

**INTERIM PROGRESS REPORT FOR CDFA AGREEMENT NUMBER 16-0510-SA:
CHARACTERIZATION OF *XYLELLA FASTIDIOSA* PLANT CELL WALL DEGRADATION AND
INHIBITION OF THE TYPE II SECRETION MACHINERY**

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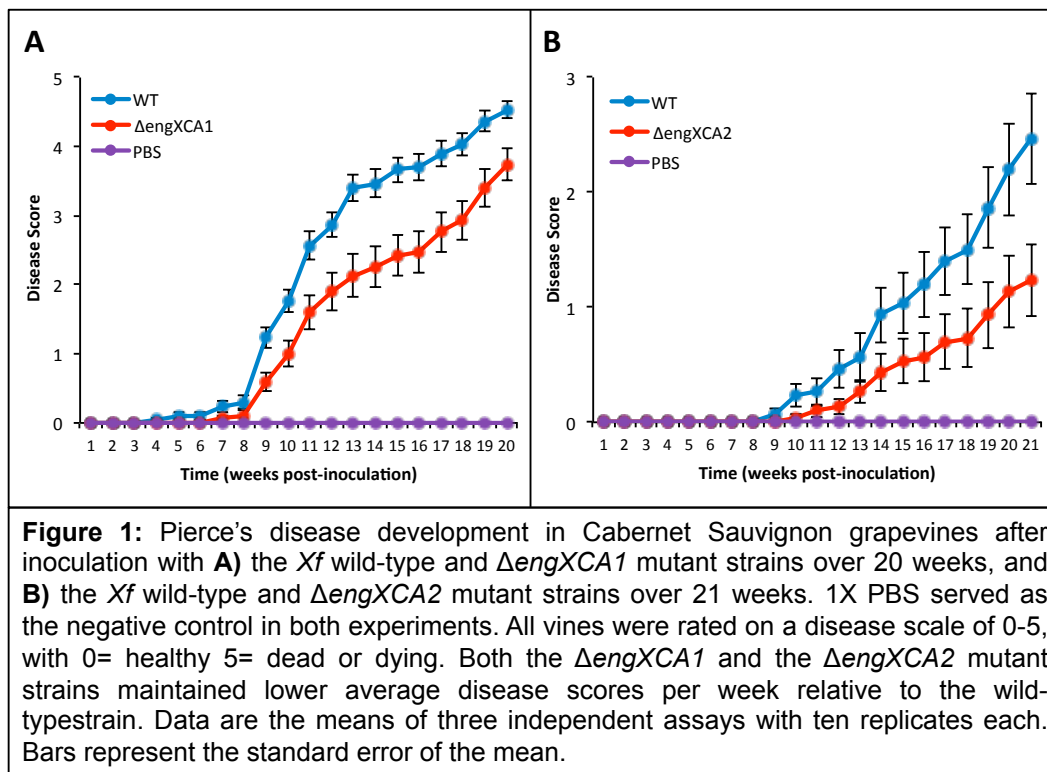
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INTRODUCTION

Xylella fastidiosa (*Xf*) is the causal agent of Pierce's Disease (PD) of grapevine, a serious and often lethal disease (Hopkins and Purcell, 2002, Chatterjee et al., 2008, Purcell and Hopkins, 1996). This xylem-limited bacterial pathogen colonizes the xylem and in doing so must be able to move efficiently from one xylem vessel element to adjacent vessels (Roper et al. 2007). Xylem conduits are separated by pit membranes (PMs) that are composed of primary cell wall and serve to prevent movement of air embolisms and pathogens within the xylem (Buchanan, 2000). More specifically, PMs are composed of cellulose microfibrils embedded in a meshwork of pectin and hemicellulose (Buchanan, 2000). The pore sizes within that meshwork range from 5 to 20 nM, which will not allow passive passage of *Xf* cells whose size is 250-500 x 1,000-4,000 nM (Perez-Donoso et al., 2010, Mollenhauer & Hopkins, 1974). Based on functional genomics and *in planta* experimental evidence, *Xf* utilizes cell wall-degrading enzymes (CWDEs) to actively digest the polymers within the PMs, thereby facilitating its movement throughout the xylem network (Simpson et al. 2000, Roper et al., 2007, Perez-Donoso et al., 2010). It is known that polygalacturonase (PG) is a major pathogenicity factor for *Xf* (Roper et al., 2007) and that it acts in concert with at least one EGase to breach the PM barrier (Perez-Donoso et al. 2010). EGases are implicated in virulence and colonization of the xylem in other bacterial phytopathogens, such as *Pantoea stewartii* subsp. *stewartii*, *Ralstonia solanacearum* and *Xanthomonas campestris* pv. *campestris* (Gough, 1988, Roberts et al., 1988, Saile et al., 1997, Mohammadi et al., 2012). In our previous study (project # 14-0144-SA), we tested the role of the *Xf* EGases *in planta* by constructing deletion mutants in two of the EGases (Δ engXCA1 and Δ engXCA2) and mechanically inoculating the modified *Xf* lines into *Vitis vinifera* cv. Cabernet sauvignon and cv. Chardonnay grapevines. Interestingly, both Δ engXCA1 and Δ engXCA2 achieved the same titers (*data not shown*) in the Cabernet sauvignon vines as wild type *Xf*, yet they were significantly less virulent and elicited fewer PD symptoms (Fig 1A,B).

PD symptom development is tightly correlated with the ability of *Xf* to degrade specific polysaccharides, namely fucosylated xyloglucans (part of the hemicellulosic component) and weakly esterified homogalacturonans (part of the pectin portion), that make up the intervessel PMs (Sun et al., 2011). In general, pectin is one of the first targets of cell wall digestion for invading pathogens and the resulting oligogalacturonides (OGs), which are smaller pieces of the pectin polymer, that are released are likely used as a carbon source for the invading pathogen. In addition, specific OGs with a degree of polymerization in the size range of 10-15 residues can also serve as signals that trigger host defense responses (Benedetti et al., 2015). These responses include accumulation of reactive oxygen species (ROS), expression of pathogenesis-related proteins, deposition of callose, activation of mitogen-activated protein kinases (MAPKs), among other defense related processes (Boller & Felix, 2009, Benedetti et al., 2015).



Tyloses are outgrowths of parenchyma cells that emerge through vessel-parenchyma pits into vessel lumen, and are common in a wide range of species (Bonsen and Kučera 1990; Esau 1977; Tyree and Zimmermann 2002). Tyloses impede fluid penetration (Parameswaran et al. 1985) and induce a permanent state of reduced hydraulic conductivity, and are triggered by abiotic and biotic stresses, such as pathogen infection (Aleemullah and Walsh 1996; Collins et al. 2009; Dimond 1955; Parke et al. 2007). Tylose formation is the predominant vascular occlusion associated with *Xf* infection (Fig 2A,B), and excessive tylose development has been linked to the extreme susceptibility of *Vitis vinifera* wine grapes to PD (Fritschi et al. 2008; Sun et al. 2013). Importantly, rates of tylose development in *V. arizonica*, a resistant species, are much lower than those in *V. vinifera*, which may reflect differing innate immune responses to the presence of *Xf* in the xylem. To our knowledge, no one has looked at the molecular mechanisms underlying the differences in response to *Xf* among different *V. vinifera* cultivars. Thus, we propose to better understand this difference in cultivar response to *Xf* in the context of host cell wall degradation and the elicitation of specific defense responses that lead to tylose formation in grapevines. Interestingly, a preliminary analysis of tylose formation in Cabernet Sauvignon vines inoculated with the $\Delta\text{engXCA1}$ mutant using a high resolution microCT technique (a kind of CAT scan) by the McElrone laboratory determined that these vines exhibited fewer tyloses than those inoculated with wild type *Xf* (Fig. 3). Therefore, our hypothesis is that enzymatic degradation of the plant cell wall by *Xf* CWDEs is generating cell wall fragments that elicit DAMP signaling defense pathways, which leads to downstream tylose production and PD symptom development in certain grape cultivars.

OBJECTIVES

- 1) Qualitative analysis of the effect of cell wall degradation on the grapevine response to *Xf*.
- 2) Quantitative analysis of plant defense pathways induced by *Xf* cell wall degrading enzyme activity: biochemical and transcriptional studies.
- 3) Inhibition of the Type II secretion system using natural products produced by grapevine microbial endophytes.

DESCRIPTION OF ACTIVITIES

Qualitative analysis of the effect of cell wall degradation on the grapevine response to *Xf*.

In the context of plant cell wall degradation, we will examine the effects that different *Xf* mutants ($\Delta\text{engXCA1}$, $\Delta\text{engXCA2}$, *egl* (all EGases and EGase/expansin hybrid) and *pglA* (a PG)) have on integrity and carbohydrate

composition of grapevine pit membranes in different varieties using both microscopic and immunological techniques coupled with fluorescence (Sun et al., 2011) and/or electron (Sun et al., unpublished) microscopy.

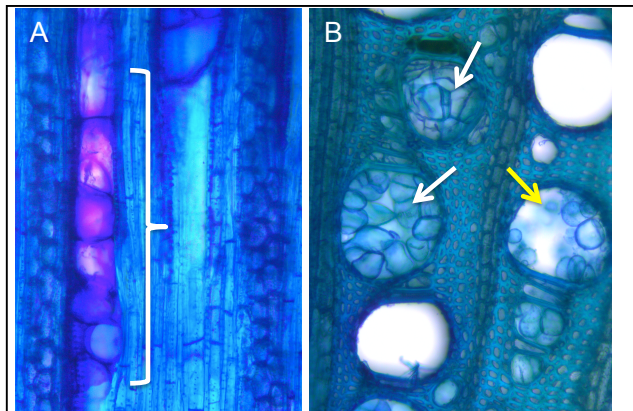


Figure 2. Xylem vessels of *V. vinifera* grapevines inoculated with *Xf*. A) Longitudinal section B) cross-section. Grapevine petiole sections were stained with toluidine blue O (0.05%). White arrows and bracket indicate vessels that are completely occluded with tyloses and yellow arrow indicates a partially occluded vessel. Images taken by J. Rapicavoli (Roper lab).

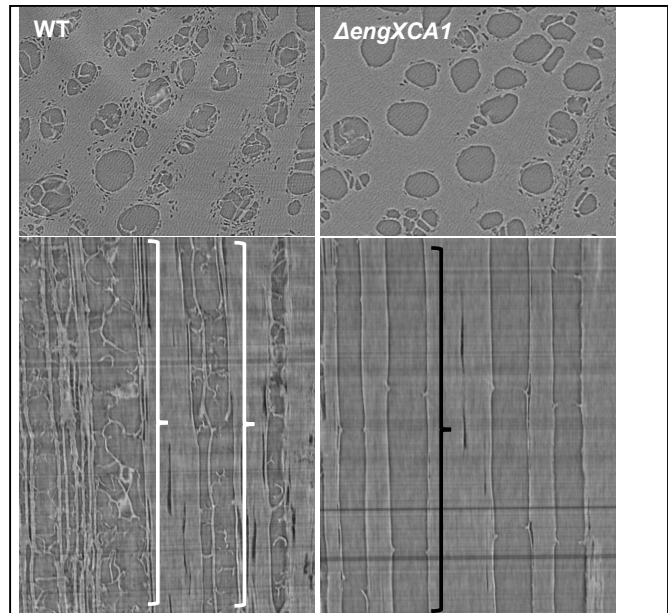


Figure 3. Images of grapevine xylem obtained using microCT. Vines inoculated with wild type *Xf* had substantial vascular occlusions, whereas, vines inoculated with $\Delta\text{engXCA1}$ had very few tyloses similar to the 1X PBS inoculated controls (not shown). Top panels are cross-sectional views and bottom panels are longitudinal views. White brackets highlight occluded vessels and black bracket highlights open vessel.

We will couple these microscopic observations with macroscopic studies of the spatial distribution of tyloses and other vascular occlusions, such as plant-derived gels and bacterial aggregates using high resolution micro-computed tomography (microCT). This non-destructive method technique uses x-rays to create cross-sections of an object that can be used to recreate a virtual model (3D model). These experiments will allow us to match degradation of specific host cell wall carbohydrates with spatiotemporal patterns of production of tyloses in 3 dimensions.

Wild type *Xf*, $\Delta\text{engXCA1}$, $\Delta\text{engXCA2}$, and ΔpglA mutants have been used to inoculate Cabernet Sauvignon and Chardonnay grapevines in the greenhouse. PBS-inoculated vines were used as negative controls. Each *Xf* strain was inoculated into 27 plants and PD symptoms were rated each week using the 0 – 5 PD rating index (Guilhabert and Kirkpatrick, 2005). Vine tissue samples are currently being collected for each of the three experiments: stem and petiole tissue for RNAseq, stem tissue for microCT analysis, and stem explants for EM analysis. Samples from three biological replications (consisting of three technical replications) per treatment have been collected at two time-points covering early and mid-infection based on the PD rating index (Early infection = 1 – 2, Mid-infection = 2 – 3).

Early time-point samples from Chardonnay were analyzed using scanning electron microscopy to study vascular occlusion, pit membrane integrity and presence/absence of *Xf* in the xylem tissue after inoculation with Wild type *Xf*, the PBS negative control, the $\Delta\text{engXCA1}$ mutant, or the ΔpglA mutant. Our results indicate that no vascular occlusions have been observed in the vines inoculated with PBS (Fig. 4), wild-type (Temecula 1, Fig. 5A, B) and ΔpglA (Fig. 7A) *Xf*, respectively. Tyloses were found in very few vessels of the vine inoculated with $\Delta\text{engXCA1}$ *Xf* (Fig. 6A, B), but were at the early developmental stages and did not occlude the vessels where they occurred. *Xf* cells were not observed in all the vines except that inoculated with $\Delta\text{engXCA1}$ *Xf* (Fig. 6D). Vessel-parenchyma pit membranes (PMs) were intact in the vines with the four different inoculums (Figs. 4B and 5C). Some broken (degraded) intervessel PMs were observed in the vine inoculated with either wild type (Fig. 5C, D) or $\Delta\text{engXCA1}$ *Xf* (Fig. 6C, D), but were rare or absent in the vine inoculated with either PBS or ΔpglA *Xf* (Fig.

7C). EM data is still being collected and analyzed for the remaining Chardonnay early timepoint samples, as well as all of the samples from the Chardonnay middle timepoint and both timepoints from Cabernet Sauvignon.

In addition to samples imaged via electron microscopy, samples from the early and middle time-points in both Chardonnay and Cabernet Sauvignon have also been analyzed by microCT. This technique is particularly resource-intensive and thus, imaging all nine samples per treatment was not feasible. Instead, three samples per treatment were chosen randomly, and singular midslice images were analyzed to determine if tyloses formed in the xylem in response to *Xf* infection. Of the analyzed stems, both Chardonnay and Cabernet Sauvignon inoculated with wild type (Temecula1) exhibited the most blocked vessels by tyloses, whereas the $\Delta engXCA1$ and $\Delta engXCA2$ mutants exhibited fewer tyloses (Fig. 8). Additionally, vines inoculated with the wild type Fetzner strain and the *pglA* mutant exhibited very few tyloses, and vines inoculated with PBS (negative control) displayed no tyloses. Transverse and longitudinal images slices of the selected samples from the early time-point in Chardonnay were also performed to visualize tylose formation (Fig. 9). Several vessels from vines inoculated with wild type (Temecula 1) displayed tyloses, while fewer vessels were occluded in vines inoculated with the $\Delta engXCA1$ mutant (Fig. 10). Vessels from vines inoculated with the PBS negative control were occlusion-free and displayed no tylose formation. Analysis of transverse and longitudinal images slices from the other time-points in both varieties is expected soon.

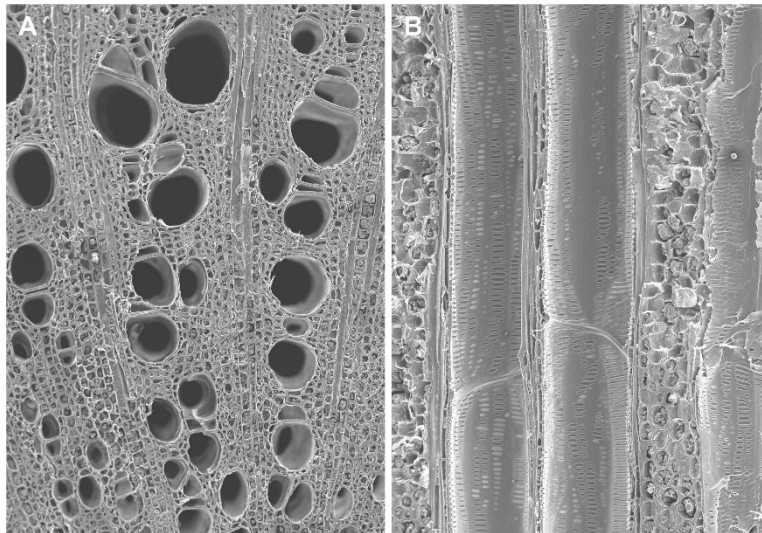


Figure 4. Vessel structural features in a Chardonnay stem inoculated with the PBS negative control. **A.** Transverse section of secondary xylem showing absence of vascular occlusion in the vessels. **B.** Tangential longitudinal section of secondary xylem, showing three transected vessels that have intact vessel-parenchyma PMs and do not contain vascular occlusions.

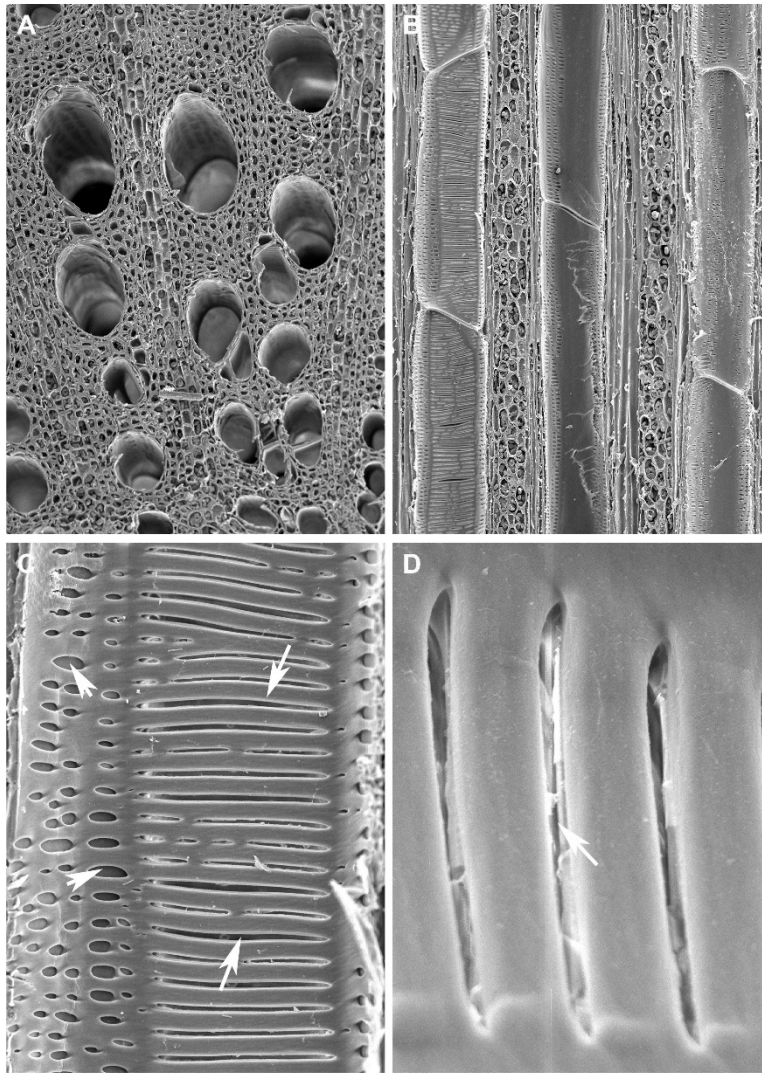


Figure 5. Vessel structural features in a Chardonnay stem inoculated with wild type *Xf* (Temecula 1). **A.** Transverse section of secondary xylem showing absence of vascular occlusion in the vessels. **B.** Tangential longitudinal section of secondary xylem. Vessels do not contain vascular occlusions. **C.** A transected vessel, showing oval vessel-parenchyma pit pairs and intact PMs (short arrows) and scalariform intervessel pit pairs (long arrows). **D.** Broken intervessel PMs.

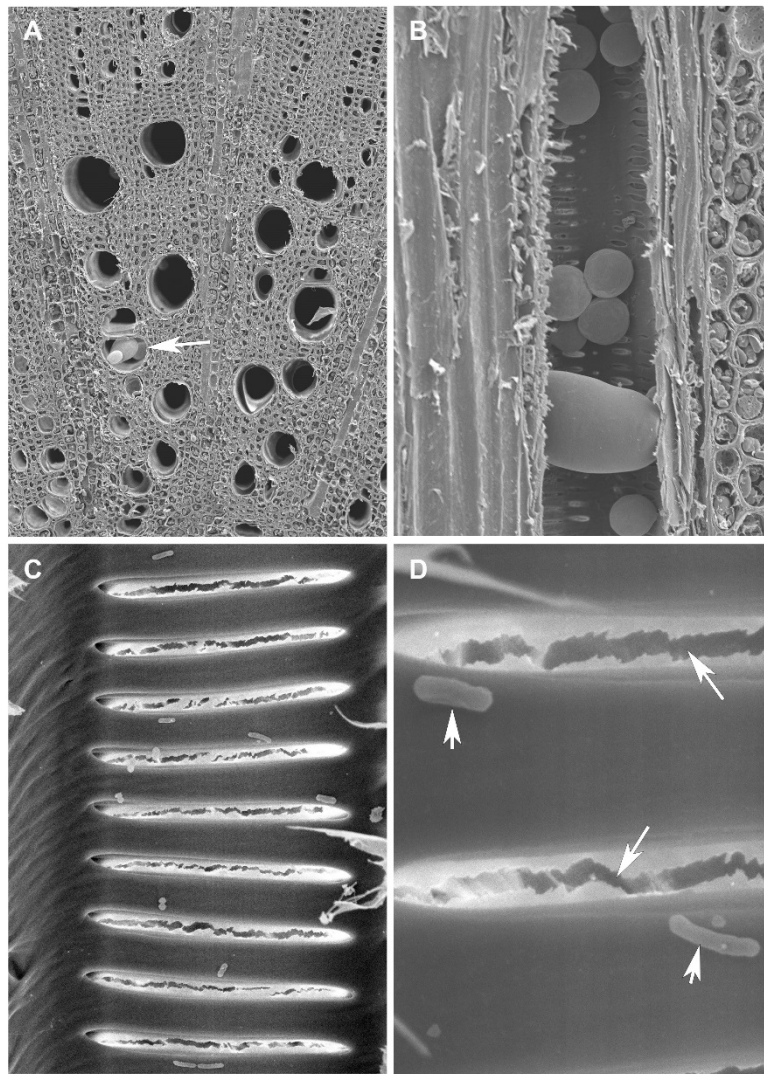


Figure 6. Vessel structural features in a Chardonnay stem inoculated with *ΔengXCA1 Xf*. **A.** Transverse section of secondary xylem. Most vessels have empty lumens but few vessels are filled with tyloses (arrow). **B.** Tangential longitudinal section of secondary xylem, showing a transected vessel with developing tyloses inside. **C.** Scalariform intervessel pit pairs in a vessel lateral wall. **D.** Enlargement of several intervessel pit pairs in a vessel lateral wall. Broken intervessel PMs (long arrows) are seen from a pit aperture and *Xf* cells (short arrows) are present on the lateral wall.

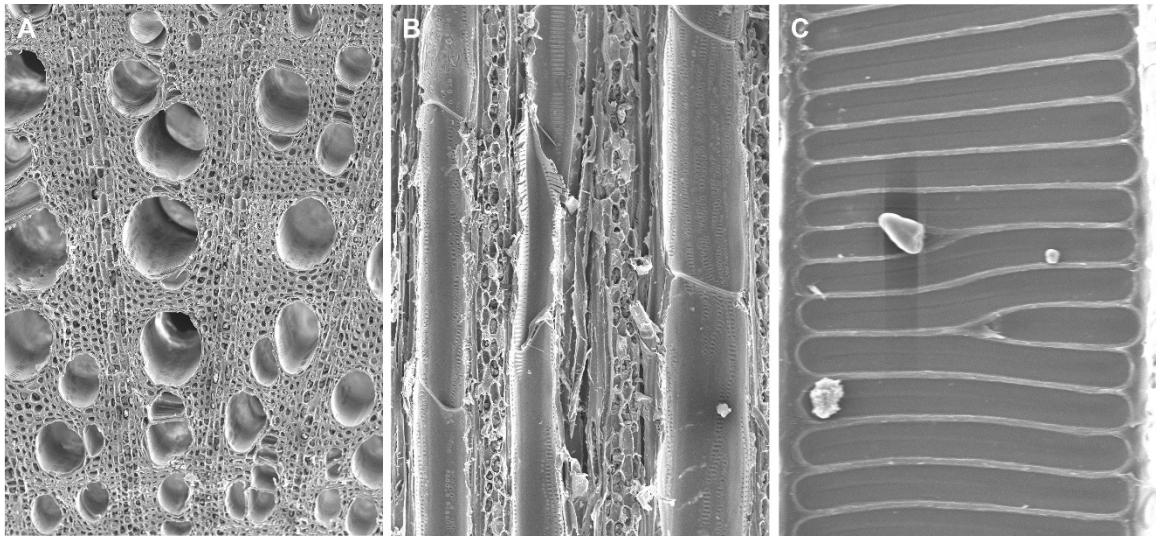


Figure 7. Vessel structural features in a Chardonnay stem inoculated with *ΔpglA Xf*. **A.** Transverse section of secondary xylem. All the vessels are free of vascular occlusions. **B.** Tangential longitudinal section of secondary xylem, showing several transected vessels without vascular occlusion. **C.** Surface view of a vessel's lateral wall. Whole intervessel PMs are visible after removal of secondary wall borders of intervessel pits. Intervessel PMs are intact and they are horizontally elongated and have a ladder-like arrangement along the vessel axial direction.

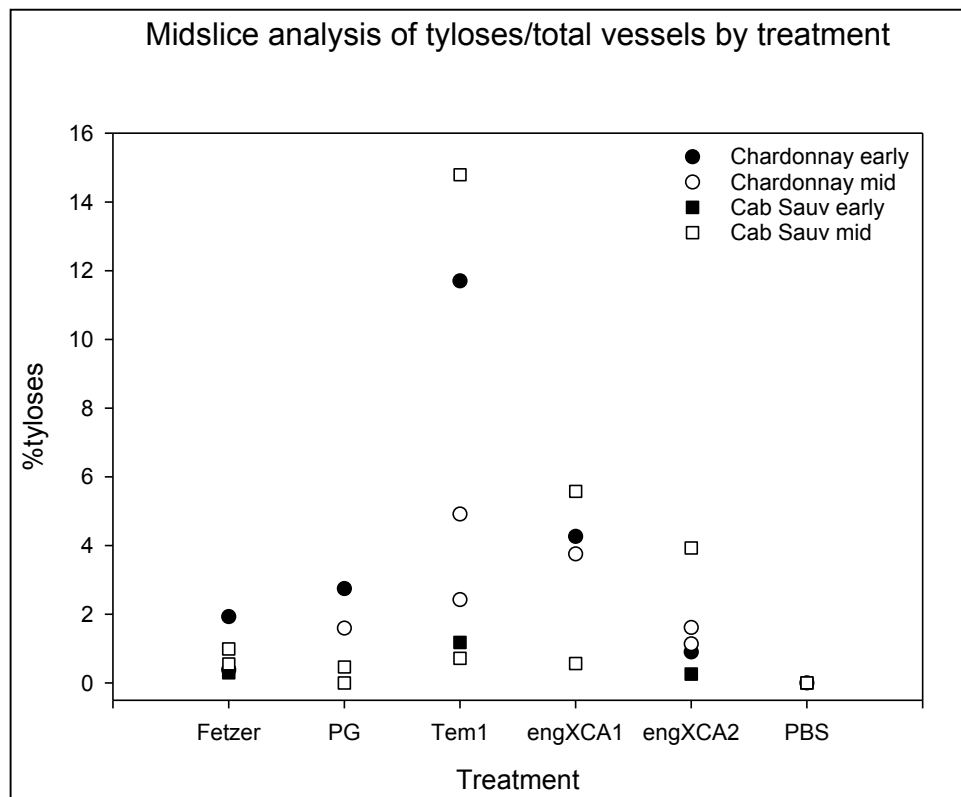


Figure 8: Manual midslice analysis of %-tyloses (occluded vessels/total vessels) per treatment. Each data point represents one analyzed stem sample (three samples per treatment). Highest %-tyloses occur in Cabernet Sauvignon inoculated with wild type Tem1 from the middle timepoint, with reduced tylose formation in *ΔengXCA1* and *ΔengXCA2*. Chardonnay from early timepoint shows a similar pattern. Relatively low tylose formation occurs in both Cabernet Sauvignon and Chardonnay inoculated with Fetzer and Fetzer-background ΔPG treatments. Negative control inoculated with PBS buffer exhibited no tylose formation.

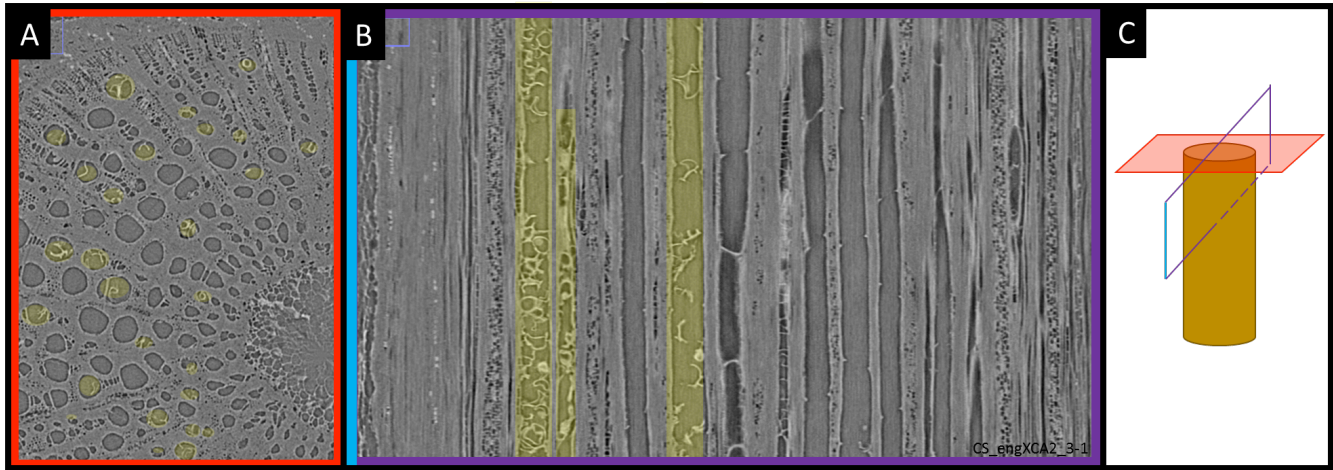


Figure 9: Improved tyloses detection/quantification. Colored outlines in **A** (xy-axis) and **B** (yz-axis) correspond with **C** to help orient the viewer. Tyloses (highlighted in yellow) are relatively small and rare features relative to empty vessels on the xy-axis, and can easily be confused with interconnected vessels, yet appear more distinctly in the yz-axis.

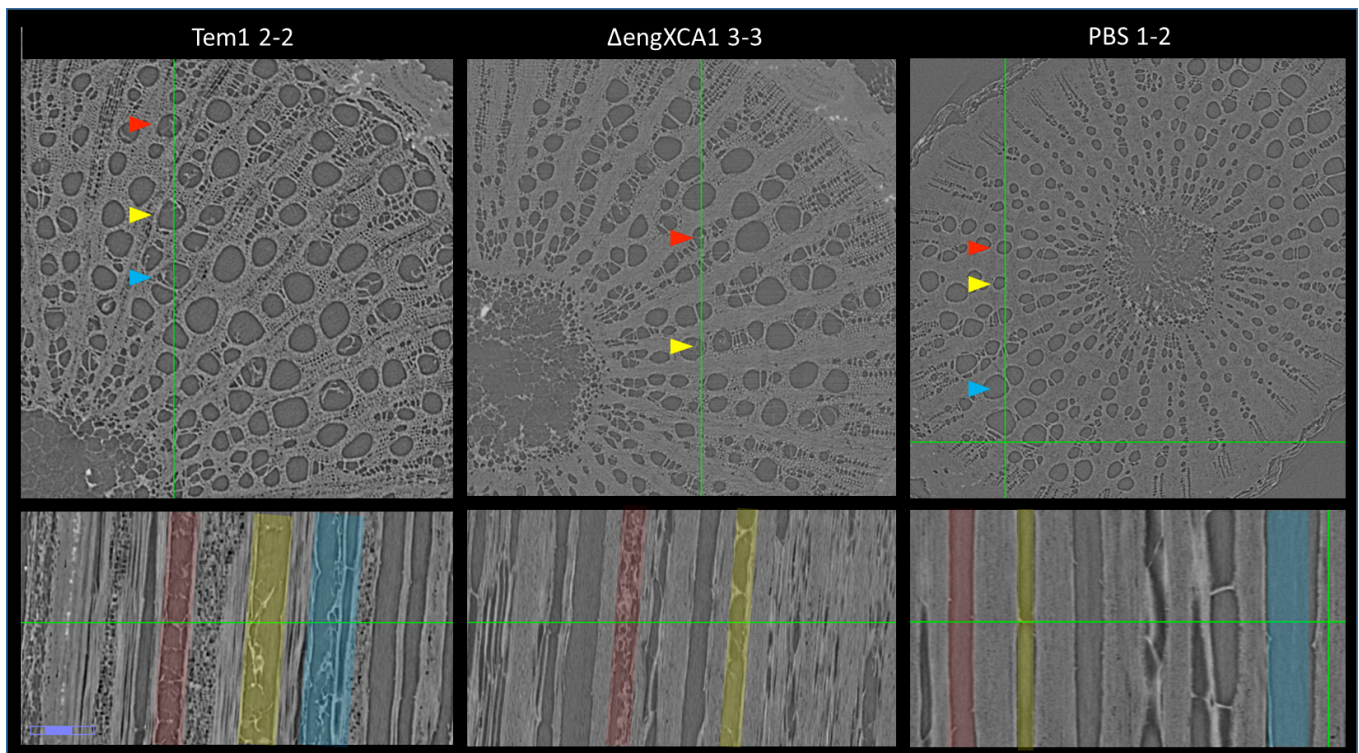


Figure 10: ImageJ orthogonal views of tyloses in Chardonnay (early timepoint) vines inoculated with wild type (Temecula 1), $\Delta engXCA1$, or PBS (negative control). Colored arrows on transverse image slices (top) correspond to highlighted vessels of same color on the longitudinal image slices (bottom), cut from the vertical green line in the transverse image. Empty vessels appear dark gray, while tyloses appear as highly branched membranes within vessel elements. PBS buffer treatment exhibits no tylose formation.

Quantitative analysis of plant defense pathways induced by *Xf* cell wall degrading enzyme activity: biochemical and transcriptional studies.

Pit membrane degradation by *Xf* CWDEs likely results in the release of small chain carbohydrates into the xylem. These oligosaccharides have been known to act as elicitors of plant immunity (i.e. damage-associated molecular patterns). It is possible that oligosaccharides released from pit membrane degradation are being recognized by associated parenchyma cells, triggering defense responses such as tylose production. To test this hypothesis, we will use RNAseq to analyze the grapevine transcriptome to determine if pit membrane degradation products act as elicitors of plant immunity and trigger tylose production. All tissue samples used for RNA extraction will be collected from the same plants and time-points as used for the qualitative experiments so that we can determine correlations between defense gene expression, pit membrane degradation, and/or tylose production. As these samples come from the same plants used in the qualitative experiments, all treatments, grapevine varieties, sample sizes, and time-points used are the same as in the previous section. Currently, stem and petiole tissue for all treatments from each time-point and variety have been collected, and are being prepared for RNAseq analysis.

Inhibition of the Type II secretion system using natural products produced by grapevine microbial endophytes.

Given that *Xf* CWDEs are important for the degradation of pit membranes (thus allowing systemic colonization), and their potential role in inducing tylose formation, it is imperative that these virulence factors are targeted for inhibition. However, inhibiting each CWDE individually as a commercial strategy for controlling *Xf* is both impractical and costly. Interestingly, these CWDEs are predicted (using SignalP software) to be secreted via the Type II secretion system (T2SS). The T2SS is a molecular nanomachine that transports pre-folded proteins from the periplasm across a dedicated channel in the outer membrane (Cianciotto, 2005, Korotkov et al., 2012). The T2SS systems of many plant and animal pathogens are either known or predicted to secrete proteins, namely polymer degrading enzymes, which are involved in nutrient acquisition (Jha et al., 2005). The *Xf* CWDEs being studied in this proposal are predicted (using SignalP software) to be secreted through the T2SS. Proteins destined for secretion by the T2SS are first delivered to the periplasm via the Sec or Tat-dependent secretion pathway where they are folded (Slonczewski, 2014). *Xf* appears to only possess the Sec-dependent secretion pathway. Because of our interest in host CWDEs and their mechanism of secretion, we created a mutation in the *xpsE* gene, which encodes the putative ATPase that powers the T2SS. Grapevines inoculated with the *xpsE* mutant never developed PD symptoms and remained healthy, a phenotype similar to the grapevine response to the *Xf* *pglA* mutant (Fig. 11).

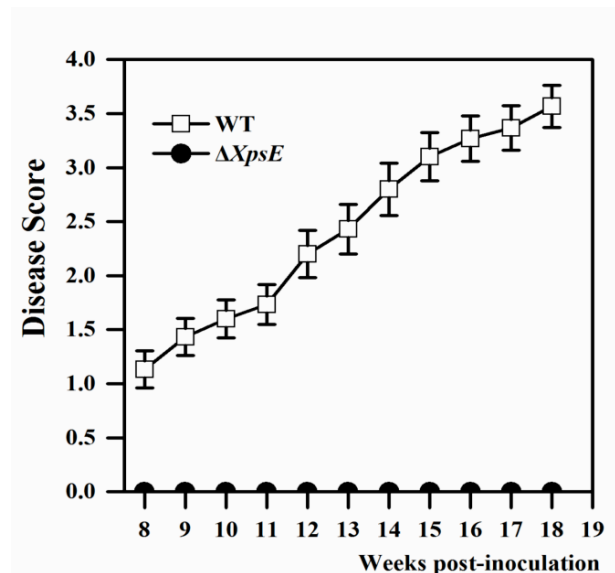


Figure 11: The *Xf* T2SS is necessary for PD development in grapevine. The $\Delta xpsE$ mutant does not induce PD symptoms in *V. vinifera* grapevines. Disease severity was based on a visual disease scale from 0 (no disease) to 5 (dead). Vines inoculated with 1X PBS (negative control) did not develop PD symptoms.

We hypothesize that this is due to the pathogen's inability to secrete the CWDEs necessary for xylem colonization. In addition, we have indirect experimental evidence that *Xf* utilizes the T2SS to secrete PG. We observed that the $\Delta xpsE$ mutant produces visibly less EPS on XFM minimal medium containing pectin as the sole carbon source, resulting in a much less mucoid phenotype (*data not shown*). However, when wild type *Xf* and $\Delta xpsE$ are grown on XFM+galacturonic acid (i.e., the monomeric sugar that makes up the pectin polymer) or on XFM+glucose, both strains produce similar amounts of EPS. We infer from this that, indeed, breakdown of the pectin substrate is necessary to produce EPS and when the T2SS is disrupted this prevents secretion of PG and the subsequent breakdown of pectin.

Thus, we have compelling *in planta* and *in vitro* preliminary data indicating that *Xf* has a functional T2SS system and the proteins secreted by T2SS are critical for the infection process. From this we reason that the T2SS represents an excellent target for disease control because disrupting this system would provide comprehensive inhibition of secretion of PG (the major pathogenicity factor for *Xf*) and the other auxiliary CWDEs (Roper et al. 2007 and recent results discussed above). Therefore, identifying molecules that can inhibit T2SS function is an excellent avenue of research to pursue to develop strategies that mitigate PD by preventing pathogen ingress.

PUBLICATIONS AND PRESENTATIONS

Poster Presentations

Brian M. Ingel, Dario Cantu, Andrew McElrone, Qiang Sun, John Labavitch, and M. Caroline Roper. Characterization of *Xylella fastidiosa* plant cell wall degradation and inhibition of the Type II secretion machinery (2016). Pierce's Disease Research Symposium, San Diego, CA.

RESEARCH RELEVANCE STATEMENT

Xf is the causal agent of PD of grapevine, a serious and often lethal disease of grapevines (Hopkins and Purcell, 2002, Chatterjee et al., 2008, Purcell and Hopkins, 1996). This xylem-limited bacterial pathogen colonizes the xylem and in doing so must be able to move efficiently from one xylem vessel element to adjacent vessels (Roper et al. 2007). Xylem conduits are separated by pit membranes (PMs) that are composed of primary cell wall that serve to prevent movement of air embolisms and pathogens within the xylem (Buchanan, 2000). More specifically, PMs are composed of cellulose microfibrils embedded in a meshwork of pectin and hemicellulose (Buchanan, 2000). The pore sizes within that meshwork range from 5 to 20 nM, which will not allow passive passage of *Xf* cells whose size is 250-500 x 1,000-4,000 nM (Perez-Donoso et al., 2010, Mollenhauer & Hopkins, 1974). Based on functional genomics and *in planta* experimental evidence, *Xf* utilizes host CWDEs to actively digest the polymers within the PMs, thereby facilitating its movement throughout the xylem network (Roper et al., 2007, Perez-Donoso et al., 2010). This previous work demonstrated that a polygalacturonase (PG), PglA, was required for movement and pathogenicity in grape (Roper et al, 2007). In addition, an EGase (EngXCA2) worked in concert with PG to breach pit membranes (Perez-Donoso et al. 2010). Based on these findings, inhibition of *Xf* PG has been identified as a top research priority by the PD research board as outlined in Attachment A of the Request for Proposals. Several other research groups are working towards inhibiting PG *in planta* as a means of PD control. In our currently supported project (project # 14-0144-SA) and in this proposal, we have outlined objectives designed to complement and augment these current research efforts that are aimed at inhibiting PG. Our central hypothesis is that *Xf* utilizes other CWDEs in concert with PG to breach the pit membranes and that the majority of these are secreted by a common mechanism, the Type II Secretion System (T2SS). We propose a project composed of two broad goals: 1) Elucidation of how the plant perceives host cell wall damage inflicted by the suite of *Xf* CWDEs during the infection process and 2) Utilization of natural products produced by grapevine microbial endophytes to inhibit the T2SS that delivers PG, and other CWDEs (EGases) to the xylem. We view this as a comprehensive approach to achieving disease control with the potential impact being to effectively disrupt systemic spread of *Xf* and vascular occlusions in the xylem and, therefore, PD development.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) relies on degradation of the plant cell wall to move within the grapevine, which occurs through cooperation between at least two classes of enzymes that target different carbohydrate components of the complex scaffold of the plant cell wall. A major goal of this project is to elucidate the mechanisms that lead to disassembly of the plant cell wall that eventually leads to systemic colonization of *Xf* in grapevines. Here we propose experiments designed to better understand what facilitates movement of the bacterium and the subsequent clogging of the water-conducting cells that worsens Pierce's Disease severity. In addition, we also outline

experiments that inhibit the secretion machinery responsible for delivering the *Xf* enzymes that are involved in *Xf* movement throughout the plant, thus, providing a comprehensive approach to restriction of *Xf* and disease development rather than targeting individual enzymes.

STATUS OF FUNDS

The funding for this project is largely going towards supporting a Ph.D. graduate student, Mr. Brian Ingel. This project is the main focus of his Ph.D. dissertation. We anticipate spending the remainder of the salary, supply, services and greenhouse recharge money associated with this project as it progresses.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY

There is no intellectual property associated with this project to date.

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