DO CELL WALL STRUCTURES LIMIT *XYLELLA FASTIDIOSA* DISTRIBUTION IN INOCULATED, PIERCE'S DISEASE SUSCEPTIBLE AND RESISTANT GRAPEVINES?

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ABSTRACT
The development of Pierce's disease (PD) in grapevines depends, at least in part, on the ability of the *Xylella fastidiosa* (*Xf*) pathogen to spread from the point of infection and ultimately develop a population that is systemic in the infected plant. This systemic spread of the pathogen is limited by the pit membranes (PMs) that separate one xylem water conduit from its neighbors and, perhaps, by the production of tyloses and polysaccharide-rich gels that are produced in and block xylem cells following infection. The work in this proposal will describe the polysaccharides of PMs, tylose cell walls and gels by using immunohistochemical tools and determine whether situations in which grapevine infection with *Xf* does not result in PD are situations in which PM integrity is not disrupted by the pathogen so that pathogen spread is limited by intact PMs and/or production of tyloses or gels.

INTRODUCTION
The introduction of *Xylella fastidiosa (Xf)* to grapevine xylem tissues often results in Pierce's disease (PD) and, ultimately, to vine death. Several studies over the past five years have indicated that the expansion of the locally introduced, relatively small population of *Xf* cells throughout the vine, creating a systemic infection, is the cause of vine death (Krivanek and Walker, 2005; Labavitch, 2007; Lin, 2005; Lindow, 2006a, b, 2007a, b; Rost and Matthews, 2007). The individual elements of the water-conducting tubes in xylem are separated from one another by the so-called pit PMs, primary cell wall "filters" whose meshwork is too small to permit *Xf* passage (Labavitch et al., 2004). Thus, it has been generally believed that the pathogen uses cell wall-degrading enzymes (CWDEs) to digest the polysaccharides of the PMs, opening the primary cell wall barrier and permitting the systemic expansion of the pathogen population.

The genome of *Xf* contains genes encoding polygalacturonase (PG) and a few ß-1,4-endo-glucanases (EGase), CWDEs that digest cell wall pectin and xyloglucan polymers, respectively. Such enzymes are good candidates for pathogen factors that facilitate *Xf* systemic movement and PD development. This supposition has been supported by several studies performed over the past several years. Roper et al. (2007) reported the generation of a PG-deficient strain of *Xf* and showed that it was unable to cause PD symptoms, thus identifying the pathogen's PG as a PD virulence factor. Labavitch et al. (2006) reported that introduction of PG and EGase into explanted stems of uninfected grapevines caused the breakage of the PM cell wall network and, subsequently (Labavitch, 2007), that substrates for these enzymes, pectins and xyloglucans, are present in grapevine PMs.

Research in the laboratory of the PI on the present proposal has shown that PG-inhibiting proteins (PGIPs) limit the development of PD in grapevines (Agüero et al., 2005). Research in Cooperator Steve Lindow's program has focused on the role of a diffusible signal factor produced by *Xf* in controlling the pathogen's expression of virulence functions that affect whether the pathogen spreads systemically in grapevines and causes PD **or does not** (Lindow, 2007a, b). Cooperator Andy Walker and his colleagues have identified a grapevine quantitative trait loci (QTL) that contains the PD resistance (*PdR1*) locus (Walker and Riaz, 2007) that eventually will be deployed in grapevine genotypes that will have enhanced resistance to PD. Walker, Lindow and Cooperator Hong Lin (Lin, 2007), have all made use of natural variations in the PD resistance/susceptibility of different grape germplasm in order to understand the factors that influence *Xf* movement in grapevines and, therefore, PD development. It is reasonable to assume that differential PD susceptibility of grape genotypes is determined by (1) genetic variation in PM barriers to pathogen movement; differences in porosity, polysaccharide composition or susceptibility to *Xf*'s CWDEs or/and (2) the post-infection deployment of tyloses and gels, factors that could restrict the pathogen to the few vessels into which it has been introduced.

While the production of gels and tyloses in response to infection has been examined in several programs (e.g., Lin, 2005; Stevenson et al., 2004), this has not been done using techniques that can specifically identify the polysaccharides that make

up the gels and tylose walls. The utility of immunohistochemical techniques in identifying the polysaccharides of grapevine PMs has recently been demonstrated by Co-PI Sun (Labavitch, 2007). These techniques may contribute to an understanding of the differences in xylem water-conducting cell structures that have been thought by many to hold the key to grapevine resistance to PD. This proposal will use these techniques in several systems where differential resistance to PD have been shown in order to obtain the detailed structural and spatial information that may help explain why some grapevine genotypes are resistant to PD while others are not. It is important to note that while the research in this program is likely to enhance our understanding of grapevine PD resistance it will not lead immediately to new approaches to PD control.

OBJECTIVES

1. Determine if the development of xylem obstructions (tyloses and pectin-rich gels) and the polysaccharide structure and integrity of pit membranes are affected by *Xf* inoculation of grapevines transformed to express the PGIP from pear and other plant species in rootstocks and in scions.
2. Determine whether there are differences in pit membrane porosity or polysaccharide structure between resistant and susceptible grapevines. To what extent are these PM characteristics and the production of tyloses and gels modified by introduction of *Xf* to PD-resistant and -susceptible genotypes?
3. Determine the extent to which changes in pathogen virulence resulting from altered production of diffusible signal factor (DSF) correlate with the appearance of tyloses, gels and damaged PMs in inoculated vines.
4. Determine whether the impacts of inoculation on PM integrity and the production of vascular system occlusions identified in tested greenhouse-cultured vines also occur in infected vines growing in the field.

RESULTS AND DISCUSSION

The primary work on this project will be performed at the University of Wisconsin-Stevens Point, where Co-PI Dr. Qiang Sun is an Asst. Professor. The project is currently funded only for the first of the two years of work that were proposed in January, 2008. Unfortunately, because Dr. Sun did not have a history of funding from the CDFA at his university, he could not be "advanced" research support from his Office of Research, thus his full effort at Wisconsin could not be started until the sub-contract could be established with UC Davis and UCD could not establish that until funding was available from the CDFA (mid-August). Nevertheless we have made considerable progress, primarily in developing the techniques required for addressing our four objectives in several grape genetic backgrounds. We have shown that PMs of these different grapes contain homogalacturonans, the target cell wall substrate for the *Xf* PG "PD virulence factor," setting the stage for the detailed studies identified under Objective 1. We have also begun describing variations in the gross vascular system architectures of the different grape germplasms.

The following five grape species/cultivars of different susceptibilities to PD have been selected for our experiment: *Vitis vinifera* cv. Chardonnay (susceptible), *V. vinifera* cv. Riesling (less susceptible), *Muscadinia rotundifolia* (highly tolerant), 89-0908 and 89-0917 (both are selected from *V. arizonica* x *vinifera* and are resistant to PD). We have succeeded in using two monoclonal cell wall antibodies (JIM 5 and JIM 7) to distinguish pectin wall components of the living parenchyma cells adjacent to water-conducting vessels of the secondary xylem tissue (wood) in Chardonnay and Riesling (**Figure 1**). Our results indicate that homogalacturonans (HGs) with different levels of methyl esterification are present in PMs between vessels as well as between vessel and axial parenchyma cells (**Figure 1A-C**). Cell walls of developing tyloses (**Figure 1D**) and inner secondary wall of xylem fibers (**Figures 1C and D**) also contained the HGs. In our next step, the protocols established will be used to identify HGs in the other grape species/cultivars, particularly in PMs that are thought to be the barriers that should limit systemic spread of *Xf* in grapevines. Some other wall antibodies (CCRC-M1 and 2F4 etc.) will also be tested to detect other possible cell wall components (pectins and xyloglucans) in all the five grape groups. Our ultimate goal is to use these tools for localized cell wall component visualization to determine whether the post-inoculation integrity of pit membranes differs between PD-susceptible, -tolerant and -resistant grape germplasm (Objectives 1 to 3).

Understanding vessel morphology is essential to elucidate any possible differences in susceptibility of these grape groups, thus we have also made some anatomical analyses of secondary xylem. Our results indicate that there are major differences among these groups in the arrangement, density and diameter of vessels. In Riesling (**Figure 2A and E**) and Chardonnay (**Figure 2D**), vessels are relatively evenly distributed in xylem, are mostly solitary and have less difference to one another in size. Vessel density is also close in these two cultivars (34.6/mm^2 in Chardonnay and 30.7/mm^2 in Riesling). However, vessel diameters in Chardonnay (68.2 μm) are generally smaller than those in Riesling (84.7 μm). In 89-0917, vessels are not uniformly distributed in xylem tissue with a density of 42.8/mm^2. They are usually solitary or in multiples of 3-5 cells. Solitary vessels are usually larger while most vessels in multiples are much smaller. Vessels in 89-0908 are more or less evenly spread through the secondary xylem and usually form radial chains of 3-6 cells. Vessels have an average diameter of 66.4 μm, but individual vessels show large size differences. In *Muscadinia rotundifolia*, vessels usually forms radial chains of 2-5 cells and individual vessel sizes (56.5 μm diameter, at average) vary less than in some other groups. The vessel density is highest (53.1/ mm^2) among the five groups. Morphological analysis of pits and pit membranes on lateral vessel walls has also been made (**Figure 3**). Two types of pits (intervessel pits and vessel-parenchyma pits) are common in all the five grape groups (**Figure 3A**). As for vessel parenchyma pits, PMs are intact in all groups (**Figure 3E**), except for 89-0917 in which PMs that are broken in a relatively regular pattern are common. No other obvious differences have been found in the

structures and distributional patterns of pits on vessel walls (**Figure 3B-D**). Further investigation is still needed to clarify any interconnection between these anatomical characteristics and susceptibility. As for vessel-parenchyma pits, PMs are intact in all the groups (**Figure 3E**) except 89-0917 in which PMs that are broken in a relatively regular pattern are common.

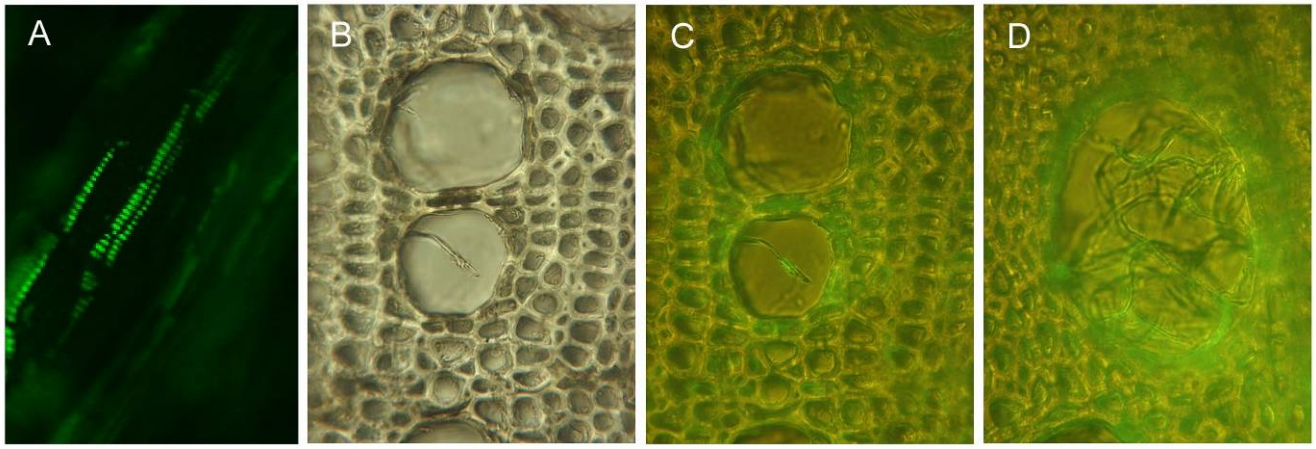

Figure 1. Cell wall composition revealed by JIM 5 (A) and JIM 7 (B-D) in Chardonnay, a susceptible cultivar. JIM 5 and JIM 7 can be used to distinguish weakly methyl esterified homogalacturonans (Me-esterified HG) and heavily Me-esterified HGs, respectively. A. Green fluorescence from the rows of pit membranes (PMs) between a vessel and axial parenchyma cells indicates the presence of weakly Me-esterified HGs. B. Image of xylem tissue under transmission illumination, showing vessels, parenchyma cells surrounding vessels, and fiber cells. C. Image of xylem tissue under both transmission light and fluorescent light. Green fluorescence is emitted from parenchyma cell walls, PMs between vessel and parenchyma cells and fiber inner wall layers, indicating the presence of heavily Me-esterified HGs in these locations. D. Transverse section of a vessel containing tyloses, showing HG presence in tylose cell walls.

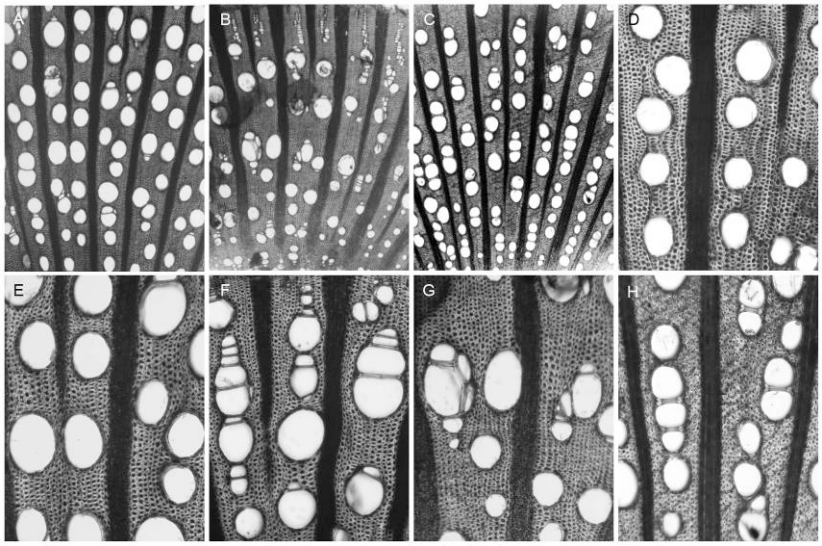

Figure 2.. Differences in the distribution, arrangement and sizes of vessels among grapes of different PD susceptibilities. A and E. Riesling. Vessels are larger in diameter than other grape groups and mostly solitary, occasionally in groups of up to 3 vessels. B and G. 89-0917 grape. Vessels are usually in multiples of 3 – 5 and individual vessels differ in size. C and H. *Muscadinia rotundifolia*. Vessels of similar size are usually in radial chains of 3-5 cells. D. Chardonnay. Vessel arrangement is similar to Riesling. F. 89-0908 grape. Radial chains of 3-6 vessels are common and vessels differ in size.

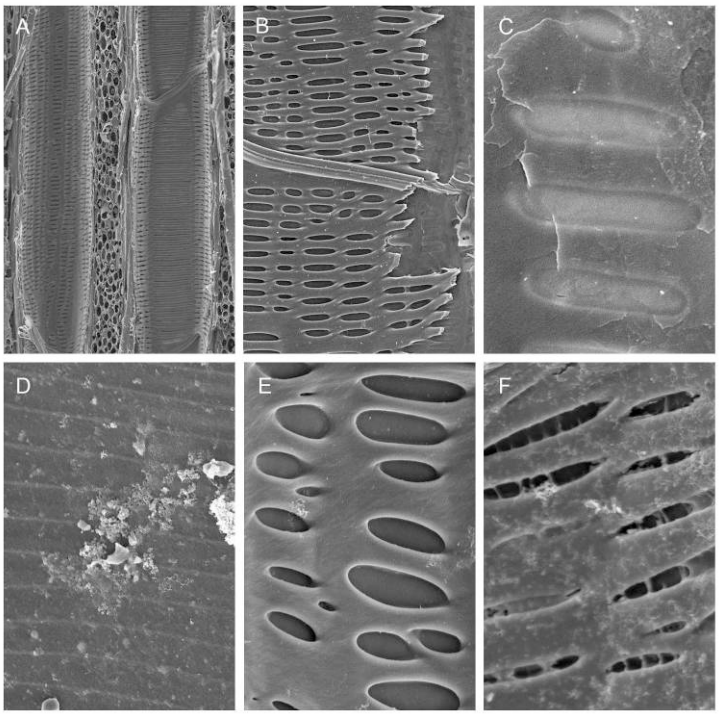

Figure 3. Pits and pit membranes in lateral vessel walls in grapes of three different susceptibilities. A-C. *Muscadinia rotundifolia* (highly resistant grape species). A. Two types of pits are present on vessel lateral walls: vessel-axial parenchyma pits (the vessel on the left) and intervessel pits (the vessel on the right). B. Vessel-axial parenchyma pits. Vessel secondary walls have been partially peeled on the right. C. Pit membranes between vessel and axial parenchyma cells are in a ladder-like arrangement (scalariform) along the vessel axis. D and E. Riesling (less susceptible *vinifera* cultivar), showing that scalariform intervessel pit membranes are arranged tightly. E. Bordered vessel-axial parenchyma pits, showing intact pit membranes. F. Bordered vessel-axial parenchyma pits in tolerant 89-0917 grape (*V. vinifera* x *arizonica*, tolerant grape). Many pit membranes are broken in a more or less regular way.

CONCLUSIONS

1. Because of the limited time that the funds have been available for this project, this report documents only a small portion of the work planned for year 1.
2. Immunohistochemical studies indicate the presence of simple homogalacturonan pectins in the cell wall fabric of pit membranes from PD-susceptible, -tolerant and -resistant grape grapevines.
3. Xylem vessel diameters and distribution patterns of vessels within the secondary xylem tissues of PD-susceptible, -tolerant and -resistant grape grapevines are described.

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