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

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# ABSTRACT

The development and progression of Pierce's disease (PD) symptoms depends largely on the ability of the pathogen to spread via xylem, more specifically, its vessel system, in the infected grapevine. We believe that to the *Xylella fastidiosa* (*Xf*) entering vessels, pit membranes (PM) separating neighboring vessels should become barriers which the pathogen must digest to enhance its systemic spread. Production of occlusions (tyloses and pectin-rich gels) in vessels in response to the presence of *Xf* may also be related to disease symptom development or the host plant's resistance. The research included in this report focuses on these two factors of the host plant which should affect *Xf*'s systemic spread in the host plant. Our data revealed that grape varieties with different PD resistance were different in some cell wall polysaccharides of PMs, that intervessel PMs may be modified in infected PD susceptible grapes and that development of the host plant. These observations provide information for understanding of the possible roles of these factors in grape's resistance to PD and are also likely to contribute to identification of an efficient approach for control of the disease.

# LAYPERSON SUMMARY

Several of the approaches currently being investigated as strategies for management of Pierce's disease (PD) in vineyards are based on studies that identified the way the disease becomes established in a grapevine. The relative resistance/susceptibility of range of grape genotypes has been studied in the past decade. The work described here asks whether the pathway used by *Xylella fastidiosa (Xf)* to spread through a grapevine, the so-called pit membranes (PMs), differ between susceptible and tolerant grape lines. It also asks whether the development of vascular system obstructions, barriers that could either prevent *Xf* spread or shut down vine water transport, or both, differ in susceptible and resistant vines. The data suggest that the polysaccharide compositions of the PMs are different (in terms of kinds or amounts of polymers present) in susceptible and resistant vines. Whether these differences are important in determining whether a given grape germplasm will be PD resistant or tolerant is not yet known.

# INTRODUCTION

Pierce's disease (PD) is a devastating grapevine disease caused by the xylem-limited bacterium, *Xylella fastidiosa* (*Xf*). It is clear that vine death is caused by the systemic spread of the locally introduced *Xf* throughout the vine (Krivanek and Walker, 2005; Labavitch, 2007; Lin, 2005; Lindow, 2006a, b, 2007a, b; Rost and Matthews, 2007). The initial introduction of *Xf* by the glassy-winged sharpshooter (GWSS) involves only in few vessels. To spread throughout the grapevine, *Xf* cells must move successively from one vessel to another. The neighboring vessels are separated from one another by the so-called pit membranes (PMs), primary cell wall "filters." Since the meshwork of PMs is too small to permit *Xf* passage, an increase in PM porosity is a prerequisite for spread of the *Xf* population in a host plant (Labavitch et al., 2004).

*Xf*'s genome contains genes encoding cell wall-degrading enzymes (CWDEs), including polygalacturonase (PG) and a few β-1,4-endo-glucanases (EGase). We believe that *Xf* cells use the CWDEs to digest the polysaccharides of the PMs, opening the primary cell wall barrier and allowing *Xf* passage. 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.

Research in the laboratories of the PIs on the present proposal has shown that the substrates for *Xf* 's CDWEs, pectins and xyloglucans, are present in grapevine PMs (Labavitch, 2007; Labavitch and Sun, 2008) and 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 (Lindow, 2007a, b). Cooperator Andy Walker and his colleagues have identified a grapevine QTL that contains the Pierce's disease 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 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 that are expressed as differences in porosity, polysaccharide composition or susceptibility to *the pathogen'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.

Grape genotypes show differential PD resistance. Most *vinifera* varieties are susceptible to PD, while wild *Vitis* species and some of their hybrids with *vinifera* varieties have been demonstrated to have PD tolerance or resistance in greenhouse and field evaluations. Quantitative analyses of the concentration and distribution of the pathogen have clarified that *Xf*'s spread from the inoculation site in resistant genotypes is limited relative the its spread in susceptible *vinifera* varieties (Lindow, 2007a), suggesting differences in PM polysaccharide composition among the genotypes with differential PD resistance. Therefore, the clarification of any possible cell wall compositional differences in PMs of those grape varieties/genotypes is essential to the better understanding of the natural PD resistance mechanisms of grapes.

While the production of gels and tyloses in response to infection have been examined in several programs (e.g., Lin, 2005; Stevenson et al., 2004), detailed information about the spatial and temporal distributions and of vascular occlusions in susceptible and resistant germplasm is still lacking. This information is crucial to clarify the role of the vascular occlusions in PD symptom development or disease resistance of host plant. An efficient system to evaluate the development of vascular occlusions in grapevines quantitatively and qualitatively has been developed by Co-PI Sun (Sun et al., 2006, 2007 and 2008) and was used in this study. The utility of immunohistochemical techniques in identifying the polysaccharides of grapevine PMs and vascular occlusions 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 grape germplasms where differential resistance to PD has 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. These results may provide the information useful for finding an effective approach for control of grape PD.

### **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?

(Note: The original proposal had four Objectives, but only Objectives 1 and 2 were approved for funding.)

# **RESULTS AND DISCUSSION**

### Differences in cell wall polysaccharide compositions of pit membranes of grapevines with differential PD resistance

In this research, we have used the following grape genotypes/varieties with different PD susceptibility: *Vitis vinifera var*. Chardonnay (susceptible), *Muscadinia rotundifolia* (highly tolerant) and 89-0908 (resistant, a hybrid of *V. arizonica* x *rupestris*). The immunohistochemical techniques and confocal laser scanning microscopy we established previously were used to identify and compare polysaccharide compositions of the vessel PMs in these genotypes/varieties. The research covered both intervessel PMs and vessel-parenchyma PMs, which exist in vessel lateral walls. The former are the barriers to Xf's systemic spread, while the latter are related to the development of vascular occlusions (tyloses and gels) and may contribute to disease resistance or symptom development.

Our experiments focused on two major groups of cell wall polysaccharides: homogalacturonans (the predominant components of pectin) which polygalacturonases may attack, and xyloglucans (XyGs, a major group of hemicellulosic polysaccharides), the substrates of endo-glucanases. We have used three different kinds of monoclonal cell wall antibodies to identify the polysaccharide composition of PMs: JIM5, JIM7 and CCRC-M1. JIM5, JIM7 and CCRC-M1 can recognize weakly methyl-esterified homogalacturonans (low Me- HGs), heavily Me-esterified HGs (high Me-HGs), and fucosylated XyGs, respectively. Our aim is to determine whether there are any differences in the presence or distributions of these two groups of polysaccharides in the PMs of the four genotypes/varieties studied.



JIM5

JIM7

В

Е

CCRC-M1

JIM7

A

D

CCRC-M1

JIM5







Figure 3. Cell wall compositions of intervessel PMs (A,C,E) and vessel-parenchyma PMs (B,D,F) in Vitis vinifera cv. Chardonnay, a PD-susceptible genotype. A. Intervessel PMs have strong fluorescence when incubated with CCRC-M1, indicating the abundant presence of fucosylated XyGs. B. Xylem tissue incubated with JIM5, showing that low Me-HGs are common components of both intervessel PMs (arrow head) and vessel-parenchyma PMs (arrow). C-D. Xylem tissue incubated with JIM7. Fluorescence is below the detectable level in intervessel PMs (arrow, C) and is strong from vessel-parenchyma PMs (D), indicating high Me-HGs is weakly present in intervessel PMs (C) but is abundantly present in vessel-parenchyma PMs (D).

Our results have indicated that the four genotypes with different PD susceptibility all have intervessel PMs and vesselparenchyma PMs in their vessel lateral walls. Individual intervessel PMs are transversely elongated across the whole surface of the shared (i.e., common) wall of neighboring vessels and are arranged in a tight scalariform pattern along the vessel long axis (Fig. 2A). Vessel parenchyma PMs are round, oval or slightly transversely elongated (**Figure 2D**). The genotypes also showed differences in the polysaccharide compositions of intervessel and vessel-parenchyma PMs. In 89-0908, both intervessel PMs (**Figure 1A**) and vessel-parenchyma PMs (**Figure 1B**) lack fucosylated XyGs In addition, their intervessel PMs do not have a detectable amount of low Me-HGs (**Figure 1C**) or high Me-HGs (**Figure 1E**). However the vessel-parenchyma PMs contain both low Me-esterified (**Figure 1D**) and high Me- HGs (**Figure 1F**). In *Muscadinia rotundifolia*, strong fluorescence signals were detected from both intervessel PMs (**Figure 2A**) and vessel-parenchyma PMs (**Figure 2B**) when incubated with CCRC-M1 (showing fucosylated XyGs) in both types of PMs. Some high Me-HGs are also present in both types of PMs (**Figure 2C**). In *V. vinifera var*. Chardonnay, fucosylated XyGs (**Figure 3A**) and low Me-HGs (**Figure 3B**) are abundantly present in both intervessel PMs and vessel-parenchyma PMs. High Me-HGs occur in a large quantity in vessel-parenchyma PMs (**Figure 3D**), but are undetectable in intervessel PMs (**Figure 3C**).



**Figure 4**. Xylem structure of control (A and B) and inoculated (C-E) vines. A-B. No vascular occlusions occurred in secondary xylem vessels (A); a closer image shows that vessel lumens are empty (B). C. Vascular occlusions developed in secondary xylem of inoculated branches and showed uneven distribution. D. A xylem region with extensive vascular occlusions, showing most vessels blocked by tyloses. E. Xylem region with fewer vascular occlusions and some empty vessels.



**Figure 5**. Comparison of vascular occlusion occurrence among different internodes of the two shoots of a same vine. "Ai" and "A" are the shoots with *Xf* inoculation and without inoculation, respectively. The number following "Ai" indicates a specific internode with the positive or negative number showing that the counting of internode started from the inoculated internode and moved upward (positive) or downward (negative), respectively. The number following "A" shows the internode in the non-inoculated shoot, counted from its base.



**Figure 6**. Other types of vascular occlusions in infected grapevines. A. Gels in a vessel lumen. B. Gels covering the lateral wall of a vessel. C. Gels sparsely attached to the vessel lateral walls. D. Crystals filling a vessel lumen.

### Comparison of vascular occlusion formation between control vines and sick vines

PD susceptible Chardonnay vines were used in our experiment. Each chardonnay vine on rootstock was pruned back with only two buds left at the base. The two buds thus develop into two branches. When the branches are six weeks-old, one branch of each treatment vine was needle-inoculated with *Xf* at the 12th internode from the base. Vines for controls were inoculated at the corresponding internode with phosphate buffer also on one of the two branches for each vine. Both

branches of each vine (control and treatment) were kept about 25 nodes in height by pruning the top off. Samples were collected from both branches of each vine for both control and treatment vines at different times after the inoculation. Included here are only the data from the vines at Week 12 after inoculation when severe external PD symptoms of the treatment vines have developed.

The vines inoculated with Xf and those inoculated with buffer showed obvious differences in secondary xylem structure (**Figure 4**). In control vines, no vascular occlusions were observed in secondary xylem, even in the internode with the inoculation of buffer (**Figures 4A and B**). In vines treated with Xf, extensive formation of vascular occlusions occurred in secondary xylem vessels (**Figure 4C**). Vascular occlusions in infected vines were not even in vessels across the transverse section. Instead, in some regions of xylem, they were present in most of the vessels (**Figure 4D**), while in other regions, some vessels were free of vascular occlusions (**Figure 4E**). The cause for patchy occurrence of vascular occlusions in secondary xylem is not known.

Investigation of the spatial distribution of vascular occlusion indicated that it occurred to the internodes of both branches of each infected vine, no matter how far away the internodes were from the inoculation site. Quantitative analysis of vascular occlusions revealed that the percentage of the vessels with one or more vascular occlusions was usually around 60% in all the examined internodes and that no big difference can be distinguished between the two branches of each vine as well as among different internodes of each branch (**Figure 5**).

When tracking through vessels in the longitudinal direction, we found that tyloses did not always continuously block a whole vessel; a given vessel may have some gaps where no occlusions developed. With this in consideration, the actual percentage of vessels affected by vascular occlusions should be higher than the value measured at any transverse section. The effect of vascular occlusion on hydraulic conductivity of xylem is to be evaluated.

Our investigation also clarified that three types of vascular occlusions excluding *Xf* formed in secondary xylem. Tyloses are the predominant type and accounted for over 95% of the occlusions in vessels (**Figures 4C and D**). Pectin-rich gels were another type of occlusion observed; these formed usually in less than 3% of the total vessels (**Figures 6A-C**). Occasionally, crystals were found in the vessels of infected vines and may partially or completely block the affected vessels (**Figure 6 D**).



**Figure 7**. Distribution of *Xf* in infected vines. A. Bacteria are mostly present freely in the internode just above the internode with the inoculation site. B. Many bacteria in an aggregate in the 9th internode (the counting started from the inoculated internode with it as zero). C. Some free bacteria and some bacteria in an aggregate in the 9th internode of the non-inoculated shoot (the counting started from the shoot base with the lowest internode as one). D. Free bacteria in the 17th internode of the non-inoculated shoot (the counting started from the shoot base with the lowest internode as one). E. A vessel filled with tyloses in the lowest internode of the non-inoculated shoot. Gels were present between tyloses. F. Enlargement of the rectangle region in E, showing bacteria embedded in the gels.

### Distribution of Xf after inoculation

In the vines with severe external PD symptoms, *Xf* cells were observed in all the examined internodes of the two branches (**Figure 7**). This indicated that the bacteria could move not only upward from the inoculation site in the shoot, but also travelled downward, from the inoculated shoot to the trunk shared by the two branches, and then moved into the non-inoculated branch and travelled up towards its top internodes.

Our observations also indicated that bacteria in the vines with severe external PD symptoms were present in very few vessels. Vessels with *Xf* were usually less than 10% and 3% of all vessels in the inoculated and non-inoculated shoots, respectively. The number of bacteria in the affected vessel was also larger in the internodes of an inoculated shoot than in those of a non-inoculated shoot. However, no vessels with enough bacteria to completely block vessels were observed, as suggested by some earlier studies . Since *Xf* are only present in few vessels in limited amount, a direct influence of bacterial inhabitation on the water transport through the vessel system should be very limited.

*Xf* were present in vessel lumens in several different forms. Most commonly, they occurred as free individuals (**Figures 7A and D**). Bacteria in this form were observed in the internodes of both inoculated and non-inoculated shoots. Aggregates of 2-6 cells were also common, in which bacteria are loosely bound together through a filamentous network (**Figure 7C**). Occasionally, aggregates formed by tens or hundreds of bacteria were observed in some vessel lumens (**Figure 7B**). Bacteria were also observed between loosely or compactly arranged tyloses (**Figures 7E and F**). In this case, bacteria were always embedded in gels whose origin (tylose or bacterium) is not clear.

### CONCLUSIONS

- 1. Grape varieties/genotypes with differential PD resistance show differences in the cell wall polysaccharide composition of intervessel PMs. The intervessel PMs of resistant genotypes lack fucosylated xyloglucans and weakly Me-esterified HGs, and contain only a little amount of heavily Me-esterified HGs, while the PMs of the more susceptible genotypes/varieties all have fucosylated xyloglucans, and contain substantial amounts of either heavily Me-esterified HGs or weakly Me-esterified HGs. The absence of polysaccharide substrates for *Xf*'s CWDEs in intervessel PMs of resistant genotypes may limit the ability of the pathogen to move away from the inoculation point and, thus, may contribute to the localized distribution of *Xf* in host plant and its PD resistance.
- 2. Multiple types of vascular occlusions (tyloses, gels and crystals) may develop in infected vines, but tyloses are the principal occlusion type which blocks the majority of vessels, contributing the symptom development.
- 3. *Xf* may occur in diverse forms (singly, or in groups) and in different parts of the vines with severe PD symptom, but the *Xf* cells are present in only few vessels where they are too low in number to block the vessels.

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