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* (*Xf*) 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 among vessels, whether vessels cavitate upon introduction of the bacterium by the insect vector or during artificial inoculation, and whether the bacterium must enter vessels in order to cause disease symptoms. 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.

Based on work that we have done (VanderMolen et al., 1983) with the *Fusarium oxysporum* vascular wilt pathogen that also causes blockage of its plant host's vascular system, we anticipate a linkage between cell wall metabolism, xylem occlusion, vine water stress and leaf abscission. If the disease develops in a manner similar to fusarium wilt, the plant may respond to oligosaccharide "signals" (Melotto et al., 1994; Ryan and Farmer, 1991) produced upon digestion of its own cell walls and, consequently, vessels that have not been infected with bacteria may nevertheless become occluded. Therefore the "occluding" material could be of grapevine origin and component of the plant defense responses to infection. There also is reason to suspect that each vessel cavitates upon bacterial entry, rendering the vessel unable to transport water (Schultz and Matthews, 1986 and 1993; Zimmerman, 1983). Thus cavitation may be the fundamental cause of impaired water transport and leaf death. The proposed work will establish whether the vessels become occluded with material of bacterial or *Vitis* origin. It will also determine the role of vessel occlusion and cavitation on the progression of PD.

OBJECTIVES

1. Determine the impact of infection by *Xylella* on the water status of grapevines using both destructive and non-destructive measurements of stem and leaf water potential and water conductivity.
2. Determine the chemical nature of the xylem-occluding material.
3. Determine the nature of the grape cell wall degradation that is caused by *Xylella*: What cell wall components are digested and what wall-digesting enzymes do the bacteria make or cause the grapevine to make?
4. Determine if oligosaccharide signals influence the progression of the disease.
5. Determine whether the plant hormone ethylene is produced when the bacterium infects grapevines and, if so, whether grape responses to the ethylene influence the development of xylem occlusions and other PD symptoms.

RESULTS AND CONCLUSIONS

We began our efforts on July 1, 2001. The approach we have taken in the first part of the study is to determine which technical approaches will be most useful to test individual components of our hypothesis about how PD progresses following infection with *Xf*. Our "model" for PD development is:

***Xf* introduction to vessels—>vessel cavitation—> initial water deficit—> *Xf* population increase—> production of enzymes by *Xf* (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**

Thus far we have performed one large scale inoculation test in which a population of Merlot vines was infected with *Xf* using the pinprick technique (Purcell and Saunders, 1999). Infection rate was low and PD symptom development was slow (little disease after 10 weeks) but we were able to make several important observations and test techniques. Initially we have used immunocapture PCR (Smart et al., 1998) to determine *Xf* presence in inoculated vines. Vines were sampled every two weeks and tissues (internodes, nodes and leaves at, above, and below the point of inoculation) were tested for *Xf* presence. In addition, water conductance through stem segments was measured. PD symptoms were clear in a few vines sampled 8 weeks after inoculation. At this time, PCR revealed a substantial bacterial population in the internodes that had been inoculated, but bacterial DNA was not found in extracts of other vine tissues. We have done tests of the PCR system's sensitivity and know that a few hundred bacteria can be readily detected. We also have shown that the inoculation technique efficiently introduces a large *Xf* population to the **interior** of the vine (i.e., bacteria are not removed from the inoculation site by scrubbing with detergent). Therefore we conclude that bacteria were introduced during inoculation, that the number of bacteria that moved from the site was quite low, and that at least one PD symptom (reduced water movement) can be detected (Figure 1) in the absence of a large *Xf* population. We have begun a second inoculation trial with a population of young Merlot vines and hope to have greater success in promoting PD development.

Among the approaches we will use to enhance PD development is controlled water stressing of the vines and alternative approaches to vine inoculation. We have found that mock pinprick inoculation using water-soluble dyes instead of bacteria leads to rapid movement of dye both up and down the cane. Thus the introduced dye (and presumably also a suspension of *Xf*) has access to the vascular system and at least some vessels remain functional. However, we have used PCR to show that *Xf* that has been introduced by pinprick does not spread out of the inoculated internode within 20 hours of inoculation. We presume that because the bacteria are particulate and in suspension (rather than in solution like the dye) they are not readily taken into the xylem and that vessels near the pinprick will have cavitated and be non-functional, at least for a time. On the other hand, bacteria introduced directly into the xylem by the sharpshooter insect vector may be more immediately mobile if damage to water-conducting elements is not extensive.

We have carried out a trial where bacteria were introduced to young canes by cutting the stems under water and placing them in an *Xf* suspension. In this situation, we were able to detect *Xf* with PCR at least four internodes above the cut internode within three hours. We will use a more quantitative PCR approach to determine the relative concentrations of *Xf* in each of the internodes, but it is clear that bacteria can move relatively freely through functional vessels. In a parallel test, dye moved to the top of the canes (>12 internodes) within minutes. This experiment also indicated that some continuous xylem vessels extend through several grapevine internodes or that the pit membranes that allow one vessel to pass water to its neighbors are not barriers to *Xf* movement. We will examine this point further because one tenet of our hypothesis is that cell wall degradation, of pit membranes within the xylem, is an important factor in bacterial movement. One difficulty that all researchers of PD must face is the slow progress of the disease. Our tests with introduction of *Xf* via cut stem ends makes clear that a substantial inoculum can be introduced into the xylem. Therefore we will begin testing an infection technique that utilizes direct "xylem feeding" of bacteria into vines. We have experience with xylem feeding of compounds into woody stems of almond. We should be able to seal grape stem flaps through which bacteria are introduced and the wounds may heal. This approach could allow more rapid testing of aspects of our PD model (above) that are downstream of the proposed "***Xf* population increase**" stage.

An important aspect of our model is the idea that the bacteria produce enzymes which digest the primary cell wall present in pit membranes and that this facilitates systemic *Xf* spread and generates oligosaccharide signals that cause grapevine responses leading to vessel blockage and reduced water transport. We have evidence that cell wall digestion occurs in canes that contain substantial *Xf* populations and show PD symptoms. Graduate student Caroline Roper and Dr. Carl Greve have found that low molecular weight sugars of the sort found in the secondary cell walls of vessels (particularly

xylose) are found in the small amount of xylem sap that is expressed from infected stems and that the amount of xylose in the cell walls of these stems is also reduced. Presumably xylan polysaccharides are being digested in these stems. One problem with this analysis is that severely infected tissue may support growth of microbes other than *Xf* and so the digestion we see may not be due to the PD causal agent. The work with inoculation by stem feeding may make clearer both that wall digestion occurs and *Xf* is responsible. We have also looked for cell wall-digesting enzymes in the medium of bacterial cultures. Thus far we have not identified any activities. However, bacterial wall-digesting enzymes are generally under close control and we do not have a good minimal medium for *Xf* growth to which we can add possible inducers of wall-modifying enzymes (generally wall digestion products). We hope to obtain guidance from other PD researchers so that we can mimic in culture the environment the bacterium would experience *in vivo* and test the potential for *Xf* to digest grapevine walls.

Many aspects of our work require that we correlate *in vivo* the presence of *Xf* and obstructions in the xylem. Work in other labs is attempting to introduce genes encoding green fluorescent protein into the *Xf* genome. This will greatly facilitate histological screening for *Xf*. However, undergraduate Phillip Bates, working with Rost and Greve has developed a number of techniques to enhance our examination of vine vascular system anatomy. The primary antibody used in our immunocapture PCR (above) recognizes and binds to a surface component of *Xf*. We have obtained a second antibody that is tagged to the histochemical dye Texas red. When this dye-antibody conjugate is used in conjunction with the primary antibody we can stain *Xf* bacteria in tissue sections (Figure 2). Additional histochemical approaches have allowed us to see tyloses (cellular "balloons" protruding through pit membranes from adjacent parenchyma cells into vessels, Figure 3) and gel-like vessel occlusions in PD-infected stems (Figure 4). Because these gels stain with both the non-specific stain methylene blue as well as the pectin-specific stain ruthenium red, we presume that they are of grape plant, rather than *Xf* origin and are like the vessel-occluding gels we identified in *Fusarium*-infected tissues several years ago. (VanderMolen et al. 1986).

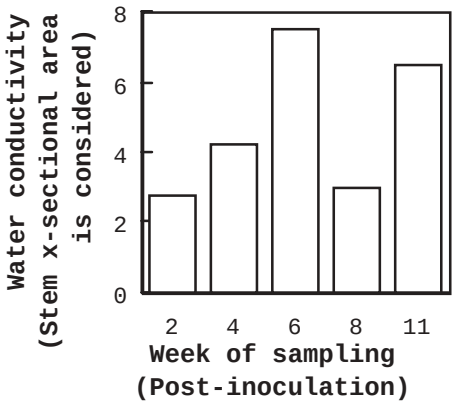

Figure 1. Water transport is reduced in infected grape stems sampled 8 weeks after inoculation. Water transport increased as stems grew (weeks 2-6). The symptomatic week 8 sample showed reduced water transport. Week 11 samples did not show symptoms.

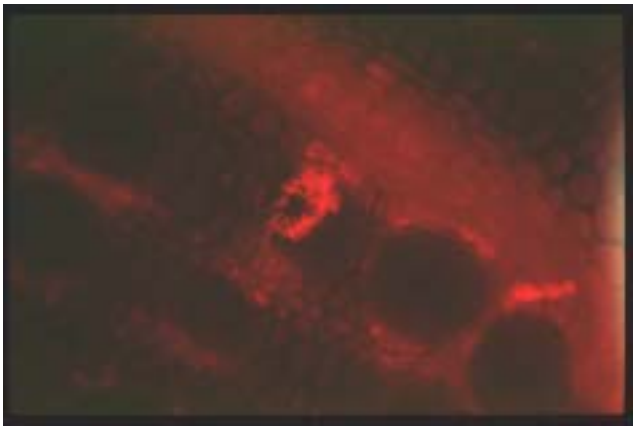

Figure 2. This section of an inoculated young grape stem cuts across three water-conducting vessels. One of the three contains *Xylella fastidiosa* cells which are seen as the red fluorescent ring revealed by immunostaining with the Texas red-anti-rabbit antibody conjugate.

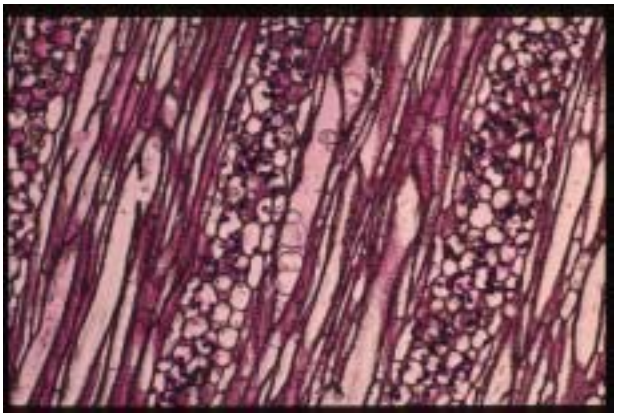

Figure 3. The longitudinal section across the xylem of a heavily infected grape stem reveals several tyloses. The section is stained with periodic acid-Schiff's reagent and the tyloses are seen as membrane surrounded "bubbles".

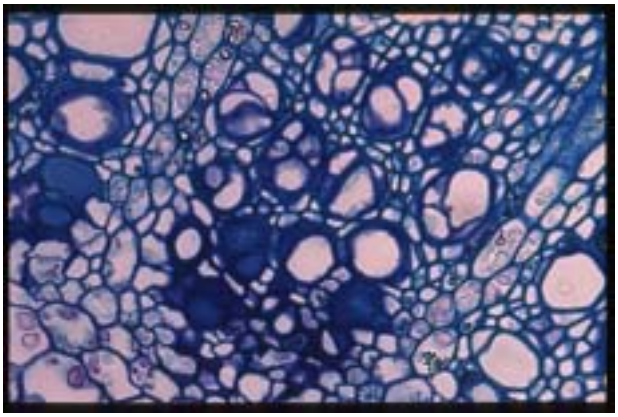

Figure 4. This section across the xylem of a heavily infected grape stem is stained with methylene blue. Tyloses are seen in section and grazing cut in some vessels and occluding "gels" are seen as amorphous stained bodies in other vessels.

Our work began in July and so we have only limited findings to describe in this report. However, we anticipate results from our second large inoculation trial and several of the specific approaches described above by the time of the December meeting. We also hope to report on results from experiments testing links between *Xf* presence, ethylene production in vines, and altered vessel anatomy and water transport. We anticipate also results from preliminary work aimed at understanding further the chemical nature of vessel occlusions.

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