Title: Mechanisms of Pierce’s disease transmission in grape vines: The xylem pathways and movement of Xylella fastidiosa

Time period covered by the report: 7/1/02 to 6/30/08

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This report is organized as a series of summaries of the issues, results, and conclusions from segments of our work, in general moving back in time from recent to past:

Xylem tension required to cause cavitation and spread embolisms from GWSS feeding and Xf-induced xylem occlusion

Plant responses to Xf that gives rise to Pierce's disease symptoms

Xf movement and xylem structure in susceptible and resistant grapevines and in alternate hosts

Movement of Xf through rootstock-scion grafts

Tylose and gel symptom development in grapevine

Xylem conduits and the path of Xylella in grapevine stems, leaves, and berries

Comparative analysis of the symptoms of Pierce’s disease and of water deficits in grapevine

Progression of symptoms of Pierce’s disease in grapevine

Analysis of the vascular anatomy that facilitates Xylella movement in grapevine
Summary:

This project addressed the diseased plant. After identifying that there was little background on the nature of PD and its progression in grapevine in both time and space (where in the plant), our approach has been to lay down some fundamental groundwork for analysis of when and where technology or conventional breeding might intervene in the disease process. We found that although much work cites the need for *Xylella fastidiosa* (*Xf*) movement and the role of xylem occlusions in Pierce’s disease, there was actually quite limited investigation into the patient – particularly structural characteristics of the movement pathways and metabolic responses to the presence of the bacteria that should form a foundation for pursuit of traits or genes that will ameliorate the devastating consequences of *Xylella* infection in cultivated grapevines.

The specific objectives have varied over the several years that we were supported. Always the overarching objectives were to discover how the plant experiences and deals with infection by *Xylella fastidiosa*; how, when, and where the bacterium moves throughout the grapevine; how, when, and where symptoms develop; and what role water deficits play in symptom development. We elucidated much of the gross xylem pathways through which the bacteria can become systemic in grapevine stems, leaves, berries, and graft unions. We discovered long open conduits through the stem and into leaves that may facilitate axial movement of bacteria – even through graft unions. And, we have obtained NSF funding to further clarify the 3D organization of xylem and test whether its organization may have evolved to limit *Xf* movement using CAT scan and NMR imaging technologies. We clarified the timing and anatomical nature of most PD symptoms: matchsticks (not proper abscission zones); green islands (result of inhibited periderm development); leaf scorch (correlates with ethylene and other indicators of programmed cell death); tyloses (dependent upon ethylene and not dependent upon cavitation).

Our lab has produced several lines of evidence that factors other than vessel occlusion are important in PD effects on grapevine growth and productivity. Resolving this debate is important because *Xf* pathogenesis has become synonymous with vessel occlusion in the Pierce’s disease literature (eg. Fry and Milholland, 1990; Hopkins, 1989; Krivanek and Walker, 2005; Newman et al., 2003) without having a single report that demonstrates a strong and positive correlation between symptoms and concentration of bacteria. *Xylella fastidiosa* is vectored to and lives in many plants at high concentrations without causing disease, but causes vessel occlusions and leaf scorch in PD-susceptible grapevine. If the disease is in essence water deficits, strategies to improve and/or maintain water transport should be pursued; but if the disease arises from a metabolic response to the pathogen as is commonly observed in plant disease, the specific metabolism involved should be elucidated and dissected for potential intervention points. Important among our lines of evidence is the possible role of ethylene in the disease process – a stress hormone whose action may be a PD intervention target. Ethylene mediates tylose formation in response to wounding, analogous to tyloses that form during PD. We developed a highly sensitive qPCR assay for *Xf* in leaf and stem tissue, but found no clear relationship between bacterial population and PD symptoms in any of our studies. Thus, high and localized concentrations of *Xf* may not be necessary for the formation of leaf scorch symptoms. On the other hand, our observations are consistent with an etiology that involves a systemic plant response. Grapevines produce copious tyloses and gels in response to specific stimuli: wounding, *Xf* infection, and exogenous ethylene treatments. Our published work demonstrated that ethylene action is required for tylose occlusions to develop in grape stems. Our unpublished data (described below) are consistent with the hypotheses that ethylene production is integral to the processes of symptom (tyloses, gels, leaf scorch) formation during PD.
Xylem tension required to cause cavitation and spread embolisms from GWSS feeding and Xf-induced xylem occlusion

The Cohesion-Tension theory predicts that sap is transported at considerable tensions through the vascular network of plants. As a result the sap is inherently unstable and will cavitate with increasing probability the greater the tension it is under. Once the sap in a xylem vessel cavitates, an embolism rapidly forms blocking the vessel and reducing the conductivity of the stem that contains that vessel. A common way to gauge how plant water transport is affected by cavitation is to construct a vulnerability curve where the percent loss in conductivity (with respect to maximum conductivity without embolism) is plotted against stem water potential. The lower the water potential (greater tension) reached for a certain percent loss in conductivity, the less vulnerable the plant.

Grapevine has been considered a vulnerable species due to its large xylem vessels. And the tyloses that form during Pierce’s disease have been thought to depend on prior embolisms. Cultivated grapevine (Vitis vinifera L.) was assessed for vulnerability to cavitation and embolism by dehydration, air-injection, and centrifuge methods. The stem water potential at which 50% loss in conductivity occurred (Ψ50) was significantly higher for the centrifuge method (-0.7 MPa) than for either dehydration (-2.23 MPa) or air-injection (-2.3 MPa) methods. Native embolism at the end of the growing season was at 17% in Chardonnay which compared to 9%, 19% and 87% predicted by dehydration, air-injection, and centrifuge respectively. The anomalous vulnerability curve predicted by the centrifuge technique was probably a result open-ended vessels spanning the entire sample length. Previous estimates of grapevine vulnerability are high because of the use of the centrifuge technique or because of high levels of initial embolism before dehydration commenced. Grapevine is not as vulnerable to cavitation as previously stated in the literature. Therefore, the extent of Xf-caused xylem occlusion and/or Xf feeding caused xylem cavitation that would be required to significantly decrease functional water transport is correspondingly greater than previously assumed.


Plant response to Xf that gives rise to Pierce’s disease symptoms

Xf infection results in a variety of symptoms the most conspicuous of which is leaf lesion formation. Previously (Gambetta et al. 2007), we showed a disconnect between leaf lesion formation and bacterial populations, suggesting the involvement of a systemic plant defense response. We investigated the kinetics and hormonal control of leaf lesion formation. In symptomatic leaves of Xf-infected grapevines there was a high incidence of TUNEL positive nuclei just proximal to the necrotic lesions, an increase in DNA degradation, and a loss of cell vitality as determined by fluorescein diacetate (FDA) staining. The rate of lesion formation was extremely variable between, but not within, individual plants and the speeds at which lesions first appear were positively correlated with leaf age. There was a large increase in ethylene evolution during leaf lesion formation and this increase corresponded to increases in providing evidence that leaf scorch is programmed cell death, perhaps involving the jasmonate-ethylene mediated signal pathway in the development of plant defense. Taken together these results suggest leaf lesion formation during Pierce’s Disease represents an accelerated form of leaf senescence. Gambetta, Shagahsi and Matthews. In preparation.
**Xf movement and xylem structure in susceptible and resistant grapevines and in alternate hosts**

In addition to grapevine, the PD bacterium is also known to be naturally present in numerous hosts other than grapevine (Freitag, 1951), spanning at least 28 families of plants (monocots and dicots). Most of them do not show symptoms and, similarly to the resistant grapevine, most of the asymptomatic hosts allow bacterial proliferation, but limited bacterial movement from the inoculation point (Hill and Purcell, 1995; Purcell and Saunders, 1999; Costa et al., 2004; Wistrom and Purcell, 2005; Baumgartner et al., 2005). Although these studies looked at bacterial presence in relation to symptom development and incorporated some assays of movement within the plants, the xylem structure itself, where the bacteria live and accumulate, and how bacteria move were not investigated.

This limited bacterial movement in certain plants may be due to physical differences in xylem structure between systemic (and symptomatic) and non-systemic (resistant) species. Shorter and narrower vessels and the spatial organization of the vessel could be a passive strategy to limit the bacterial movement. If the majority of vessels in current-year shoots are short and narrow, the bacteria must move through many intervessel pits in order to become systemic. There is evidence that Xf is capable of digesting pit membranes in order to move between vessels (e.g., Newman et al., 2003; Scarpari et al., 2003). Although not well characterized, the movement of bacteria over several grape internodes apparently takes place over time periods of several weeks suggesting that movement along the vessel surface and/or digestion pit membranes is a relatively slow process. We recently demonstrated in grapes that Xf can move fairly long distances very fast through long xylem conduits (Chatelet et al., 2006; Thorne et al., 2006). Isolated vessels or small groups of vessels separated by parenchyma cells could limit the systemic spread of the bacteria in alternate hosts. Another strategy in the non-systemic species could be to confine the bacteria to a limited area by a more timely production of tyloses by the paratracheal parenchyma cells. Tyloses are produced by paratracheal parenchyma cells, outgrowing into the vessel lumen via vessel-parenchyma pits (Esau, 1977). They can be induced by environmental stimuli such as wounding and pathogen infection (Biggs, 1987; Cochard and Tyree, 1990; Pearce, 1990; Sun et al., 2006). While tyloses may impair xylem function by blocking vessels, they are also involved in wound healing and may inhibit the intrusion and spread of pathogens (Bonsen and Kučera, 1990; Pearce, 1991; Salleo et al., 2002), and infected tolerant grapevine species had more tyloses in their vessels than susceptible species (Mollenhauer and Hopkins 1976).

Consequently, we examined 4 varieties of grapevine with different susceptibility to Xf infection and 12 alternate plant species categorized in two groups: those that move the bacteria (systemic) and those that don’t (non-systemic), to determine whether gross xylem anatomical differences play a role in tolerance to Xf colonization. The results showed that there were little differences between the grapevine varieties or between the systemic and non-systemic alternate hosts. Among the grapevines, the stem of the tolerant variety Sylvaner had smaller vessel diameters and 20% more parenchyma rays than the other three varieties. Among the alternate hosts, the results showed that the open conduits in leaves and the open conduits connecting the stem to the leaves of systemic hosts were slightly longer compared to the non-systemic hosts. From this study, it appears that the basic xylem characteristics (vessel length, stem-petiole-leaf connectivity) are not responsible for the limited bacterial movement in the tolerant species. In grape, there are very few xylem differences between susceptible and tolerant varieties, except for smaller vessel diameters and more parenchyma rays in the tolerant variety examined. However, our findings (narrower vessels and higher number of parenchyma rays) suggest a restriction to bacterial movement imposed at the level of the vessels. Differences in the vessel network, vessel overlapping, spatial organization of the pit fields, structure (thickness, porosity) of the pit membrane, need to be considered if the xylem is to have a major role in bacterial movement. Patterns of colonization should also be investigated.
by inoculating the plants with $Xf$ and following closely its movement. **An important result is the existence of open conduits in all examined species and potentially in every plant species.** If such characteristic is present, it could enable any pathogen present in the xylem to spread rapidly in the entire plant and to reach its target, before the plant defense mechanism could react and block it. In addition, tylose formation didn’t seem to the cause for the limited Xf movement in the non-systemic plants.  

**Movement of Xf through rootstock-scion grafts**

In grapevines, scion-rootstock grafting is a common practice to impart pathogen resistance and to manipulate aspects of grapevine physiology including vigor, yield, and fruit composition. Successful grafting requires the integration of the scion and rootstock vascular networks, and this integration has the potential to facilitate pathogen transmission. Indeed, scion-rootstock grafting has led to the inadvertent transmission of pathogenic viruses. Similarly, natural root grafts provide a potential mechanism for vine-to-vine transmission of pathogens (Epstein 1978). However, surprisingly little is known about the anatomy at the graft union in grapevine, and more specifically, the integrated vascular anatomy across the graft.

Pierce’s disease may be limited to environments that allow successful overwintering which may occur in roots (Feil and Purcell 2001, Purcell and Saunders 1999). We (Chatelet et al. 2006) used the flow of air, xylem-mobile dyes, and xylem-specific bacteria to show that the unwounded grapevine stem forms long open conduits that offer no barrier to pathogen movement. However, shoot-root transitions and graft unions were not investigated. The formation of a graft union originates with the joining of two wounded (excised) stem sections. This wounding leads to extensive vascular occlusions in the phloem (Evert et al. 2006) and xylem (Sun et al. 2006) that may limit vascular integration. We used various methodologies described by Chatelet et al. (2006) to determine to what extent the vascular anatomy of the graft union limits movement across the graft in Vitis vinifera. In addition, we investigate the possibility of passive movement of $Xf$ across the graft union. This work builds upon the understanding that $Xf$ can rapidly move long distances through stems and leaves, suggesting that in Vitis there is limited structural impediment to the systemic movement of vascular pathogens.

Using both xylem mobile dyes and the xylem-limited bacterial pathogen Xylella fastidiosa, we demonstrated that the graft union contains open xylem conduits providing for passive pathogen movement. These open conduits may facilitate bacteria overwintering in below-ground vine tissues of grafted vines and systemic infection in subsequent growing seasons. Thus, from our results, graft unions in stems do not fully restrict the movement of $Xf$ in Vitis vinifera cv. Chardonnay. Although the length of open vessels is reduced by about $\frac{1}{2}$, open vessels cross the graft union as determined by air movement. However, the numbers of vessels that cross the graft are less than 10% in distribution when compared with non-grafted plants (data not shown). In order for $Xf$ to move from a cane or leaf across a graft it would need to be inoculated into a vessel that happens to extend through the graft union, or the bacteria would need to degrade membranes to move into adjacent vessels through bordered pits. Studies with paint and PCR detection of $Xf$ confirmed these results.

**Tylose and gel symptom development in grapevine**

Tyloses are outgrowths of parenchyma cells through vessel-parenchyma pit pairs into the lumen of tracheary elements (Esau, 1977), where they may obstruct sap flow. There are several reports of tyloses in xylem of *Xf* infected grapevines, and we described the temporal development of tyloses as an internal symptom of Pierce’s disease (Stevenson et al. 2004). Although long recognized (Boehm, 1867; von Reichenbach 1845, described in Zimmermann, 1979), little has been resolved regarding the functional significance (Bonsen and Kučera, 1990; Pearce, 1991; Canny, 1997) and spatial and temporal details of tylose formation. Investigations on tylose development have been based mostly on plants infected by or inoculated with pathogens, and in response to wounding. These studies have made clear that in many species the formation of tyloses is a common response to infection by vascular fungi (e.g., *Fusarium*) and bacteria (e.g., *Pseudomonas*) and to mechanical injury; and that in plants of some genera (including *Vitis*) there is genotypic variation in the propensity for tylosis (Fry and Milholland 1990) – suggesting a potential role in susceptibility and resistance.

Tyloses form in xylem vessels in response to various environmental stimuli, but little is known of the kinetics or regulation of their development. Our preliminary investigations indicated that wounds seal quickly with tyloses after pruning grapevine shoots. In this study, qualitative and quantitative analyses of tylose development were made at different depths and times from pruning cuts at basal, middle, and apical stem regions along current-year shoots of grapevines to determine the process of tylosis. Tylose development occurred simultaneously within a single vessel but much separated in time among vessels. The fraction of vessels forming tyloses was greatest in basal (85%) and least in apical (50%) regions. The depth of maximum density of tyloses was 4 mm from the cut in the basal region and 2 mm from the cut in the middle and apical regions. The results show that pruning caused prodigious tylosis in vessels of grape stems that extended to approximately 1 cm deep and to 7 days after the wound, but that about half of the vessels did not become completely occluded. The pattern of tylose development suggests tylosis is generally too slow to prevent movement of mobile pathogens such as *Xf*.

Sun, Matthews and Rost. 2006. Amer J Bot 93, 1567-1576.

Vascular occlusions in xylem conduits is a common response to environmental stresses, and plant species are recognized as primarily tylose-forming or gel-forming. These stresses occur throughout the year, but there is little information on the plant responses to stresses throughout the year and in growing and dormant tissues. In grapevine, both gel and tyloses accumulate during Pierce’s disease and are thought to restrict sap flow. Because we found that wounding quickly produces tyloses, wound-induced vascular occlusions were evaluated by type (tylose or gel), temporal progress, and spatial distribution for grape stems pruned in four seasons through an entire year. Predominantly tyloses and gels were formed in summer and winter, respectively. Cytohistological analyses indicated that wound-induced gels were pectin-rich. Both gel formation and tylose development were complete within 7 days and 10 mm from the cut regardless of the season of the wounding. Most vessels were affected by wounding, but a higher fraction of vessels developed occlusions in summer and autumn (over 80%) than in winter and spring (about 60%). The study is the first to show a single species is capable of producing primarily either tyloses or gels, and that the type of wound-induced occlusion is dependent upon the season in which wounding occurs. Winter conditions limit the wound response to reversible gel formation that may contribute to refilling of embolized vessels in the spring. Thus, *Xf*-induced gels may be a reversible consequence of infection, and tylosis is apparently an irreversible consequence that is the primary effect during the growing season.

Tyloses may impair xylem function by blocking vessels, but they are also a component in wound healing and may inhibit the intrusion and spread of pathogens (Bonsen and Kučera, 1990; Pearce, 1991; Salleo et al., 2002). Several hypotheses have been advanced to explain tylose initiation (Talboys, 1958; Sequeira, 1965; Wallis and Truter, 1978; VanderMolen et al., 1987). One persistent idea is that tyloses form in response to the presence of air embolisms in xylem vessels (Klein, 1923; Zimmermann, 1978; Canny, 1997), although most of the pertinent literature is old. Induction of ethylene biosynthesis is a common response of plants to many of the same biotic and abiotic factors that induce tylose formation (Abeles et al., 1992). In addition to wounding and pathogen infection, natural senescence (Dute et al., 1999), heartwood formation (Chattaway, 1949; Parameswaran et al., 1985), frost (Cochard and Tyree, 1990), and flooding (Davison and Tay, 1985) are conditions stimulating tylose development. All of these stimuli are also known to increase ethylene production. Ethylene production in response to wounding has been demonstrated in a wide range of species (Nilsen and Orcutt, 1996). Infection by pathogens often leads to an increase ethylene production by host plants (e.g. Pegg, 1976; Boller, 1991), and some pathogens have themselves been found to produce ethylene (Fukuda et al., 1984; Arshad and Frankenberger, 1991). Little has so far been revealed about role of ethylene in tylose development, although some research suggested its involvement.

We found a direct relationship between the formation of tyloses in decapitated grapevine stems and ethylene synthesis and action in the wounded tissues. Pruning actively growing grapevines (Vitis vinifera L.) resulted in xylem vessel embolisms and a stimulation of tylose formation in the vessels below the pruning wound. Pruning was also followed by a ten-fold increase in the concentration of ethylene at the cut surface. The wound-induced tylose development and ethylene evolution was similar in stems cut in air or water. Embolisms were present when grapevine stems were decapitated in air, and were absent when the wounding was done in water and the cut end remained in water. The presence and absence of air embolisms was confirmed by cryo-SEM of vessel lumen in severed stems. The resultant tylose formation showed no obvious differences in temporal progress or morphology between these treatments. At least in grapevine, vessel embolisms are not required for wound-induced tylose development. Thus, our data do not support the long-standing notion that tyloses are induced by embolisms. Treatment of the stems with inhibitors of ethylene biosynthesis (AVG) and/or action (STS) delayed and greatly reduced the formation of tyloses in xylem tissue, and the size and number of those that formed in individual vessels. Our data are consistent with the novel hypotheses that wound ethylene production is the cause of tylose formation. The possible role of ethylene in the formation of tyloses and other symptoms of Pierce’s disease should be investigated.


Xylem conduits and the path of Xylella in grapevine stems, leaves, and berries

Studies investigating the interconnectedness of xylem vessels in terms of the movement of xylem-borne microbes have largely focused on single organs, and have shown little or no movement of bacteria or suspended particles between adjacent organs. Xylem-dwelling pathogens have been shown to become systemic, which suggests that microorganisms must move in the xylem, either by digesting vessel member pit membranes, or by somehow moving unimpeded through long vessels to different locations in the plant, or a combination of both. To gain a better understanding of xylem pathways and how bacteria move within the xylem, three different techniques were used to study xylem vessel connectivity between stems and leaves of two grapevine varieties: 1) uptake of light-producing bacteria (Yersinia enterocolitica, Ye), 2) fluorescent bead uptake, and 3) low-pressure air movement. Bacteria, beads and air moved through long and branched xylem vessels from the petiole into primary, secondary and tertiary
veins in the leaf blade of both grape varieties. From the stem, bacteria and air traveled into leaf blade primary and secondary veins of leaves one, two and three nodes above the bacteria or air loading point. This study showed that Xf-sized particles and air can move unimpeded through continuous xylem conduits from within the stem axis into leaf blades, and that bacteria are able to move long distances within minutes from organ-to-organ passively without having to cross pit membranes. Such complex open xylem conduits have not been reported before, and these findings have implications for understanding mechanisms for the systemic spread of pathogens.

This work is the first to show long, branched xylem vessels that facilitate the rapid and passive movement of particles across multiple organs in grapevine.

_Thorne, Young, Young, Stevenson, Labavitch, Matthews and Rost._ 2006. Amer J Bot 93, 497-504.

Most published studies on xylem connections between organs focus on the movement of water (Orians et al., 2000; Zwieniecki et al., 2003; Tyree and Zimmermann, 2002, Martre et al., 2000). The movement of water, however, and the movement of bacteria within the xylem are entirely different matters. While there is a water continuum from roots to leaves (Honert, 1948; Slatyer and Taylor, 1960), several studies showed that particle movement is limited by the frequency of vessel endings, especially at the stem-leaf junction, where most vessels have been thought to end, with a few exceptions (Andre, 2002; Larson and Isebrands, 1978; Tyree and Zimmermann, 2002; Wiebe et al., 1984). Such vascular organization is thought to act as a protection against the movement of potential air embolism or pathogens (Zimmermann, 1983; Aloni and Griffith, 1991; Tyree and Ewers, 1991). A vessel end signifies that water and everything that flows passively with it (minerals, particles, bacteria) must cross the intervessel pit membrane through pores that are smaller than 0.2 µm in diameter (Siau, 1984). Indeed a bacterium such as _Xylella fastidiosa_ is too big to move passively from one vessel to another with water flow (0.1-0.5 x 1-5 µm, Nyland et al., 1973).

Pierce’s disease bacterium, _Xylella fastidiosa_ multiplies and spreads within the vascular system, reportedly plugging the xylem vessels (Hopkins, 1981; Tyson et al., 1985). In addition, xylem vessels become occluded with pectins, tyloses, and gums produced by the plant in response to invasion by the bacterium (Fry and Milholland, 1990; Hopkins, 1981; Huang et al., 1986; Teackle et al., 1975, Stevenson et al., 2004). The colonization of the plant requires the bacteria being able to move within the xylem from one organ to another (Stevenson et al., 2004), implying that the bacteria has to be able to move from vessel to vessel through the pit membrane. Indeed, the favored hypothesis to explain how bacteria become systemic is that the bacteria digest the pit membrane cell wall (Roper et al., 2002; Stevenson et al., 2004). Another more recent twist in the mechanism is that bacteria might also move through torn or remnant pit pore membranes (Carlquist and Schneider, 2004; Stevenson et al., 2004). The propagation by digestion would be rather slow if vessels are short and if numerous membranes have to be crossed. Bacterial movement in the stem is relatively easy because vessels are plentiful and can be very long, up to 1m (Sperry et al., 1987). However, there is still the problem of bacterial passage into leaves if most of the vessels end at the stem-petiole and petiole-lamina junctions.

Air, latex paint and green fluorescence protein (GFP)-Xf were loaded into leaves and followed to confirm and identify these conduits. Leaf xylem anatomy was studied to determine the basis for the free and sometimes restricted movement of Ye, beads, air, paint and GFP-Xf into the lamina. Reverse loading experiments demonstrated that long, branched xylem vessels occurred exclusively in primary xylem. They were observed in the stem for three internodes before diverging into mature leaves. However, this stem—leaf connection was an age-dependent character and was absent for the first 10–12 leaves basal to the apical meristem.
Free movement in leaf blade xylem was cell-type specific with vessels facilitating movement in the body of the blade and tracheids near the leaf margin. Air, latex paint and GFP-Xf all moved about 50–60% of the leaf length. GFP-Xf was never observed close to the leaf margin. The open vessels of the primary xylem offered unimpeded long distance pathways bridging stem to leaves, possibly facilitating the spread of bacterial pathogens in planta. GFP-Xf never reached the leaf margins where scorching appeared, suggesting a signal targeting specific cells or a toxic build-up at hydathodes. This is the first evidence showing that continuous, open vessels exist between multiple organs within grapevine shoots which allow bacterial movement. Using three distinctly different methods provided a means of determining potential xylem pathways through which Xf could move unimpeded. Ultimately, bacteria must pass through pit pores in order to become systemic. However, this study shows that the opportunity for Xf to move from one organ and into another passively and unimpeded exists. Further, when these open vessels exist, Xf are able to move rapidly and over long distances between leaf blades and the cane, greatly facilitating the systemic spread of bacteria. While the open, continuous vessels are transient, they occur during a point in plant development which coincides with high sharpshooter feeding in early summer, resulting in the maximum chance of bacterial infection. Preliminary studies to repeat these experiments using Xf containing GFP (green fluorescent protein) and confocal microscopy, have yielded similar results (Sun Q & Rost TL, unpublished).


Xylem conduits and xylem development in the berry
Transport of Xf via fruit shipments is a potential hazard in the spread of Pierce's disease. The pathway into the fruit from the parent plant would be the xylem conduits. Most reports of xylem connections to and in berries refer to tracheids, single conductive cells that would greatly restrict bacteria movement, rendering movement into fruit unlikely or at least very slow. However, the reports appear to be from casual observations as there is no systematic study of berry xylem in the literature. During the development of many fleshy fruits, water flow becomes progressively more phloemic and less xylemic. In grape (Vitis vinifera L.), the current hypothesis to explain this change is that the tracheary elements of the peripheral xylem break as a result of berry growth, rendering the xylem structurally discontinuous and hence nonfunctional. Recent work however, has shown that movement of apoplastic dye through the xylem of postveraison berries can be caused if an appropriate driving force is applied, indicating that the xylem should remain structurally intact throughout berry development. To corroborate this, peripheral xylem structure in developing Chardonnay berries was investigated via maceration and plastic sectioning. Macerations revealed that, contrary to current belief, the xylem was comprised mostly of vessels with few tracheids. In cross-section, the tracheary elements of the vascular bundles formed almost parallel radial files with later formed elements toward the epidermis and earlier formed elements toward the center of the berry. Most tracheary elements remained intact throughout berry maturation, consistent with recent reports of vascular dye movement in postveraison berries. Since most of the xylem was intact despite the growth of the berry, we hypothesize that vessels stretch and/or that more xylem is added to accommodate postveraison berry growth.

Measurements of the intergyre distance of tracheary elements in macerated tissue were used to test for stretching, and the number of tracheary elements per vascular bundle and number of branch points of the peripheral xylem network were analyzed to test for continued differentiation from 18 to 120 days after anthesis in greenhouse grown Chardonnay berries. We found that new tracheary elements continue to differentiate within the existing vascular bundles throughout berry development, and that additional vascular bundles also differentiate until after veraison, thereby increasing the complexity of the peripheral vascular network. Our results also
confirmed that tracheary elements stretched by about 20%, but this was not as much as that predicted based on berry growth (40%). These results complete a comprehensive evaluation of grape berry peripheral xylem during its development and show that tracheary development continues further into berry maturation than previously thought, and that most xylem conduits are vessels rather than tracheids as previously thought. Thus, open functioning vessels late into development would allow movement of bacteria into ripening fruit.


Comparative analysis of the symptoms of Pierce’s disease and of water deficits in grapevine
The overwhelming consensus among researchers is that the fatal nature of PD is a result of the bacteria becoming systemic, blocking xylem conduits, and causing water transport to become progressively impaired (California 2004, Goodwin et al. 1988a,b, Goodwin and Purcell 1992, McElrone et al. 2001, 2003, Newman et al. 2003, 2004). Although it is clear that in vines with PD, xylem vessels become occluded by bacteria, tyloses, and/or gums (Mollenhauer and Hopkins 1976, Stevenson et al. 2004b), most research has addressed xylem occlusion by the bacteria (Newman et al. 2003) and attributes PD symptoms to restricted water transport. At the time of this publication, websites on PD at the University of California, Davis, Texas A&M University, and the University of Florida all carry this same interpretation, attesting to the nationwide extent of this view. Indeed, Pierce’s disease has become nearly synonymous with plant water deficits. This view is largely based on correlative evidence. Hopkins (1989) showed a strong association between reduced water conductance in stems of citrus seedlings and Xf-caused disease symptoms. Low leaf water potential and turgor, impaired hydraulic conductance, and higher stomatal resistance were correlated with PD symptoms in grapevines (Goodwin et al. 1988a). While reduced leaf water potential, stomatal conductance, and stem hydraulic conductivity are characteristic of water deficit, these same features also occur in flooded plants (Kramer and Boyer 1995). In grapevine, typical visual symptoms of PD are “green islands” (areas of green epidermis on an otherwise brown stem), patchy or marginal leaf necrosis (often called leaf scorch), and “matchsticks” (petioles that remain attached to the stem after the laminae have fallen off) (Purcell 1986, Goheen and Hopkins 1988, Stevenson et al. 2005). These symptoms are not characteristic of water deficit symptoms in grapevines (Williams and Matthews 1990). Therefore, it is important to determine which PD symptoms, if any, are a result of water deficits and what role water shortage actually plays in symptom development and vine death.

Detailed comparisons of the visual symptoms of PD and water deficit revealed that conclusions reached from earlier work, stating that water deficit causes PD symptoms, were not completely correct. The visual characteristics of Xf-infected vines were unique to PD and distinctly different from uninfected control vines experiencing extended water deficit. When vines were inoculated with Xf and exposed to water deficits, more extensive PD symptoms developed throughout the plant than when infected vines were well watered. However, vines infected with Xf exhibited symptoms unique to PD that included inhibited periderm development in stems (green islands), leaf blade separation from the petiole (matchsticks), and irregular leaf scorch – symptoms that were not observed with water deficits. Vines exposed to water deficits and not Xf displayed accelerated periderm development, basal leaf abscission at the stem/petiole junction, and uniform leaf chlorosis – symptoms not observed with Xf inoculation alone. Considering that only well-watered, Xf infected vines developed green islands, water deficit could have masked the green island symptom of PD by inducing accelerated and complete periderm and overwhelming the Xf-induced conditions necessary to
impair periderm activity leading to green islands. This suggests that the bacteria are in some way inhibiting periderm activity at seemingly random locations. Water deficits induced the development of an abscission zone at the stem/petiole junction, but PD did not. Pierce’s disease symptoms could not be produced with any of several water-deficit treatments, including severing all but one secondary vein near the leaf tip.

In summary, water deficit clearly had an exacerbating effect on the symptom development of PD. Water-stressed, Xf-infected vines displayed more extensive PD symptoms throughout the plant than did well-watered vines. Matchstick and leaf scorch symptoms moved up the canes more rapidly than in well-watered vines implying that the bacteria spread more rapidly throughout the plant under water deficit conditions, assuming bacterial proximity is necessary for symptom development. Importantly, with the exception of green islands, extended water deficit did not affect the nature of the PD symptoms. The results indicate that factors other than water deficits are involved in producing the symptoms of PD. The widely accepted hypothesis that PD-infected plants develop water deficits that cause green islands, matchsticks, localized leaf scorch, and eventual death of vines should be reevaluated.


In susceptible cultivars of grapevine, Xf infection results in leaf scorch, premature leaf senescence, and eventually vine death. Earlier studies correlated high virulence of Xf strains, or high susceptibility of host plants, with high Xf populations at the point of inoculation (107-108 CFU/g) and Xf movement away from the point of inoculation (Fry and Milholland, 1990; Hill and Purcell, 1995; Hopkins, 1985b). In a comparative study of grape hybrids, Krivanek and Walker (2005) correlated Xf concentration with susceptibility, as predicted. However, Xf lives as a harmless endophyte in most plant species, and many resistant hosts (including species of grapevine) harbor high concentrations and support systemic movement of Xf (Baumgartner and Warren, 2005; Wistrom and Purcell, 2005). Thus, no symptoms occur in the presence of high concentrations of Xf in some plant hosts. Together these studies demonstrate that high populations of Xf, along with Xf movement, are not sufficient for pathogenesis in many plant species. A similar conclusion has been reached for other vascular pathogens (Grimault and Prior, 1993; McGarvey et al., 1999).

The vascular occlusion hypothesis of pathogenesis depends on the development of water deficits, and despite their central role in the Xf literature plant water status has seldom been measured. Inoculation with Xf in both grapevine (Goodwin et al. 1988b) and Parthenocissus quinquefolia (McElrone et al. 2003) resulted in leaf scorch symptoms that were correlated with reductions in hydraulic conductance, stomatal conductance, and leaf water potential. Thorne et al. (2006) did not observe reduced leaf water potential in infected and symptomatic grapevines, although stomatal closure may have obscured differences in plant water status. More importantly, that study clearly demonstrated that there are qualitative and quantitative differences between the visual symptoms resulting from experimentally imposed water deficits and Xf inoculation. Although water deficits are clearly a component of wilt diseases their role in other vascular diseases such as CVC and PD, where wilting is not generally observed, are less clear. Furthermore, the presence of low leaf water potentials is not always sufficient to conclude the cause of the low water status. Biotic stressors in general induce premature leaf senescence (reviewed in Guo and Gan, 2005); a process during which there are also decreases in leaf hydraulic conductance and leaf water potential (reviewed in Sack and Holbrook, 2006). Thus, it is possible that changes in the water relations of symptomatic leaves do not result from vascular occlusion by the pathogen per se.
Our knowledge of the nature of the plant-pathogen interaction and the plant immune system has evolved, and we now understand that plants have a variety of ways to recognize and respond to pathogens (reviewed in Dangl and Jones, 2001; Jones and Dangl, 2006). Many molecules that previously were described as “phytotoxins” are now recognized to bind specific plant receptors inducing the defense response (Deboer et al., 1989; Meyer and Dubery, 1993). This knowledge has guided the research of some xylem pathogens to a new nuanced understanding of the mechanisms of pathogenesis. For example, in Verticillium numerous small molecules have been identified and isolated that contribute to pathogenicity and evoke many of the observed wilt symptoms (Chu et al., 1999; Davis et al., 1998; Meyer and Dubery, 1993; Nachmias, 1985; Wang et al., 2004). In the case of Ralstonia, yet to be characterized elicitors certainly play a role in pathogenesis (Pfund et al., 2004).

Resolving this debate is important because *Xf* pathogenesis has become synonymous with vessel occlusion in the Pierce’s disease literature (eg. Fry and Milholland, 1990; Hopkins, 1989; Kriyanek and Walker, 2005; Newman et al., 2003) without having a single report that demonstrates a strong and positive correlation between symptoms and concentration of bacteria. Understanding the nature of the pathogenesis clearly has important implications in designing screens for resistance or other approaches to ameliorating the effects of the disease. This is illustrated in Verticillium and Ralstonia where resistance is strongly correlated with the absence of disease symptoms, but not with reduced accumulation or movement of the fungi (Mace et al., 1981; Grimault and Prior, 1993). The studies of *Xf* resistant hosts discussed above suggest the same situation may be present for *Xf*, and a recent study provides some evidence of an alternative mode of *Xf* pathogenicity involving toxins (Reddy et al. 2007). In this study we sought to determine the relationship between *Xf* concentration and the development of leaf scorch symptoms during PD. To this end we developed a novel, robust quantitative PCR (qPCR) assay to quantify *Xf* in planta. *Xf* populations were quantified and related to symptom development in both artificially inoculated, greenhouse grown and naturally inoculated, field grown Chardonnay grapevines, a PD susceptible variety. This study provides the first detailed look at *Xf* populations across leaf lamina and among leaves exhibiting various severities of symptoms, and demonstrates that there is little or no correlation between localized *Xf* concentrations and symptom formation.

We developed a qPCR assay to determine bacterial concentrations in planta and related these concentrations to the development of leaf scorch symptoms. The concentration of *Xf* in leaves of experimental grapevines grown in the greenhouse was similar to the concentration of *Xf* in leaves of naturally infected plants in the field. The distribution of *Xf* was patchy within and among leaves. Some whole leaves exhibited severe leaf scorch symptoms in the absence of high concentrations of *Xf*. Despite a highly sensitive assay and a range of *Xf* concentrations from 102 to 109 cells/gram fresh weight, no clear relationship between bacterial population and symptom development during Pierce’s Disease was revealed. Thus, high and localized concentrations of *Xf* are not necessary for the formation of leaf scorch symptoms. The results are interpreted as being consistent with an etiology that involves a systemic plant response.


The work of Goodwin et al. (1988a ;1988a) demonstrated that PD was associated with reductions in petiole hydraulic conductance, stomatal conductance, photosynthesis, and leaf water potential (ΨL) in symptomatic leaves relative to healthy leaves growing under the same environmental conditions. These results, combined with observations that the number of xylem vessels colonized is higher in symptomatic than non-symptomatic leaves (Hopkins 1989; Newman et al. 2003) has lead to the consensus that the symptoms of PD result from occlusion.
of xylem conduits by *Xf* bacteria or associated gels and tyloses that impose water deficits on leaves distal to the blockage (California 2004). However, the low overall level of xylem vessel occlusion observed in these studies, e.g., 2-4% in (Newman et al. 2003), along with the absence of a correlation between *Xf* concentrations and leaf scorch symptoms (Gambetta et al. 2007), suggests that *Xf* populations alone are insufficient to induce water-stress. Furthermore, we (Thorne et al. 2006) demonstrated that there are qualitative and quantitative differences between visual symptoms resulting from experimentally imposed water deficits and PD in greenhouse-grown Chardonnay. Indeed, in those experiments stomatal conductance and leaf water potential were not reduced in infected plants. These observations raise questions as to the basic mode of action by which PD kills grapevines. Previous measurements relating to PD infection have not incorporated possible changes in the hydraulic conductance of the leaf (kLeaf), which has been shown to contribute at least 30% of whole plant hydraulic resistance (Sack and Holbrook 2006) and is a site in which *Xf* can be prolific. Previous studies have also indicated that gel and tylose formation associated with *Xf* infection are initially restricted to the leaves and petioles (Stevenson et al. 2004). Therefore, evaluating the extent to which the hydraulic capacity of the leaf vasculature is impaired by PD is an important step in clarifying the role of changes in hydraulic conductance in symptom formation. In this study, the relationships between watering regime, bacterial population growth, hydraulic capacity of leaves and symptom formation (leaf scorch) were examined in Vitis vinifera cv. Chardonnay.

We tested the hypothesis that the symptoms of Pierce’s Disease result from the occlusion of xylem conduits by *Xylella* fastidiosa. Four treatments were imposed on greenhouse-grown *V. vinifera* cv Chardonnay: well-watered and deficit-irrigated plants with and without petiole inoculation with *Xf*. The hydraulic conductance of the stem-petiole junction (kjun) and leaves (kleaf) were measured, and *Xf* concentrations were established by quantitative PCR. Leaf hydraulic conductance decreased with increasing leaf scorch symptoms in both irrigation treatments. The positive relationship between *Xf* concentration and symptom formation in deficit-irrigated plants suggests that water-stress increases susceptibility to PD. However, in well-watered plants, increasing *Xf* concentrations in leaves were not correlated with changes in kleaf nor leaf scorch symptoms. The development of symptoms in leaves of well-watered plants with low *Xf* concentrations indicates that high bacterial populations and bacterial occlusion of xylem conduits are not required for symptom formation. In field-grown vines, water relations of symptomatic leaves were similar to naturally senescing leaves but differed from green control leaves. Overall, these results suggest that the development of PD symptoms represents a form of accelerated senescence as part of a systemic response of the plant to *Xf* infection.

*Choat, Gambetta, Wada, Shackel, and Matthews. 2009. Physiol Plant: [published online 11 March 2009].*

**Progression of symptoms of Pierce’s disease in grapevine**

Grapevines were inoculated with *Xylella* in one of the two shoots per plant. Six weeks following inoculation, symptoms consistent with PD were observed on the leaves (marginal leaf necrosis) and the stem (incomplete cork formation on stem) of the inoculated shoot, but the opposite, non-inoculated shoot was asymptomatic. Anatomical examination of stem, petiole, and midrib xylem with both light and electron microscopy revealed the internal progression of PD corresponding to external symptoms. The petioles and midribs of leaves displaying external PD symptoms contained tracheary elements with abundant gummosis and accumulation of bacteria, but few tyloses. Bacteria observed in symptomatic leaf midrib xylem, and to a lesser extent in petiole xylem, were embedded within a globular matrix. Stem xylem proximal to leaves showing PD symptoms included tracheary elements with abundant tyloses, but little gum formation. Bacteria were rare in the proximal stem and bacterial cells were not contained within
a matrix. Observations of fully expanded asymptomatic leaves and stem tissue distal to the inoculation site showed similar anatomical pathology to nearby symptomatic leaves. Consequently, internal progression of PD appears to precede external symptoms. Six weeks following inoculation, no anatomical symptoms of PD were found in the basal main shoot subtending the inoculated shoot, nor in the opposite non-inoculated shoot. Eight weeks following inoculation, PD symptoms manifested in the opposite, non-inoculated, shoots. Anatomical examination showed the same pathology as was observed two weeks earlier in the inoculated shoot, including the accumulation of bacteria and embedding matrix in the xylem vessel members of leaf midribs and petioles.

Following these observations, a working hypothesis of the progression of PD within a young grapevine shoot can be proposed. Xylella inoculation of stem xylem precedes a relatively rapid movement of bacteria through the hydraulic network to distal stem regions, petioles, and leaf vascular tissue. The rapid movement is potentially facilitated by one, or a combination, of three mechanisms: 1. Grapevine vessels are long and few vessel-vessel transitions are needed to reach distal tissues, 2. pit membranes of grapevine are frequently damaged, either in development, or as a result of frequent cavitation/refilling cycles, or 3. bacteria are able to quickly digest pit membranes of terminal vessel elements. Once bacteria moving in the transpirational stream enter regions of the hydraulic network that contain many narrow tracheary elements and more frequent terminal tracheary elements (i.e. shorter vessels in petioles and leaves), bacteria are ‘filtered out’ and accumulate, and become embedded in a surrounding matrix which effectively blocks water flow in that conduit. It is unknown whether this matrix is secreted from the bacteria itself, from the plant either as a defense reaction or responding to bacterial stimulus, or a combination of the two. Tylose formation in the stem coincides with bacterial infection, but at least initially, is not present to such a degree that bacterial movement is prevented or that the water supply to distal tissues is restricted to levels causing visual symptoms. Additionally, bacteria can move from an inoculated shoot to another shoot via the subtending trunk relatively quickly. Consequently, in can be proposed that the PD symptoms observed in multiple shoots of a grapevine are not a symptom of a whole-plant response to a localized infection, but rather are an indication of a systemic Xylella presence. For this to occur, bacteria must move basipetally from the site of inoculation, into the basal stem and then acropetally into the opposite shoot. Whether bacteria are moving against the transpirational stream in an intact water column, or whether the downward bacterial movement is facilitated by the release of tension in a cavitated water column is unknown.


Analysis of the vascular anatomy that facilitates Xylella movement in grapevine
Examination of the vascular structure of the node of Vitis vinifera Chardonnay grapevine confirmed many aspects of prior investigations, however, no evidence was found that the traces of each leaf are distinct for four internodes before they leave the stele (Fournioux and Bessis 1979), or that trace fusion necessarily occurs between pairs of dorsal and ventral leaf traces (Fournioux and Bessis 1974). Additionally, variability in number of leaf traces present at each node presented here was not suggested in previous published reports (Fournioux and Bessis 1979).

No distinct traces were observed in serial sections either immediately before a node, or immediately after a node. Leaf traces of fully expanded leaves were visibly and conductively distinct only a few millimeters before divergence from the stele. Leaf gaps closed within a few millimeters of trace divergence, and after gap closure no distinct traces for successive nodes
were observed. If traces are not visibly distinct across even one node, there is little support for the idea that traces are distinct for four nodes of mature tissues. This is a significant finding for the study of the spread of Pierce's disease within a grapevine as bacteria must move through leaf traces to colonize the leaf lamina and vice versa. If leaf traces are distinct for four nodes then \( Xf \) present in a specific leaf would have to be directly inoculated, inoculated within a trace that supplies that leaf, or enter a trace four nodes basal to a leaf from adjacent stem xylem. If leaf traces are only distinct for a short length many more sources of inoculation are possible.

Fusion between mature traces is subjective based on the juxtaposition of two or more adjacent traces. Dorsal and ventral traces were observed from the point of single traces diverging from the stele and progressing into the base of the petiole in both transverse and tangential planes. Frequently these traces did not appear to fuse at all, and when the case for fusion could be argued it was likely that traces were simply juxtaposed with little or no ground tissue between them. If leaf trace fusion does not, or rarely occurs then bacterial colonization of the petiole and perhaps subsequently the leaf lamina may be segregated depending on the location of the source of bacteria from the stem. If specific leaf traces supply specific regions of the leaf lamina then a lack of fusion between traces may make a uniform dispersion of bacteria throughout the leaf unlikely.

The stem of the grapevine can be described as sectored based on the consistent location of large parenchymatous gaps, separate from leaf gaps, in the vascular cylinder. These gaps are created by the regular divergence of xylem into the tendril on one side, and into the summer lateral shoot and compound bud on the other. The result of the regular repeating gap pattern creates 4 sectors in the stem; a dorsal sector, a ventral sector, and two opposite lateral sectors. The consequence of having the large gaps lacking vascular tissue in the lateral sector(s) of each node is that long distance conductance beyond one internode may only occur in the dorsal and ventral sectors of the stem. Consequently, depending on the location of sharpshooter feeding, bacteria introduced within dorsal and ventral sectors would be more likely to move to distal regions of the shoot than bacteria introduced to lateral sectors. Additionally, the wider vessels of the dorsi-ventral sectors would provide a lower resistance pathway for long distance movement of bacterial aggregations.

The characteristics of the tracheary elements within grapevine wood and primary tissues may contribute to the level of susceptibility to PD. The relatively wide vessels found within stem wood possess simple perforation plates with scalariform intervascular pitting (Metcalf and Chalk 1950). Simple perforation plates likely provide a low resistance pathway for bacterial cells between consecutive vessel elements allowing the bacteria to move relatively unimpeded through a single vessel. Resistance to bacterial movement would occur at the end of a vessel within the terminal vessel element. The very wide scalariform lateral wall pits within the terminal vessel elements create a large pit membrane surface area which may be weaker and susceptible to bacterial breach by digestion, or physical damage due to the physical stress of cavitation and refilling cycles within the vessel. The combination of these traits may allow bacteria to move through the stem system very quickly until the vessel lamellae in which they are located diverges into a lateral organ. Once bacteria are in a leaf trace or petiole, much narrower and shorter vessels and tracheids may act to filter bacteria from the conductive stream.


This project produced no intellectual property.
Publications:

This collaboration produced 14 peer-reviewed publications and 2 further manuscripts in preparation. Three postdoctoral scholars were trained and moved into faculty positions at University of Nevada – Reno, Brigham Young University, and University of Wisconsin – Stevens Point, and a fourth is a postdoc at University of Tennessee.


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