was more diverse than the fungal community. *Proteobacteria* (54%) was the major phylum, followed with

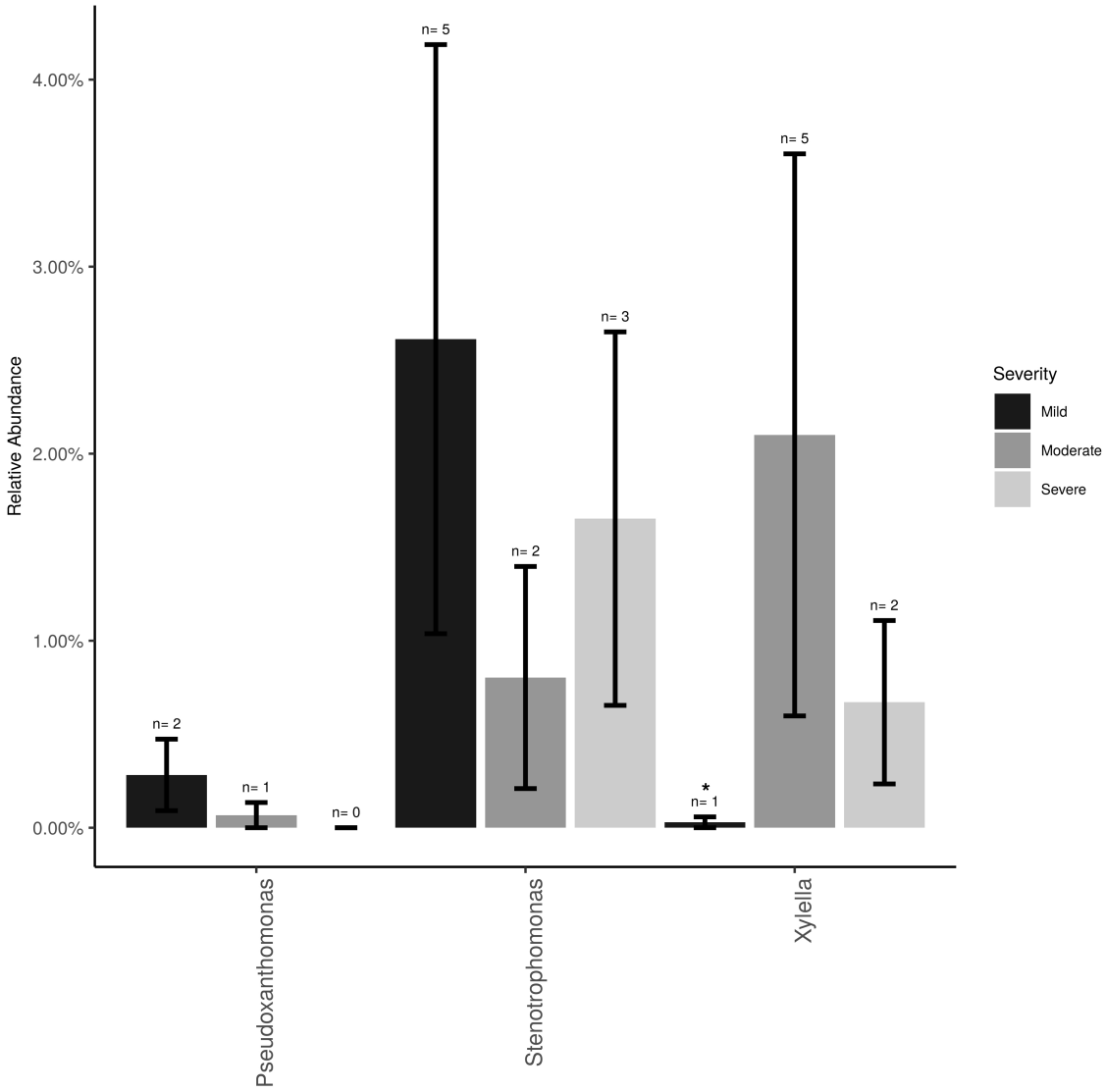

Figure 3.2: Mean relative abundance and incidence for *Xylella*, *Stenotrophomonas* and *Pseudoxanthomonas* (Family *Xanthomonadaceae*) in the total sampled grapevines (n = 26) showing no/mild (n = 12), moderate (n = 7) or severe (n = 7) PD symptoms at post-harvest. Error bars represent standard error of the mean. n value above the bar represent the number of vines that tested positive for *Xylella*.

Firmicutes (24%), *Actinobacteria* (12%), and *Bacteroidetes* (8%). At the genus level an unidentified genus in the Enterobacteriaceae family was most abundant (19%). *Streptococcus* represented (10%) followed with *Bacteroides* (6%), *Bacillus* (6%), *Acinetobacter* (4%) and *Pseudomonas* (3%). The culturable bacteria were mostly in the genus *Bacillus*, but *Paenibacillus*, *Virgibacillus*, *Curtobacterium*, and *Micrococcus* were also re-isolated.

The *Xanthomonadaceae* (the family that *X. fastidiosa* belongs to) represented 3% of the overall dataset and was comprised of three genera, *Stenotrophomonas* (2%), *Xylella* (0.5%), and *Pseudoxanthomonas* (0.5%). *Xylella*, the causal agent of PD, was found at low frequencies and low incidence (11 samples total) and mostly at post-harvest (eight samples of the total 26 post-harvest samples = 31%). *Xylella* was also detected at bloom (one sample) and veraison (two samples). Relative abundance of *Xylella* at the post-harvest time point was only significantly higher ($P < 0.05$) in vines showing moderate PD symptoms compared to healthy vines (i.e., showing mild or no symptoms; Figure 3.2). *Stenotrophomonas* incidence and abundance was the highest in healthy vines although not statistically different from the other disease conditions.

Alpha-diversity showed that there were approximately twice as many bacterial than fungal families in grapevine sap (Figure 3.3A). Bacterial family richness increased significantly at post-harvest in aging grapevines between 2016-17 and 2018 ($P < 0.001$). In addition, bloom and veraison time-points displayed significant higher family richness compared to post-harvest in both years for bacteria ($P < 0.001$) and in 2018 for fungi ($P < 0.01$). Moreover, vine condition impacted microbial diversity (Figure 3.3B). Vines categorized as moderately PD symptomatic at post-harvest harbored significantly higher

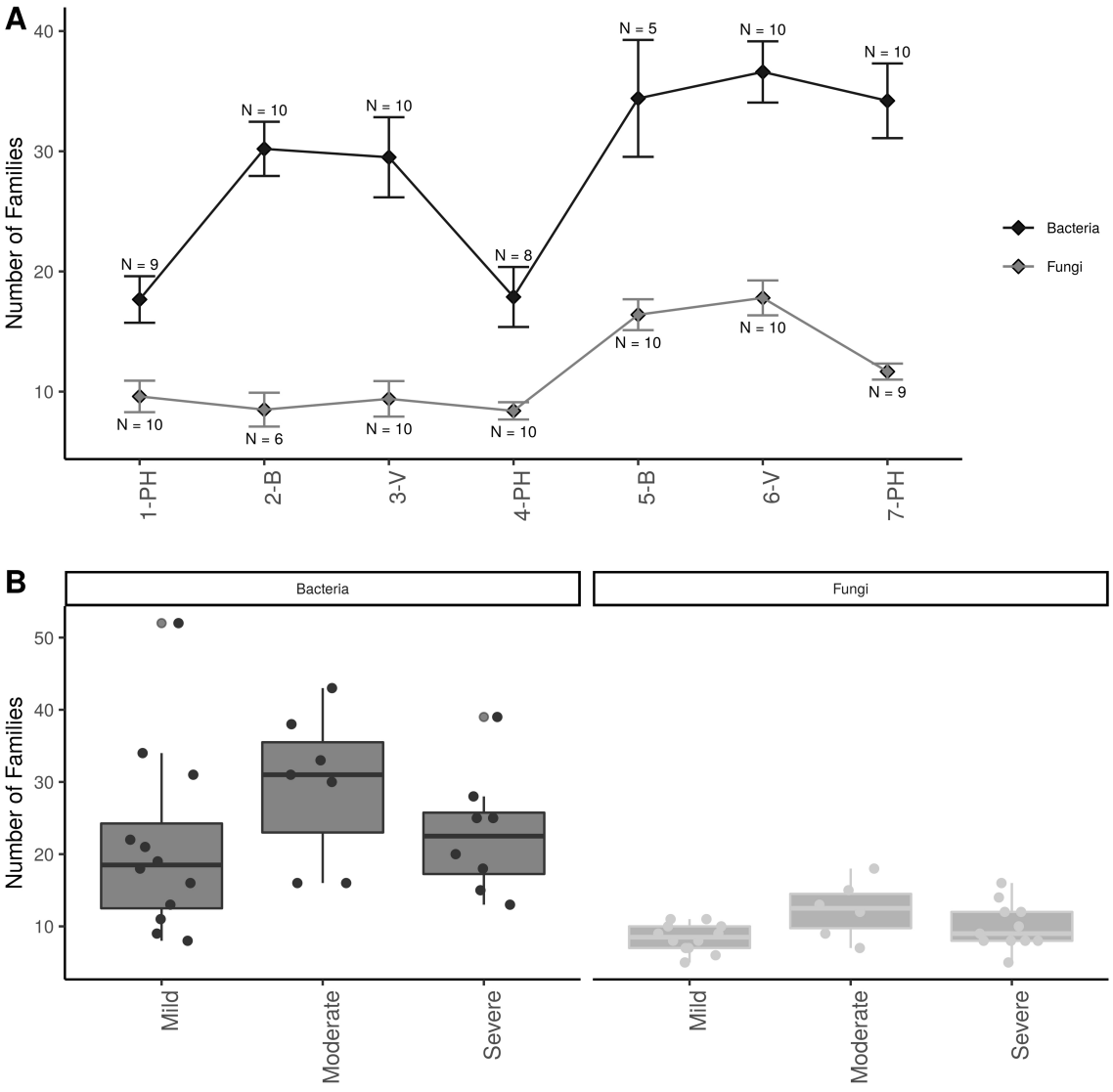

Figure 3.3: Alpha diversity plots; (A) Familial richness in bacterial and fungal sap microbiomes across seasonal timepoints; 1-PH : post-harvest 2016, 2-B : bloom 2017, 3-V: veraison 2017, 4-PH : post-Harvest 2017, 5-B : bloom 2018, 6-V: veraison 2018, and 7-PH : post-harvest 2018. Timepoint scheme is the same across graphs. Error bars represent standard error of the mean. n values represented the number of samples for each time point. (B) Number families in bacterial and fungal within different disease conditions at the PH time point. Point represent individual samples.

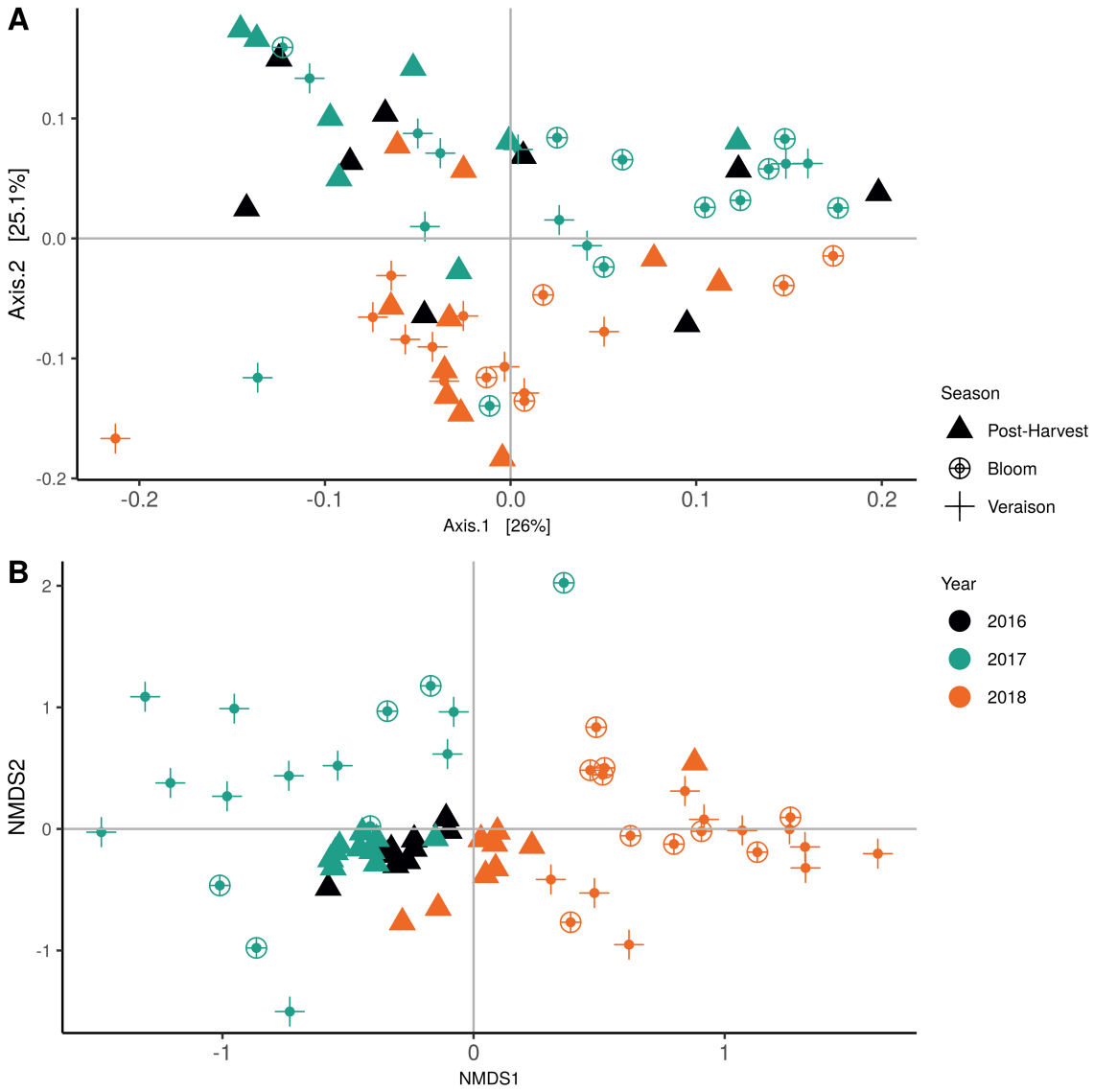

Figure 3.4: Beta diversity plots for seasonal time points based on: (A) Weighted UniFrac distance for bacteria and (B) Bray-Curtis dissimilarity for fungi. Points represent one vine community. Point are colored by year, shaped by season.

number of bacterial and fungal families compared to mildly symptomatic vines ($P < 0.05$). Severely symptomatic vines also had significantly fewer unique bacterial families compared to moderately symptomatic vines ($P < 0.05$).

Weighted UniFrac of bacterial beta-diversity plots (Figure 3.4A) indicated a significant clustering due to the year of sampling ($P < 0.001$) and the time of sampling in a given year ($P < 0.01$). Pairwise PERMANOVA revealed that bloom was distinct from the other two phenological stages ($P < 0.05$) and that 2018 was distinct from 2016 and 2017 ($P < 0.01$). Fungal Bray-Curtis beta-diversity plots (Figure 3.4B) indicated that both year of sampling ($P < 0.01$) and time of sampling ($P < 0.01$) were significantly different in the clustering of fungal communities. There was a strong clustering of the samples collected at post-harvest for all three years in comparison to other sampling year and time. A pairwise PERMANOVA revealed that post-harvest was significantly different to both bloom and veraison ($P < 0.05$) and 2018 was significantly different from both 2017 and 2016, and that 2016 was significantly different than 2017 ($P < 0.05$). Prevalence Venn diagrams indicated that seven bacteria (*Streptococcus*, *Micrococcus*, *Pseudomonas*, *Bacteroides*, *Massilia*, *Acinetobacter* and *Bacillus*) and five fungi (*Cladosporium*, *Mycosphaerella*, *Alternaria*, *Aureobasidium*, and *Filobasidium*) were ubiquitous across season at all three plant phenological stages (Figure 3.5). In contrast, other taxa were more likely associated with specific seasonal time points likely contributing for the significant differences in alpha diversity (Figure 3A). Hence, *Sphingobium*, *Novosphingobium*, *Methylobacterium* and *Streptomyces* were more specific to bloom and *Actinomyces*, *Neisseria*, and *Hygrogenophilus* to veraison. We used Random Forest analyses to identify predictors (fungal and bacterial taxa)

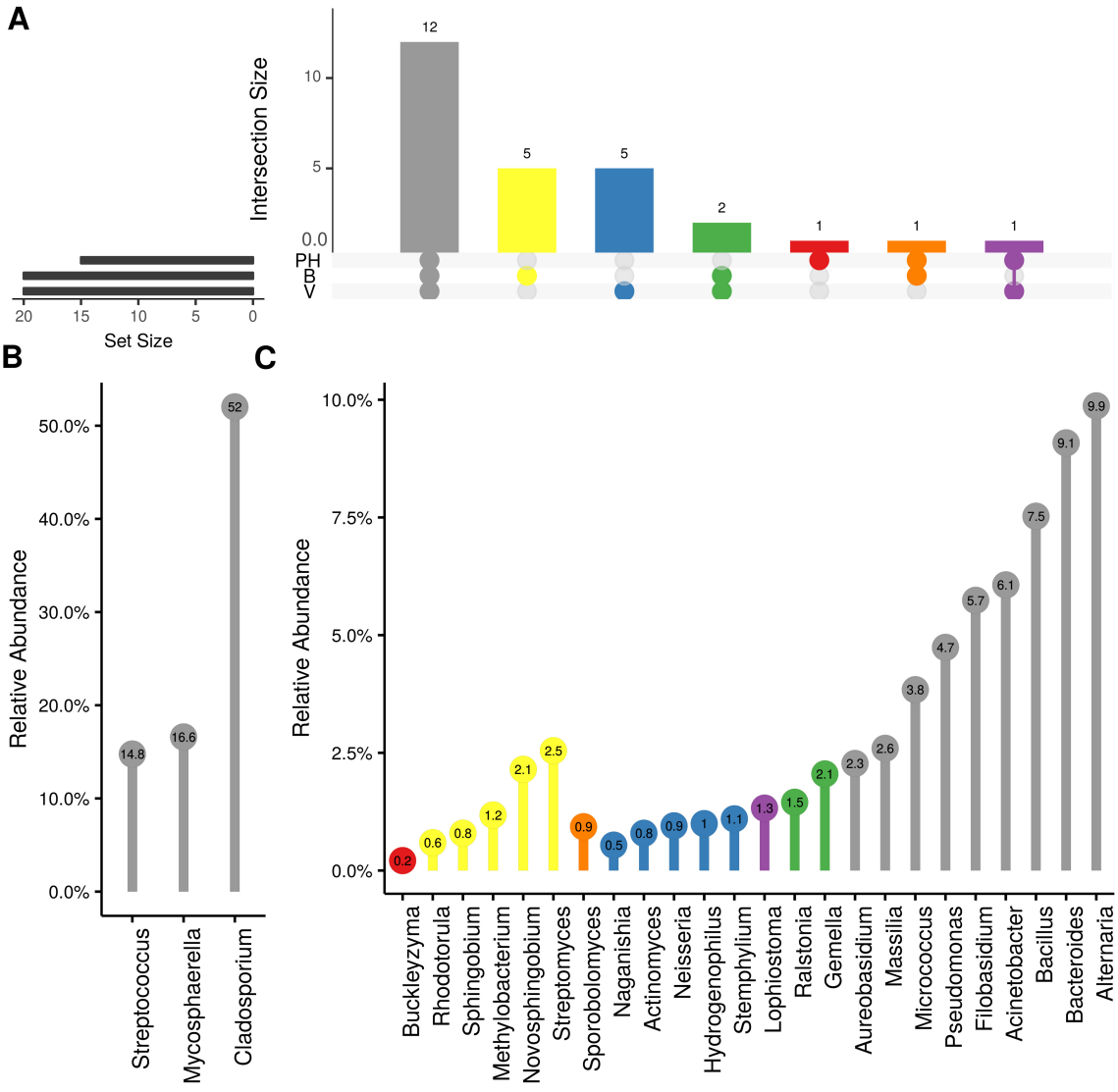

Figure 3.5: Prevalence Venn diagrams. Genera must occur in 50% of samples for each phenological stage to be considered. (A) quantities of genera intersection between seasonal time points. (B) High and (C) low relative abundant genera associated with a category. Color scheme is the same as (A).

responsible for the clustering in the beta-diversity metrics (Figure 3.4) and identified that the most abundant taxa (*Alternaria*, *Cladosporium*, *Filobasidium*, *Mycosphaerella*, *Streptococcus*, *Micrococcus*, *Bacteroides* and unknown *Enterobacteriaceae*) were the likely drivers (Figure 3.6).

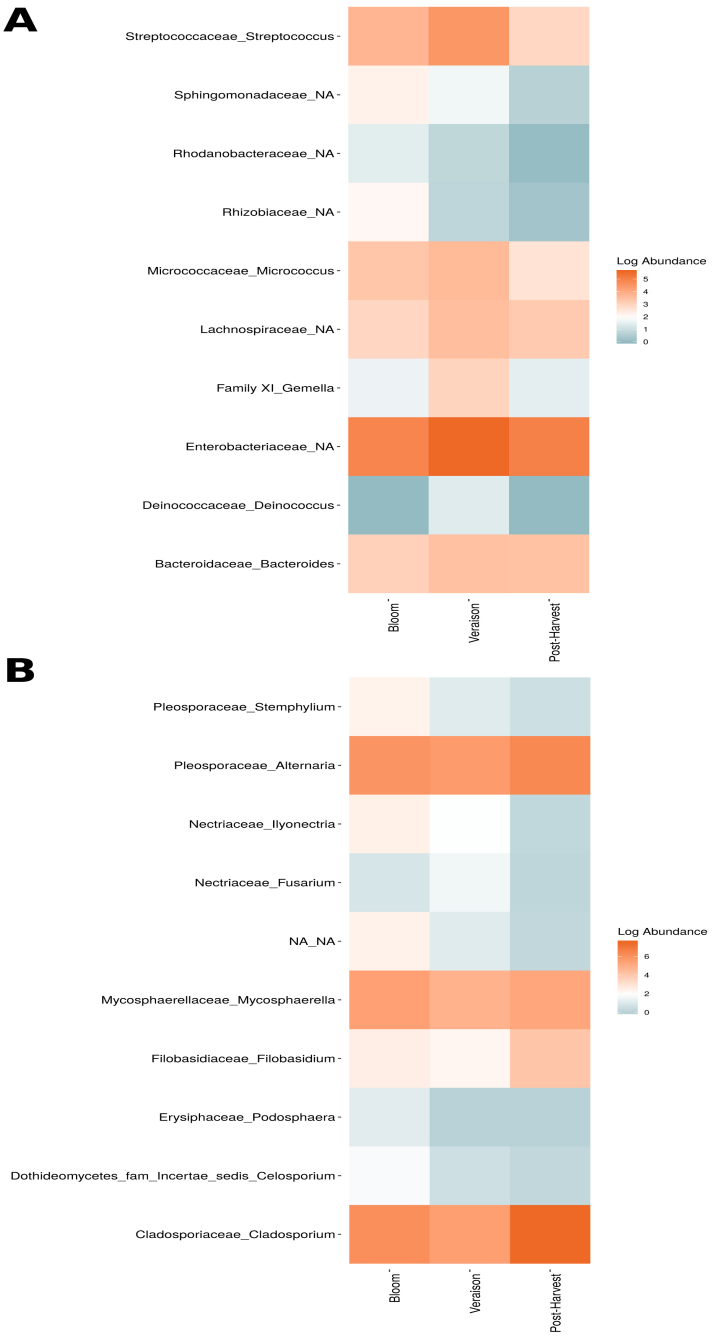

Figure 3.6: Log abundance of the top 10 random forest important genera for (A) Bacteria and (B) fungal across seasonal time points.

In order to evaluate the impact of disease on microbial communities, we only considered taxa from the post-harvest time points. Beta diversity weighted UniFrac metrics showed that bacterial community clustering was significantly affected by disease severity ($P < 0.05$; Figure 3.7A). In addition, Bray-Curtis metrics indicated that fungal clustering was not affected by disease condition of the vines but was rather attributed to year ($P < 0.05$; Figure 3.7B). Prevalence Venn diagrams showed that several microbes identified in the core microbiome were also found across all grapevines regardless of their disease condition (Figure 3.8). In addition, eleven bacteria (*Corynebacterium*, *Methylobacterium*, *Rothia*, *Actinomyces*, *Meiothermus*, *Neisseria*, *Ralstonia*, *Xylella*, *Hydrogenophilus*, *Gemella*, and *Pseudomonas*) were specific to moderate PD symptoms severity likely contributing to the significant differences in alpha diversity (Figure 3B). Random Forest analysis suggested that the clustering in bacterial beta-diversity metrics (Figure 7A) was driven by the most abundant bacterial taxa (*Streptococcus*, *Pseudomonas*, *Micrococcus*, *Bacteroides* and unknown *Enterobacteriaceae*), disease condition-specific taxa (*Rothia* and *Meiothermus*) and the pathogen *Xylella* (Figure 3.9).

3.0.5 Discussion

This study is, to the knowledge of the authors, the first comprehensive culture-independent amplicon-based metagenomics microbiome analysis from the sap of a plant. It establishes a reference framework for the bacterial and fungal communities living in the xylem sap of grapevine and captures temporal microbial shifts during the annual cyclic changes of the host phenology. In addition, it provides insightful information about the im-

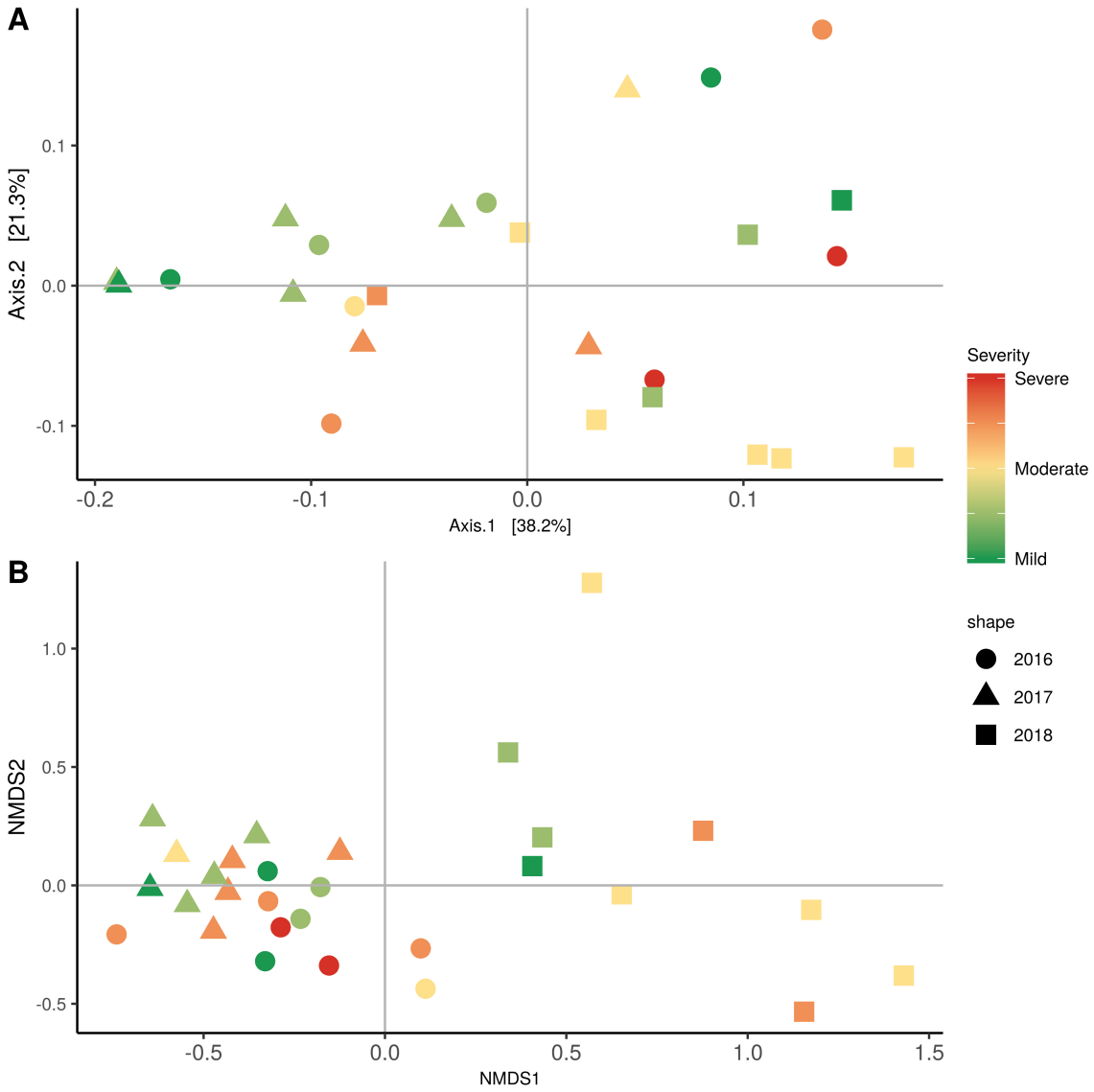

Figure 3.7: Beta diversity for disease condition plots at post-harvest based on: (A) Weighted UniFrac distance for bacteria and (B) Bray-Curtis dissimilarity for fungi. Points represent one vine community. Point are colored by PD severity, shaped by year.

pact of the xylem-dwelling pathogen *X. fastidiosa* on the microbiome profile of the grapevine sap.

The grapevine sap mycobiome was most exclusively composed of taxa in the *Ascomycota* in addition to a few yeasts from the phylum *Basidiomycota*. The sap bacteriome was similar to that of other biocompartments with a high abundance of *Proteobacteria*, and lower abundance of *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (Deyett *et al.* 2017; Faist *et al.* 2016; Marasco *et al.* 2018; Zarraonaindia *et al.* 2015). Noticeably, no *Acidobacteria* were detected, whereas it was a significant member of the rhizosphere, and root endosphere community in other studies. However, the sap microbiome profile at the phylum level was more similar to the root than the cane, despite the fact that the sap was collected from the later tissue. In addition, we found evidence for the presence of many genera known to be associated with rhizosphere and root endosphere (*Rhizobium*, *Streptomyces*, and *Pseudomonas*) including several pathogenic fungi known to cause root diseases (*Campylocarpon*, *Ilyonectria*, *Fusarium*) (Brum *et al.* 2012; Cabral *et al.* 2012). Together these data support the evidence that the microbial makeup of the sap is of belowground origin. However, one should also consider that some organisms, such as *X. fastidiosa*, can also be introduced to the host vascular system by insect feeding and become part of the sap microbiome (Lopez-Fernandez *et al.* 2017; Redak *et al.* 2004), while other airborne microbes may enter the plant endosphere through wounds, and plant surfaces via stomatas (Compant *et al.* 2011).

The core microbiome of grapevine sap, that was defined as the microorganisms that occured in at least 50% of all samples regardless of seasonal time points, was composed of seven bacterial (*Streptococcus*, *Massilia*, *Bacteroides*, *Micrococcus*, *Pseudomonas*,

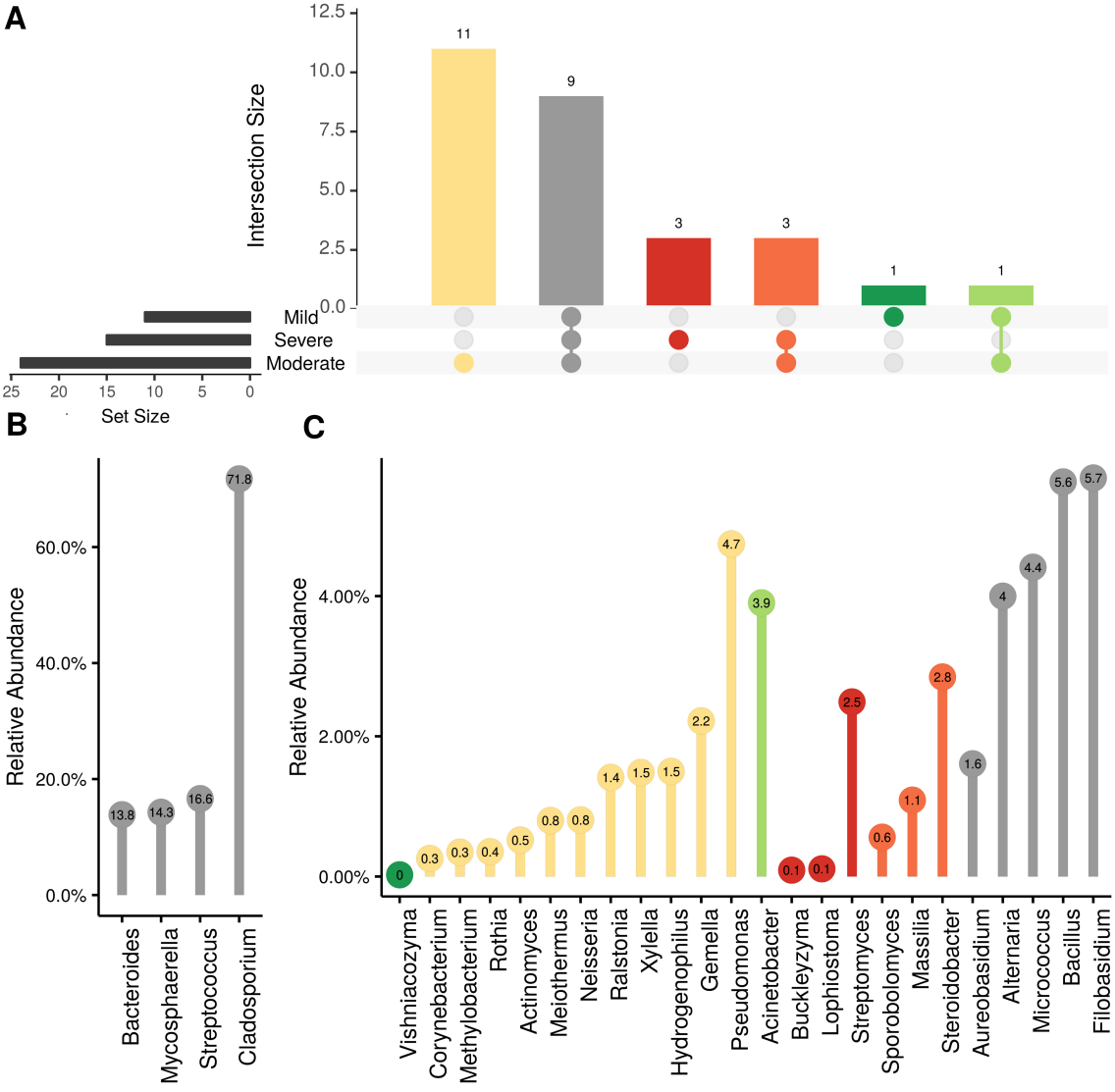

Figure 3.8: Prevalence Venn diagrams. Genera must occur in 50% of samples for each disease condition at post-harvest to be considered. (A) quantities of genera intersection between disease condition. (B) High and (C) low relative abundant genera associated with a category. Color scheme is the same as (A).

Acinetobacter and *Bacillus*) and five fungal (*Cladosporium*, *Mycosphaerella*, *Alternaria*, *Aureobasidium*, and *Filobasidium*) taxa. Together these make up over 51% and 92% of the bacterial and fungal datasets, respectively. *Pseudomonas*, *Bacillus*, *Micrococcus*, *Acinetobacter*, *Cladosporium*, *Aureobasidium Mycosphaerella*, *Filobasdium* and *Alternaria* have been identified in and/or on many plants organs of grapevine (Baldan *et al.* 2014; Brysch-Herzberg and Seidel 2015; Compant *et al.* 2011; Deyett *et al.* 2017; Dissanayake *et al.* 2018; Kraus *et al.* 2018; Nigris *et al.* 2018; West *et al.* 2010). More surprising bacterial taxa included *Massilia*, *Bacteroides* and *Streptococcus*. *Streptococcus* is a facultative-anaerobe bacterium and a well-studied human pathogen. However, it was previously found to be associated with many grapevine biocompartments (soil, root, grape leaf) and across several viticulture areas (Zarraonaindia *et al.* 2015). *Massilia* is an aerobic bacterium found in a range of habitats (i.e., desert, glacier, water and soils) and was reported to colonize the rhizosphere of plants (Ofek *et al.* 2012). *Bacteroides* is an anaerobic bacterium that metabolize mono or polysaccharides and is known to colonize the guts of animals and survive in water habitats (Wexler 2007). One should consider that the more unique bacteria (*Massilia*, *Bacteroides*), might be specific to the viticulture region, including its water quality and the soil properties. If additional vineyards had been sampled in California, perhaps those taxa would not have arose as part of the core microbiome. As previsouly demonstrated for grapevine epiphytes (Bokulich *et al.* 2014), each viticulture area possess unique indigenous microbial taxa that shape the characteristics of the region, and our data suggests that this paradigm also applies to endophytic microbes. For the other core microbiome taxa, the fact that several of them have been found in association with many grapevine biocompart-

ments both as epiphytes and endophytes, suggest that those could use xylem (and perhaps phloem) as transport routes as previously suggested (Compant *et al.* 2010; Compant *et al.* 2011).

Our alpha and beta-diversity metrics showed that the sap microbiome assemblage was shaped by both the plant phenology and disease condition, although the later mainly affected bacterial communities. One should not rule out that the other vascular pathogens also detected in this study (*Campylocarpon*, *Ilyonectria*, *Fusarium*) may have increased background noise in our analyses. Interestingly we measured a peak in microbial richness during bloom, a key phenological stage for pollination and fertilization. Especially four taxa surfaced at this time point (*Streptomyces*, *Rhodotula*, *Sphingobium* and *Novosphingobium*), and those are known to possess plant growth promoting capabilities (El-Tarabily 2004; Hahm *et al.* 2012; Kim *et al.* 2019; Seipke *et al.* 2012), suggesting that the plant could recruit microbes to participate in key physiological processes. Similarly, vines with moderate PD symptoms displayed higher microbial diversity than in either healthy or severely symptomatic vines. Plants are known to drive microbial assemblage in order to cope with biotic or abiotic stresses and increase environmental fitness (Berendsen *et al.* 2012; Turner *et al.* 2013). Perhaps our data indicated a plant-driven microbial response to the pathogen infection. In contrast, in severely symptomatic vines, the toxic environment (e.g., occlusion of xylem vessel with tyloses and decrease of hydraulic conductivity; Deyett *et al.*, 2019) is not conducive to microbial survival.

Asymptomatic grapevines at post-harvest displayed overall a lower pathogen incidence and abundance. The relatively low incidence of *X. fastidiosa* (31%) measured in

Figure 3.9: Log abundance of the top 10 random forest important genera for (A) Bacteria and (B) fungal across disease conditions at post-harvest.

vines exacerbating PD symptoms was comparable to a previously published report (Deyett *et al.* 2017). These results can be attributed to the sampling design (only 3 canes per vine canopy), the heterogeneous distribution of *X. fastidiosa* in the vine canopy and the occlusion of vessels in severely symptomatic vines make it challenging to recover the bacterium (Deyett *et al.* 2019). Interestingly, *Stenotrophomonas* abundance was inversely proportional to that of *Xylella* in all three PD rating categories.*Stenotrophomonas* is a closely related bacterium to *Xylella* but is known to benefit grapevine by acting as a biofertilizer and biocontrol agent to nematodes (Aballay *et al.* 2011; Karagoz *et al.* 2012). *Pseudomonas* was also found in vines with moderate PD symptoms, although this was a much lower abundance (5%) than previously reported (80%) from grapevine canes (Deyett *et al.* 2017; Faist *et al.* 2016). As previously discussed, *Pseudomonas* likely originates from the roots and colonizes the developing vegetative organs early in the growing season, but unlike most of the other microbial inhabitants of the sap, it appears to have the ability to become established in the lignified structural component of the grapevine vascular system. Deyett *et al.* (2017) showed that *Pseudomonas* correlated negatively with *Xylella* in PD-affected vines, suggesting that it could be used as a biocontrol agent given its known properties for secreting antimicrobial and plant growth promoting compounds (Gruau *et al.* 2015; Khmel *et al.* 1998; Loper *et al.* 2012; Pieterse *et al.* 2014). *Paraburkholderia phytofirmans* (formerly known as *Burkholderia phytofirmans*) has also been recognized as a potential biocontrol agent to grapevine PD because it has been identified as an endophyte of grapevine leaf and showed the ability to colonize the grapevine endosphere upon inoculation (root, vascular system and leaf) and prime host disease resistance pathways (Baccari

et al. 2019; Compant *et al.* 2008; Compant *et al.* 2005; Lo Piccolo *et al.* 2010). Our data did not support the presence of this specific bacterium in the sap of grapevine but identified several taxa from the family *Burkholderiaceae* (about 5% abundance), including *Massilia* and *Ralstonia* that were a part of the core microbiome and a biomarker for vines showing moderate PD-symptoms, respectively. Finally, *Methylobacterium* also surfaced as a common bacteria in grapevines with moderate PD symptoms. Araujo *et al.* (2002) described that in the Citrus Variagated Chlorosis (CVC) pathosystem, *Xylella fastidiosa subsp. pauca* and *Methylobacterium* acted in synergy and caused an increase in disease severity. Our data support the evidence that *Methylobacterium* is an endophyte of grapevine and that perhaps similar synergistic mechanisms occur in the CVC and PD pathosystems.

This study highlights several potential fungal and bacterial targets with antimicrobial and plant growth promoting properties. Future studies should focus on isolating those potentially beneficial strains and test their biological functions *in vitro* and *in planta* bioassays. The broad host range of *X. fastidiosa* combined with the movement of plant material has led to the introduction of the pathogen to new agricultural areas and made it an emerging global threat (Rapicavoli *et al.* 2018; Sicard *et al.* 2018). PD management strategies has relied most exclusively on vector control and the breeding of PD-resistant varieties in grapevine (Daugherty *et al.* 2015; Krivanek *et al.* 2006). Few resources have been invested in developing commercial bioproducts that do not select for disease resistance or cause environmental pollution. We hope that this microbiome approach will lay the foundation for innovative research and lead to novel product development and marketing.

3.0.6 Referecnes

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Chapter 4

Microbial Landscape of the Grapevine Endosphere in the Context of Pierce's Disease

4.0.1 Abstract

Microbial community structure and composition in the plant vascular endosphere has not been studied extensively especially in the context of vascular diseases. Pierce's Disease (PD) of grapevine is caused by *Xylella fastidiosa*, a xylem-limited bacterium. In PD-impacted vineyards, there are observations of vines that remain asymptomatic despite being under high disease pressure. Because these vines are clonal, we hypothesized that the microbial community inhabiting the grapevine vascular endosphere is a major contributor to the disease escape phenotype. We used a next generation sequencing Illumina MiSeq-based platform to characterize the bacterial and fungal microbiome residing in the cane endosphere of grapevine that displayed severely symptomatic, to mildly symptomatic or asymptomatic disease phenotypes. Our results provide evidence that the endophytic grapevine microbial community is composed primarily of *Proteobacteria* and *Ascomycota* with *Pseudomonodales* and *Pleosporales* as the main bacterial and fungal orders, respec-

tively. Endophytic *Pseudomonas fluorescens* and *Achromobacter xylosoxidans* showed significant negative correlations with *X. fastidiosa* titer. Our data suggest that the clustering of bacterial communities appeared to be driven by the abundance of both *P. fluorescens* and *X. fastidiosa*. *Pseudomonas fluorescens* emerged as a potential driver of the disease-escape phenotype and a promising biological control agent of PD.

4.0.2 Introduction

Plants host a diverse community of microorganisms and the plant-associated microbiome is defined as the collection of all microbes associated with the rhizophere (the soil-root interface), the phyllosphere (epiphytic, aerial surfaces) and the endosphere (internal tissues) (Turner *et al.* 2013). The structures of the tissue specific communities can have shared members, but also support unique groupings of microbes within each of the tissue types or biocompartments (Compant *et al.* 2011; Martins *et al.* 2013). Many factors can shape the microbiomes of the rhizosphere, phyllosphere and endosphere. Rhizosphere microbiomes are derived from the soil and can be recruited by the plant host (Bais *et al.* 2006; Berendsen *et al.* 2012; Compant *et al.* 2010; Turner *et al.* 2013; van der Heijden and Schlaeppi 2015). The microbial community landscape of the root endosphere can also be derived from the rhizosphere (Turner *et al.* 2013), which further shapes the microbiome of the plant vascular system (Compant *et al.* 2010; van der Heijden and Schlaeppi 2015). In contrast, the phyllosphere is a transient environment with fluctuations in nutrient and water availability as well as extreme variations in temperature and ultraviolet radiation that all shape the microbial landscape on leaf surfaces (Rastogi *et al.* 2013; Vorholt 2012). Both rhizosphere and phyllosphere microbiomes are known to provide protection against plant pathogens through a variety of mechanisms ranging from niche displacement, production of antimicrobial compounds and interference with microbial cell-cell signaling systems (Compant *et al.* 2010; Rastogi *et al.* 2013; Vorholt 2012). In addition, plant growth promoting bacteria in the rhizosphere, such as *Pseudomonas* or *Bacillus spp.*, can activate induced systemic resistance (ISR) and sensitize the plant immune system for enhanced defense re-

sponses leading to systemic defense priming and tolerance to pathogen challenge (Bolwerk *et al.* 2003; Gruau *et al.* 2015; Kloepper *et al.* 1992; Pieterse *et al.* 2014).

Grapevine (*Vitis vinifera* L.) is an important agricultural crop worldwide. Grape berries are prized for their nutritional value as well as their oenological properties that contribute to high quality wine production. Thus far, studies on the microbial communities associated with *V.vinifera* grapevines have primarily focused on characterizing the communities that are present on epiphytic parts of the plant (leaves, berries, and roots) and the dynamics of these epiphytic microbiomes in response to external factors such as fungicide applications (Bokulich *et al.* 2014; Campisano *et al.* 2015; Perazzolli *et al.* 2014; Pinto *et al.* 2014; Zarraonaindia *et al.* 2015). Two recent studies have also looked at the impact of root and collar infection with *Agrobacterium* and the sap-feeding leafhopper *Scaphoideus titanus* on the endosphere bacterial community composition in grapevine (Faist *et al.* 2016; Lopez-Fernandez *et al.* 2017).

Pierce's Disease (PD), caused by the Gram-negative, xylem-limited bacterium, *Xylella fastidiosa* (Wells *et al.* 1987) is one of the major threats to grapevine production in the Americas (Roper and Lindow 2015; Varela *et al.* 2001). In addition, *X. fastidiosa* has recently been reported in olives in Italy and ornamentals in France iterating the importance of *X. fastidiosa* as a global re-emerging pathogen (Almeida and Nunney 2015; Saponari *et al.* 2013). The bacterium is limited to the xylem tissue compartment where it multiplies to high titer, which leads to vascular occlusion caused by bacterial aggregates, bacterial exopolysaccharide and host-produced tyloses (Roper *et al.* 2007; Sun *et al.* 2013). External symptoms of PD include leaf scorching, irregular wood maturation, raisining of

Figure 4.1: A disease escape grapevine in a vineyard under high PD pressure. Note all the grapevines that were replanted due to mortality attributed to PD that surround the one PD-escaped grapevine (white arrow)

berries and overall stunting (Hopkins and Purcell 2002). Once infected, vines can succumb to the disease in as little as two years, and all *V.vinifera* cultivars are susceptible to PD. *X. fastidiosa* is exclusively vectored by xylem-feeding insects belonging to the *Cicadellidae* and *Cercopidae* families, primarily sharpshooters. Insecticide sprays are the primary means of management of the disease aside from severe pruning or rogueing of infected vines. Currently, there are no sustainable control measures for PD and, hence, this disease remains a serious threat to California and worldwide grape production, and results in millions of dollars of annual economic losses related to damage to existing vineyard plantings, vector and disease management, and replanting (Alston *et al.* 2013; Tumber *et al.* 2014).

In vineyards under high PD pressure (Figure 4.1), individual asymptomatic or mildly symptomatic vines can be found amongst many heavily infected vines (Darjean-Jones 2004) and are referred to as disease escape vines. All grapevines in vineyard blocks are clonal, which suggests a non-genetic mechanism for the observed host tolerance to PD. Because of the diversity of microbes in any given ecosystem and the array of biological functions inherent to microbes, we based this study on the hypothesis that the observed PD tolerance is driven by the endophytic microorganisms living in the plant endosphere that either directly or indirectly suppress the growth of *X. fastidiosa* in the xylem. An understanding of how endophytic microbial communities modify plant health by affecting disease outcomes through antagonizing or facilitating disease (Busby *et al.* 2016), particularly for vascular-dwelling pathogens, is just beginning. Many comprehensive plant microbiome studies have focused on the bacterial communities and from this a breadth of valuable knowledge about how bacterial communities interact with plants has been derived (Sessitsch *et al.* 2012). Fungi are also important interactors in the context of the plant-microbiome holobiont. The initial goal of this study was to characterize the core microbial structure and composition of the grapevine vascular endosphere including both bacteria and fungi. Stemming from that information, the second objective was to gain a better understanding of the microbial community structure under disease pressure and, in particular, in vines that escape PD despite being in areas of high disease pressure. The long-term goal is to develop this information into translational studies that test the application of endogenous microbes as tools for sustainable crop protection.

4.0.3 Materials and Methods

Plant sample collection

Samples were collected from a total of ten vineyards in California (Table 4.1). Five commercial vineyards with cultivars, Syrah, Petite Sirah, Chardonnay, Tempranillo, and Mourvedre were located in Temecula Valley, in southern California. This hot and arid viticulture area has been severely impacted by PD, because of the introduction of the invasive glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Blua *et al.* 1999). In addition, four commercial vineyards with cultivars, Merlot, Riesling and Chardonnay (two vineyards) were located in Napa Valley in northern California. This viticultural area is wetter and cooler than the southern area. In northern California, *X. fastidiosa* is commonly vectored by the California native blue-green sharpshooter (BGSS), *Graphocephala atropunctata*.

Historically, the incidence of PD in northern California vineyards has been primarily isolated to vineyards adjacent to riparian areas, which serve as reservoirs of the BGSS (Hopkins and Purcell 2002). These areas have high incidences of PD and are referred to as PD hotspots. Because of the distinctly different feeding and breeding behavior of the GWSS in southern California, entire vineyards are impacted with severe PD rather than being isolated to hotspots (Perring *et al.* 2001). In northern California, disease escape and infected vines were sampled from within PD hotspots and in southern California disease escape and infected vines were sampled from within vineyards highly impacted with PD (adjacent to citrus groves that serve as reservoirs for the GWSS).

Grapevine cane tissue was sampled over three years in August/September, when grapevines express typical PD symptoms. A total of 68 samples were collected from those nine vineyards in Napa or Temecula (Table 4.1), with half from grapevines expressing PD symptoms (PD-symptomatic) and the other half from healthy to mildly symptomatic grapevines (PD-escape). Canes were randomly selected from PD-symptomatic grapevines and from PD-escape grapevines.

Following sampling, canes were stored in a cooler on ice and brought back to the laboratory. In addition, five one year-old grape cuttings cultivar Chardonnay (three samples) and Merlot (two samples) originating from one vineyard at the Foundation Plant Services (FPS), UC Davis, were included in our pool of samples. These FPS samples are certified to be free of *X. fastidiosa* and were mainly used to build the endosphere microbiome profile of grapevine canes and were not included in the Pearson correlation analyses.

In the laboratory, one internode was harvested with sterile pruning shears from each cane. Internode samples were dipped in 100% ethanol and surface sterilized by flaming. Subsequently, the bark was removed with a sterile blade and these de-barked samples were stored at -80°C.

DNA extraction from de-barked cane tissue

Frozen tissues were lyophilized for 36 h with a FreeZone 2.5 l Benchtop Freeze Dry System (Labconco, Kansas City, MO). Following this, the samples were finely chopped with sterilized knives and ground at room temperature using a Retsch MM300 grinder (90 s, 25 oscillations per second) in a 35 ml stainless-steel grinding jar (Retsch, Haan, Germany)

Location	Number of Vineyards	Cultivar	Year collected	Number of samples
Temecula	5	Chardonnay	2013	10
		Mouvdre	2013	10
		Petite Sirah	2013	10
		Syrah	2010	12
		Tempranillo	2013	10
Napa	4	Chardonnay	2011	8
		Merlot	2011	4
		Riesling	2011	4
FPS Davis[a]	1	Chardonnay	2014	2
		Merloe	2014	3

Table 4.1: Scheme of the 73 grapevine cane samples. [a] Foundation Plant Services, University of California, Davis.

with 20 mm stainless steel balls as previously described (Pouzoulet *et al.* 2014). Total DNA was extracted from 50 mg of dried tissue using a Qiagen DNeasy plant mini kit (Qiagen, Valencia, CA) according to manufacturers instructions with the exception of using CTAB (hexadecyltrimethylammonium bromide, Sigma-Aldrich, St. Louis, USA) extraction buffer (Doyle and Doyle 1987) as the lysing buffer instead of the AP1 buffer provided with the kit. The DNA for all samples was aliquoted and stored at -20°C before use.

Bacterial rRNA Gene Sequencing

Illumina bacterial 16S rRNA gene libraries were constructed as previously described (Ruegger *et al.* 2014). One hundred microliter amplification reactions were performed in a MJ Research PTC-200 thermal cycler (Bio-Rad Inc, Hercules, CA, USA) containing: 50 mM Tris (pH 8.3), 500 μg/ml bovine serum albumin (BSA), 2.5 mM MgCl2, 250 μM of each deoxynucleotide triphosphate (dNTP), 400 nM of each PCR primer, 4μl of DNA template, and 2.5 units JumpStart *Taq* DNA polymerase (Sigma-Aldrich, St. Louis,

MO, USA). In brief, the PCR primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) targeted the 16S rRNA gene containing portions of the hypervariable regions V4 and V5, with the reverse primers including a 12-bp barcode (Caporaso *et al.* 2010). Thermal cycling parameters were 94°C for 5 min; 35 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s, and followed by 72 °C for 5 min. PCR products were purified using the MinElute 96 UF PCR Purification Kit (Qiagen, Valencia, CA, USA).

DNA sequencing was performed using an Illumina MiSeq (Illumina, Inc, San Diego, CA). Clusters were created using template concentrations of 1.5 pM and PhiX at 65 K/mm^{-2} and 301 base read lengths were truncated after the 260th base where sequence quality was diminished. Sequences were then processed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.8.0 (Caporaso *et al.* 2010). De-multiplexing and quality filtering were performed using default parameters except that the minimum number of consecutive high quality base calls to include a read as a fraction of input read length was raised to 0.99. Chimeric sequences were detected using the denovo method of USEARCH 6.1 (Edgar 2010) and then removed. Denovo OTUs were picked at 97% similarity and singlet OTUs were removed. OTUs having non-bacterial DNA were identified by performing a local BLAST search (Altschul *et al.* 1990) of their sequences against the nt database. OTUs were removed if any of their highest scoring BLAST hits contained taxonomic IDs within the grapevine family *Vitaceae*, fungi or PhiX, or if they contained no hits to any bacteria. Taxonomic assignments to bacterial OTUs were made using the May 5th, 2013 version of the Greengenes reference database (DeSantis *et al.* 2006) using QIIMEs default parameters. For statistical and graphical representation of the data, OTU tables were rarified to an even

depth of 6000 reads per samples. Samples with less than 6000 reads were removed from the OTU table. The bacterial sequences can be found at NCBI under the accession number PRJNA361009.

Fungal rRNA Gene Sequencing

Illumina fungal rRNA ITS libraries were constructed as follows. One hundred μl amplification reactions were performed in a MJ Research PTC-200 thermal cycler (Bio-Rad Inc., Hercules, CA, USA) and contained 50 mM Tris (pH 8.3), 500 μg ml-1 BSA, 2.5 mM MgCl2, 250 μM of each deoxynucleotide triphosphate (dNTP), 400 nM of each primer, 4μl of DNA template, and 2.5 units JumpStart *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA). The PCR primers gITS7 (GTGARTCATCGARTCTTTG) and ITS4-1 (TCCTCCGCTTATTGATATGC) targeted the ITS2 region of the ribosomal rRNA gene operon, with the reverse primers including a 7-bp barcode (Ihrmark *et al.* 2012; White *et al.* 1990). Thermal cycling parameters were 94°C for 5 min; 35 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s, and followed by 72°C for 10 min. PCR products were purified using a Qiagen QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturers instructions.

DNA sequencing was performed using an Illumina Miseq (Illumina, Inc., San Diego, CA, USA). Clusters were created using template concentrations 1.0 pM and PhiX at 65 K/mm^{-2}. Raw read lengths were 301 bases. Raw sequences were processed using QIIME version 1.8.0 (Caporaso *et al.* 2010). De-multiplexing and quality filtering were performed using default parameters except that the minimum allowed Phred quality score was raised to Q30. Chimeric sequences were detected using the denovo method of USEARCH 6.1

(Edgar 2010) and then removed. Denovo OTUs were picked at 99% similarity and singlet OTUs were removed. OTUs having non-fungal DNA were identified by performing a local BLAST search (Altschul *et al.* 1990) of their sequences against the nt database. OTUs were removed if any of their highest scoring BLAST hits contained taxonomic IDs within the grapevine family *Vitaceae* or PhiX, or if they contained no hits to any fungi. Taxonomic assignments to fungal OTUs were made with the RDP Classifier (Wang *et al.* 2007) using the July, 2014 UNITE fungal database (Koljalg *et al.* 2005). BLASTN 2.4.0 was used to identify the top 50 genus level sequences that were unable to be identified through RDP database. Genus level names were adopted for each sequence if there was a 97% identity and had a cut-off E-value of E-120. The newly identified genera were added to the original OTU table obtained through QIIME. For fungal taxonomy barplots, OTU tables were rarefied to 1000 reads per sample. Samples with less than a 1000 reads were removed from the dataset. The fungal sequences can be found at NCBI under the accession number PRJNA361126.

Microbiome data analyses

Using the rarified OTU tables from QIIME, R 3.4.0 (https://www.R-project.org/) was employed for the graphical and statistical analysis. The Phyloseq package 1.20.2 (McMurdie and Holmes 2013) and ggplot2 2.2.1 (Wickham 2009) were utilized for further parsing the OTU tables and graphical representations. Taxonomy plots for bacteria and fungi were generated from 72 and 34 samples, respectively, by pruning OTUs for only those representing at least 1% of the bacterial or fungal OTU table. Venn diagrams were also created with the R package VennDiagram (Chen and Boutros 2011) version 1.6.17 (https://cran.rstudio.com/web/packages/VennDiagram/VennDiagram.pdf) and

demonstrated the presence/absence of number of bacterial OTUs occurring in various metadata categories including cultivar (60 samples), sampling year (72 samples), vineyard location (72 samples) and grapevine condition (72 samples). Wilcoxon test corrected by FDR values were used to statistically compare *X. fastidiosa* abundance and incidence in PD-symptomatic vs. PD-escape grapevine. Pearson correlation analyses from 67 samples (excluding FPS plant samples) between the numbers of *X. fastidiosa* sequencing reads and the relative abundance of the other bacterial taxa were performed using the R package Hmisc 4.0-3 (http://biostat.mc.vanderbilt.edu/Hmisc). FDR was used to adjust P-values. Hellinger distance matrix was calculated using 72 samples with QIIME (Caporaso *et al.* 2010) and used to create PCoA plots. The Hellinger distance matrix was also used on all 72 samples to create the Canonical analysis of principal coordinates (CAP) plot using the Vegan package (https://cran.r-project.org/web/packages/vegan/index.html). Statistics for the PCoA was carried out with an Adonis test. Adonis was run on *Xylella* and *Pseudomonas* abundances. Similarly, ANOVA like permutation test for constrained analysis was run to determine statistical significance between *Xylella* and *Pseudomonas* abundance among samples.

Chromosome primer walking

To obtain the sequences of a greater portion of the 16S rRNA gene along with the internal transcribed spacer (ITS) region for selected OTUs, chromosome walking was conducted as follows. Samples that showed relative high abundance of the most abundant *Pseudomonas* OTU #646559 and *Achromobacter* OTU #558264 based on Illumina sequencing were selected. PCR was performed on the total DNA using a nested approach. The first

PCR used primers targeting the small-subunit rRNA gene and the large-subunit rRNA gene: 530F (GTGCCAGCMGCCGCGG) and 23SR (GGGTTBCCCCATTCRG). PCR products were diluted 1:99 and then subjected to sequence-selective PCRs where the reverse primer was 23SR (GGGTTBCCCCATTCRG) and the following sequence-selective forward primers were: *Achromobacter* (TTTAACTACCGAGCTAGAG), *Pseudomonas* (AACTGACTGACTAGAGTAT). Amplification products were gel isolated and cloned as described previously (Bent *et al.* 2009), and the nucleotide sequences were obtained using ABI Cycle Sequencing (Applied Biosystems, Foster City, CA). We analyzed 16 different samples and obtained bands of the predicted size from 14 samples. Six of these bands were cloned and sequenced, resulting in eight sequences from three different samples that had 100% identity to the sequence of Illumina *Pseudomonas* OTU #646549. These sequences were examined further by a phylogenetic tree analysis described below. For the *Achromobacter* analyses, we analyzed 16 different samples and obtained bands of the predicted size from 13 samples. Six of these bands were cloned and sequenced, resulting in four sequences from two different samples that had 100% identity to the sequence of Illumina *Achromobacter* OTU #558264. The best match to all four of these sequences was *Achromobacter xylosoxidans* as determined using BLAST analyses.

Phylogenetic analysis

To better identify the species name of the most abundant *Pseudomonas* OTU#646549, a multi-loci phylogenetic tree was created. BLASTN was used to find homologous sequences in the *Pseudomonas* genome sequence database (Loper *et al.* 2012). The sequences averaged 1524 base pairs in length and included 16S, ITS and 23S loci. A total of 32 *Pseu-*

domonas sequences were aligned using ClustalW (Geneious v. 6.1.6; Biomatters Ltd.). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated. There were a total of 1408 positions in the final dataset. Bootstrap values were inferred using 1,000 replicates. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016).

4.0.4 Results

The grapevine endophytic microbiome was characterized from lignified shoot samples collected from ten vineyards across California, including five commercial vineyards in the Temecula valley, four commercial vineyards in the Napa valley and one vineyard from the Foundation Plant Services at UC Davis. A total of 60% and 1.1% of the total number of reads were of grapevine origin for bacteria and fungi, respectively. After removing plants reads, we obtained 2209462 and 462294 total high quality reads for the bacterial and fungal data sets. After processing the rarefied data, 918 and 4437 bacterial and fungal OTUs (Operational Taxonomic Units) were identified respectively.

Community analysis was performed with only OTUs representing greater than 1% of the data and transformed to relative abundance for taxonomy plots. For fungal taxonomy, barplots (Figure 4.2) were constructed with 34 samples because 39 samples with less

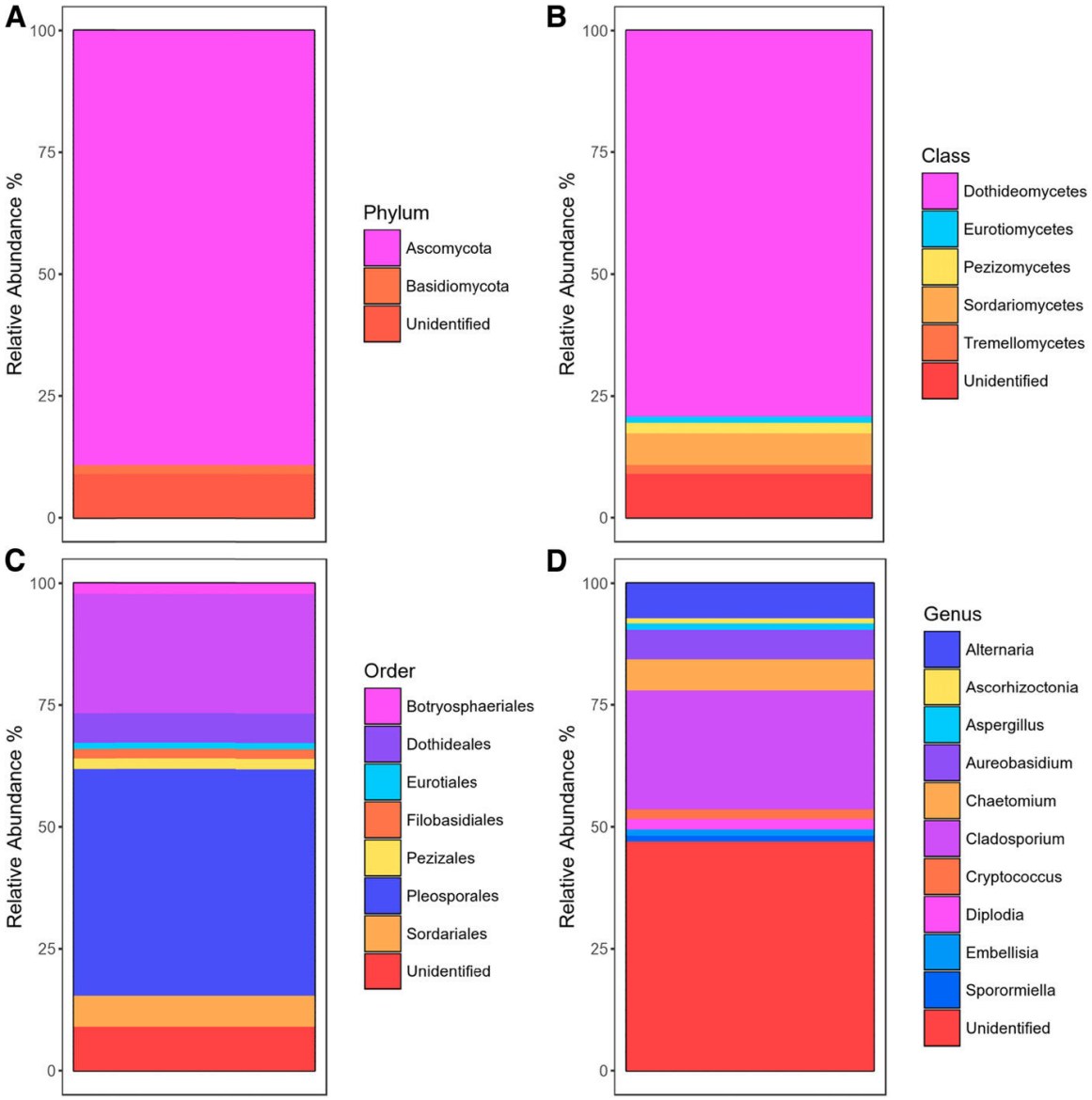

Figure 4.2: Relative abundance (percent) of the fungal taxa inhabiting the grape vascular system endosphere: A, phylum; B, class; C, order; and D, genus. The operational taxonomic units (OTUs) that did not occur in at least 1% of the dataset were filtered out and the remaining OTUs are presented in relative abundance (n = 34)

than 1000 reads were removed from the dataset. Our results showed that fungal communities were predominately comprised of phyla belonging to the *Ascomycota* (89.2%), which was mostly composed of four classes including *Dothideomycetes* (79.3%), *Sordariomycetes* (6.4%), *Pezizomycetes* (2.2%) and *Eurotiomycetes* (1.3%). Within the *Dothideomycetes*, the *Pleosporales* (46.5%) was the most abundant order and was comprised of four genera including one unassigned genera *Alternaria*, *Sporomiella*, and *Embellisia*. The *Dothidiomycetes* also included three additional orders *Capnodiales* (24.5%), *Dothideales* (6%), and *Botryosphaeriales* (2.1%) and were represented predominantly by *Cladosporium*, *Aureobasidium*, and *Diplodia*, respectively. The *Sordariomycetes* was represented predominantly by the order *Sordariales* (6.4%) with one genus, *Chaetomium*. The *Eurotiomycetes* was represented predominantly by the order *Eurotiales* (1.3%) comprised of mainly *Aspergillus*. *Pezizomycetes* was represented by the single order *Pezizales* and one unassigned genus and the genus *Ascorhizoctonia*. Organisms belonging to the phylum *Basidiomycota* represented only 1.9% and were mostly composed of organisms belonging to the class *Tremellomycetes*, order *Filobasidiales* and genus *Cryptococcus*. Interestingly, 46.9% of the remaining fungi could not be assigned at the genus level indicating they are likely undescribed species. Because only 34 of the 73 initial samples could be used in our analysis after rarefaction, we did not further analyze *X. fastidiosa* associations in the context of the fungal community.

In comparison, the bacterial dataset was more robust following rarefaction method allowing us to use 72 of the 73 samples. Our results showed that the bacterial communities (Figure 4.3) were predominately comprised of organisms belonging to the phylum *Proteobacteria* (99.5%) and especially two classes, *Gammaproteobacteria* (89.3%) and *Be-*

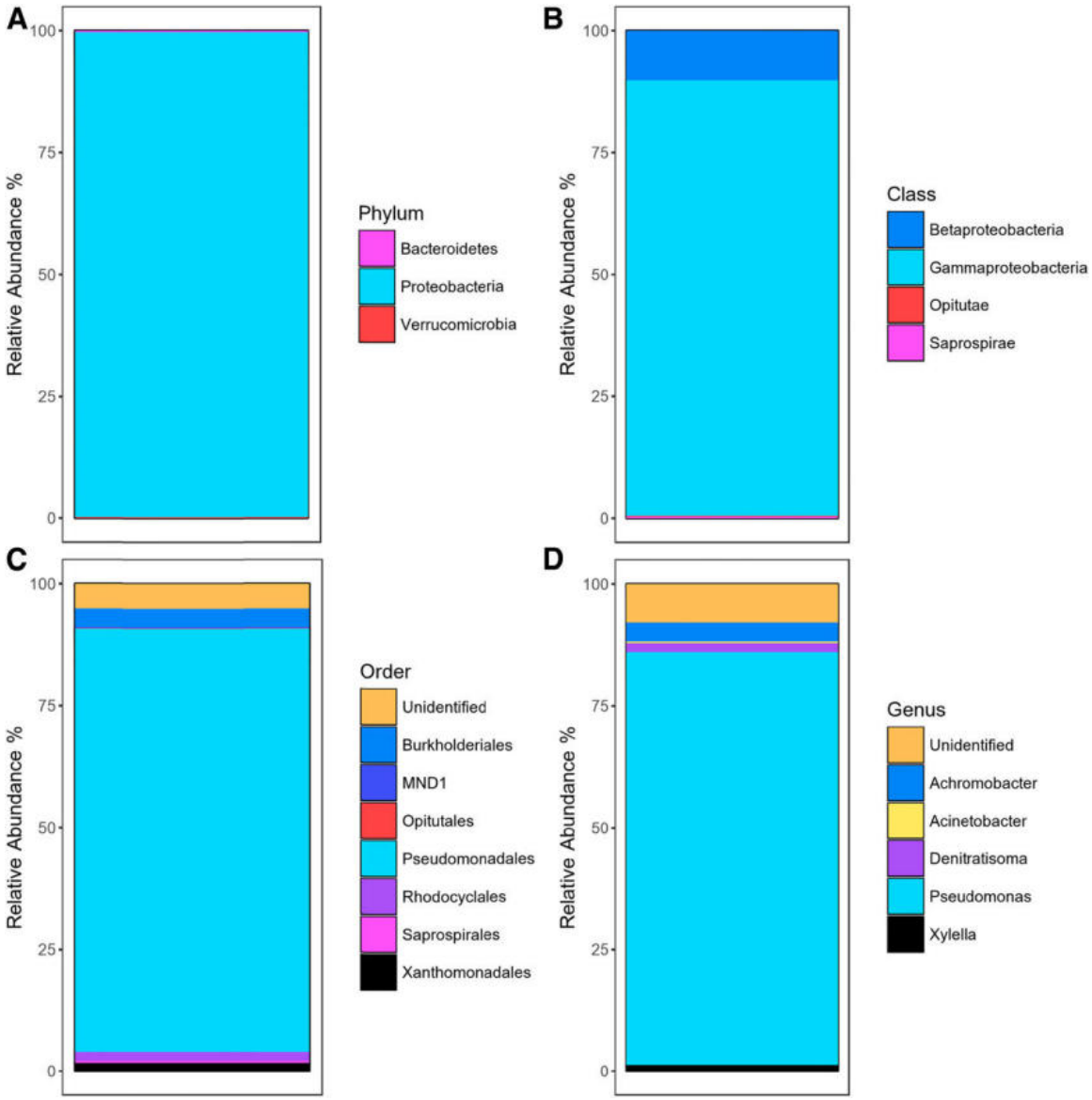

Figure 4.3: Relative abundance (percent) of the bacterial taxa inhabiting the grape vascular system endosphere: A, phylum; B, class; C, order; and D, genus. The operational taxonomic units (OTUs) that did not occur in at least 1% of the dataset were filtered out and the remaining OTUs are presented in relative abundance (n = 72).

taproteobacteria (10.2%). Among the *Gammaproteobacteria* the two dominant orders were *Pseudomonadales* (86.8%) and *Xanthomonadales* (1.7%) and were predominantly represented by *Pseudomonas* and *Xylella*, respectively. Among the *Betaproteobacteria* the two dominant orders were *Burkholderiales* (3.8%) and *Rhodocyclales* (1.9%) and were predominantly represented by the genera *Achromobacter* and *Denitratisoma*, respectively. We also identified additional phyla but at a very low abundance. For example, *Bacteroidetes* (0.4%) was the second most abundant phylum followed by *Verrucomicrobia* (0.1%).

Venn diagrams were generated to assess the common and different bacterial OTUs among four variables (sampling year, vineyard location, vine condition, and grapevine cultivar) (Figure 4.4). OTUs had to occur in a minimum of 10% of the vines in any group to be included. Among five grape cultivars with at least 10 samples (Table 4.1), we identified 107 shared OTUs. Syrah had the most unique OTUs (n=47) followed by Chardonnay (n=38), Tempranillo (n=14), Mourvedre (n=13), Petite Sirah (n=11). Among the 72 samples collected from all four years, 112 OTUs were shared between sampling dates. The year 2010 had the most unique OTUs (n=123), followed by 2014 (n=47), 2011 (n=18) and 2013 (n=10). In addition, 120 OTUs were shared between sampling locations. FPS samples from Davis had 57 unique OTUs, Napa had 54 and Temecula had 12 unique OTUS. Similarly, 120 OTUs were shared between vine conditions with 65, 16 and 26 unique OTUs for the certified *X. fastidiosa* free plants, escape and symptomatic grapevines, respectively. Ninety-eight OTUs were shared among all the inner circles of the four Venn diagrams (condition= 154; year= 112, location= 120; variety= 107).

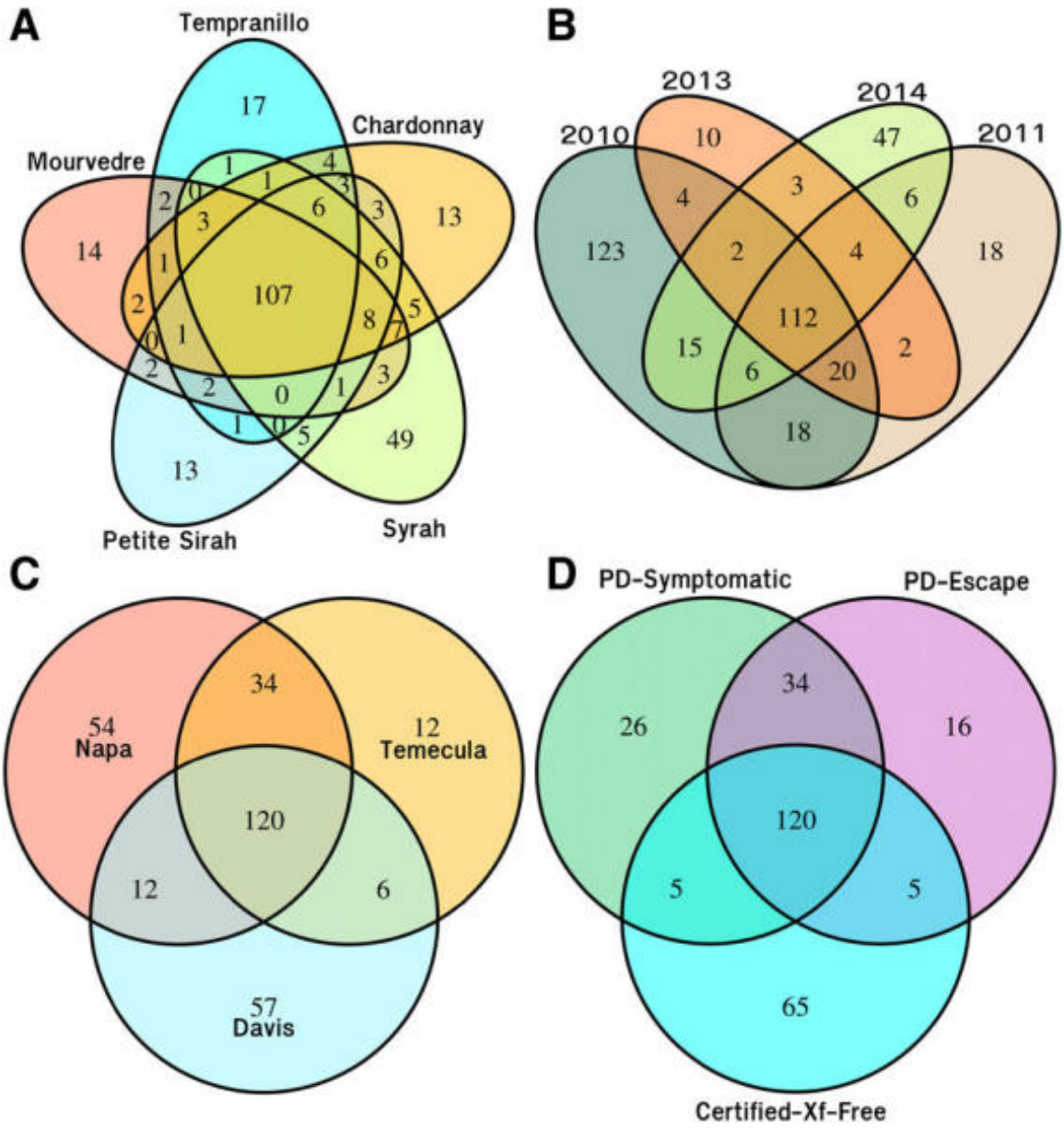

Figure 4.4: Venn Diagrams depicting the number of shared and different operational taxonomic units based on four variables: A, grapevine cultivar (n = 60); B, sampling year (n = 72); C, vineyard location (n = 72); and D, grapevine condition (n = 72).

Bacterial community composition was impacted by the condition of the vine (Figure 4.5A). Statistical analyses showed that *X. fastidiosa* was found both in significantly higher abundance ($P<0.01$; Figure 4.5B) and at a significantly higher incidence ($P<0.01$; Figure 4.5C) in PD-symptomatic than PD-escape grapevines. Symptom severity on the scale of a whole plant directly correlates with an increase in *Xylella* titer and the reason that *X. fastidiosa* incidence is not reported at 100% in symptomatic vines is likely due to the heterogeneous distribution of *X. fastidiosa* within the xylem architecture of grapes (Clifford *et al.* 2013). In addition, four bacterial taxa with very low abundance correlated positively with *Xylella* including *Bacillus*, *Pediococcus*, *Caulobacter*, and *Dialister* (Table 4.2). Most notably, *Pseudomonas* and *Achromobacter*, two of the predominant phylotypes in the cane endosphere (Figure 4.3), showed significant negative correlations with *Xylella* (Table 4.2). Because of the potential application of those bacteria as biological control agents for management of PD, these taxa were further identified to the species level. Chromosomal walking was used to obtain a greater portion of *Pseudomonas* and *Achromobacter* 16S rRNA gene sequence, along with their rRNA ITS sequence, producing sequences that were approximately 1500bp in length. From this we determined that the species designations for these taxa were *P. fluorescens* or *P. chlororaphis* and *A. xylosoxidans*. Because we could not assign with confidence a species name to the *Pseudomonas* OTU #646549 (i.e., the most abundant *Pseudomonas* OTU) using nucleotide BLAST search, we used a Maximum Likelihood analysis to resolve species identity (Figure 4.6). Results showed that our *Pseudomonas* sequences clustered with *Pseudomonas fluorescens* with strong bootstrap support (98%).

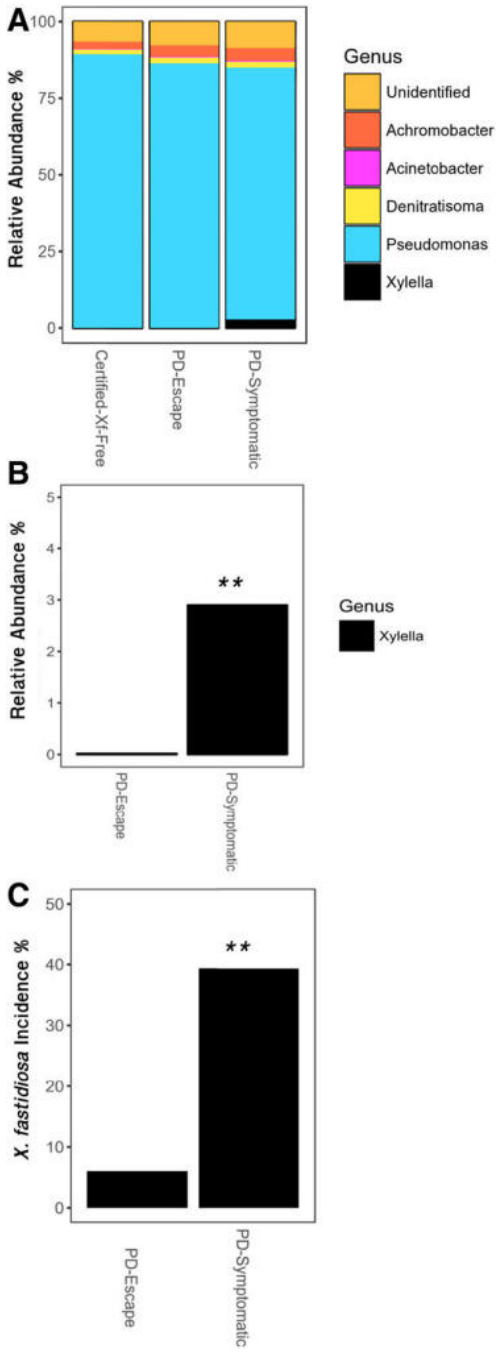

Figure 4.5: A, Abundance of genus level taxa identified in certified *Xylella fastidiosa*-free, PD-escape and PD-symptomatic vines (n = 72). B, *X. fastidiosa* relative abundance (mean percent) in PD-escape and PD-symptomatic grapevines; ** indicates a statistical significant difference (P <0.01; n = 67). C, *X. fastidiosa* incidence (percentage of vines where *X. fastidiosa* reads could be detected) in PD-symptomatic versus PD-escape; ** indicates a statistical significant difference (P <0.01; n = 67).

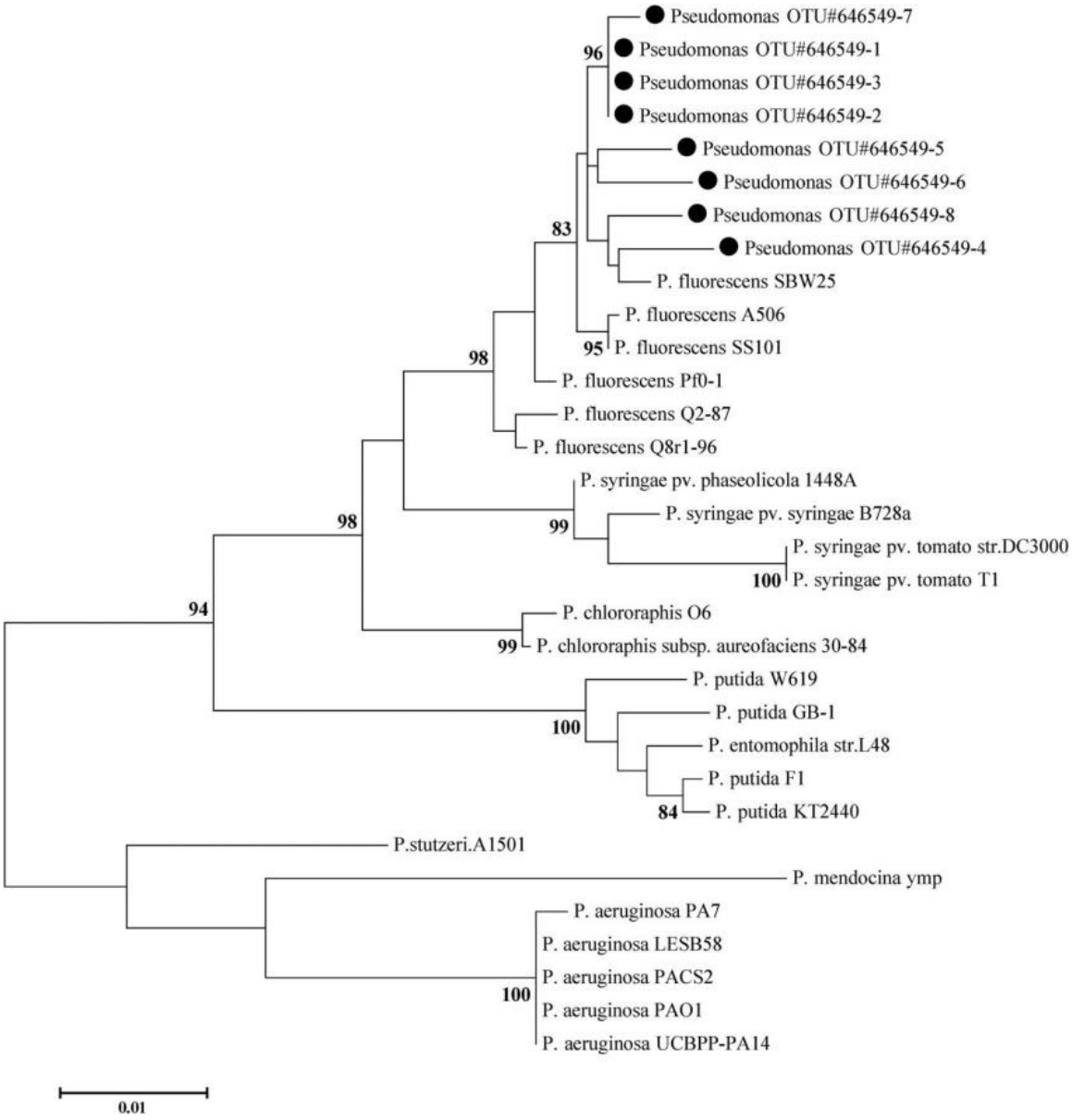

Figure 4.6: Molecular phylogenetic analysis by maximum likelihood method. The tree with the highest log likelihood is shown. Bootstrap values were inferred using 1,000 replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The eight *Pseudomonas* sequences that displayed 100% match with the dominant operational taxonomic unit (OTU) 646549 identified from the grape cane endosphere are labeled with a full circle.

OTUs	**P**	**FDR corrected**	**r[a]**	**Number of OTUs[b]**	**Relative abundance(%)**
Psuedomonas	0.00	0.00	-0.84	84	84.2
Achromobacter	0.043	0.043	-0.25	12	3.89
Bacillus	0.009	0.013	0.32	9	0.01
Caulobacter	0.0433	0.043	0.37	1	1e-03
Pediococcus	4.8e-05	5.6e-05	0.49	1	5e-04
Dialister	0.000	0.000	0.81	1	2e-04

Table 4.2: Bacterial operational taxonomic units (OTUs) correlating negatively and positively with *Xylella*. [a] Pearson correlation analyses were performed between the numbers of *Xylella fastidiosa* sequencing reads and the relative abundance of other bacterial taxa. Standard (P) and flase discovery rate (FDR) corrected probability values are presented along with the correlation coefficient (r) n=67. [b] Total number of OTUs with the same genus and a significant P value

Beta-diversity plots using a Hellinger distance matrix showed evidence of compositional divergence when looking at abundance of *Xylella* and *Pseudomonas* counts (Figure 4.8). The clustering of bacterial communities appeared to be primarily driven by the abundance of *Xylella* and *Pseudomonas*. Adonis test revealed a significant P value and correlation coefficient for both *Xylella* ($P < 0.001$, $R^2 = 0.34$) and *Pseudomonas* ($P < 0.001$ $R^2 = 0.28$) beta-diversity metrics. A canonical correspondence of principal coordinate analysis was run to better identify the factors contributing to the variance in the dataset (Figure 4.7). Results reiterate that abundance of *Xylella* and abundance of *Pseudomonas* carry the heaviest weight on the data set with these two forces driving the data in divergent directions ($P < 0.001$). The data points in Figure 4.8 are colored by condition but the data were analyzed by *X. fastidiosa* abundance. Beta-diversity using Hellinger distance matrix did not show any compositional divergence belonging to any other metadata variable (i.e. condition of vine, sampling time, vineyard location; data not shown).

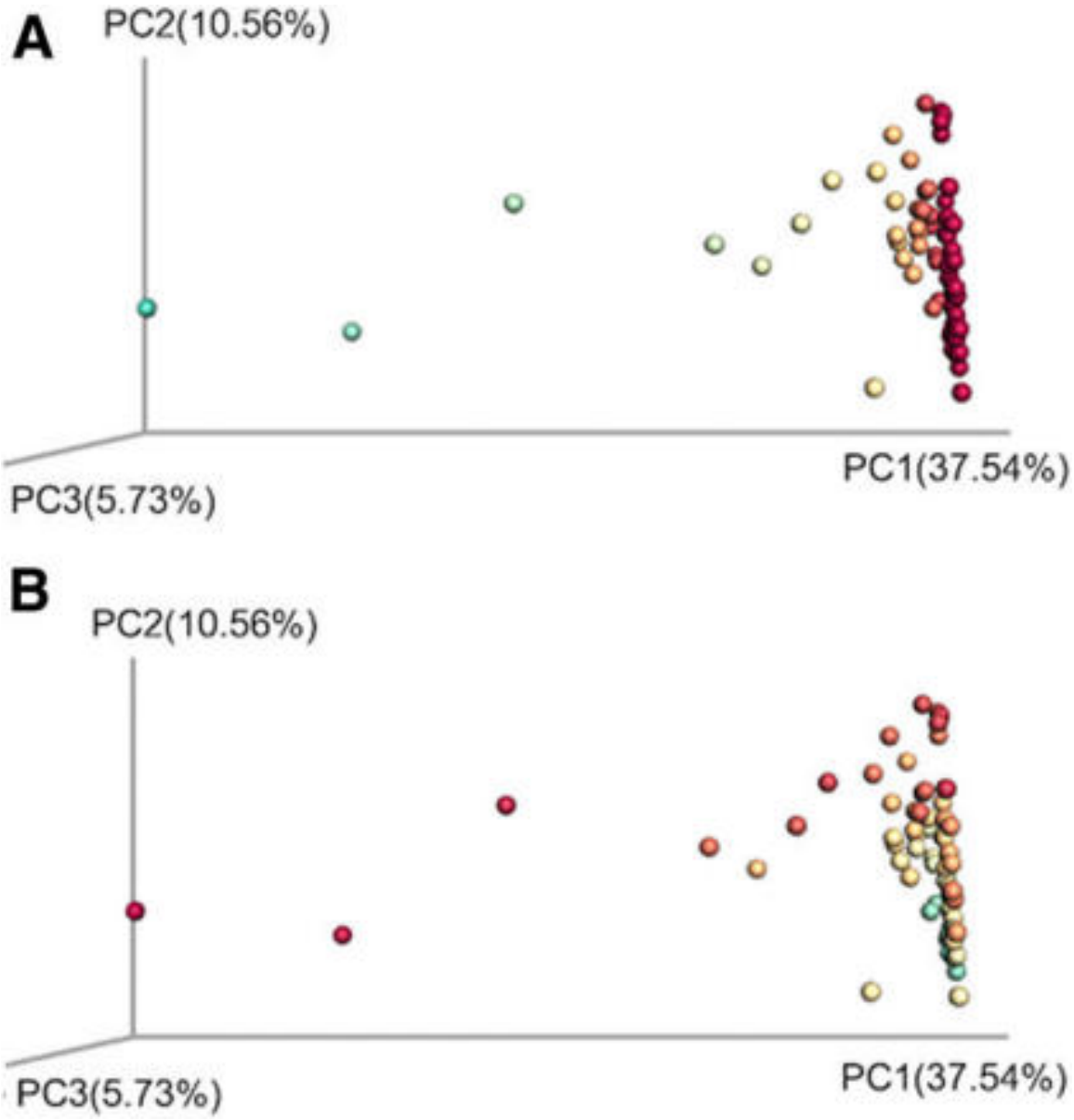

Figure 4.7: Principal coordinates analysis (PCoA) plots based on Hellinger distance matrix plotted with the first three dimensions. Comparison of bacterial communities by A, abundance of *Xylella fastidiosa* and B, abundance of *Pseudomonas*. Points represent individual samples in the data set. Red indicates lower abundance and blue indicates higher abundance. Adonis test revealed a significant P value and coefficient correlation for abundance of both *Pseudomonas* ($P < 0.001$; $R2 = 0.28$) and Xylella ($P < 0.001$, $R2 = 0.34$)

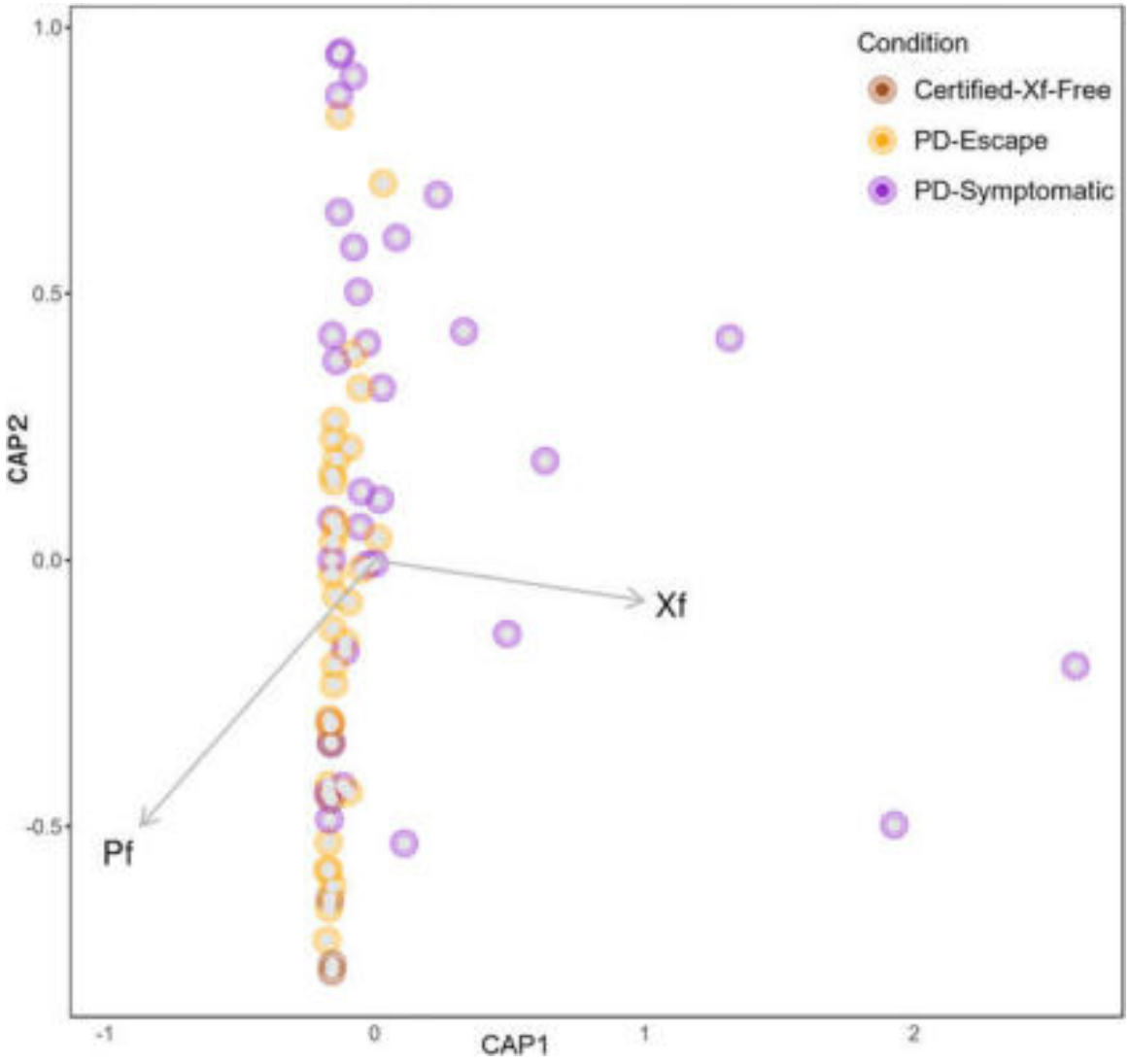

Figure 4.8: Bacterial community variation represented by canonical analysis of principal coordinates (Hellinger distance matrix) plotted on the first two dimensions. Top contributors of community variation are represented by arrows, Pf = *Pseudomonas fluorescens*; Xf = *Xylella fastidiosa*. Arrows are in divergent directions (P <0.001). Dots are colored by condition but the data were analyzed by *X. fastidiosa* abundance.

4.0.5 Discussion

Grapevine has become a model system for investigations of microbial communities associated with agricultural crops and different grape plant organs support distinct microbial communities. Berry flora has been a major focal point (Bokulich *et al.* 2014; Zarraonaindia *et al.* 2015) because of the interest in indigenous microbes as contributors of the regional organoleptic properties (i.e., terroir). Zarraonaindia *et al.* (2015) determined that above ground grape bacterial communities are significantly different than the cognate belowground soil bacterial communities. However, specific above ground (leaves, berries and flowers) communities were more similar to below ground soil communities than they were to each other. Here we focused our study on the endosphere of the above ground cane tissue. Canes are lignified shoots that bear leaves and grape clusters. Canes are seasonal, annually produced vine tissues that emerge from buds in the spring and are pruned off in the fall after harvest. Our data indicated that the grapevine cane endosphere hosts a bacterial community structure with species of similar relative abundances to communities described for other annually produced aerial plant parts (i.e, grape flowers berries and leaves) with a high abundance of *Proteobacteria* and a low incidence of *Firmicutes*, *Acidobacteria* and *Bacteriodetes* (Zarraonaindia *et al.* 2015). Faist *et al.* (2016) also supported these results although they found a higher abundance of *Firmicutes*, *Actinobacteria* and *Bacteriodetes* in canes likely due to the difference with sampling location and design, grapevine cultivar and rootstock and environmental conditions. In addition, we found that *Verrucomicrobia* was represented in the cane endosphere, albeit low abundance. Zarraonaindia *et al.* (2015) showed that *Verrucomicrobia* were absent in leaves, berries or flowers but were abundant

in the soil, grape rhizosphere and root endosphere. This suggests that the *Verrucomicrobia* found in the internal cane endosphere is likely derived from the soil or rhizosphere.

Fungi are an important segment of the plant microbiome and a key determinant to plant health (Pieterse *et al.* 2014; Shoresh *et al.* 2010). Deciphering their biological functions could lead to new strategies for disease control and integrated pest management. For example, fungi produce a diversity of structurally distinctive compounds with antimicrobial properties (Aldrich *et al.* 2015; Porras-Alfaro and Bayman 2011). Culture-independent grape microbiome studies have primarily been centered on bacterial community composition and to our knowledge, our study is the initial report of culture-independent analyses of the fungal communities associated with grapevine cane endosphere. There are other culture-independent studies that focused on fungal communities on pre-harvest phyllosphere (Perazzolli *et al.* 2014; Pinto *et al.* 2014) and post-harvest grape berries just before fermentation (Bokulich *et al.* 2014). Our data showed that overall the fungal community composition was more diverse than the bacterial community composition in terms of total number of OTUs. Several taxa (*Cryptococcus, Alternaria, Sporomiella*, and *Aureobasidium*) inhabiting the grape cane endosphere were also previously reported in the grape phyllosphere (Perazzolli *et al.* 2014; Pinto *et al.* 2014). In addition, we found that several fungal taxonomic groups remained unclassified suggesting that the conditions of the plant vascular system (i.e., low oxygen, negative pressure, nutrient availability) have selected for unique taxa that have adapted to this particular niche that are not well represented in publicly available databases.

At the genus and species level, the cane endosphere bacterial community was composed primarily of *Pseudomonas*, which aligns with previous results from Faist *et al.* (2016). We determined that *P. fluorescens* was the main species inhabiting the grapevine cane endosphere and we also found that it was in significantly higher abundance in PD-escape vines vs. PD-symptomatic vines. In fact, the clustering of bacterial communities appeared to be driven by the incidence of these *P. fluorescens* and *X. fastidiosa* in the endosphere. This suggests that perhaps *P. fluorescens* has the ability to mitigate the establishment of *X. fastidiosa* and confer the host escape phenotype. *P. fluorescens* is commonly found in association with the rhizosphere, phyllosphere and endosphere of other plant species. Moreover, it is a known biological control agent in many pathosystems, but can also protect against abiotic stresses and stimulate plant growth (Bardas *et al.* 2009; Lugtenberg and Kamilova 2009; Maksimov *et al.* 2011; Olanya *et al.* 2016; Samavat *et al.* 2014; Shen *et al.* 2013; Srinivasan *et al.* 2009). This bacterium produces a diverse array of secondary metabolites that have antimicrobial properties, such as phenazines, lipopeptides, hydrogen cyanide and 2,4-diacetylphloroglucinol and antibiosis is described as a mechanism underlying how this bacterium imparts broad spectrum disease control (Arseneault *et al.* 2016; Loper *et al.* 2012; Michelsen *et al.* 2015; Rezzonico *et al.* 2007). In addition, *P. fluorescens* is a well-described plant growth promoting rhizobacteria. It has been shown to actively colonize the plant rhizosphere and the root endosphere and activate Induced Systemic Resistance (ISR) (Kloepper *et al.* 1992) that can confer increased immunity against a broad range of plant pests and diseases in some plant species (Bolwerk *et al.* 2003; Cabanas *et al.* 2014; Maksimov *et al.* 2011; Pieterse *et al.* 1996; Pieterse *et al.*

2014). In grapevine, *P. fluorescens* can become established in the grape rhizosphere and suppress the growth of the pathogenic bacterium *Agrobacterium* spp., the causal agent of crown gall (Khmel *et al.* 1998). Moreover, it can actively colonize the root endosphere and trigger a systemic induced response that provide enhanced resistance against the leaf and berry pathogen *Botrytis cinerea*, the causal agent of gray mold (Aziz *et al.* 2016; Gruau *et al.* 2015). Future biological and functional genomic studies will allow us to decipher the molecular basis underlying *P. fluorescens* putative antagonistic or ISR-inducing mechanism of action against *X. fastidiosa in planta*.

Achromobacter xylosoxidans was also an abundant member of the microbial community occupying the grape cane endosphere that correlated negatively with *X. fastidiosa* suggesting that this bacterial taxa could also play a role in the disease escape phenotype. *A. xylosoxidans* is a known plant growth promoting rhizobacteria (Forchetti *et al.* 2010; Mayak *et al.* 2004) and a biological control agent of many plant pathogens (de Boer *et al.* 2015; Triki *et al.* 2012). *A. xylosoxidans* is commonly found in aqueous environments and soils. This organism can metabolize nitrate and glyphosate (Ermakova *et al.* 2010; Felgate *et al.* 2012), which are commonly used in grape production systems as part of fertilization regimes and weed management, respectively. We speculate that this bacterium has been selected for in vineyard soils because of these standard viticultural practices and may have been introduced through the irrigation water.

Plants can acquire microorganisms from soil and those selected microbes can move to the rhizosphere and root endosphere to fulfill specific biological functions (Berendsen *et al.* 2012). It was also proposed that organisms that inhabit the vascular system originate

in part from the roots through the lumen of xylem vessels via the plant evapotranspiration stream (Compant *et al.* 2010). Other entry points to the plant vascular system may also include insect feeding (Lopez-Fernandez *et al.* 2017) wounding (Munkvold and Marois 1993) and perhaps plant natural openings (i.e., stomata). The origin of the endosphere-associated *A. xylosoxidans* and *P. fluorescens* or any of the other phylotypes, aside from *X. fastidiosa*, is presently unknown and understanding how these microbes are introduced into the endosphere will rely on future, detailed niche partitioning experiments. In addition, collecting more information on microbial community dynamics during the grapevine annual phenological cycle with relation to pre- and post-*X. fastidiosa* infection events following sharpshooter feeding, pathogen colonization and PD symptoms development will provide insightful information especially with regards to the colonization dynamics of *P. fluorescens* and *X. fastidiosa*. This information coupled with functional experimental data may provide practical solutions to PD management.

4.0.6 Referecnes

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Chapter 5

Assessment of Pierce's Disease Susceptibility in *Vitis vinifera* with Different Pedigrees

5.0.1 Abstract

Pierce's Disease (PD) of grapevine is caused by the bacterium *Xylella fastidiosa*. In this study, we applied an integrated approach to assessing PD susceptibility among different *Vitis vinifera* cultivars that incorporates disease severity, bacterial pathogen abundance and loss of stem xylem hydraulic conductivity. We hypothesized that levels of PD susceptibility in *V. vinifera* can be attributed in part by the host anatomical features that is shaped by its pedigree background. We initially selected two popular wine grape cultivars from the occidentalis group, Merlot and Cabernet Sauvignon and one from the orientalis group, Thompson Seedless. We also included the more recently bred table grape cultivar Scarlet Royal that has mixed pedigree parentage. We compared PD susceptibility to the known PD resistant b43-17 *V. arizonica/candicans* wild grape species from North America. Our data indicate that Thompson Seedless was ranked as the most susceptible to PD because it significantly exhibited the most severe disease symptoms at 12 weeks post-inoculation and

hosted the highest *X. fastidiosa* titer than the other cultivars, and lost over 90% of its stem hydraulic conductivity. In contrast the other 3 cultivars displayed less susceptibility to PD. We discuss in what capacity xylem anatomy could impact PD susceptibility in *V. vinifera* cultivars and how grape pedigrees and their cognate center of domestication may have influenced xylem anatomical features. This work provides a reference framework to further test the hypothesis that *V. vinifera* cultivars with wide-xylem vessels may be more prone to PD decline.

5.0.2 Introduction

Pierce's Disease (PD) is a severe vascular disease of grapevine caused by the Gram-negative bacterium *Xylella fastidiosa* (Wells *et* al. 1987) and costs $104 million in annual losses to the California grape industry (Tumber *et* al. 2014). This disease was first described in California vineyards in the late 19th century by Newton Pierce (Pierce 1892) and its bacterial causal agent likely originated from Central America (Nunney *et* al. 2010). The pathogen is transmitted by a number of xylem feeding insects (Redak *et* al. 2004). The recent introduction of the invasive glassy-winged sharpshooter (GWSS) to southern California altered the epidemiology of PD and increased disease incidence to epidemic proportions (Purcell and Saunders 1999).

X. fastidiosa is limited to the xylem tissue of its plant host and migrates both acropetally and basipetally through the xylem, in part by using Type IV pili-mediated motility (Meng *et* al. 2005). The xylem network is a collection of xylem vessels that are interconnected both horizontally and vertically by pit membranes, which are composed of primary plant cell wall. The pathogen subsequently spreads to new connected vessels by breaching the pit membrane barrier in a cell wall degrading enzyme-dependent manner (Perez-Donoso *et* al. 2010). The bacteria can multiply to high population numbers in susceptible hosts and these bacterial aggregates and biofilms can occlude xylem vessels (De La Fuente *et* al. 2008). In addition, there are several plant-derived vascular occlusions (i.e., tyloses, gums, and pectin gels) that occur in infected vines that further impede plant hydraulic conductivity, with tyloses being the predominant vascular occlusion associated with PD (Sun *et* al. 2013). At the whole plant level, PD symptomology manifests into

scorched leaves, irregular periderm development, irregular petiole abscission, raisining of berries, canopy stunting and vine death (Varela *et* al. 2001).

PD-resistance has only been identified in several wild grape species endemic to the Americas including *V. arizonica/candicans* and *Muscadinia rotundifolia*, whereas all *V. vinifera* genotypes from Eurasia are susceptible to this disease (Fritschi *et* al. 2007; Riaz *et* al. 2018). Three major pedigree lineages have been recognized within the cultivated grapevine *Vitis vinifera*; *occidentalis*, the small-berried wine grapes of western Europe; orientalis, the large-berried table grapes of West Asia; and pontica, the intermediate type from the basin of the Black Sea and eastern Europe (Aradhya *et* al. 2003). Field observations and greenhouse experiments indicated that there was varying degrees of susceptibility to PD among the Genetic pool of cultivated grapevine (Purcell 1974; Rashed *et* al. 2011), but none of these studies linked PD-susceptibility to *V. vinifera* cultivar pedigree. Because cultivated grapevines evolved under different climates and were domesticated under different practices, populations developed different anatomical traits and strategy regarding water transport, which was illustrated by striking differences in xylem morphology (Pouzoulet *et* al. 2014; Pouzoulet *et* al. 2017a).

It is hypothesized here that domestication of grapevine and selection for specific phenotypic traits have shaped PD susceptibility levels in *V. vinifera*. This hypothesis was initially tested by selecting grape cultivars for which we had previous knowledge on wood anatomy and specifically vessel diam *et*ers (Pouzoulet *et* al. 2014; Pouzoulet *et* al. 2017a). These included two winegrape cultivars from the *occidentalis* group, Merlot and Cabernet Sauvignon; and two table grape cultivars, Thompson Seedless (a.k.a. Sultanina, an ancient

cultivar used for breeding of many commercial seedless table grapes) from the *orientalis* group and Scarlet Royal a recently released cultivar from the USDA breeding program (i.e., 2006) with mixed pedigree parentage that includes *orientalis* (e.g., Sultanina) and *pontica* (e.g., Muscat of Alexandria, Emperor). We compared susceptibility of those cultivars to the PD resistant b43-17 *V. arizonica/candicans* grape genotype from North America. We measured host susceptibility in *in planta* bioassays as a function of disease severity and *X. fastidiosa* abundance and integrated these data with hydraulic conductivity for selected *V. vinifera* cultivars. This work provides a benchmark for PD-susceptibility levels for some of the most widely planted table and wine grape cultivars. It also warrants additional research efforts to test a greater number of *V. vinifera* cultivars from different Genetic pools and collect information on their vascular anatomical features in order to validate our stated hypothesis. Ultimately, this research may aid in the development of tools to assess and predict PD susceptibility for cultivated grapevines cultivars and facilitate recommendations to industry stakeholders with regards to the planting of PD-tolerant grape cultivars when confronted with high PD pressure.

5.0.3 Materials and Methods

In planta evaluation of PD susceptibility

The four commercial *V. vinifera* cultivars used in this study were obtained from the Foundation Plant Service at UC Davis, and included two wine grape (Merlot selection #06 and Cabernet Sauvignon selection #31) and two table grapes (Scarlet Royal selection #01 and Thompson Seedless selection #02A). We selected those cultivars because of their

different pedigrees (Aradhya *et* al. 2003) and also because we had accumulated substantial amount of data on plant vascular anatomy as it relates to susceptibility to another xylem-dwelling fungal pathogen *Phaeomoniella chlamydospora* (Pouzoulet *et* al. 2014; Pouzoulet *et* al. 2017a). We also included as a reference, the PD resistant b43-17 *V. arizonica/candicans* type (Krivanek *et* al. 2006), a wild PD-resistant grapevine found in North America. Cuttings of *V. arizonica/candicans* cuttings were kindly provided by A. Walker (Dept of Viticulture and Enology, UC Davis, California, USA).

One-bud cuttings were rooted and potted into a mixture of soil and sand (1:1) amended with 5 g of controlled release fertilizer (Scotts Osmocote Classic, N-P-K: 14-14-14, Marysville, USA). A single shoot per grape cutting was trained vertically onto a stake. Inoculations were performed as follows: *X. fastidiosa subsp. fastidiosa* strain Temecula 1 was cultured on PD3 solid medium at 28C for 7-10 days. Cells were harvested from PD3 plates and suspended in 1X Phosphate-buffered saline (PBS) after which cell suspensions were adjusted to $OD_{600\ nm}$ 0.25 (approximately 1 x 10^8 CFU/mL $^{-1}$). Plants were inoculated on both sides of the stem with 10μ łof cell suspension of *X. fastidiosa* between the first and second nodes at the base of the shoot by mechanical needle inoculation as previously described (Hill and Purcell 1995). Control plants were needle-inoculated with a 10μ ł1X Phosphate-buffered saline (PBS). Twenty-four plants were used per genotype and half inoculated with *X. fastidiosa* and the other half with 1X PBS buffer. Thus, a total of 120 plants were utilized for each replicate. The experiment was replicated two times in two separate years. For each trial, all plants were kept under the same greenhouse conditions with the same watering regime equally over the 12-week incubation period. All vines were

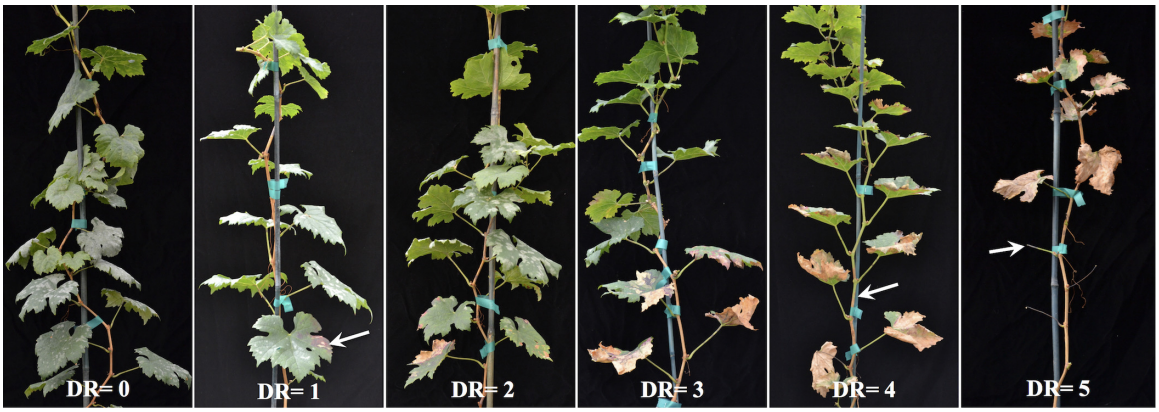

Figure 5.1: Disease rating (DR) scale used to evaluate Pierce's disease (PD) severity. 0 = no disease; 1 = 12 leaves showing initial PD symptoms with leaf scorching (white arrow); 2 = 34 leaves showing PD symptoms; 3 = <50% of the leaves expressing PD symptoms; 4 = >50% of the vines expressing PD symptoms including formation of green islands (white arrow); 5 = vines are dead or dying showing matchstick petioles (white arrow)

visually rated by the same scientist at 12 weeks post-inoculation on an PD symptom severity scale ranging from 0-5 (Figure 5.1) adapted from Guilhabert and Kirkpatrick (2005).

Detectionand quantification method for *X. fastidiosa in planta*

Three petioles were harvested per vine and pooled to quantify *X. fastidiosa* abundance in planta, starting at the first PD symptomatic leaf sampling downward. When no PD symptoms were observed (i.e., in the negative controls) the three petioles were harvested randomly and pooled. petioles were kept at -20°C until further processing for qPCR analysis. The frozen petioles from each treatment were lyophilized for 36 hrs with a FreeZone 2.5 liter Benchtop Freeze Dry System (Labconco, Kansas City, MO). Samples were then finely ground at room temperature using a R *et*sch MM300 grinder (45 s, 25 oscillations per second) in a 35 ml stainless-steel grinding jar (R *et*sch, Haan, Germany) with 20-mm stainless steel balls. Ground tissue was incubated at 65C for ten minutes in a heat block

with hexadecyltrim *et*hylammonium bromide (CTAB, Sigma-Aldrich, St. Louis, USA) extraction buffer. Total DNA was extracted from the 100 mg of petiole tissue using a Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to manufacturers instructions, with the exception of using CTAB extraction buffer as the lysing buffer. The DNA for all samples was stored at -20C before use. DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) according to the manufacturers protocol.

A set of primers targ *et*ing a 90-bp long fragment of the internal transcribed spacer (ITS) region of the ribosomal rRNA genes of *X. fastidiosa* was developed using the PRISE 2 software (Huang *et* al. 2014). *X. fastidiosa* has two copies of the ribosomal operon (Simpson *et* al. 2000). The designed primers were XfITSF6 (5-GAGTATGGTGAATATAATTGTC-3), XfITSR6 (5-CAACATAAACCCAAACCTAT-3). Amplification reactions were performed using a Biorad CFX96 Real-Time PCR cycler and Biorad CFX manager software v3.1 (Bio-Rad, Irvine, CA, USA) using default s *et*tings for amplification curve analysis. Reactions conditions (i.e. primer concentrations, annealing temperature) were optimized for amplification selectivity and PCR efficiency according to the MIQE guidelines (Bustin *et* al. 2009). Reactions proceeded in a final volume of 25μ łand reactions mixtures contained 12.5μ łof Quantitect SYBR Green Master Mix reagent (Qiagen, Venlo, The N *et*herlands) 0.4 um of each primer, and 2μ łof DNA template. The cycling program consisted in (1) 95C for 15 min; (2) 40 cycles of 55C for 15 s, 72C for 45 s; 95C for 15 s; (3) a melt curve analysis from 65-95C (0.5C increments every 5 s) to verify the amplicons identity. In all qPCR plates, a standard curve was generated from a DNA solution from *X. fastidiosa* cultured on PD3 solid medium. Limit of Detectionand

limit of quantification was determined by running absolute quantities of *X. fastidiosa* from pure cultures as previously described by Pouzoulet *et* al. (2017b), using *X. fastidiosa* DNA amounts ranging from up to 4.2 ng and as low as 1.7 fg. Specificity of the qPCR method was also validated against several bacterial strains. This included the grapevine strain, *X. fastidiosa subsp. fastidiosa* Temecula1; the almond strain, *X. fastidiosa subsp. multiplex* M12 (provided by Dr. Bruce Kirkpatrick, UC Davis); and the oleander strain, *X. fastidiosa subsp. sandyi* Ann-1 (provided by Dr. Donald Cooksey, UC Riverside). All three *X. fastidiosa* strains were cultured on PD3 solid medium at 28C for 7-10 days prior to DNA extraction. We also included a closely related organism to *X. fastidiosa*, *Xanthomonas campestris* pv. *campestris* 0198-18 (provided by Dr. Donald Cooksey, UC Riverside). In addition, we tested several phyloGenetically diverse bacteria: (i.e., *Bacillus megaterium, Pantoea agglomerans, Pseudomonas syringae, Agrobacterium tumefaciens*, and *Pseudomonas fluorescens*) representing taxonomic groups of bacteria associated with grapevines (Dey *ett et* al. 2017). *X. campestris* was cultured on yeast extract-dextrose-$CaCO_3$ agar (YDC) at 28C for two days before DNA extraction. All the other bacterial strains were propagated on Luria-Bertani (LB) medium at 28C for up to two days before DNA extraction. Absolute quantification of *X. fastidiosa* DNA by qPCR from total DNA extracted from plant samples was done by using standard DNA solution from *X. fastidiosa* cultured on PD3 solid medium. Non-template controls were included in each plate and each sample was tested in triplicate. For each reaction, the identity of the amplicon was verified through its melt-curve profile.

The average quantity (femtograms of *X. fastidiosa* DNA) of the three replicates was standardized by the amount of total DNA input, and used for further statistical analysis.

Measurement of stem hydraulic conductivity

Hydraulic conductivity was determined on three of the total five grape genotypes that displayed contrasting symptoms severity and included b43-17 *V. arizonica/candicans* (resistant), *V. vinifera* Thompson Seedless (highly susceptible) and Merlot (moderately susceptible). Ten plants were sampled for each grape genotype (30 samples total) with 5 for the 1X PBS control vines and 5 for the *X. fastidiosa* inoculated vines, at the end of the experiment (i.e., 12 weeks post-inoculation) following the recording of disease ratings. The day before shoot sampling, plants were watered to saturation to assure maximum stem hydration. Petioles were cut with a safety razor blade and wounds were sealed immediately with cyanocrylate glue (The Gorilla Glue Company, Sharonville, Ohio, USA). Subsequently, shoots were cut at the basal end and immediately immersed in water. The apical part of the stem above the eighth node was cut off under water to avoid embolism. Each stem fragment that was further used to measure hydraulic conductivity was cut under water to 15 cm in length from the basal end. Stem hydraulic conductivity (K_h; kg m $^{-1}$ s $^{-1}$ MPa $^{-1}$) was determined by connecting the 15 cm long stem to a tubing system with filtered (0.2 um), de-gassed 20 mM KCl solution flowing from an elevated source, through the stem, and into a reservoir on a balance (0.1 mg; Denver Instrument P-214; Sartorius, Gttingen, Germany) that was interfaced with a computer to record flow rate, allowing the calculation of conductivity (Sperry and Tyree 1988). We corrected for stem passive water uptake by beginning and ending each conductivity measurement with a background measurement

(Torres-Ruiz *et* al. 2012). Stem hydraulic conductivity (K_h; kg m^{-1} s^{-1} MPa $^{-1}$) was calculated as: $K_h = F \ L/dP$; where F is the flow rate (kg s^{-1}), L is the stem length (m), and dP is the driving force (MPa). The head pressure was 1.2 kPa. Immediately after K_h was measured, the stem fragment was infiltrated with a solution of safranine O (0.1%) until the dye was allowed to cross along the stem fragment (appearance of a red cloud at the tip of the fragment being placed under water). Then the excess of dye was flushed by dH_2O until no more stain efflux could be seen. Seventy microm *et*re-thick cross-sections of the middle part of the stem were sliced with a microtome, and micrographs of the specimens were obtained as previously described (Pouzoulet *et* al. 2017a). Trans-sectional xylem area was determined with a stereo-microscope (M165C, Leica microsystems CMS GmbH, W *et*zlar, Germany) and LAS v 4.2 software (Leica microsystems CMS GmbH, W *et*zlar, Germany) and was used to calculate the sapwood-specific hydraulic conductivity (K_h; kg m^{-1} s^{-1} MPa $^{-1}$). Vessel diam *et*er data were obtained as described by Pouzoulet al. (2014). In parallel, high contrast micrographs showing the vessel infiltrated with dye were obtained by subtracting the green from the blue filter using *ImageJ* v 1.48. (https://imagej.nih.gov/). Diameter data from stained vessels were collected and used to calculate theorical stem conductivity (K_{th}) using the Hagen-Poisseuille equation as described by Santiago *et* al. (2004).

Statistical analyses

Statistical analyses were conducted using R v. 3.4.2 (http://www.R-project.org/). Kruskal-Wallis rank sum statistical testing was used to determine statistical differences between PD rating, *X. fastidiosa* quantity, hydraulic conductivity and grapevine genotypes.

A pairwise Wilcoxon test was used to calculate the pairwise statistical differences between the 5 grapevine genotypes and the visual PD ratings, hydraulic conductivity or *X. fastidiosa* quantities. P-values were corrected with the false discovery rate, fdr, method.

5.0.4 Results

To validate the *X. fastidiosa* detection assay, primers were evaluated for specificity and reproducibility using real time qPCR SYBR-Green technology. Primers consistently showed robust efficiencies and R^2 values as defined by MIQE guidelines (Bustin *et* al. 2009). The assay was able to accurately quantify *X. fastidiosa* DNA amounts ranging from 4.2 ng to 8.4 fg, and the limit of detection (LOD) of the assays was 16.8 fg of *X. fastidiosa* DNA (Figure 5.2). The *X. fastidiosa* genome size is 2.68 Mbp and has two copies of the ribosomal operon (Simpson *et* al. 2000). Considering that the average molecular weight of a base pair is 650 Da, the LOD of this assay represents approximately six *X. fastidiosa* genomes, and twelve copies of the target DNA sequence. Melt curve profiles indicated all amplicons had a single melting peak at 75.2C 2 further verifying specific binding to the primers and the presence of other byproducts, such as primer dimers, were not observed through melt curve analysis including from plant samples (Figure 5.2b). Amplicons were not detected for closely related and off-target bacteria that commonly associated with grapevines. The assay could not segregate the three *X. fastidiosa* subspecies tested from one another (data not shown), indicating that this qPCR was not specific to *X. fastidiosa* subsp. *fastidiosa*.

V. vinifera vines inoculated with *X. fastidiosa* exhibited characteristic PD symptoms that initiated with scorching of the basal leaves, close to the point of inoculation

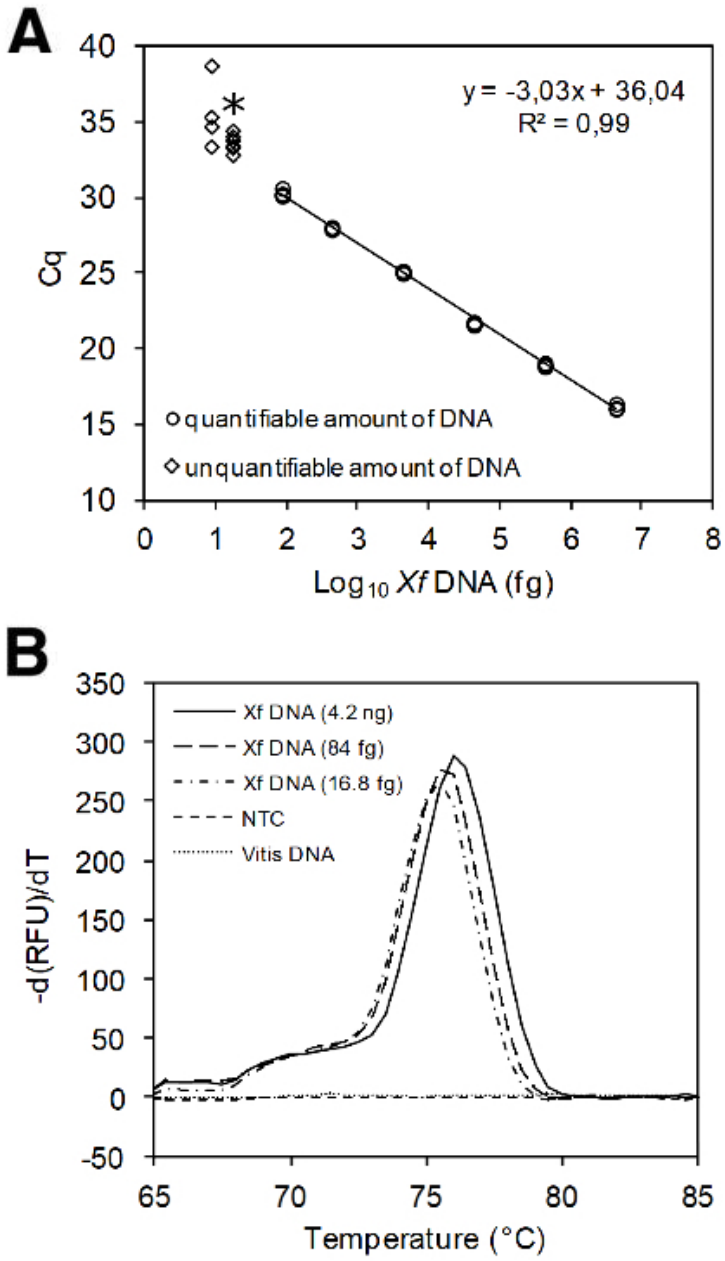

Figure 5.2: Performance of the *Xylella fastidiosa* qPCR assay. (A) The calibration curve displayed and associated PCR efficiency (i.e. 104.6%) using serial dilution of *X. fastidiosa* DNA. Decreasing amounts of bacterial DNA displayed are 4.2 ng-1.7fg. An amount of DNA was considered quantifiable when the standard deviation associated with its measurement was below 33% (SD Cq <0.41, n = 5). The lowest limit of quantification was 84 fg *X. fastidiosa* DNA. The limit of detection (*), determined as the minimum amount of bacterial DNA being detected with a probability superior or equal to 95%, was 16.8 fg. (B) Example of melt profiles of amplification products observed with *X. fastidiosa* assay.

(Figure 5.1). As the disease severity progressed, leaf scorching expanded to the apical part of the vine with appearance of match stick petioles (i.e., leaf drop with only petiole attached) and uneven wood lignification causing formation of green islands. Non-param *et*ric Kruskal-Wallis testing detected a significant effect with grape cultivar ($P < 0.0001$) and trial ($P < 0.01$). Post hoc analyses confirmed that b43-17 *V. arizonica/candicans* was resistant to PD with very few isolated leaves developing typical symptoms. Among the *V. vinifera* cultivars tested, Thompson Seedless was significantly more susceptible to PD than the other three cultivars (Figure 5.3). The negative buffer-inoculated controls did not develop typical PD symptoms.

The results of the PD ratings were further supported by quantification of *X. fastidiosa* titer by qPCR (Figure 5.4). *X. fastidiosa* was not detected in any of the PBS-inoculated grape genotypes. Kruskal-Wallis nonparametric testing detected a significant effect of grape cultivar ($P < 0.0001$) and trial ($P < 0.0001$). The PD-resistant b43-17 *V. arizonica/candicans* displayed the lowest amount of pathogen DNA. Among the *V. vinifera* cultivars, Thompson Seedless significantly harbored the highest *X. fastidiosa* DNA amount but no significant statistical differences were found between the cultivars Merlot, Scarlet Royal and Cabernet Sauvignon.

No significant effect of *X. fastidiosa* inoculation on hydraulic conductivity (K_s) was measured for the resistant b43-17 *V. arizonica/candicans* in comparison to the cognate PBS control (Figure 5.5). In contrast, a significant decrease in K_s was measured in the *X. fastidiosa*-infected *V. vinifera* Merlot and Thompson Seedless with a reduction of 62% and 94%, respectively. Thompson Seedless was also the most susceptible based on disease rating

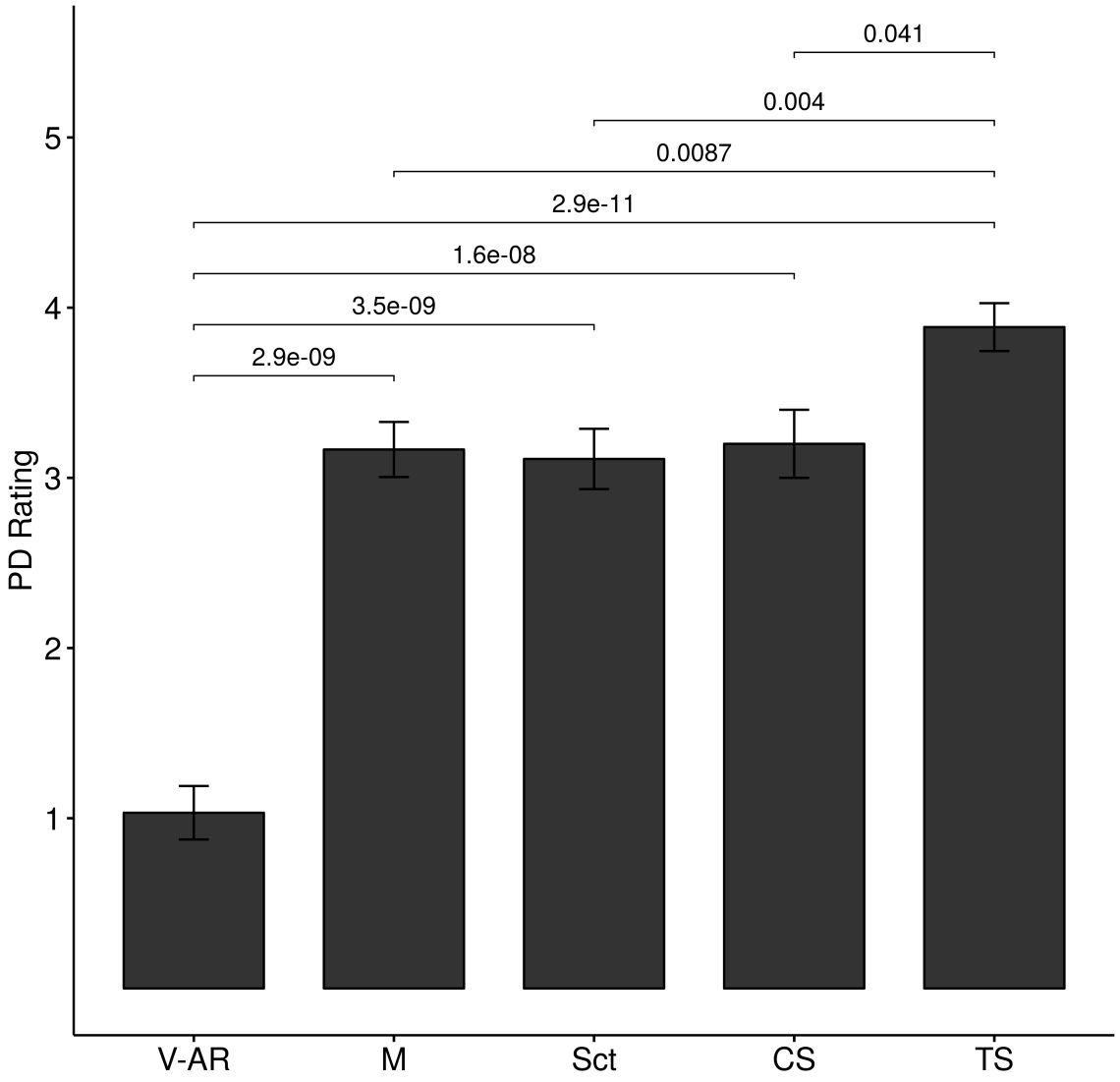

Figure 5.3: Mean disease rating for individual grape genotypes 12 weeks post-inoculation with *Xylella fastidiosa*. V-AR, b43-17 *Vitis arizonica/candicans*; M, *Vitis vinifera* Merlot; Sct, *V. vinifera* Scarlet Royal; CS, *V. vinifera* Cabernet Sauvignon; TS, *V. vinifera* Thompson Seedless. Bars represent standard errors.

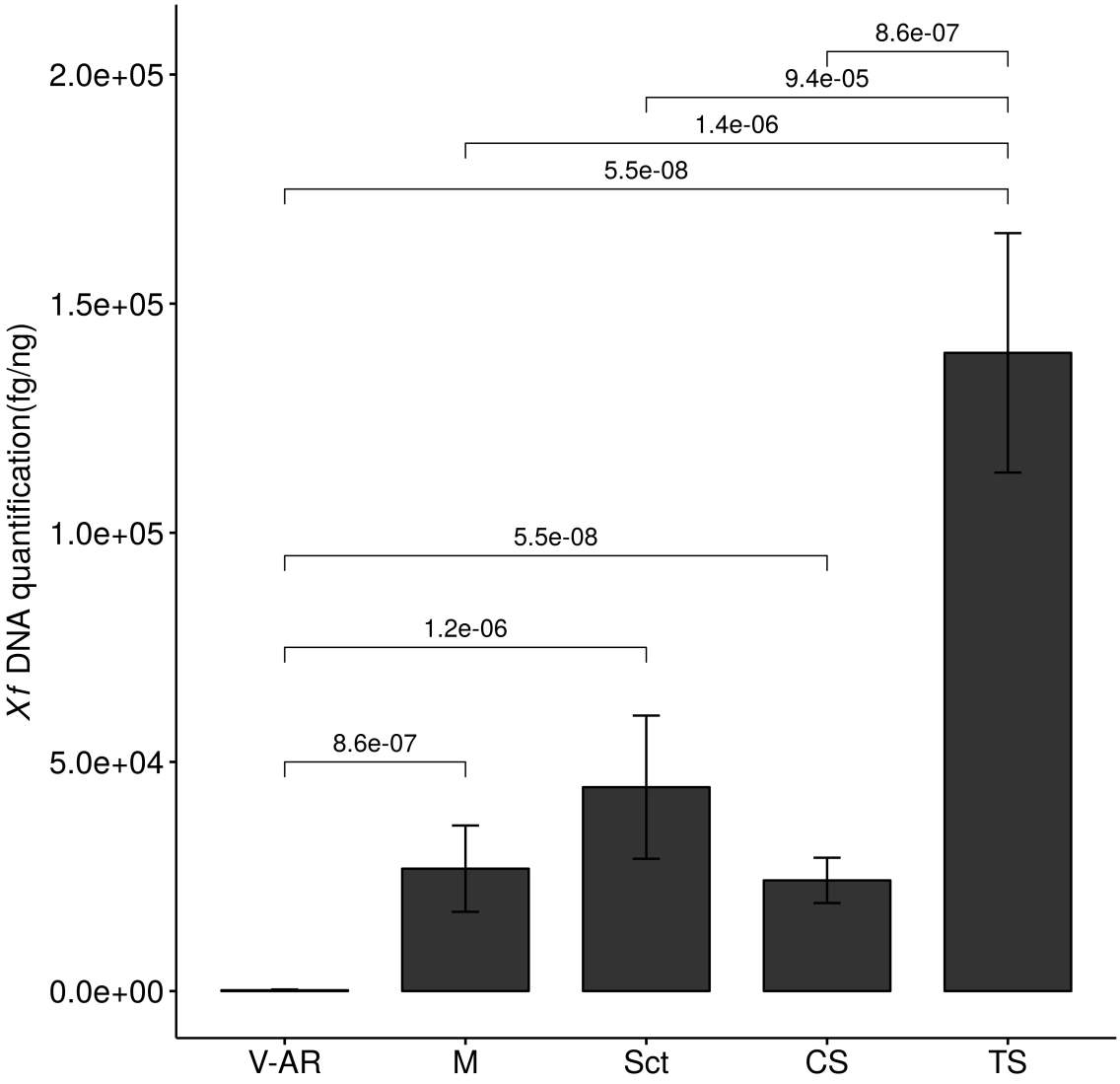

Figure 5.4: Mean *Xylella fastidiosa* quantification (*X. fastidiosa* fg DNA per ng of plant DNA) in petioles of individual grape genotypes 12 weeks post-inoculation. V-AR, b43-17 *Vitis arizonica/candicans*; M, *Vitis vinifera* Merlot; Sct, *V. vinifera* Scarlet Royal; CS, *V. vinifera* Cabernet Sauvignon; TS, *V. vinifera* Thompson Seedless. Bars represent standard errors.

(Figure 5.3) and harbored the highest levels of *X. fastidiosa* (Figure 5.4). K_s values were not significantly different across the three genotypes following mock-PBS inoculation. To further assess the effect of *X. fastidiosa* on stem hydraulic conductivity, the functional vessels within stem samples were visualized following a dye vacuum infiltration method (Figure 5.6). Our results confirmed that xylem vessel function was compromised in highly PD symptomatic grapevines because we observed a significant decrease in staining of conductive xylem vessels likely due to vascular occlusion. Finally, significant correlations ($P < 0.05$) were found between predicted (K_{th}) and measured (K_s) values of water conductivity (Figure 5.7), supporting that differential loss in K_s of genotype was driven by the number of vessels remaining functional following *X. fastidiosa* establishment.

Our data suggested that the progression of PD symptoms as observed in our *in planta* bioassay (Figure 5.1) from a DR 2 (3 to 4 leaves showing PD symptoms) to DR 3 (less than 50% of leaves with PD symptoms) was the critical point for both *X. fastidiosa* titer and hydraulic conductivity (Figure 5.8). Overall, *X. fastidiosa* abundance increased with appearance of disease symptoms but DR 3 was associated with the highest detectable *X. fastidiosa* DNA amount for all grapevine genotypes. Interestingly, the detection of *X. fastidiosa* DNA did not correlate with disease rating in a linear fashion because pathogen detection was compromised in severely symptomatic plants (with a DR of 4 and 5). We measured a sharp decrease in hydraulic conductivity as the diseased progressed from a visual rating of 2 to 3 and the pathogen titer increased.

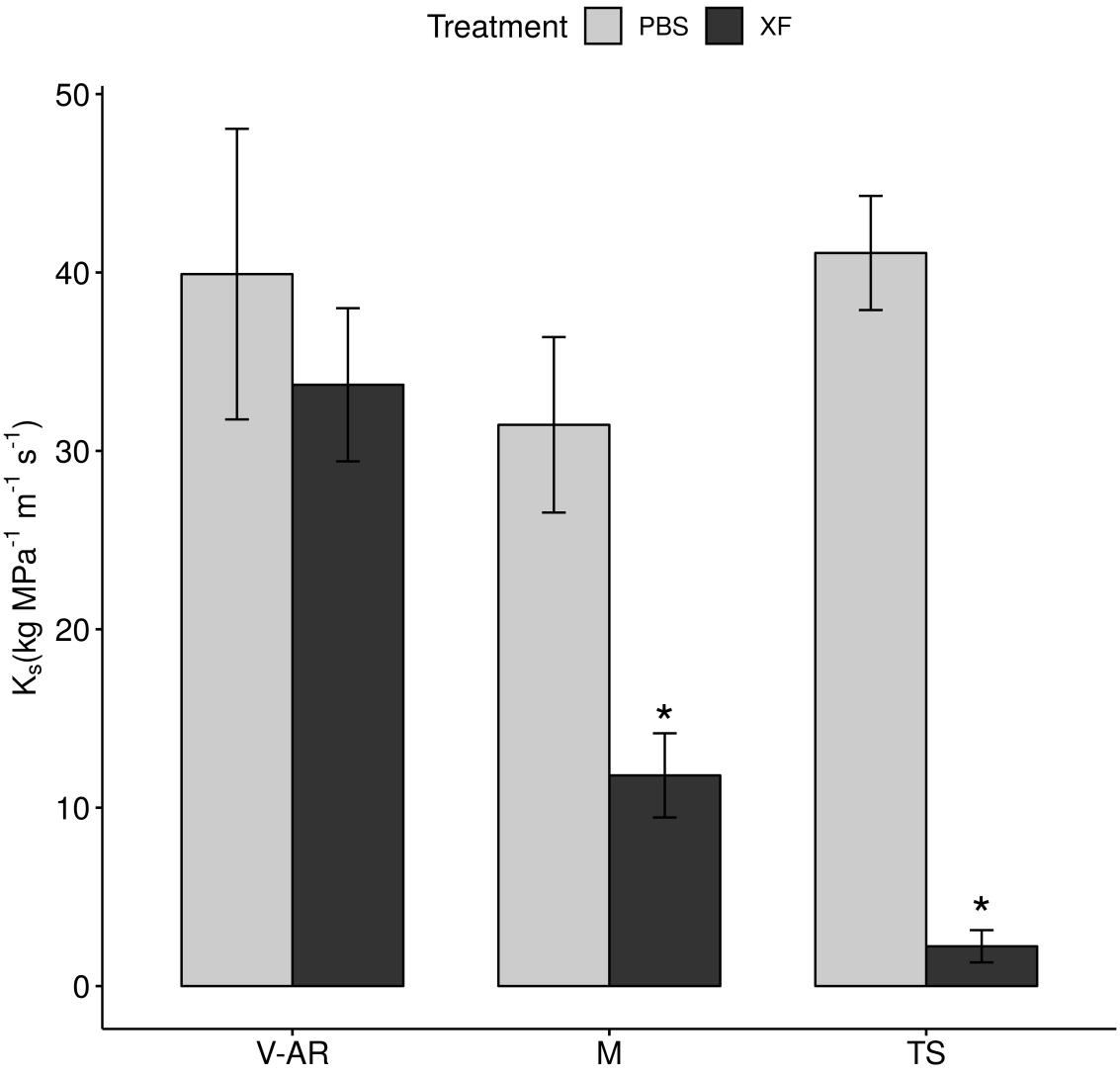

Figure 5.5: Specific stem hydraulic conductivity K_s (kg kg m $^{-1}$ s $^{-1}$ MPa $^{-1}$) measured for three grapevine genotypes, b43-17 *Vitis arizonica/ candicans* (V-AR), *Vitis vinifera* Merlot (M) and Thompson Seedless (TS), 12 weeks post-inoculation with phosphate-buffered saline (PBS) and *Xylella fastidiosa* (XF). * indicates statistically significant differences ($P < 0.05$; $n = 30$).

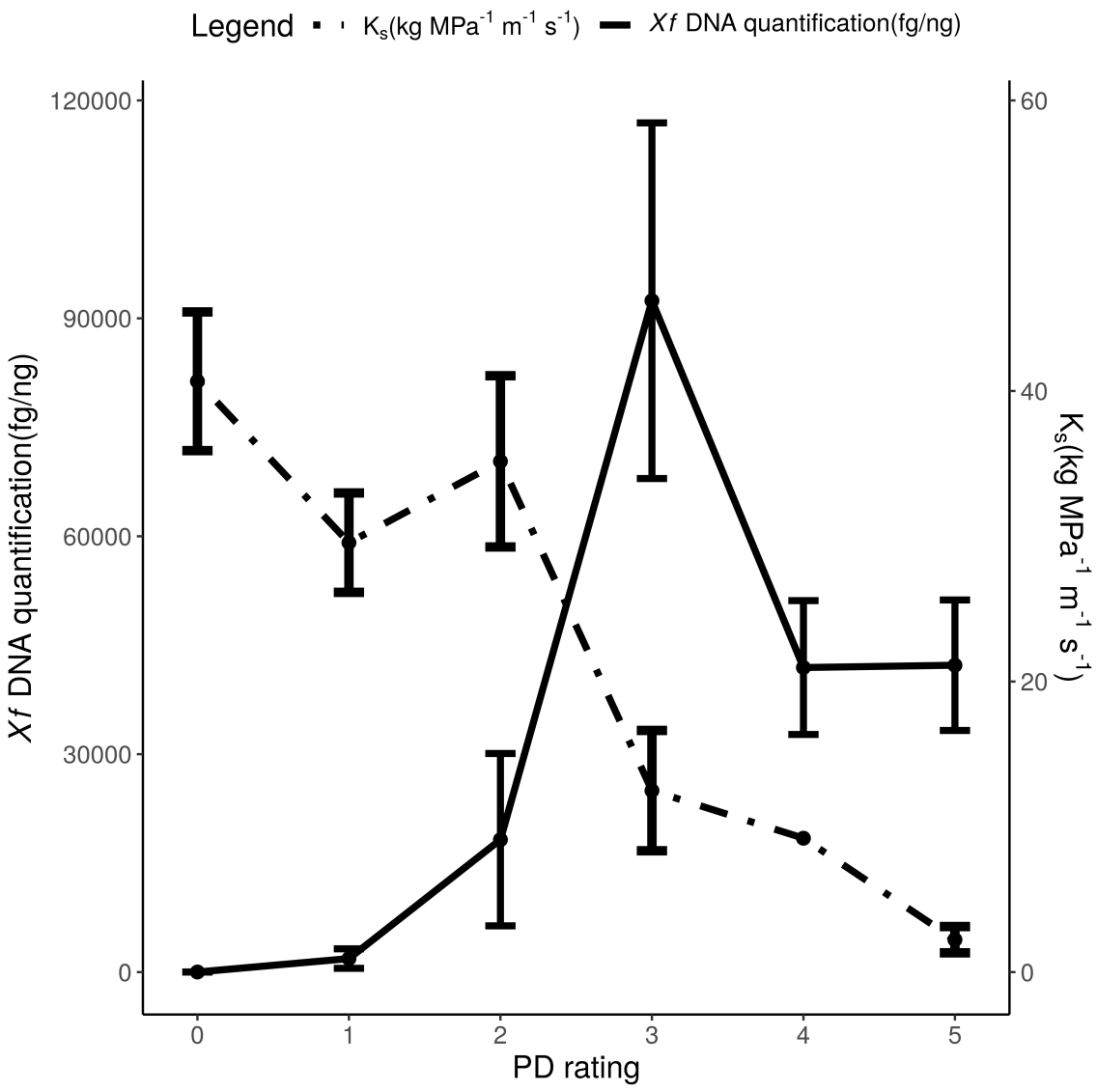

Figure 5.6: Relationship between Pierce's disease (PD) rating and *Xylella fastidiosa* DNA quantification (fg *Xylella fastidiosa* DNA per ng of plant DNA) and plant hydraulic conductivity measured as K_s (kg m $^{-1}$ s $^{-1}$ MPa $^{-1}$). Bars represent standard errors.

5.0.5 Discussion

PD is a multi-faceted pathosystem and manifestation of disease in susceptible *Vitis* genotypes is associated with systemic movement of the bacterium and extensive occlusion of the vascular system. Breeding programs focused on evaluating PD resistance of wild *Vitis* species and introgressing resistance into existing susceptible *V. vinifera* cultivars are well established (Fritschi *et* al. 2007; Krivanek and Walker 2005; Riaz *et* al. 2018). However, experiments designed to comprehensively assess PD susceptibility in *V. vinifera* cultivars have been limited in scope. In this study we have used published protocols and developed new tools to fully evaluate *V. vinifera* cultivars with different pedigree backgrounds for PD susceptibility. Our qPCR detection method amplified the three *X. fastidiosa* subspecies tested. *X. fastidiosa* subsp. *pauca* was not included in our validation assay because it is a quarantine pathogenic organism in the US. Other detection methods for *X. fastidiosa* have used TaqMan probes (Harper *et* al. 2010) and ELISA (Krivanek and Walker 2005) with a LOD of 10 copy numbers of DNA targeted sequence and about 10^4 CFU/mL^{-1}, respectively. We had initially developed a TaqMan probe for our assay but switched to the SYBR-Green technology, because the sensitivity levels were comparable and the latter allowed to processing of large volume of samples at lower cost. Likewise, Luvisi *et* al. (2017) adapted the TaqMan primer sets developed by Harper *et* al. (2010) to a SYBR-Green assay for screening *X. fastidiosa* subsp. *pauca* from olive samples and showed similar sensitivity between the two methods.

Our assay is quick, specific, more sensitive than an ELISA, and more cost-effective than TaqMan qPCR assays while displaying similar LODs (Harper *et* al. 2010) deeming it a reliable and reproducible quantification method for *X. fastidiosa in planta*.

Our results indicated that the bacterium moved systemically and achieved high population numbers in all *V. vinifera* cultivars, albeit at significantly different levels across *V. vinifera* cultivars. *X. fastidiosa* was also able to colonize b43-17 *V. arizonica/candicans* at a distance from the point inoculation and the manifestation of PD symptoms was isolated to a few leaves. The bacterial population was low in b43-17 *V. arizonica/candicans* relative to the susceptible cultivars, which is similar to previous reports (Fritschi *et* al. 2007; Krivanek and Walker 2005). Our classification of *V. vinifera* cultivar susceptibility based on *X. fastidiosa* DNA load was also supported by Rashed *et* al. (2011). Pathogen abundance strongly correlated with disease symptom severity and based on those criteria we established that Thompson Seedless was highly susceptible whereas Merlot, Cabernet Sauvignon and Scarlet Royal were moderately susceptible. Our data also showed that severe PD symptoms does not correlate with high pathogen titer and supported the results of Gambetta *et* al. (2007). This suggests that the optimum time to quantify *X. fastidiosa in planta* and to show differences in disease susceptibility is when plants display mild to average symptoms severity.

The basis of what dictates PD susceptibility versus PD tolerance/resistance is not well understood. PD-resistance has only been identified in the pool of wild North American Vitis species whereas all *V. vinifera* cultivars endemic to Eurasia are susceptible. In b43-17 *V. arizonica/candicans* the sequence analysis of the *PdR1* locus indicates that it contains

putative candidate resistance genes belonging to the leucine-rich repeat resistance gene family (Riaz *et* al. 2018). However, the biological function of the protein(s) encoded by *PdR1* has to our knowledge, not been characterized. In wild *Vitis* genotypes, host chemical, physical and anatomical traits appeared to be key determinants of the PD-resistance. Xylem sap extracted from the PD-resistant *V. champinii* and *V. aestevalis* did not support viable *X. fastidiosa* populations when compared to PD-susceptible *V. vinifera* suggesting that xylem sap chemistry could potentially impact bacterial viability *in planta* (Hao *et* al. 2016). The xylem sap of *V. vinifera* also contains significantly higher soluble sugars and free amino acids than PD-tolerant *V. rotundifolia* suggesting that *V. vinifera* xylem sap provides a more nutritionally favorable environment for *X. fastidiosa* to multiply and move systemically. In addition, plant defense-related proteins, -1,3-glucanase, peroxidase, and oxygen-evolving enhancer protein, were more abundant in the PD-resistant species suggesting it can mount a more effective defense response to *X. fastidiosa* invasion (Basha *et* al. 2010).

Pit membrane chemical composition is also hypothesized to influence pathogen movement *in planta* because the bacterium must breach this barrier in a cell-wall degrading enzyme-dependent manner to colonize new neighboring xylem vessels. The susceptible *V. vinifera* Chardonnay and Riesling pit membranes have higher amounts of fucosylated xyloglucans and weakly methyl esterified homogalacturonan, whereas these ratios were inverted in the PD-resistant *Vitis arizonica x V. rupestris* and *V. vinifera* x V. arizonica (Sun *et* al. 2011). It is reasonable to speculate that the chemical composition of the pit membranes in the b43-17 *V. arizonica/candicans* vines used in our study share similar

chemical characteristics with the related PD-resistant *V. arizonica* hybrids described by Sun *et* al. (2011), which may explain the restricted development of PD symptoms we observed in b43-17 *V. arizonica/candicans.*

Xylem vessel occlusion via production of host-produced tyloses and gels in response to wounding or infection by xylem-dwelling pathogens is a conserved mechanism in plants to wall-off compromised vessels (Beckman and Roberts 1995; Pouzoulet *et* al. 2017a). As the disease progresses, more vessels become occluded, which compromises plant hydraulic functions, and those functions are hindered even more under water deficit (Choi *et* al. 2013). Sun *et* al. (2013) showed that over 60% of the vessels were occluded in the *X. fastidiosa*-infected *V. vinifera* Thompson Seedless and compared to less than 30% in different resistant genotypes *V. arizonica x Vitis rupestris* and *V. vinifera* x *V. arizonica*. Our data mirror their results in that K_s was reduced by 62% and 94% in Merlot and Thompson Seedless, respectively, but b43-17 *V. arizonica/candicans* maintained hydraulic function following pathogen ingress.

X. fastidiosa moved systemically in all *V. vinifera* cultivars we tested, yet differences in PD severity were significant among some of the cultivars. This suggests that factors independent of systemic movement influence the degrees of susceptibility observed across *V. vinifera* cultivars. *V. vinifera* Sylvaner is described has PD-tolerant and has interestingly smaller vessel diameters than susceptible *V. vinifera* cultivars indicating that the plant vascular system anatomy could be a determinant of level of PD susceptibility (Chatelet *et* al. 2011). Vessels with larger diameters also harbor a greater number of tyloses, which may be linked to PD symptom severity (Sun *et* al. 2013). Similar results were found for

the fungal wilt disease of grapevine caused by the xylem-dwelling fungus, *Phaeomoniella chlamydospora*, where grapevine cultivars with wider xylem vessel diameters were less efficient at restricting and compartmentalizing *P. chlamydospora* movement and that number of tyloses correlated with vessel size (Pouzoulet *et* al. 2017a). The authors concluded that xylem vessel diameter influences timing of vessel occlusion, a key feature to compartmentalization in response to xylem-dwelling pathogen, and that the density of xylem vessels above 120 um correlated with increased disease susceptibility. Based on these criteria, the decreasing order of susceptibility to the fungal wilt disease *P. chlamydospora* was classified as followed, Thompson Seedless, Cabernet Sauvignon and Merlot (Pouzoulet *et* al. 2017a), which is comparable to the PD susceptibility levels presented in this manuscript. From this, we speculate that xylem vessel diameter size is a key determinant to effective defense against *X. fastidiosa* infection and cognate degree of susceptibility to PD in *V. vinifera*.

In conclusion, the cultivated table grape variety Thompson Seedless with the *orientalis* pedigree was highly susceptible to PD, whereas the *occidentalis* wine grape cultivars Merlot and Cabernet Sauvignon displayed lower susceptibility to the disease. Interestingly, the recently bred table grape cultivar Scarlet Royal showed similar PD susceptibility levels to Merlot and Cabernet Sauvignon, perhaps due to introgression of multiple Genetic backgrounds with contrasting pedigrees. Future experiments should be conducted with an expanded set of cultivated *V. vinifera* cultivars from various pedigree lineages as well as its wild ancestor *V. sylvestris* in order to determine if traits of xylem morphology a reliable marker to predict PD susceptibility. This could help breeding programs with the selection of plant Genetic materials with disease-tolerant traits. In addition, trials should

evaluate how those phenotypes identified in a semi-controlled environment hold under field conditions when subjected to standard cultural practices (e.g., cultivars grafted on different rootstocks, irrigation regimes).

5.0.6 Referecnes

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Chapter 6

Green-house evaluations of biocontrols

6.0.1 Abstract

Xylella fastidiosa, the causal agent of Pierce's Disease (PD), is a xylem-limited bacterium for which effective management strategies are limited. *Pseudomonas spp.*, including *P. viridiflava, P. fluorescence*, and *P. putida* as well as *Achromobacter xylosoxidans* are in high relative abundance in the endosphere of grapevines and have previously been shown to be negatively correlated with *X. fastidiosa* titer and PD symptom severity. In this study, we evaluated these four native endophytic microbes for their ability to mitigate *X. fastidiosa* both *in vitro* through an antagonism and enhancement assay as well as *in planta* both through visual ratings and pathogen titre. Our results indicated that the grapevine-associated *P. viridiflava* and *A. xylosidans* are potential biocontrol candidates against *X. fastidiosa* and significantly suppresses PD symptoms. Our *in vitro* assays indicated that direct antagonism is not the mechanism of protection but rather that *P. viridiflava* may enhance *X. fastidiosas* biofilm formation via a siderophore-mediated iron sequestration mechanism. We hypothesize that by effectively locking *X. fastidiosa* in an

adhesive biofilm, the rampant spread and systemic movement throughout the plant will be prevented, thereby effectively slowing disease progression. We also hypothesize that *A. xylosidans* may be priming the plant's immune system for the biotic stress. This work is the first step to assessing these endophytic microbe's ability to suppress Pierce's Disease, providing the foundation for future research to better understand plant-microbe-pathogen interactions and develop integrated management strategies for this disease.

6.0.2 Introduction

Xylellafastidiosa has been a constant plague to California vineyards since the 1880s. It is estimated that this disease costs the $6.25 billion California grape industry an average of $104 million per year(Tumber *et al.*, 2014). This pathogen is vectored mainly by xylem feeding insects such sharpshooters in the *Cicadellidae* family, such as *Draculacephala minerva* (green sharpshooter) and *Graphocephala atropunctata* (blue-green sharpshooter). However, the introduction of the invasive species, *Homalodisca vitripennis* (glassy-winged sharpshooter (GWSS), has been associated with the more recent disease outbreaks (Janse & Obradovic, 2010). This Gram-negative, xylem-limited bacterium has been found in over 63 plant families but has only been known to cause disease in a number of plants, most notably, citrus, olive, alfalfa, peach, plum, almond, elm, and coffee(Chatterjee *et al.*, 2008; Rapicavoli *et al.*, 2017).

After inoculation into the plant, the pathogen will systematically colonize the nutrient poor xylem environment throughout the vine(Sun *et al.*, 2011). Many vessels of the vine can host small or no quantities of the pathogen making it hard to quantify(Sun *et al.*, 2013). The pathogen may also form a biofilm, adhering to the walls of the xylem. In field

settings, symptoms progress slowly over a season and often resembles water deprivation but with distinct characteristics such as matchstick petioles, uneven maturation, leaf scorching, berry raisening, stunted growth and eventually death. However, not all *Vitis vinifera* cultivars are equally susceptible to the disease and a combination of the plant defense response, xylem anatomy, inherent microbiota and pathogen virulence factors play an important role in disease outcome (E. Deyett *et al.*, 2019; Ingel *et al.*, 2019; Sun *et al.*, 2013).

Pierce's Disease (PD) management strategies can be grouped into two types, therapeutic and prophylactic. The former focuses on curing a vine that already has PD and uses tools like antagonistic bacteria and antibacterial substances that target the pathogen(Kyrkou *et al.*, 2018). The latter strategy is to prevent the plant from contracting the disease. Insecticide application is the most widely used prophylactic management strategy in vineyards (Daugherty *et al.*, 2015). However, these tactics can be harmful, expensive and are often off-target (Pathak *et al.*, 2017; Sarigiannis *et al.*, 2013/8). Other management strategies include breeding programs where resistant cultivars are bred into *V. vinifera* lines (Krivanek & Walker, 2005). A third strategy is to use microbes to prime or prevent pathogenesis and has been increasing in popularity over recent years(Baccari *et al.*, 2019; Philippe Rolshausen Caroline Roper, 2017).

The innate microbiome is a potential pool of viable biocontrol candidates for the suppression of plant diseases and adds an additional layer of complexity to the host-pathogen dynamic that has yet to be fully understood. Microbes are associated with the plant and may aid in the protection of the plant or in its demise. Microbes within the plant environment can trigger induced systemic resistance (ISR), a phenomenon known as priming, and

as a result the plant is more resilient to abiotic and biotic factors(Aziz *et al.*, 2015; Santoyo *et al.*, 2016). In addition, microbes may directly interact with each other, indirectly protecting the host, through a variety of mechanisms including antimicrobial metabolites production, nutrient/niche exclusion or quorum quenching activities (Brader *et al.*, 2014; Hartmann & Schikora, 2012; Sarrocco & Vannacci, 2017). One example of nutrient exclusion often seen in biocontrol agents (BCA) is the production of siderophores. Siderophores are small iron-chelating compounds released from microbes to transport iron across cell membranes and their production is important for the biosynthesis, pathogenesis, motility and biofilm formation(Saha *et al.*, 2016)(Silva-Stenico *et al.*, 2005). Siderophores are often thought to deplete the environment of iron and suppressing pathogen population due to lack of this essential nutrient. In nutrient poor environments, such as the xylem vessels, these exclusion events may pose a greater threat to other inhabitants than direct antagonism.

Biocontrol agents such as *P. phytofirmans*, and avirulent strains of X. *fastidiosa* have been tested to mitigate Pierce's Disease but with varying degrees of success(Baccari *et al.*, 2019; Hao *et al.*, 2017). Deyett *et al.* also found that *Pseudomonas spp.* and *Achromobacter spp.* were commonly found in the grapevine endosphere including the xylem lumen where *X. fastidiosa* resides. They were shown to negatively correlate with pathogen abundance through observational amplicon metagenomics of field vines (Deyett *et al.*, 2017). In this study, four native microbes, *Pseudomonas fluorescens*, *Pseudomonas viridiflava*, *Pseudomonas putida* and *Achorombacter xylosoxidans* were assessed for their ability to mitigate Pierce's disease. We conducted *in vitro* assays to elucidate potential mechanisms of the biocontrols that may cause disease suppression. We used *in planta* bioassays to

validate our hypothesis and assessed disease as a measure of both symptom severity and pathogen titer.

6.0.3 Materials and Methods

Identification of Biocontrols

Four bacterial strains were assessed in this study, *Pseudomonas fluorescens*, *Pseudomonas viridiflava*, *Pseudomonas putida*, and *Achromobacter xylosoxidans*. Each strain was previously isolated from a grapevine host and chosen based on negative correlation to the pathogen (Deyett *et al.*, 2017) . For all assays, *Pseudomonas spp.* were grown on Tryptic Soy Agar (TSA) for 24 h at 28°C . *Achromobacter* was grown on Potato Dextrose Agar (PDA) for 48 h at room temperature. *Xylellafastidiosa* subsp. *fastidiosa* strain Temecula 1 was grown on PD3 media for 5 days at 28°C . All plates were assessed for monoclonal growth and showed no signs of contamination.

To verify species identification, Sanger sequencing was performed. First DNA was extracted from overnight grown cultures streaked for isolation using the Qiagen Blood and Tissue kit per manufacturer's protocol. DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) 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). To differentiate between strains of *Pseudomonas* spp. additional primers rpoDf (5'-ACTTCCCTGGCACGGTTGACCA-3') and rpoDr (5'TCGACATGCGACGGTTGATGTC-3')(Mulet *et al.*, 2009) as well as primer pair 16SPSEfluF (5'TGCATTCAAAACTGACTG-3') and 16SPSER

(5'-AATCACACCGTGGTAACCG-3') were utilized(Scarpellini *et al.*, 2004). All PCRs were performed in a 25 μl volume with the following ingredients: 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. For 16S PCR the following thermocycling run was performed: 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 . The same parameters were used for the 16SPSEfluF/16SPSER PCR but the annealing temperature was reduced to 52°C . For rpoD PCR the following thermocycling run was performed: 94°C for 5 min; 35 cycles of 94°C for 30 s, 60°C 30 s, 72°C for 60 s; followed by a final elongation step for 10 min at 72°C .

Amplificons were visualized by electrophoresis at 110 V in 1% agarose gel with gel red in 1x TBE buffer. Product was then cleaned using the Qiagen QIAquick PCR Purification kit and quantified as previously described. PCR products were then Sanger sequenced at the UCR genomics core. Sequences were checked for quality before using NCBI BLAST nucleotide database to obtain identifications.

Growth Curve Analysis

Each of the bacterial strains were grown overnight in 2 ml of their respective media and incubated for 16-18 h in a table top shaker. Cultures were normalized to an OD_600 of 0.1 and 150 μL of starting 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 replicates and the plate was incubated at 28 °C.

For *X. fastidiosa*, wild type (WT) *X. fastidiosa* was prepared by plating onto PD3 solid medium and incubating at 28 °C for 5 days and harvesting cells from plates using sterile 1x PBS buffer. The OD_600 was brought to 0.25 (108 CFU/ml). 4.5 mLs of PD3 and TSA were inoculated with 0.5 mL of inoculum at an OD_600 of 0.25, bringing the final OD_600 of each tube to 0.025. Cultures were grown in a shaker incubator at 28 °C for 7 days. OD_600 measurements were taken every 24 hours for 6 days.

in planta assessment of biocontrol efficacy to PD

For all *in planta* assays, *V. vinifera* cv. Cabernet Sauvignon selection #31 was obtained from the Foundation Plant Service at UC Davis. One-bud cuttings were rooted and potted using UCR soil mix and amended with 5 g of controlled release fertilizer (Scotts Osmocote Classic, N-P-K: 14-14-14). Vines were trained vertically onto a stake and allowed to mature prior to bacterial inoculations.

Inoculations were prepared as follows: Cells were scraped from the plates and suspended in sterile 1x PBS and adjusted to an OD_600 of 0.25. For each trial 10 plants were inoculated with one of 4 treatments: *Pseudomonas putida* (PP), *Pseudomonas viridiflava* (PV), *Pseudomonas fluorescens* (PF), *Achoromobacter xylosoxidans* (ACH), or 1X PBS, as the control. Plants were inoculated with 10 μ l of biocontrol or 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). *Xylellafastidiosa* suspension (10 μ l) was needle-inoculated into the plant between the second and third node (above the biocontrol inoculant) at 8 hours and 7 days post BCA inoculation. Ten plants were used for each treatment; thus for 5 treatments at two different time points a 100 plants were utilized. Plants were grown

under greenhouse conditions with the same watering regime for 14 weeks. Visual PD severity symptom ratings (0-5 (Deyett *et al.*, 2019)) were taken for the last 5 weeks with sample collection occurring at week 12. Briefly, vines rated as 0 exhibited no symptoms while a 5 indicated a dead or dying vine.

Detection and quantification of X. *fastidiosa in planta*

Detection and quantification of X. *fastidiosa* was determined through the method described by (Deyett *et al.*, 2019). Briefly, three petioles were harvested from the point of inoculation (POI) and at one and two nodes above the POI and pooled. Petioles were frozen, lyophilized for 36 h with a FreeZone 2.5 L benchtop freeze dry system (Labconco) and ground to a powder at room temperature using a MM300 grinder (Retsch; 45 s 25 oscillations per second) in a 35 mL stainless-steel grinding jar (Retsch) with 20 mm stainless steel balls. DNA was extracted from 100 mg of ground petiole tissue using a DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's instructions with the substitution of hexadecyltrimethylammonium bromide (CTAB; Sigma-Aldrich) as the lysis buffer. DNA was stored at -20°C until further processing and quantified using a Qubit 2.0 fluorometer (Invitrogen) according to the manufacturer's protocol.

Xylellafastidiosa ITS primers (XfITSF6: 5'-GAGTATGGTGAATATAATTGTC-3'and XfITSR6: 5'-CAACATAAACCCAAACCTAT-3') were utilized to quantify pathogen abundance in each sample. qPCR was performed in a CFX96 Real-Time PCR cycler and 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), 0.4 μ M of each primer, and 2 μL of DNA template. The

cycling programme 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 from 65 to 95 °C (0.5 °C increments every 5 s) to verify the amplicon's identity and no off target amplification. Each qPCR plate was accompanied with both a negative control to avoid contamination and a standard curve consisting of known quantities of *Xylellafastidiosa* DNA procured from a monoclonal growth from a plate. Each sample was tested in triplicate and the average amount of *Xylellafastidiosa* was used and standardized to the amount of total DNA input. These quantities were used for further statistical analysis.

Enhancement Assay

Inoculum of WT *X. fastidiosa* was prepared by plating onto PD3 solid medium and incubating at 28°C for 5 days. Cells were harvested from the plate and normalized to an OD_600 of 0.25 (108 CFU/ml). Inocula of *A. xylosoxidans*,*P. fluorescens*,*P. putida*, and*P. viridiflava* were prepared by plating onto TSA solid medium and incubating at 28°C for 3 days. Following incubation, one loopful of each strain was plated onto TSA solid medium plates in 4 cm streak. The 30 μL of WT *X. fastidiosa* inoculum was drip plated 1.5 cm away from the other endophytic microbes. After incubating for 21 days, *X. fastidiosa* CFUs were recorded.

Antagonism Assay

To assess antimicrobial competition between microbes the deferred growth inhibition assay was adapted from Moran *et. al* (Moran *et al.*, 2016). Briefly, 25-μl of overnight cultures of each treatment was placed in the center PD3 agar plates and allowed to dry

completely before incubating at 28°C for 24h. WT *X. fastidiosa* was grown in liquid PD3 media for 5 days at 28°C before being transferred to a sterile spray bottle. Approximately 250 μl (3 sprays) of*X. fastidiosa* was sprayed at approximately 15 cm above each of the previously mentioned biocontrol inoculated PD3 agar plates. Plates were allowed to incubate for 5 days at 28°C before a zone of inhibition (ZOI) was measured. This assay was performed for each treatment against the pathogen in triplicate and repeated three times.

Statistical Analysis

All statistical analysis and graph visualizations were done using R v. 3.6.1 (http://www.R-project.org/). Differences in Pierce's Disease 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 biocontrols and time periods. P-values were adjusted with 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 biocontrols and analysis was adapted from Schandry, 2017(Schandry, 2017).

6.0.4 Results

Growth curve analysis showed all four endophytes reached stationary phase within 24 hours with *Pseudomonas putida* having the steepest exponential phase. All endophytes were normalized to an OD_600 of 0.25 before being inoculated into plants, which is within the exponential phase of all four endophytes (Figure 6.1A). *X. fastidiosa* growth curves indicate slow and limited growth with general media TSA and proficient growth in preferred media

PD3 reaching stationary phase around day 5 (Figure 6.1B).

The four biocontrol candidates were tested for their ability to provide the vine protection from X. *fastidiosa in planta* . Vines inoculated with just biocontrols did not exhibit any plant health decline or Pierce's Disease symptoms (data not shown). However, *V. vinifera* vines inoculated with X. *fastidiosa* did show typical disease symptoms of Pierce's Disease including scorched leaves, matchstick petioles, and uneven periderm maturation. Vines were rated on a scale 0-5 as described by Deyett *et. al.* (Deyett *et al.*, 2019). A visual rating of 3 was considered a critical point in terms of disease symptoms beyond which it was difficult to accurately measure *X. fastidiosa* titer within the petiole(Deyett *et al.*, 2019). Ten weeks after initial inoculations, the first vines (n=2) reached this fatal disease rating. By week eleven, twelve, thirteen, 22%, 47% and 60% of plants had passed this threshold respectively (Figure 6.2). At the time of harvest (12 weeks post inoculation), all control and *P. fluorescens* treatments regardless of time between inoculations and plants with pathogen inoculated one day after treatment of *A. xylosidians* had a greater than 50% mortality rate. *P. putida* plants inoculated 7 days prior to pathogen also exhibited over 50% mortality whereas *P. putida* treated plants inoculated 1 day prior to pathogen exhibited a less than 50% mortality; however at the end of week 14, both *P. putida* treated plants (1 day and 7 day prior to pathogen inoculations) had a 50% or greater mortality rate. Vines treated with*P. viridiflava* 1 day prior to pathogen inoculations had a low mortality rate at week 12 but by the end of the experiment mortality rates had risen by 50%. Plants treated with either *A. xylosoxidans* or *P. viridiflava* 7 days prior to pathogen introduction exhibited a less than 50% mortality throughout the experiment (Figure 6.3A &B).

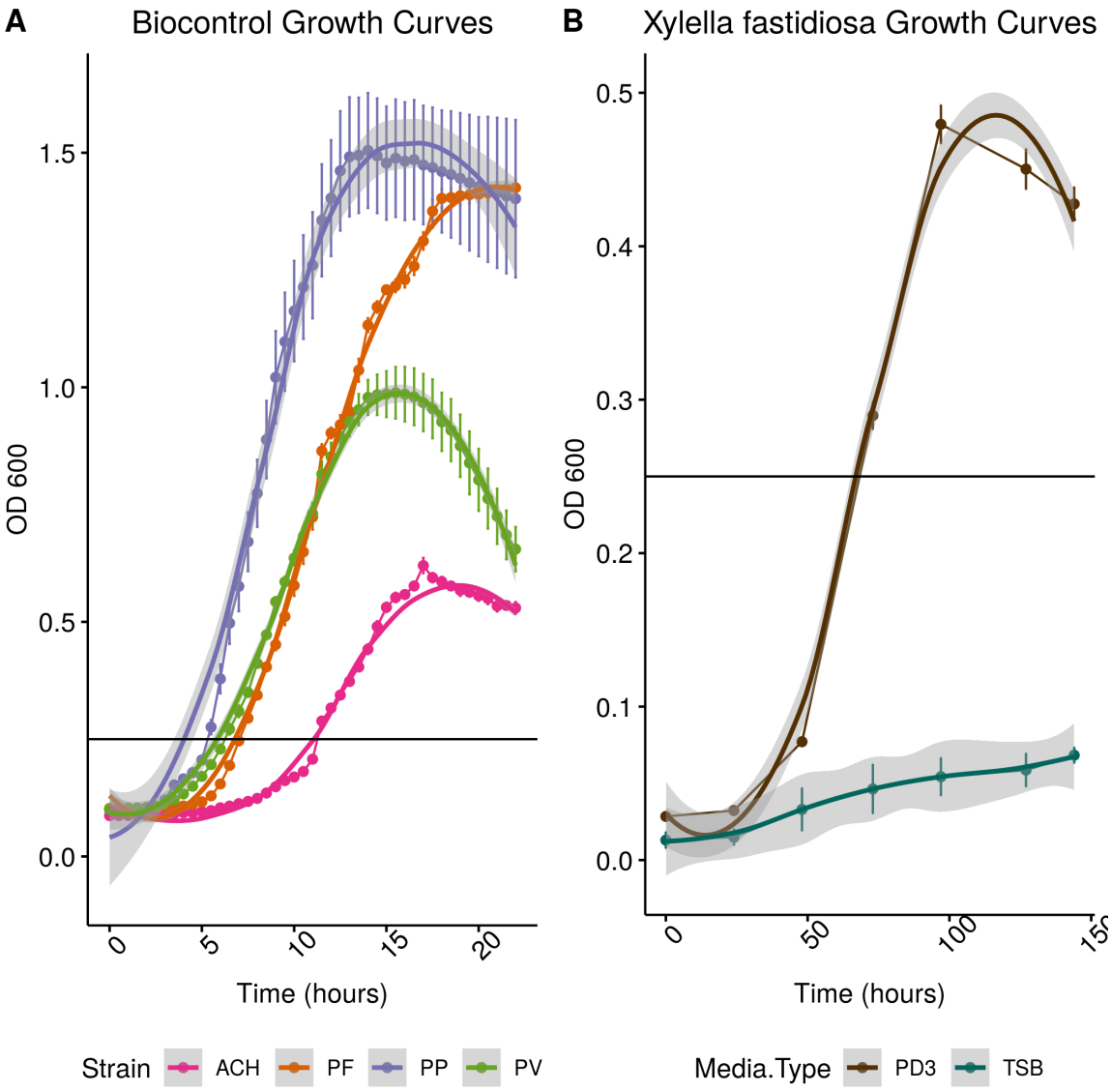

Figure 6.1: Growth curve analysis for (A) the four tested grapevine endophytes and (B) *X. fastidiosa* grown on preferred media (PD3) and general media (TSA). Gray horizontal lines indicate the OD_600 all microbes were normalized to before plant inoculations. Error bars represent standard error.

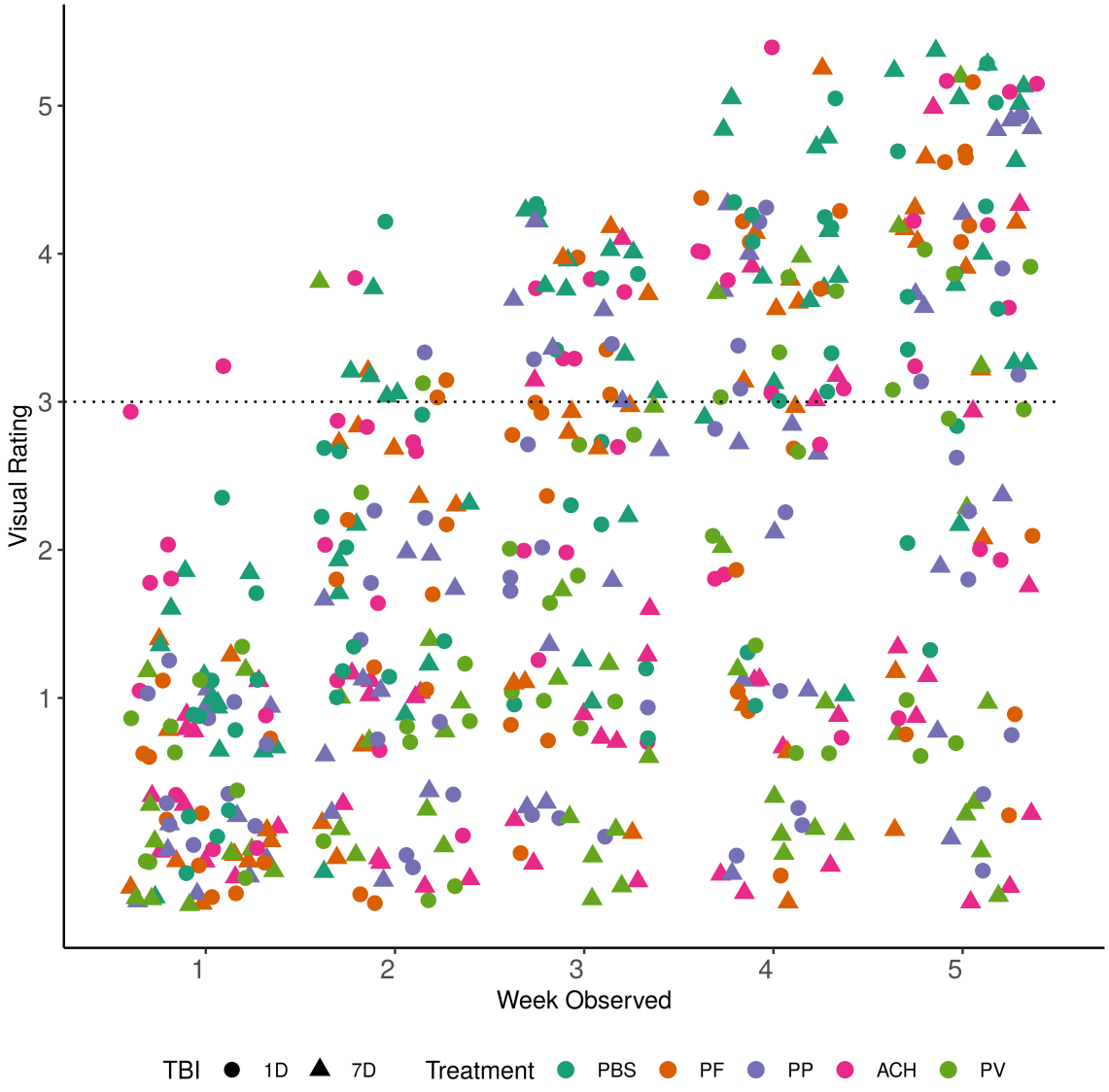

Figure 6.2: Scatter plot depicting the distribution of visual ratings across the last 5 weeks of the experiment. Points represent a single vine which are colored by treatment. Shapes represent time between endophyte and pathogen inoculation. Dotted line represents the fatal threshold cut-off used throughout visual rating analysis.

PD ratings were significantly different between treatments [Kruskall-Wallis, P<0.0001] and time between inoculations (TBI) [Kruskall-Wallis, P<0.05]. At the time of harvest, vines treated with BCAs 1 day prior to being challenged with the pathogen did not show significant differences of symptoms [Kruskall-Wallis], however; plants incubated with biocontrols for 7 days prior to pathogen introduction showed significant differences [Kruskall-Wallis, P<0.01]. Specifically, plants inoculated with *P. viridiflava* showed significantly lower disease rating than *P. fluorescens* and control [Wilcox, P<0.05]. *A. xyloxidians* also had lower observational symptoms than control [Wilcox, P<0.05] (Figure 6.3C).

No significant difference in pathogen titre was observed at this time [Kruskall-Wallis]. However, similar to our visual ratings, *P. viridiflava* and *A. xyloxidians* treated vines that were challenged 7 days after with *X. fastidiosa* had the lowest overall mean of pathogen titre compared to other treatments. *P. fluorescens* performed the worst with several plants having a higher pathogen titre then the control (Figure 6.4). Timing between biocontrol and pathogen inoculations did show significant differences [Kruskal-Wallis, P<0.001], with 7 day separation between inoculations being significantly lower than 1 day between inoculations (Figure 6.4 &5). Pathogen populations appear to grow nearly linearly until visual rating of 3 at which point population declines (Figure 6.5).

Survival analysis was conducted to better visualize the survival rate of each treatment over time. Control plants and plants treated with*P. fluorescens* had a steep and steady decline at both TBI. *A. xylosidans* and *P. putida* treated plants challenged with *X. fastidiosa* one day after treatments followed a similar stepwise pattern as the control. Interestingly, plants inoculated with *P. putida* and challenged with *X. fastidiosa* 7 days

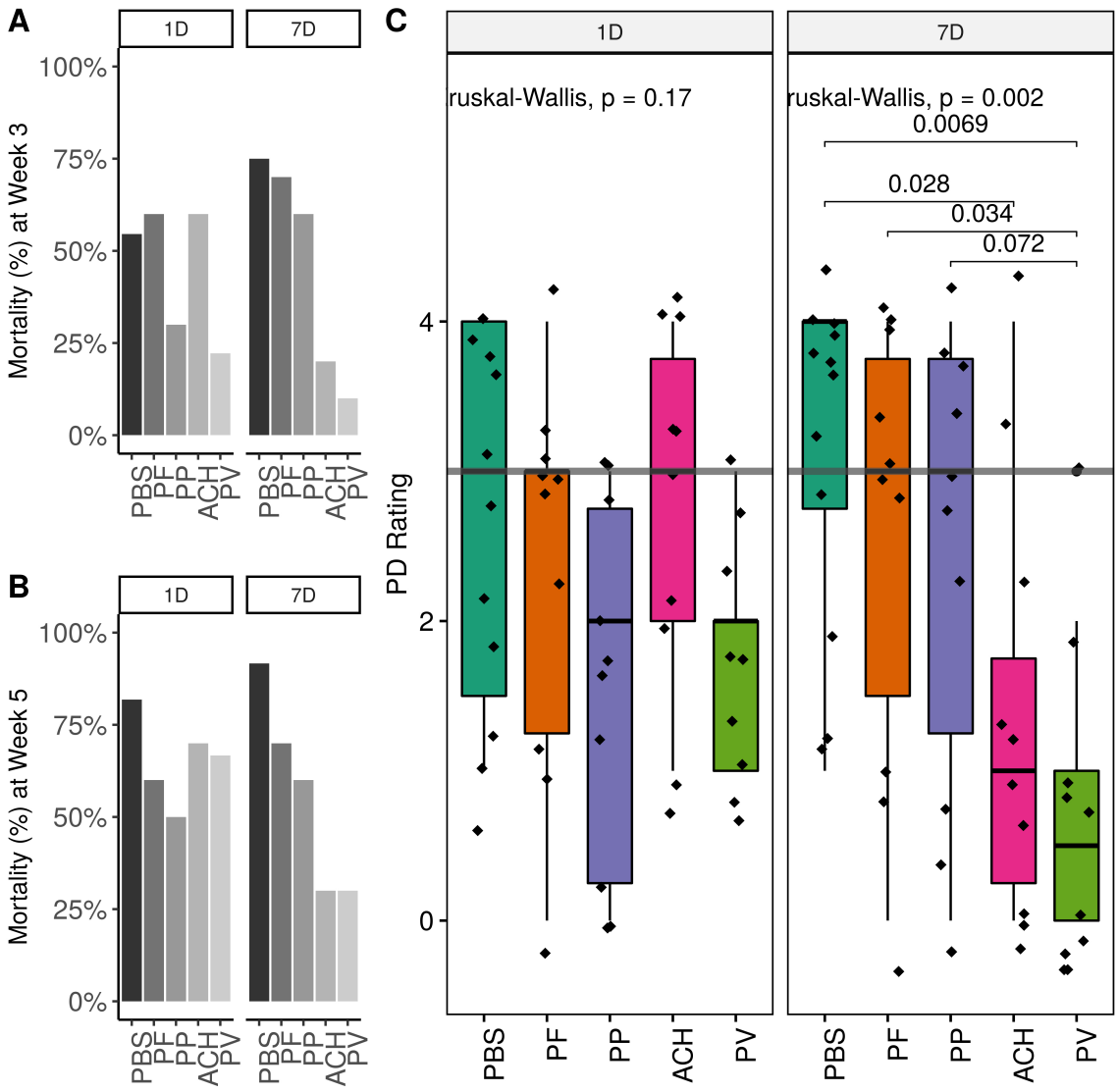

Figure 6.3: Incidence of vines for individual treatments which passed the fatal threshold (PD rating =3) (A) at the time of harvest and (B) at the end of the experiment. (C) Disease rating for individual ednophyte treatments 12 weeks post-inoculation. PBS, 1x phosphate buffer saline; PF, *Pseudomonas fluorescens*; PP, *Pseudomonas putida*; PV, *Pseudomonas viridiflava*; ACH, *Achromobacter xylosoxidans*; 1D, one day between endophyte and pathogen inoculations; 7D, seven days between endophyte and pathogen inoculations. Gray line represents fatal threshold point.

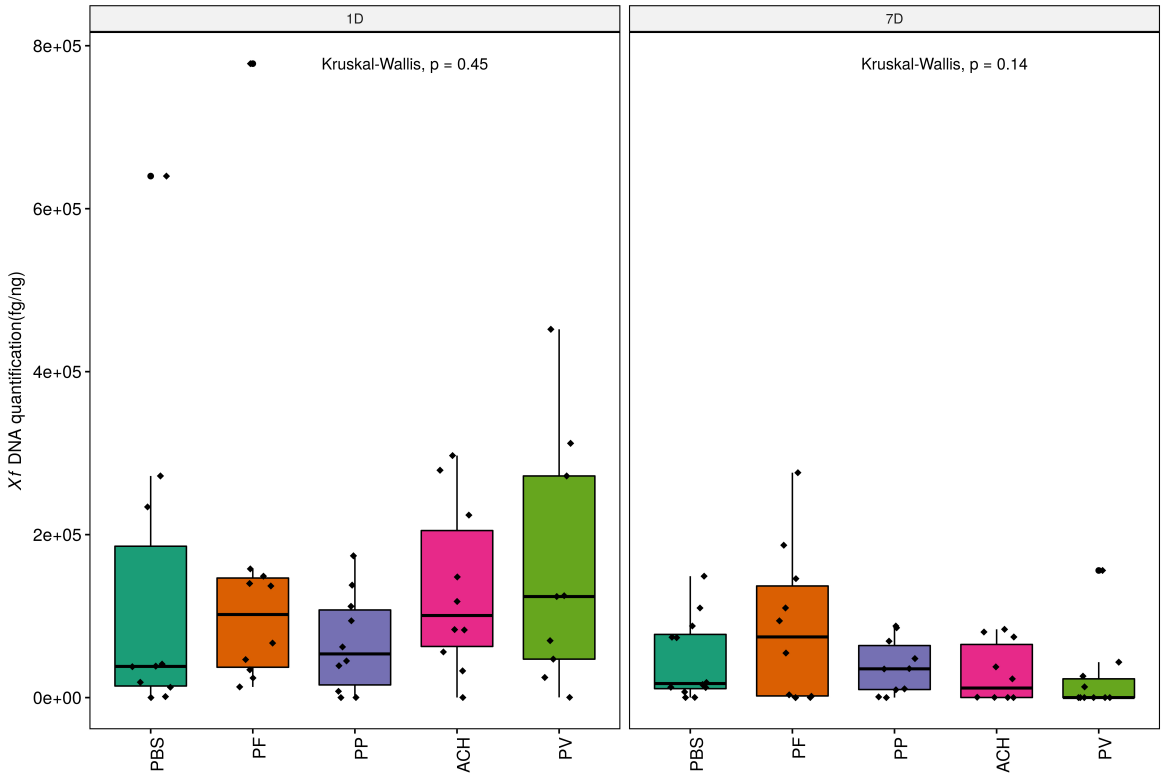

Figure 6.4: *X. fastidiosa* quantifications (*X. fastidiosa* fg DNA per ng of plant DNA) in petioles of individual treatments 12 weeks post-inoculations. PBS, 1x phosphate buffer saline; PF, *Pseudomonas fluorescens*; PP, *Pseudomonas putida*; PV, *Pseudomonas viridiflava*; ACH, *Achromobacter xylosoxidans*; 1D, one day between endophyte and pathogen inoculations; 7D, seven days between endophyte and pathogen inoculations.

after saw a drastic drop in survival rates the week of petiole collection. Plants inoculated with either *A. xylosidians* or *P. viridiflava* and challenged with *X. fastidiosa* 7 days after treatments had the best rate of survival (Figure 6.6). A lognormal distribution was used to create survival regression lines. Pairwise comparisons of survival regression showed at the one day time between inoculations, *P. putida* and*P. viridiflava* treated vines had a significantly higher rate of survival compared to control plants [Tukey, $P<0.01$]. *A. xylosidans* had a significantly lower rate compared to all other biocontrols tested [Tukey, $P<0.01$]. At the seven days between inoculations, all biocontrols accept *P. fluorescens* performed better than the control [Tukey, $P<0.05$] and other treatment had higher survival rates when compared to *P. fluorescens* [Tukey, $P<0.05$]. *A. xylosidans* and *P. viridiflava* treated plants survived significantly better than *P. putida* treated plants [Tukey, $P<0.01$]. No significant differences were observed between *A. xylosidans* and*P. viridiflava* treated plants with 7 days between inoculations.

To better understand microbe-pathogen interactions and potential antagonistic behavior two *in vitro* assays were conducted (Figure 6.7). The first assessed the ability of the BCAs to inhibit growth of *X. fastidiosa* by measuring zones of inhibition (ZOI) which were significantly different among BCAs (Figure 6.8; [Kruskal-Wallis, $p<0.0001$]). *P. fluorescens* exhibits complete inhibition, with no colonies of *X. fastidiosa* observed and had significantly greater zones of inhibitions compared to *A. xylosidans* and*P. viridiflava* [Wilcox, $p<0.01$]. *P. putida* had a significantly greater zone of inhibition compared to *A. xylosidans* and *P. viridiflava* [Wilcox, $p<0.05$]. *A. xylosidans* had nearly no zone of inhibition and was significantly less than *P. viridiflava* [Wilcox, $p<0.05$] (Figure 6.7A&B). The four microbes

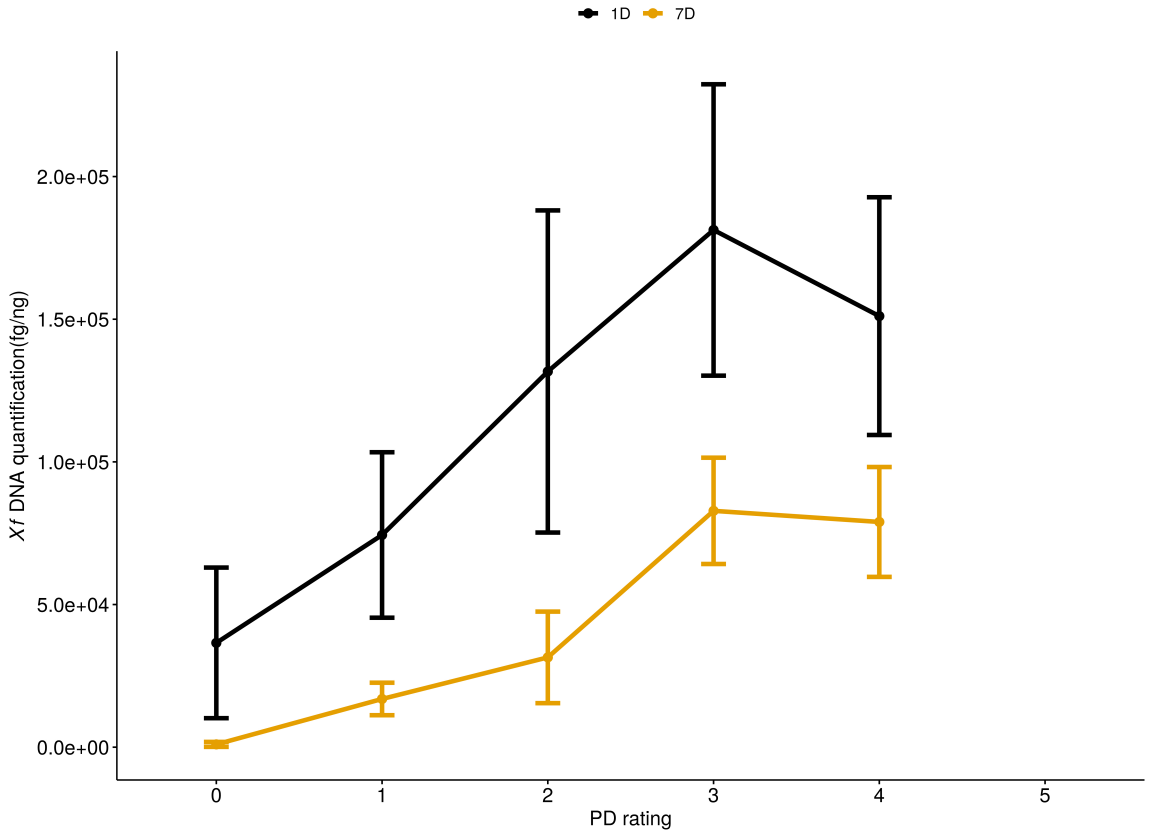

Figure 6.5: Relationship between Pierce's Disease rating and *Xylella fastidiosa* DNA quantification (fg *Xylella fastidiosa* DNA per ng of plant DNA. Bars represent standard errors.

were also co-inoculated with *X. fastidiosa* on TSA. *X. fastidiosa* was unable to form a biofilm on TSA media when inoculated by itself or co-inoculated with *A. xylosoxidans*, *P. fluorescens*, or *P. putida*. Interestingly, colonies of *X. fastidiosa* were observed when co-inoculated with *P. viridiflava* on TSA (Figure 6.7B). To assess the importance of iron as an essential element to growth of the pathogen, *X. fastidiosa* was grown on media containing iron concentrations equivalent to xylem sap (10 μm) and on iron enhanced media (100 μm) . *X. fastidiosa* was seen to grow more prolifically in higher iron concentrations (Figure 6.9).

6.0.5 Discussion

We have assessed four native grapevine microbes for their ability to mitigate Pierce's Disease *in planta* and have shown *A. xylosidans* and *P. viridiflava* have potential time dependent biocontrol qualities and confer a level of protection to *V. vinifera* from *X. fastidiosa*. *Pseudomonas spp.* and *Achromobacter spp.* are native grapevine inhabitants and have been found to persist within the microbiome across years and tissue types (Deyett, 2020). They have also been shown to live in the same niche as our pathogen and to be negatively correlated to *X. fastidiosa*(Deyett *et al.*, 2017). *in vitro* assays have shown it is unlikely the microbes showing the greatest amount of protection against Pierce's Disease are acting directly antagonistically to *X. fastidiosa* but rather their presence may be changing the behavior of the pathogen within the vine or priming the plant.

X. fastidiosa is an economically relevant pathogen and responsible for the deaths of several important crops every year. The pathogen resides within the nutrient poor

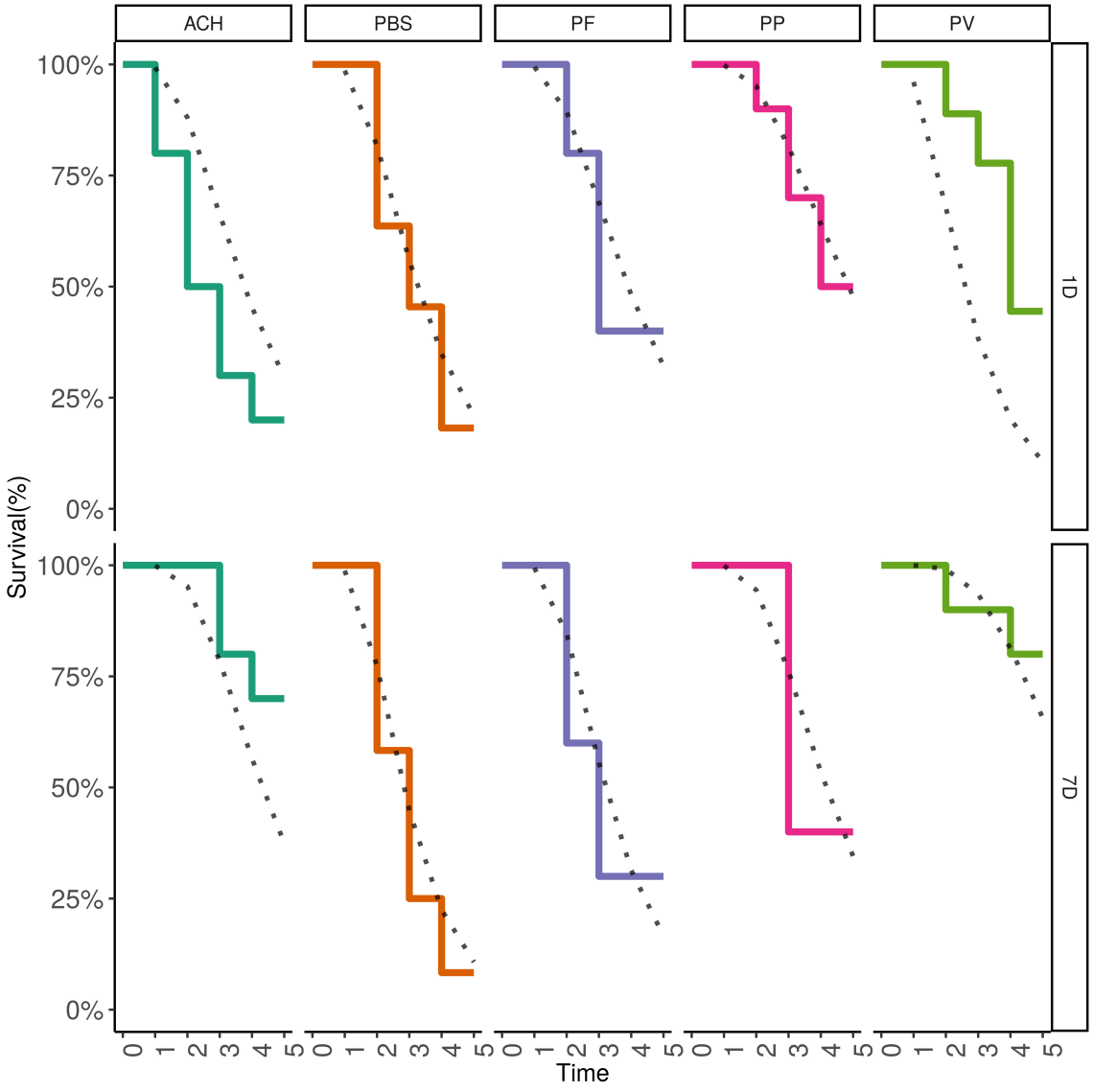

Figure 6.6: Survival estimate are shown in stepwise lines with different colors representing different treatments. Dotted lines represent survival regression fit to lognormal distribution across replicates. Survival analysis was calculated with death event defined as Pierce's Disease rating of three.

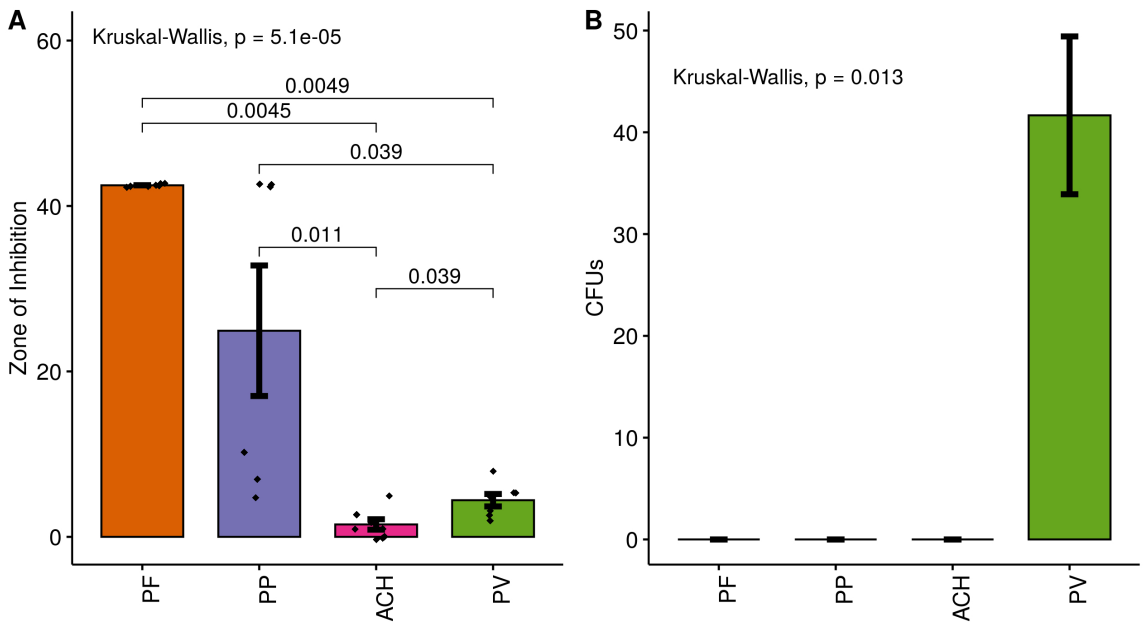

Figure 6.7: *In vitro* interaction of *X. fastidiosa* and the four grapevine endophytes tested. (A) Antagonistic assay depicts the zone of inhibition (mm) produced by each microbe against *X. fastidiosa*. (B) Number of *X. fastidiosa* colonies (CFUs) observed when pathogen was co-inoculated with the four endophytes on general media, TSA. Error bars represent standard error.

environment of the xylem. Iron is an essential element for life and is found in small quantities within the xylem environment usually less than 10 μm making this a limited resource and highly competitive nutrient for xylem dwelling microbes(de Macedo Lemos *et al.*, 2003). For example, *X. fastidiosa* was observed to grow 20% less on iron-free media and not at all on several general media types(Silva-Stenico *et al.*, 2005). Our results also show an increase in growth due to heightened iron concentration. Furthermore, *X. fastidiosa* genome analysis reveals the microbe has over 65 genes dedicated to iron metabolism and 5 iron membrane receptors but does not appear to be able to produce siderophores on its own(Pacheco *et al.*, 2006; Simpson *et al.*, 2000). Iron is implicated in several virulence factors released by X. *fastidiosa* including type IV pilus and bacteriocins. X. *fastidiosa*, has also been shown to be highly sensitive to a number of antibiotics and antimicrobial products (Kuzina *et al.*, 2006). Making direct antagonism to the pathogen either through nutrient/niche exclusion or through antimicrobial products a viable pathway to endophyte mitigation of pathogen population.

Pathogen titer correlated with disease symptoms severity until a disease rating of 3, after which pathogen populations began to decline as visual ratings progressed, a distribution also observed in other published Pierce's Diseases assessments (Deyett *et al.*, 2019; Gambetta *et al.*, 2007). The decline in pathogen population is likely due to the toxic environment in the host xylem (Ingel *et al.*, 2019; Sun *et al.*, 2013). However, the time between biocontrol inoculation and *X. fastidiosa* impacted the overall titre of the pathogen and the amount of protection the biocontrols gave to the plant. Timing between inoculation of biocontrols and pathogen has been shown to play a vital role in the ability to provide

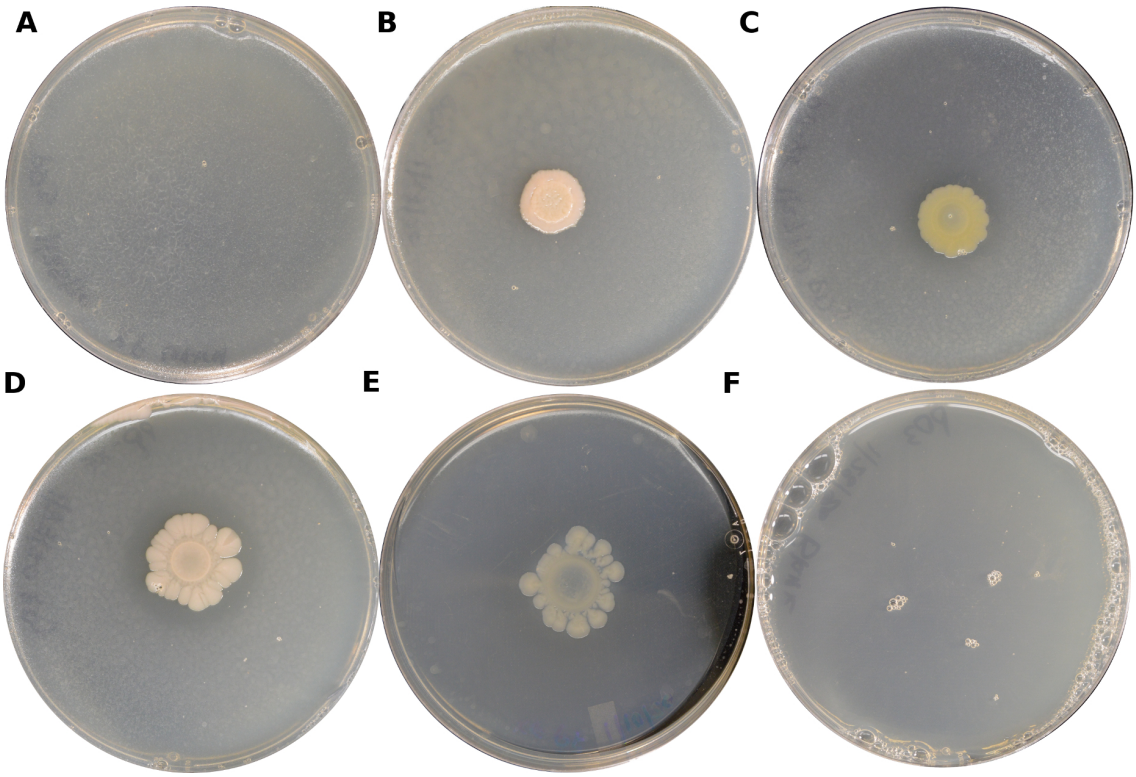

Figure 6.8: *X. fasitiodosa* inhibition assay. Images of antagonism assay plates where BCAs were innoculated in the center of plates and *X. fastidiosa* was sprayed across the plates. (A) *X. fastidiosa* spray, (B/C) *A. xylosoxidans* and *P. Viridiflava* shows no or little zone of inhibtion (ZOI) to *X. fastidiosa*, (D) *P. putida* shows limited inhibition, (E) *P. fluorescence* shows complete inhibition to *X. fastidiosa*, (F) sterility check of spray bottle before *X. fastidiosa* culture was added.

plant protection and has been assessed in several pathosystems (Chow *et al.*, 2018; Sugar & Basile, 2008). *Paraburkholderia phytofirmans* has recently been tested as a biological control for Pierce's Disease and found the timing of BCA introduction to be important for disease suppression(Baccari *et al.*, 2019). The 7-day incubation period of the biocontrols before challenged with the pathogen, likely allows the BCAs to establish residency within the vine improving their competitiveness and efficacy over the one day incubation period. Many mechanisms of defense such as ISR and nutrient/niche exclusion may require an adequate time for plant and microbes to interact, allowing for the production of salicylic acid, siderophores, and nutrient acquisition to induce resistance or deprive the pathogen of essential nutrients. Other modes of antagonistic behavior such as degradation of pathogen virulence factors, antibiotic production, or ability to out compete may rely less on timing of induction into the host and more on the direct interaction of microbe and pathogen (Latha *et al.*, 2019). Having only hours between biocontrol and pathogen introduction is likely inadequate for these host-microbe interactions to occur before pathogen takeover and may explain why *P. viridiflava* and *A. xylosidans* provided a greater level of protection when inoculated 7 days prior to *X. fastidiosa*. Understanding the interaction between the biocontrol, the pathogen and the host will help improve the timing of BCA inoculation to improve *X. fastidiosa* management.

Pseudomonas fluorescens has been heavily studied for its biocontrol abilities, harboring genes important for phytohormone production, priming (ISR) and production of antimicrobial substances (Latha *et al.*, 2019; Sahu *et al.*, 2018). It is a well known plant growth promoting rhizobacteria in a number of systems and thus is capable of penetrating

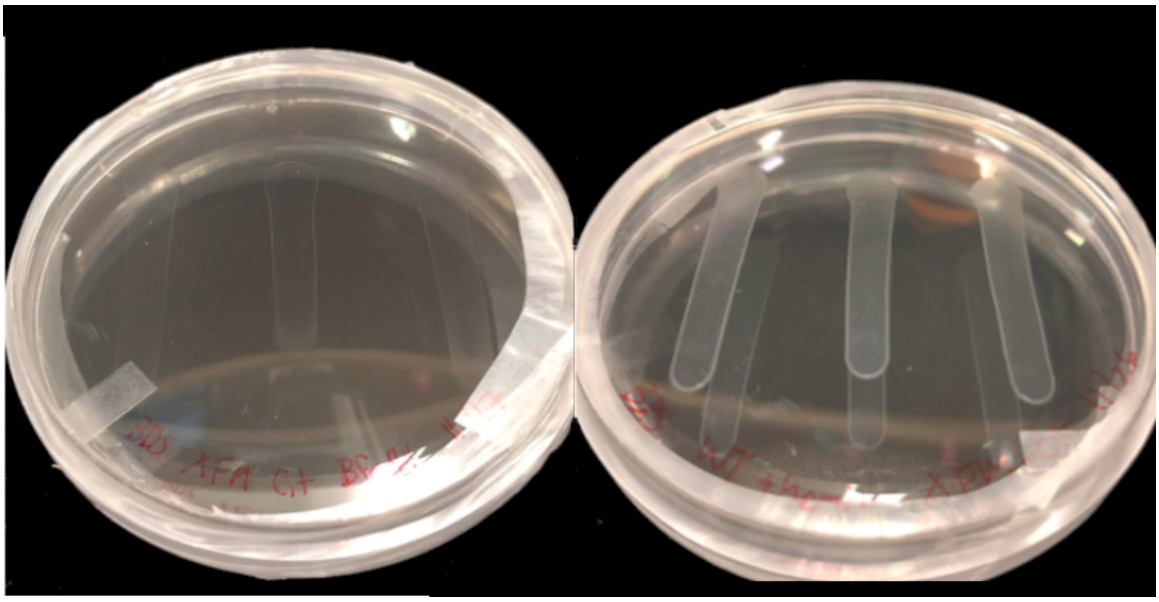

Figure 6.9: *X. fastidiosa* grown at iron concentration equivalent to xylem sap and at iron concentrations 10X sap equivalent

the roots and living competently as an endophyte making nutrient/niche exclusion another viable tactic of protection (Sahu *et al.*, 2018). Based on our antagonistic assay, it appears that our *P. fluorescens* and *P. putida* may be producing antimicrobials which kill the pathogen *in vitro*. *P. fluorescens* is known for creating a number of antimicrobial substances with the capability to suppress a number of phytopathogens including *Thielaviopsis basicola* and *Gaeumannomyces graminis* (Keel *et al.*, 1992). *in planta P. fluorescens* has been shown to delay symptoms in olive trees suffering from *Verticillium dahliae* likely not from antimicrobial production but from enhancing plant growth and induced systemic resistance(Mercado-Blanco *et al.*, 2004). Grauau *et al.* also concluded ISR to be the reason why *P. fluorescens* conferred protection to grapevine against *Botrytis cinerea* (Gruau *et al.*, 2015). However, *P. fluorescens* and *P. putida* did not mitigate disease symptoms, pathogen titre or prolong survival within our vines. Suggesting that although they have

the capabilities to suppress *X. fastidiosa in vitro*, colonize the plant and have been shown to induce systemic resistance, these qualities are either not triggered by our strain or are not sufficient to control the pathogen population *in planta* . The reason for this antibiosis breakdown is unknown but is not uncommon and emphasizes the importance for *in planta* experiments in both control and field settings (Kavroulakis *et al.*, 2010). Environmental factors, host-microbe interactions, metabolites and available resources may all play a role in the inability of these strains to provide protective antagonism to *X. fastidiosa in planta*.

The two best performing biocontrols, *Achromobacter xylosoxidans* and *Pseudomonas viridiflava* both produced lower pathogen titre and visual ratings of Pierce's Disease. Due to the *in vitro* results, it is unlikely either candidate was acting directly antagonistically against *X. fastidiosa in planta*. Strains from *Pseudomonas viridiflava* have been reported to have a number of plant growth promoting and pathogen suppression attributes including phosphate solubilization, indole acetic acid, hydrogen cyanide, ACC deaminase, quorum sensing and siderophore production(Samad *et al.*, 2017). Siderophores may act antagonistically and mitigate pathogen population through nutrient exclusion which is believed to be the case for *Trichoderma asperellum* control over *Fusarium* wilt in tomato (Segarra *et al.*, 2010). However, many microorganisms have lost their ability to produce siderophores and are unable to grow *in vitro* without the presence of the communities'siderophores (Lewis *et al.*, 2010; Saha *et al.*, 2016). *X.fastidiosa*, has been known to accumulate metals differently for its planktonic and biofilm states, particularly an increase in copper and manganese(Cobine *et al.*, 2013) and does not appear to produce its own siderophores. In addition, depriving *X. fastidiosa* of iron has shown inhibitory effect on pathogen biofilm formation(Toney &

Koh, 2006). In *X.fastidiosa* mutants unable to form biofilm, hyper virulence phenotypes were observed(Chatterjee *et al.*, 2008; Guilhabert & Kirkpatrick, 2005; Ingel *et al.*, 2019), suggesting *X. fastidiosa* produces biofilm to attenuate its own virulence, keeping the host alive longer. We believe *X. fastidiosa*'s ability to grow on TSA in the presence of *P. viridiflava* might be due to metabolite (possibly siderophore related) release by *P. viridiflava*. It is thus possible that *X. fastidiosa* is 'stealing'elements from the *P. viridiflava* to quickly build its biofilm, reducing its virulence and possibly allowing the vine's defense time to react to the pathogen's presence and produce targeted tylose release. *X. fastidiosa* would be limited to the lower portions of the vine, produce less cell wall degrading enzymes as well as other virulence factors and thus block fewer vessels. Petioles were collected from the bottom of the vines for qPCR analysis which did not show significant differences in pathogen population. In this scenario, *X. fastidiosa* would not necessarily have a lower pathogen population at the base of the vine but perhaps would not have systemic colonization in vines co-inoculated with *P. viridiflava*. Future work should further investigate this interaction by creating a mutant of *P. viridiflava* which is incapable of siderophore production to verify this as the mode of action. Transcriptomic analysis will also provide insight into the expression of defense genes and reveal if priming within the plant is occuring. Finally, future assays should assess the titre of pathogen and number of symptomatic leaves both at the point of inoculation and at the end of the vine to determine if systemic colonization of *X. fastidiosa* is occuring.

Previous research has noted the presence of *Achromobacter* within the grapevine and have alluded to its capabilities as a potential biocontrol against *X. fastidiosa* (Deyett *et*

al., 2017). *Achromobacter xylosidans* has been reported to produce inhibitory effects and is capable of producing antibiotics (Yan *et al.*, 2004). Here, we do not see any zone of clearing in our antagonism assay inferring there is no direct antimicrobial behavior *in vitro* against *X. fastidiosa*. Yet, we measured a significant suppression in disease severity in plants inoculated with *Achromobacter* seven days prior to being challenged by the pathogen. This rhizobacteria is known for stimulating plant growth through mineral uptake in particular nitrogen and nitrate(Bertrand *et al.*, 2000). In addition, *Achromobacter* had been evaluated for its biological control potential against *Fusarium* wilt of tomato and authors note iron competition to likely be the mechanism of protection (Moretti *et al.*, 2008). Unlike *P. viridiflava*, *X. fastidiosa* does not grow on TSA in the presence of *A. xylosidians*. It is thus unlikely, the mechanism of protection is the same as *P. viridiflava*, but could be limiting iron availability within the vine to a degree which limits the growth of *X. fastidiosa*. *Achromobacter* resides in the same order as *Paraburkholderia* which has also been evaluated for its ability to suppress *X. fastidiosa* in grapevine. Authors attribute this protective quality of *P. phytofirmans* PsJN to priming of the grapevine evidenced by the higher abundance of PR1 compared to control plants. They also note the ability of *P. phytofirmans* to rapidly replicate to a high population within the plant increasing its probability of detection and triggering plant defenses (Baccari *et al.*, 2019). Although, *A. xylosidans* exhibited the slowest growth rate of the BCAs it is still significantly faster then *X. fastidiosa* and could thus be outcompeting *Xylella* for nutrients and space while simultaneously triggering an immune response of the host. Although the mechanism of protection is not known at this time, future transcriptomic work can help better define this interaction and elucidate the

mechanism of protection.

Work is still in progress with these strains to better determine the specific mechanisms of interaction with *X. fastidiosa* and its impact on disease outcome. Full genome sequencing of BCAs combined with functional genomics and transcriptomics experiments will help further decipher the role that these microbes play and enhance our understanding of their biocontrol capabilities in order to develop new biotechnologies designed for commercial application.

6.0.6 Referecnes

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Chapter 7

Conclusions

In conclusion, this research represents the first comprehensive culture-independent microbial study of the grapevine and includes previously unexplored plant niches, sap and wood. This research has developed novel methods for detection of *X. fastidiosa*, assessment of Pierce's Disease *in planta* and processing pipeline for endophytic communities. We have defined a core microbiome as well as biomarkers for specific tissue types and have explored microbial changes in a commercial vineyard over time, tissue type and condition. Finally we have developed the foundation to new management strategies including new information on grapevine pedigrees susceptibility to Pierce's Disease and the potential for two endophytes of grapevine, *Pseudomonas* and *Achromobacter* to protect *in planta* against *X. fastidiosa*.

This was the first study to profile the microbial fingerprint of 6 different biocompartments (root, rhizosphere, soil, cane, cordon and sap) in an agriculturally relevant crop. Overall the bacteriome was more diverse than the mycobiome and belowground tissues had higher diversity than above ground tissues. Each niche harbors distinct taxonomic groups selectively adapted to the habitat. The roots serve as a bottleneck to microbial diversity, releasing resources to cultivate and enrich plant growth promoting microbes. In response,

microbes must be able to react to these resources and be highly versatile to live both within the ectosphere and endosphere of a plant. The grapevine microbiome was dominated by *Proteobacteria, Ascomycota* and to a lesser degree *Basidiomycota, Actinobacteria, Bacteroides, Verrucomicrobia,* and *Firmicutes*. Core genera consists of 6 bacteria (*Pseudomonas*, *Streptomyces, Bacillus, Novosphingobium, Steroidobacter, and Rhizobium*) and 2 fungi (*Cladosporium, Mycosphaerella*). Of these *Pseudomonas*, *Streptomyces, Novosphingobium, Rhizobium* and *Bacillus* are often praised for their plant growth promoting/stress relieving potential. Beside the roots, microbial assemblage could occur through horizontal transfer in a number of routes including via insect feeding, through open wounds or natural openings either in the commercial or nursery vineyards or during the propagation process in nurseries. Understanding microbial assemblage will help with managing diseases and promoting a healthy plant-microbiome.

The plant sap microbiome is a unique tissue previously unexplored by culture-independent methods. Our data shows the sap microbiome to cluster in between the 'above'and 'belowground'niches. Sap transports microbes including several vascular pathogens making it an interesting habitat to explore in the context of Pierce's Disease. The core sap microbiome consisted of seven bacterial genera (*Streptococcus, Massilia, Bacteroides, Micrococcus*, *Pseudomonas*, *Acinetobaceter* and *Bacillus*) and five fungal genera (M*ycosphaerella, Alternaria, Aureobasidium and Filobasidium*). Sap microbiome was affected by plant phenology and disease severity. Hence, in the growing season or when a vine expresses severe PD symptoms, microbial diversity is decreased likely due to unfavorable and/or toxic environments (dormancy, occlusion of vessels, fewer food resources). Bloom time points were

highly associated with spikes in plant growth promoting microbes such as *Streptomyces, Rhodotorula, Sphingobium, Novosphingobium*). Suggesting the plant may recruit beneficial microbes for key physiological processes. Moderately symptomatic vines harbored the highest quantities of *X. fastidiosa* and were also associated with *Methylobacterium*, known to be associated with the pathogen in citrus variegated chlorosis, another *X. fastidiosa* disease. Further research is needed to elucidate the nature of this interaction within the two pathosystems. Although sap was extracted from the cane, the two tissue types harbor distinct microbial fingerprints. The largest difference being a higher abundance of *Pseudomonas* in the cane compared to the sap. Through microbiome analysis of the cane, *Pseudomonas* and *Achromobacter* were found to be negatively correlated to the pathogen and thus may suppress pathogen population or heighten the plant's response to an attack. These results have also shown *X. fastidiosa* to be an elusive endophyte, which does not ubiquitously colonize the host, making it difficult to identify through 16S microbial profiling. The microbiome results show the highly dynamic association between the plant and its microbes.

To better understand host susceptibility to this pathogen an *in planta* greenhouse experiment including four V. vinifera cultivars and one resistant variety, *V. arizonica/candidans*, was conducted. This project created novel qPCR primers for the detection of *X. fastidiosa* in greenhouse settings. This assay is relatively quick, specific and sensitive, producing reliable and reproducible quantification method for *X. fastidiosa* in greenhouse grown vines. Physical disease ratings, pathogen titre and stem hydraulic conductivity were all utilized to assess susceptibility. These measurements were all highly correlated par-

ticularly stem hydraulic conductivity and disease ratings. Pathogen titre dropped after disease ratings of three, likely due to toxic environments; however, disease symptoms still progressed as water was unable to be transported due to occlusion and tylose formations. Thompson seedless was the most susceptible variety while Merlot was the least susceptible. This cultivar had the greatest xylem vessel diameter which may influence vessel occlusion, tylose abundance, conductivity and overall susceptibility to Pierce's Disease. This Pierce's Disease susceptibility knowledge on different *Vitis vinifera* pedigrees could help breeding programs to select genetic material that harbors higher disease tolerant traits, and provide recommendation to growers with regards to planting PD-tolerant varieties in areas under disease pressure.

Equipped with a reliable pathogen quantification method and an established plant bioassay we were able to test in vitro and *in planta* a handful of beneficial endophytic microbes for their ability to mitigate disease. *P. viridflava* and *A. xylosidians* proved to be the best candidates; however, the mechanism is unknown at this stage. It does appear that neither microbe is acting directly antagonistically to the pathogen through the production of antimicrobials. However, the two microbes tested that did not confer protection *in planta*, *P. fluorescens* and *P. putida*, were capable of producing antimicrobials and acting antagonistically to the pathogen in vitro. *X. fastidiosa* growth and biofilm formation appear to increase in the presence of *V. viridiflava* likely from the pathogens ability to 'steal'siderophores from other microbes, acquiring more iron and thus attenuating its own virulence.

This project looked at one of the several currently available 'omic'techniques: amplicon-based metagenomics. These techniques, including metatranscriptomics, metabolomics and metaproteomics, supply intensive surveys into the composition of the natural world, helping with the selection of candidates for probiotics in agriculture. Future work should attempt to integrate these omic techniques to better understand plant-microbe interactions and discover new disease management strategies. Metatranscriptomics can reveal how the host or microbe is perceiving the other through identifying what is being translated during a particular time. Metaproteomics and metabolomics give a complete profile of what is being transferred/communicated between host and microbes. Taken together these Omic techniques not only identify a pool of candidate microbes for probiotic discovery but help reveal the mechanism behind the symbiosis, be it mitigating disease or plant growth promotion. Applying these techniques in the greenhouse assay will help determine the mechanism(s) of protection conferred by *A. xylosidians*, and *P. viridiflava* and could convey the reason why *P. fluorescens* and *P. putida* inhibit the growth of *X. fastidiosa* in vitro but do not appear to provide protection *in planta*.

This research provides fundamental knowledge of the grapevine microbiome through a variety of variables and assesses the susceptibility of *V. vinifera* pedigrees to *X. fastidiosa*. It establishes new tools for pathogen detection and the foundation of novel management strategies such as deploying novel biocontrols and planting/breeding PD-tolerant cultivars. These methods can be extended to other crop systems, given the host range of the bacteria. Coupling 'omic'technologies with *in vitro* and *in planta* assays will propel this field from its current descriptive stage to assessing function and a higher level understanding of

plant-microbiome interactions. Microbial solutions have the potential to fuel bioengineering projects that could result in novel technological bioproducts for commercial applications which would not select for resistance or cause environmental harm.