THE DEVELOPMENT OF PIERCE'S DISEASE IN XYLEM: THE ROLES OF VESSEL CAVITATION, CELL WALL METABOLISM, AND VESSEL OCCLUSION

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INTRODUCTION

This proposal is directed toward discovering the plant responses to infection that are fundamental to the progression of Pierce's disease (PD) in grapevine. The disease is caused by the growth of the bacterium *Xylella fastidiosa* (*X.f.*) in the xylem vessels of stems, petioles and leaf blades. The disease progresses rapidly, causing severe water deficits in infected shoots and vine death–often within two years. However the progression of the disease and the mechanism(s) by which the disease produces water deficits and death in infected tissues have not been well established.

The prevailing notion is that vessels become occluded with bacteria or products of metabolism. However, it is unclear how the bacterium moves through and between vessels, whether vessels cavitate upon introduction of the bacterium by the insect vector or artificial inoculation, and whether PD symptoms can be found in tissues at a distance from local concentrations of bacteria. The bacterium is reported to be larger than the openings in pit pore "membranes." Thus, it is likely that cell wall digestion is necessary for movement of the bacteria through the vine. This digestion may be a key component of disease progression. The studies in our project are designed to test the following hypothetical "model" of the events contributing to the development of PD.

X.f. introduction to vessels—>vessel cavitation—> initial water deficit—> *X.f.* population increase—> production of enzymes by *X.f.* (signals ?) —> cell wall digestion —> oligosaccharide signals —> ethylene synthesis rise—> a "wave" of vessel occlusion beyond the infection site —> global collapse of vine water transport—> leaf abscission—>vine death

OBJECTIVES

For this research period, the work in our project has been closely coordinated with work in the new project led by Rost and Matthews. Our project follows several aspects of PD development following introduction of *Xylella fastidiosa* (*Xf*) to grapevines by hypodermic injection to basal stem internodes. The focus of our effort in this period has been on factors that limit systemic spread of the *Xf* population and contribute to reduced water movement in the xylem.

1. Determine the "porosity" of the pit membranes that regulate movement from one xylem vessel to the next.
2. Determine whether digestion of cell wall polysaccharides in the pit membrane is required for passage of *Xf* through the xylem.
3. Determine how quickly, post-inoculation with *Xf* obstructions occur in the xylem.

RESULTS AND CONCLUSIONS

Factors limiting movement of bacteria and water in grapevines:

Using PCR-based detection of bacterial DNA sequences, we observed the rapid (within 14 days) spread of *Xf* up inoculated shoots of small grapevines (30 – 40 cm in height). This raised the possibility that the bacteria were moving unimpeded through the xylem. This could occur because a significant population of the vessels was sufficiently long that bacteria could be swept along in the transpirational stream without encountering a vessel end and, hence bordered pit. Alternatively, the pits might not offer the expected restriction to bacterial movement among vessels. Analysis to determine the length of the longest vessels in shoots of other experimental material (80 - 100 cm shoots) has indicated a 30 - 50 cm maximum, with a mean of 34 ±12 cm . By comparing the air flow rates through stems from which apical segments were repeatedly excised, it is possible to observe the relative distribution of vessel lengths. This analysis indicated that most vessels were less than 15 cm long (Figure 1). Thus, there appear to be very few paths longer than about 10 – 15 cm that do not include a vessel end. However, we have not performed the same analysis on the shorter stems used in the experiment showing rapid systemic spread of *Xf* (above).

Electron micrographs published by others have given the impression that *Xf* size is too large to pass through the cell wall meshwork in the xylem "pit membranes" that fill the pit passageway from one water-conducting xylem element to the next. *Xf* dimensions appear to be ca. 0.5 by 1.5 μm while the gaps between the cell wall elements that comprise the pit membrane appear to be no larger than 0.3 μm in size. It was important that we be certain that the pit membrane "pores" would block bacterial passage. Our hypothesis is that passage through pits could occur only if bacterial wall-degrading enzymes were able

to digest a pathway through the polysaccharides of the pit membrane. That would be unnecessary if its normal pore size did not limit *Xf* movement.

The distal cut end of an explanted stem of a healthy vine was attached to a vacuum pump that was adjusted to apply a negative pressure of 0.5 atmospheres. The proximal cut end was then placed in a flask containing water plus various test materials. We first followed water movement through the stem for a set period of time by measuring the volume of water that exited the distal end. After drawing water for a time, the proximal end was placed in 10 mM KCl in water. When a steady state of flow was reached, the water movement was measured again. The test was repeated using a 50 mM KCl solution. With each increase in salt concentration, the volume of water moved increased. This result reflects earlier reports that concluded that the increasing ion concentration had reduced the water shell around the polysaccharides in the pit membrane and this, in turn, had decreased the resistance of the membrane to water flow.

The experiment was repeated, this time with red-stained polystyrene beads of defined dimensions. The idea was that the beads would serve as useful surrogates for the non-motile *Xf* cells that had been introduced to grapevines by the glassy-winged sharpshooter or the "needle stab" inoculation technique we were using. We used beads of 1.0, 0.5, 0.3 and 0.029 μm average diameter. No beads of any size were moved the length of the stem segments tested, no matter which test solution was used (Figure 2). These experiments were conducted with shoots that were longer than the longest vessel in the test shoots, so a bead would have had to pass through at least one pit membrane on its path from one end of the stem explant to the other. This test was repeated using soluble, naturally colored proteins of known molecular weight and predictable, average molecular diameters. Cytochrome c (MWt of 14.8 kD, diameter of 0.005 μm) was not drawn up the stems when it was dissolved in water; it did move slowly when it was in 10 mM KCl and more quickly when it was in 50 mM KCl. Hemoglobin (MWt of 64 kD, diameter of 0.088 μm) was drawn up the xylem only when it was dissolved in 50 mM KCl and the rate of movement was quite slow compared to that for cytochrome c.

The beads of 0.029 μm diameter represent less than 2% of the estimated volume of a *Xf* cell and have a diameter that is less than 10% of the bacterial “width.” Therefore, these experiments confirm that the cell wall mesh of the pit membrane represents a substantial barrier to the movement of *Xf* from one vessel to the next as long as it is intact. The experiments also indicate that the pit membrane mesh provides much smaller pores than had been suggested in some earlier reports and that the chemistry of the xylem fluid can have an effect on the resistance to water flow in the xylem.

Do bacterial populations spread systemically because they produce enzymes that digest pit membranes?
The Xf genome contains DNA sequences that are predicted to encode cell wall-degrading enzymes like polygalacturonase (PG, a cell wall pectin-digesting enzyme) and endo-β-1,4-glucanase (EGase, sometimes called cellulase). Graduate student Caroline Roper has cloned the PG-like sequence of the PD-causing bacterium and is now attempting to get the cloned gene to be expressed in *E. coli*. Once the protein is isolated, we will confirm its activity and then introduce it into explanted stems to see if it opens the xylem to passage of beads or killed *Xf* cells, presumably by breaking the pit membrane cell wall mesh. Currently we have a few non-*Xf* PGs to use in the same sort of test. The experiment is made more complicated because the PG protein is too large to pass the intact pit membrane “barrier.” The result of this test may be available by the time of the Symposium.

Xf-induced xylem obstructions:
Last year, we reported on microscope-assisted observations that showed both tyloses and plant cell wall-derived "gels" obstructing many of the vessels in PD-infected grapevines. We have observed tyloses in vessels of *Xf*-inoculated grapevines as early as 4 weeks after introduction of an aliquot of bacterial suspension (Figure 3). Dr. Josh Stevenson (in the Rost/Matthews project) has developed these investigations further and will report additional details about observations of tyloses and gels in the vessels of stems and leaf petioles and midribs of PD-infected vines. Briefly, reduced hydraulic conductance in petioles is correlated with the accumulation of bacteria and gums in the petiole. Data in Table 1 indicate the correlation of PD leaf symptoms with reduced hydraulic conductance following stem inoculation with *Xf*. A related study, focusing on *Xf*'s impact on grapevine water movement is focusing on the hypothetical production, by *Xf*, of exo-polysaccharides in xylem vessels. Based on Dr. Stevenson's observations, we are now using a direct extraction and chemical analysis approach to determine whether the amorphous gels occluding vessels in petioles and leaves of infected vines contain bacterial polysaccharides like those predicted to be produced by *Xf*, based on gene sequences identified in its genome. Our interest is in coincident microscopic observation of gels and chemical measurement of bacterial polysaccharide component sugars.

In stems, few bacteria and little gum has been found to accumulate. There is an increased frequency of tyloses in stems of PD-infected and symptomatic vines. Our hydraulic conductance measurements have seldom revealed reduced water transport in stem segments. However, our samples may have been of young tissue not yet competent for extensive tylose development. Also, our hydraulic assay methods could have repaired cavitated vessels. Therefore we are developing other approaches to quantify cavitation, including a pneumatic assay that should prevent refilling, ultrasonic acoustic emissions, and the imaging technique described below.

Attempts to "see" points of reduced water flow in intact grapevines:
Typical tests of grapevine water-conducting capacity require that the stem be explanted and then tested. The decommissioned McClellan Air Force base in Sacramento houses a nuclear reactor that is a source of fast neutrons. This source is being made available for use in fundamental research. It may provide an opportunity for visualizing water flow through intact grapevines. Preliminary tests using well-watered and water-stressed vines confirmed that images (analogous to X-rays) can show differences in water moving through the stems. We are continuing pilot studies, working with the very cooperative McClellan staff scientists in attempts to enhance contrast in the photographic images that constitute the data record of these experiments. Our hope is to use the observation of points of reduced water flow in intact healthy and diseased vines to guide us in the "destructive" sampling for bacterial presence and observations of xylem obstructions. Consistent observations of obstructions in regions of reduced water transport, whether or not significant populations of *Xf* are co-localized, will be of importance in developing our ideas about progression of PD symptoms and *Xf* populations in the xylem.

Preliminary conclusions:
The picture that is emerging is that PD leaf symptoms are seen in inoculated vines at times when bacterial populations are small and that reductions in water flow may occur when no bacteria are detected. This seems to suggest that PD symptoms can develop in advance of the systemic spread of *Xf* because of the acropetal movement of thus far undefined signals that trigger responses in the xylem. We continue to address questions that are relevant to this preliminary conclusion, a conclusion that is at the center of the hypothetical PD "model" that formed the core of our proposal.

Table 1. Estimated hydraulic conductance (Kh), presence or absence of PD symptoms, and presence or absence of Xf for petioles of leaves from PD-infected and control plants**.** Kh was determined as flow rate through excised petioles at pressures of 0.8 – 1.6 bars. Presence of bacteria was determined from SEM micrographs of similar and adjacent leaves.

Treatment	**Kh (ml/bar/sec) n = 3 or 4**	**+/- presence of *Xf.***	**+/- leaf chlorosis symptoms**
- PD Control	0.53 – 0.83	-	-
+ PD Inoculated*	0.74 – 1.53	not assayed	-
+ PD Inoculated**	0.13 – 0.17	+	-
+ PD Inoculated	0.01 – 0.1	+	+

*These leaves were located (6 – 14) nodes acropetal to the symptomatic leaves.
**These leaves were located 1 or 2 nodes acropetal to the symptomatic leaves.

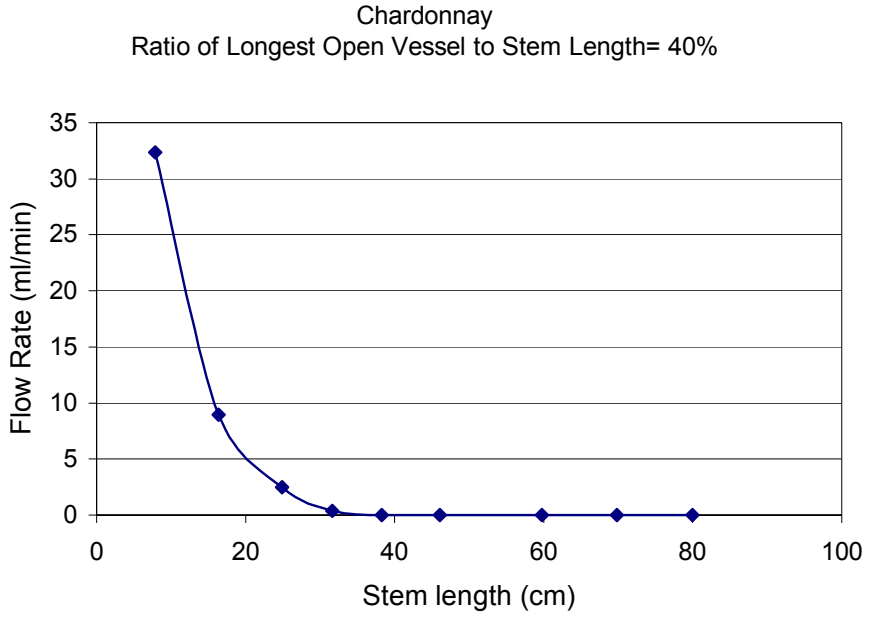
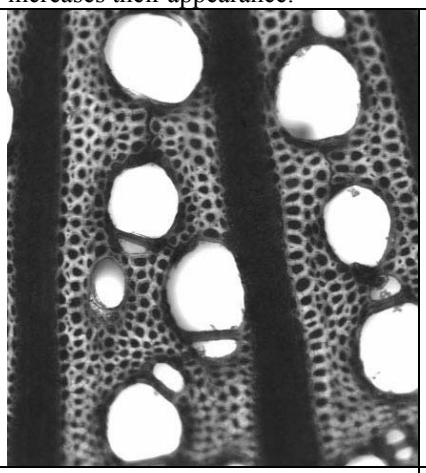
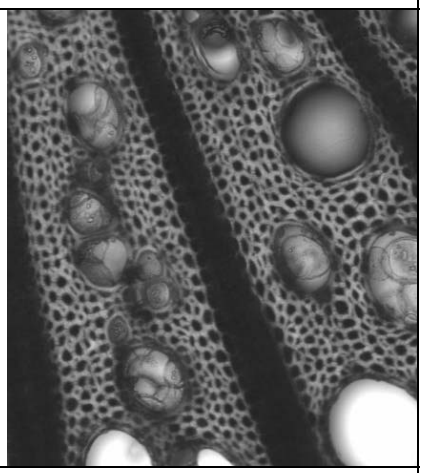


Figure 1. Rate of air flow through stem segments of varying length. Air was supplied to basal internode at pressures of 0.4 – 0.6 bar, and its rate of escape from the apical end was recorded after repeated excision of stem segments from the apical end of the shoot. The low pressure should not have passed pit pore membranes. Therefore, the flow rate should reflect the number of open vessels present.

Figure 2. Polystyrene beads in a grapevine xylem vessel.	**Figure 3.** Sections through the stems of young grapevines reveal extensive xylem blockage in vessels of all developmental ages. While tyloses are occasionally seen in uninoculated vines, introduction of *Xf* substantially increases their appearance.	
		
Red polystyrene beads (0.3 µm diam.) filling one vessel in this light microscope view of a cross-section of a grapevine stem. Note that there are many other "open" vessels which contain no beads. This suggests that the other vessels in this view did not extend all the way to the basal, cut end of the stem where beads were introduced.	Light microscopy examination of a section through the stem of an uninoculated grapevine. Vessels are seen to be unobstructed.	Light microscopy examination of a section through the stem of a grapevine 4 weeks after inoculation with *Xf*. Most of the vessels are obstructed by one or many tyloses. PCR analysis of other individuals in this set of test plants showed *Xf* throughout the vine. Typical PD leaf symptoms did not appear in these vines for 4 more weeks.

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