

Vascular Occlusions in Grapevines with Pierce's Disease Make Disease Symptom Development Worse¹[OA]

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Vascular occlusions are common structural modifications made by many plant species in response to pathogen infection. However, the functional role(s) of occlusions in host plant disease resistance/susceptibility remains controversial. This study focuses on vascular occlusions that form in stem secondary xylem of grapevines (*Vitis vinifera*) infected with Pierce's disease (PD) and the impact of occlusions on the hosts' water transport and the systemic spread of the causal bacterium *Xylella fastidiosa* in infected vines. Tyloses are the predominant type of occlusion that forms in grapevine genotypes with differing PD resistances. Tyloses form throughout PD-susceptible grapevines with over 60% of the vessels in transverse sections of all examined internodes becoming fully blocked. By contrast, tylose development was mainly limited to a few internodes close to the point of inoculation in PD-resistant grapevines, impacting only 20% or less of the vessels. The extensive vessel blockage in PD-susceptible grapevines was correlated to a greater than 90% decrease in stem hydraulic conductivity, compared with an approximately 30% reduction in the stems of PD-resistant vines. Despite the systemic spread of *X. fastidiosa* in PD-susceptible grapevines, the pathogen colonized only 15% or less of the vessels in any internode and occurred in relatively small numbers, amounts much too small to directly block the vessels. Therefore, we concluded that the extensive formation of vascular occlusions in PD-susceptible grapevines does not prevent the pathogen's systemic spread in them, but may significantly suppress the vines' water conduction, contributing to PD symptom development and the vines' eventual death.

Pierce's disease (PD) of grapevines (*Vitis vinifera*), currently jeopardizing the wine and table grape industries in the southern United States and California, as well as in many other countries, is a vascular disease caused by the xylem-limited bacterium *Xylella fastidiosa* (Hopkins, 1989; Varela et al., 2001). The pathogen is transmitted mostly via xylem sap-feeding sharpshooters (e.g. *Homalodisca vitripennis*; Redak et al., 2004) and inhabits, proliferates, and spreads within the vessel system of a host grapevine (Fry and Milholland, 1990a; Hill and Purcell, 1995). PD symptom development in grapevines depends on the interactions between the pathogen and the host vine's xylem tissue, through which the pathogen may achieve its systemic spread (Purcell and Hopkins, 1996; Krivanek and Walker, 2005; Pérez-Donoso et al., 2010; Sun et al., 2011). Since the path for this spread is the host's xylem system, xylem tissue and its vessels have become the major focus for studying potential *X. fastidiosa*-host vine

interactions at the cellular or tissue levels (Fry and Milholland, 1990b; Stevenson et al., 2004a; Sun et al., 2006, 2007; Thorne et al., 2006).

One major issue related to this host-pathogen interaction is the relationship of a vine's xylem anatomy to the *X. fastidiosa* population's spread. Sun et al. (2006) did a detailed anatomical analysis of the stem secondary xylem, especially the vessel system. Stevenson et al. (2004b) described xylem connection patterns between a stem and the attached leaves. Other studies reported the presence of open continuous vessels connecting stems and leaves, which represent conduits that might facilitate the pathogen's stem-to-leaf movement (Thorne et al., 2006; Chatelet et al., 2006, 2011). Chatelet et al. (2011) also suggested that vessel size and ray density were the two xylem features that were most relevant to the restriction of *X. fastidiosa*'s movement. These studies indicate the importance of understanding the grapevine's xylem anatomy in order to characterize the grapevine host's susceptibility or resistance to PD.

Another focus of PD-related xylem studies is the tylose, a developmental modification that has important impacts on a vessel's role in water transport and, potentially, its availability as a path for *X. fastidiosa*'s systemic spread through a vine. Tyloses are outgrowths into a vessel lumen from living parenchyma cells that are adjacent to the vessel and can transfer solutes into the transpiration stream via vessel-parenchyma (V-P) pit pairs (Esau, 1977). Tylose development involves the expansion of the portions of the parenchyma cell's wall that are shared with the neighboring vessels, specifically

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the so-called pit membranes (PMs). Intensive tylose development may eventually block the affected vessel (Sun et al., 2006). Since tyloses occur in the vessel system of PD-infected grapevines (Esau, 1948; Mollenhauer and Hopkins, 1976; Stevenson et al., 2004a; Krivanek et al., 2005) that is also the avenue of *X. fastidiosa*'s spread and water transport, a great deal of effort has been made to understand tyloses and their possible relations to grapevine PD as well as to diseases caused by other vascular system-localized pathogens. One major aspect is to clarify the process of tylose development itself, in which an open vessel may be gradually sealed (Sun et al., 2006, 2008). Our investigations of the initiation of tylose formation in grapevines have identified ethylene as an important factor (Pérez-Donoso et al., 2007; Sun et al., 2007). In terms of the relationship of tyloses to grapevine PD, studies have so far led to several controversial viewpoints that are discussed below (Mollenhauer and Hopkins, 1976; Fry and Milholland, 1990b; Stevenson et al., 2004a; Krivanek et al., 2005). However, more convincing evidence is still needed to support any of them.

Another issue potentially relevant to PD symptom development is the possibility that *X. fastidiosa* cells and/or their secretions contribute to the blockage of water transport in host vines. The bacteria secrete an exopolysaccharide (Roper et al., 2007a) that contributes to the formation of cellular aggregates. Accumulations of *X. fastidiosa* cells embedded in an exopolysaccharide matrix (occasionally identified as biofilms, gums, or gels) have been reported in PD-infected grapevines (Mollenhauer and Hopkins, 1974; Fry and Milholland, 1990a; Newman et al., 2003; Stevenson et al., 2004b). However, a more detailed investigation is still needed to clarify if and to what extent these aggregates affect water transport in infected grapevines.

The xylem tissue in which *X. fastidiosa* spreads can be classified as primary xylem or secondary xylem, being derived from procambium or vascular cambium, respectively. Primary xylem is located in and responsible for material transport and structural support in young organs (i.e. leaves, young stems, and roots), while secondary xylem is the conductive and supportive tissue in more mature stems and roots (Esau, 1977). It should be noted that most of the earlier experimental results have been based on examinations of leaves (petioles or veins) or young stems of grapevines, which contain mostly primary xylem with little or no secondary xylem. However, *X. fastidiosa*'s systemic spread generally occurs after introduction during the insect vector's feeding from an internode of one shoot. The pathogen then moves upward along that shoot and also downward toward the shoot base. The downward movement allows the bacteria to enter the vine's other shoots via the shared trunk and then move upward (Stevenson et al., 2004a; Sun et al., 2011). These upward and downward bacterial movements occur through stems that contain significant amounts of secondary xylem but relatively dysfunctional primary xylem. Secondary and primary xylem show some major differences in the structure and arrangement

of their cell components (Esau, 1977). In terms of the vessel system that is the path of *X. fastidiosa*'s spread, the secondary xylem has a large number of much bigger vessels with scalariform (ladder-like) PMs (and pit pairs) as the sole intervessel (I-V) PM type, compared with the primary xylem, which contains only a limited number of smaller vessels with multiple types of I-V PMs (Esau, 1948; Sun et al., 2006). Vessels in secondary xylem are also different from those in primary xylem in forming vessel groups and in the number of parenchyma cells associated with a vessel (as seen in transverse sections of xylem tissue). These features of secondary xylem can affect the initial entry and subsequent I-V movement of the pathogen and the formation of vascular occlusions, respectively, in stems containing significant amounts of secondary xylem. Recently, the *X. fastidiosa* population size only in stems with secondary xylem was found to correlate with the grapevine's resistance to PD (Baccari and Lindow, 2011), indicating an important role of stem secondary xylem in determining a host vine's disease resistance. Despite these facts, little is known about the pathogen-grapevine interactions in the stem secondary xylem and their possible impacts on disease development.

This study addresses *X. fastidiosa*-grapevine interactions in stem secondary xylem and examines the resulting impacts on overall vine physiology, with a primary focus on vine water transport. We have made use of grapevine genotypes displaying different PD resistances and explored whether differences in the pathogen's induction of vascular occlusions occur among the genotypes and, if so, how the differences impact *X. fastidiosa*'s systemic spread. Our overall, longer-term aim is to elucidate the functional role of vascular occlusions in PD development, an understanding that we view to be essential for identifying effective approaches for controlling this devastating disease.

RESULTS

Inclusion Types of Vascular Occlusions in *X. fastidiosa*-Inoculated Grapevines

Vascular occlusions in stem secondary xylem were compared between phosphate-buffered saline (PBS; control)- and *X. fastidiosa* (treatment)-inoculated grapevines 12 weeks post inoculation. Obvious differences in the extent of occlusion development were seen in response to introduction of the PD pathogen (Fig. 1). Almost all secondary xylem vessels in control vines, regardless of their resistance state, were free of occlusions (Fig. 1, A and B). This was true for all internodes examined throughout each vine, including the internode where the buffer inoculation was carried out. However, in the treatment vines, secondary xylem vascular occlusions were always observed (Fig. 1, C and D), although there were differences in the percentages and distribution patterns of occluded vessels in PD-resistant and -susceptible genotypes (to be described below). From this, we conclude

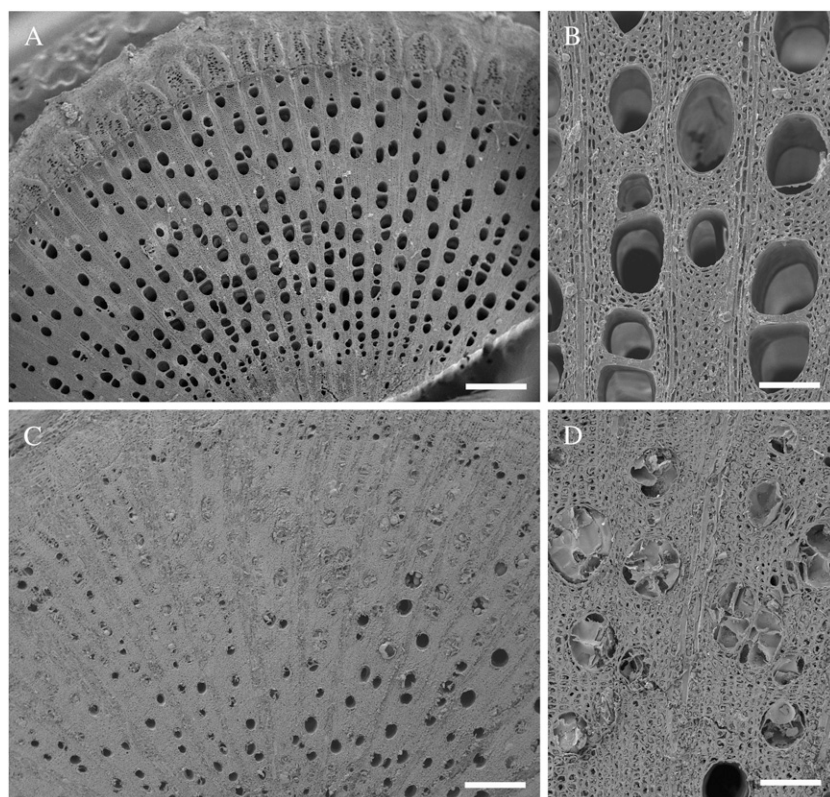


Figure 1. Representative SEM images of grapevine stem secondary xylem inoculated with PBS (A and B) or *X. fastidiosa* (C and D) 12 weeks post inoculation. A, Open vessels throughout secondary xylem in a stem transverse section of a PD-resistant 89-0917 vine. B, Enlargement of the xylem tissue in A, showing absence of vascular occlusions in vessels. C, A majority of the vessels in the secondary xylem of an *X. fastidiosa*-inoculated, PD-susceptible Chardonnay vine are occluded. D, Enlargement of the xylem tissue in C, showing masses of occlusions in most vessels. Bars = 500 μm (A), 50 μm (B), 300 μm (C), and 25 μm (D).

that the occlusions in the secondary xylem are a response to *X. fastidiosa*'s introduction.

In addition to masses of *X. fastidiosa* cells, three types of inclusions (tyloses, pectin-rich gels, and crystals) were found in secondary xylem vessels in the *X. fastidiosa*-inoculated vines, and each type appeared to be able to occlude a vessel lumen on its own (Fig. 2). Tyloses were the predominant type of inclusion and accounted for the obstruction in approximately 90% of the occluded vessels (Table I). When a vessel occluded by tyloses was viewed in a transverse section, three to 12 tyloses were often closely appressed, completely blocking the vessel lumen (Fig. 2A). The diameter of a vessel and the number of tyloses it contained were correlated, with a greater number of tyloses in a larger vessel. However, no matter what the number of tyloses was in a vessel, a vessel with tyloses was generally completely occluded by them, whether in a PD-resistant or -susceptible grapevine. When tracking along the axis of a vessel, tyloses did not always continuously block the vessel. Sometimes, gaps of open vessel lumen were interspersed between fully occluded vessel elements along the vessel (Fig. 2B).

Pectin-rich gels were found in some vessel lumens as amorphous masses of different sizes (Fig. 2C) or a more or less thick coating on a vessel's lateral walls (Fig. 2D). In either case, they were usually constantly present over one to five (up to 14 in rare cases) consecutive vessel elements of a vessel. In both light microscopy of water-saturated samples and scanning electron microscopy (SEM) of dehydrated samples, gels alone were

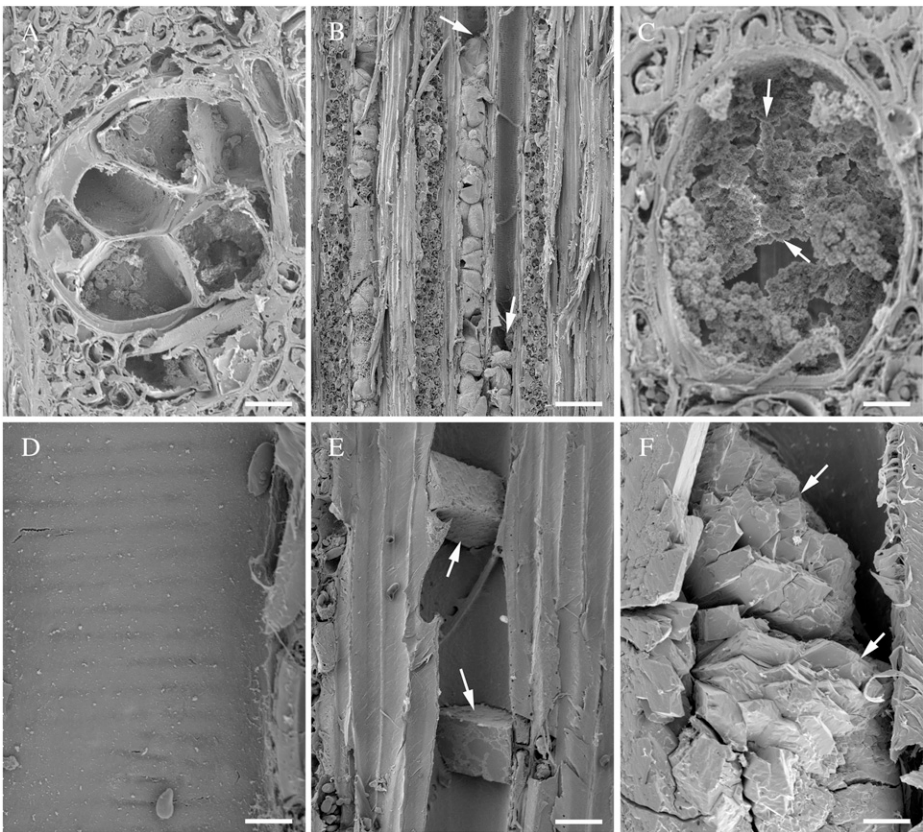
found to block the vessels in just a few cases. Vessels with gels represented less than 10% of the total vessels with occlusions (Table I). Occurrence of gels in a vessel was not correlated with the size of the vessel. In most cases, no *X. fastidiosa* cells were found to be associated with the vessels containing gels, indicating the plant's origin of the gels.

Crystals were observed in less than 4% of the occluded vessels (Table I). They occurred usually as single prismatic particles (Fig. 2E) 30 to 85 μm in diameter, but occasionally had a druse-like form (Fig. 2F). Multiple crystals were often present in a single vessel, with crystals spaced up to a few vessel elements apart from each other (Fig. 2E). Although the sizes of crystals in vessels differed, their sizes were generally sufficient to clog the vessels where they were. The three types of vessel inclusions occurred in *X. fastidiosa*-inoculated vines of both the PD-susceptible and -resistant genotypes, and the proportions of the different inclusion types found in obstructed vessels were similar whether an inoculated vine was susceptible or resistant (Table I).

Distribution and Quantity of Vascular Occlusions in Grapevines Displaying Differential PD Resistance

Tyloses were the predominant type of inclusion found in stem secondary xylem vessels of *X. fastidiosa*-inoculated grapevines. However, tyloses were not distributed uniformly in vessels throughout a transverse section (Fig. 3). Instead, some xylem regions had tyloses

Figure 2. Types of inclusions in stem secondary xylem vessels of *X. fastidiosa*-inoculated grapevines 12 weeks post inoculation: tyloses (A and B), gels (C and D), and crystals (E and F). A, Transverse section of a vessel, showing that the vessel lumen is filled by multiple compactly arranged tyloses. B, Tangential longitudinal section of xylem tissue, showing that vessels occluded with tyloses may have inclusion-free gaps in their lumens along the vessels' axis (arrows indicate where a gap starts). C, Amorphous masses (arrows) of gels filling a vessel lumen. D, Gels cover a vessel's lateral wall as a thin layer, under which outlines of scalariform I-V pit pairs can be seen. E, Prismatic crystals (arrows) in a vessel lumen. F, Druse-like crystals (arrows) in a vessel lumen. Bars = 20 μ m (A), 150 μ m (B), 10 μ m (C, D, and F), and 30 μ m (E).



filling most vessels and other regions with open vessels. In PD-resistant grapevines, the tylose-occluded regions were small in area, showing a patchy distribution throughout the larger stem transverse-sectional area with open vessels (Fig. 3, A and B). In PD-susceptible grapevines, the patterns of occluded versus open vessel regions across the xylem sections tended to be reversed (Fig. 3, C and D). The distribution and quantity of vessel occlusions throughout a host vine also differed among the grapevine genotypes based on their different PD resistance (Figs. 3 and 4). Extensive vascular occlusions were found in all examined internodes of the PD-susceptible genotypes, including both the *X. fastidiosa*-inoculated shoot and the uninoculated shoot of vines that had been trained to have two main branches (Figs. 3, C and D,

and 4, A and B). As for each PD-resistant grapevine, vascular occlusions were seen in a relatively small fraction of the vessels in internodes close to the inoculation site in the inoculated shoot and were seldom found in other internodes of the vine (Figs. 3, A and B, and 4, C and D). Quantitative comparison of vascular occlusions in the susceptible versus resistant genotypes showed that over 60% of the vessels throughout vines of the former were blocked (Fig. 4, A and B), while in the resistant vines, only 5% to 27% and less than 5% of the vessels were blocked in inoculated (Fig. 4C) and uninoculated (Fig. 4D) shoots, respectively. These quantitative data were based on the analyses of vascular occlusions in the transverse sections of stems. As indicated above, tyloses, which represented the majority of the occlusions seen, were not always

Table 1. Comparison of relative percentages of vessels with tyloses, gels, and crystals among grapevines with different PD resistance

The blockage of vessels in secondary xylem was caused by one of the three forms of inclusions: tyloses, gels, and crystals. Shown here is a comparison of the relative ratio of the vessels with each inclusion among two PD-susceptible and two PD-resistant grapevines 12 weeks following *X. fastidiosa* inoculation by using the internode just above the internode with the point of inoculation. Each datum is presented as the mean with its sd and based on the measurement of three or four plants.

Genotype	Relative Percentage of Vessels with Each Inclusion Form		
	Tyloses	Gels	Crystals
Chardonnay (PD-susceptible)	88.2 \pm 2.9	8.2 \pm 2.2	3.6 \pm 0.8
Riesling (PD-susceptible)	90.2 \pm 3.3	7.8 \pm 3.0	2.0 \pm 0.6
U0505-01 (PD-resistant)	87.8 \pm 2.4	9.8 \pm 2.5	2.4 \pm 0.5
89-0908 (PD-resistant)	88.3 \pm 3.4	8.6 \pm 2.4	3.1 \pm 1.7

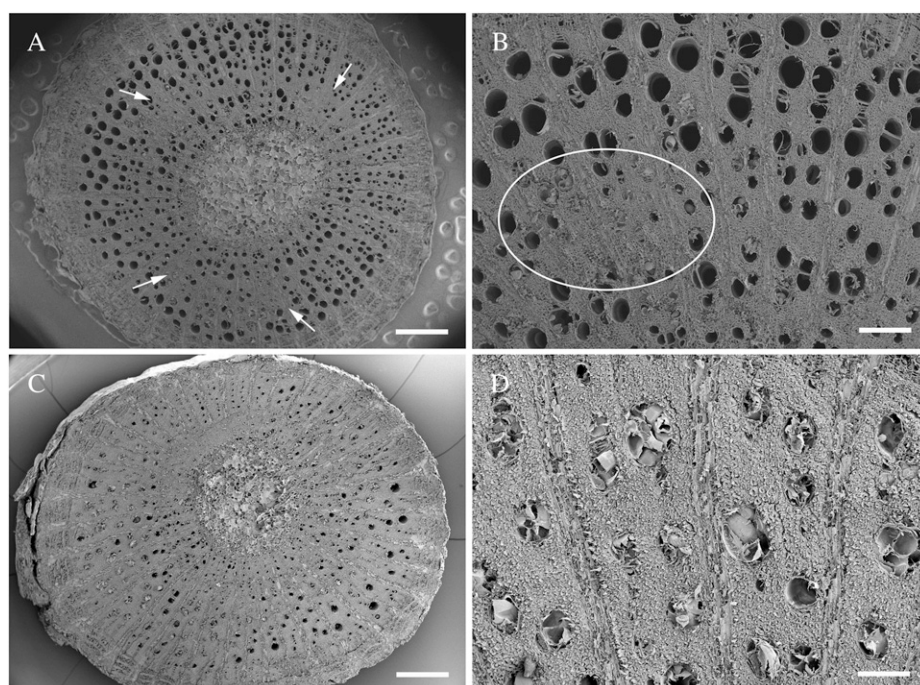


Figure 3. Extent of vascular occlusions in the eighth internode from the shoot base (*X. fastidiosa* inoculation was in the sixth internode) in representative PD-resistant (A and B) and -susceptible (C and D) grapevines 12 weeks post inoculation. A, Most vessels in a U0505-01 vine stem transverse section are open, and occluded vessels occur in small, scattered patches (arrows). B, Enlargement of xylem tissue in A, showing a patch of xylem with occluded vessels (oval frame). C, Chardonnay stem transverse section showing many occluded vessels with a few open vessels interspersed. D, Occluded vessels are filled with tyloses. Bars = 700 μ m (A and C), 250 μ m (B), and 150 μ m (D).

continuously present along individual vessels, so an open vessel observed in one stem transverse section could have been occluded at other positions along its length. Therefore, the actual percentages of occluded vessels are likely to be higher than the values presented here.

Impact of Vascular Occlusions on Hydraulic Conductivity of Grapevines with Different PD Resistance

The extent to which water transport in a grapevine was affected by *X. fastidiosa* inoculation-induced

vascular occlusions was also investigated with both PBS- and *X. fastidiosa*-inoculated grapevines of two PD-susceptible genotypes (Chardonnay and U0505-35) and two PD-resistant genotypes (89-0917 and U0505-01). The investigation compared the specific hydraulic conductivity of the inoculated shoot (with the point of inoculation included) of each grapevine 12 weeks after inoculation. In all of the four genotypes, the specific conductivity was lower in bacterium-inoculated vines than in PBS-inoculated vines (Fig. 5), indicating a decline in hydraulic conductivity in *X. fastidiosa*-infected vines regardless of the vines' PD resistance status. However, the extent of the decline in conductivity was

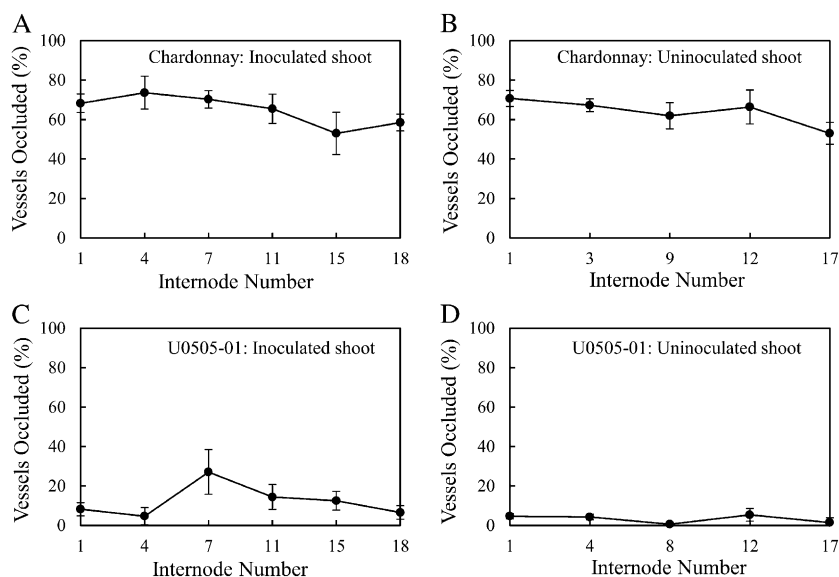


Figure 4. Quantitative comparison of vessels with occlusions in stem transverse sections. Compared are observations of PD-susceptible (A and B) and -resistant (C and D) genotypes made 12 weeks after *X. fastidiosa* inoculation. Each grapevine had two shoots. The numbering of the internodes of each shoot is from the shoot base upward (lowest internode as number 1). Inoculation was only to one shoot at internode number 6, also counting from the shoot base. A and C, Percentages of vessels with occlusions in the indicated internodes (x axis) along the inoculated shoot. B and D, Percentages of occluded vessels in the indicated internodes along the uninoculated shoots. Each datum is based on samples from three grapevines and presented as the mean with sd.

much greater in infected, susceptible grapevines. In these PD-susceptible genotypes, the specific hydraulic conductivity averaged $1.45 \times 10^{-2} \text{ g cm kPa}^{-1} \text{ min}^{-1} \text{ mm}^{-2}$ in the vines that were inoculated with PBS and averaged $1.19 \times 10^{-3} \text{ g cm kPa}^{-1} \text{ min}^{-1} \text{ mm}^{-2}$ in those inoculated with *X. fastidiosa*, a 92% conductivity loss. By contrast, the reduction in specific hydraulic conductivity of the tested PD-resistant genotypes was much less, averaging $1.94 \times 10^{-2} \text{ g cm kPa}^{-1} \text{ min}^{-1} \text{ mm}^{-2}$ in PBS-inoculated vines and $1.28 \times 10^{-2} \text{ g cm kPa}^{-1} \text{ min}^{-1} \text{ mm}^{-2}$ in *X. fastidiosa*-inoculated vines, a 34% decline in hydraulic conductivity. Thus, water conduction was significantly suppressed following introduction of *X. fastidiosa* in the PD-susceptible grapevines that were tested, but the reduction was much less in the PD-resistant grapevines.

Distribution and Quantity of *X. fastidiosa* in Host Grapevines with Different PD Resistance

The distribution and quantity of pathogen cells in a host plant are important for analysis of host plant resistance and determinations of the pathogen's role in disease symptom development. Therefore, these also were examined 12 weeks after inoculation of the PD-susceptible and -resistant test grapevine genotypes. Bacterial cells were observed in both sets of grapevine genotypes, although (as expected) the numbers and distributions of *X. fastidiosa* cells differed according to grapevine PD resistance. The bacterial cells were found in vessels, either as individuals or in aggregates of multiple cells (Fig. 6, A–F). The individuals had a relatively smooth surface (Fig. 6, A and B) or bore

multiple-branched or unbranched thin filaments, usually less than $5 \mu\text{m}$ in length (Fig. 6C). Cells found in I–V pit cavities (i.e. cells that had moved into or through the secondary cell wall “windows” that overarch the primary cell wall of pit membranes that, if disrupted, are a potential pathogen pathway from one vessel to the next; Sun et al., 2011) were always free and displayed relatively smooth surfaces (Fig. 6B). *X. fastidiosa* aggregates were composed mostly of a few to tens of cells and could be further distinguished into two morphologically different types. The aggregates of one type were loosely bound together through a filamentous network (Fig. 6D); those in the other type appeared to be glued together with “gel-like” material to form amorphous masses (Fig. 6, E and F). Both the networks and the masses were usually less than $20 \mu\text{m}$ in their largest dimension. All of these associations of *X. fastidiosa* cells were found in both PD-susceptible and -resistant grapevines with the free, smooth-surfaced individuals observed most often.

Despite their different forms, *X. fastidiosa* cells were present only in small amounts in vessels and were relatively sparsely distributed along the vessels' axes. The amounts of the bacterial cells varied significantly among the vessels from a few cells to a number of bacterial aggregates. In no case was the accumulation of bacterial cells per se great enough to block the vessels. *X. fastidiosa* cells were observed in both open and tylose-occluded vessels (Fig. 6, A, E, and G). The bacterial cells in the occluded vessels occurred individually and were associated with small amounts of gels present in the narrow spaces between tyloses (Fig. 6, G and H). These gels may have originated from the expanding primary walls of the tyloses and/or *X. fastidiosa*, indicating its production of exo-polysaccharide. Spatially, vessels with *X. fastidiosa* cells were more or less clustered or close to one another in xylem tissue, as might have been predicted from the observed patchiness of xylem occlusions (Fig. 3), although occasionally, *X. fastidiosa*-containing vessels were more separated. Different from those gels that could block a vessel on their own, the gels associated with *X. fastidiosa* cells and/or tyloses occurred much less often in secondary xylem vessels. Immunohistological analysis (Roper et al., 2007a; Sun et al., 2011) would be required to determine whether they are products of the host or pathogen.

A more quantitative analysis was conducted to explore the spatial distribution of *X. fastidiosa* cells throughout an inoculated vine. In the PD-susceptible genotypes, the bacteria were found in the examined internodes in both inoculated and uninoculated shoots of the same host vines, indicating the pathogen's successful systemic spread. However, the percentage of the vessels containing the bacterium was less than 14% in the inoculated shoot and around 5% in the uninoculated shoot of the same vine (Fig. 7). In the PD-resistant grapevines, bacteria were restricted to several internodes above or below the internode inoculated, and the percentage of the vessels colonized by the bacterium was less than 3% even in those internodes; however, no bacteria were observed in

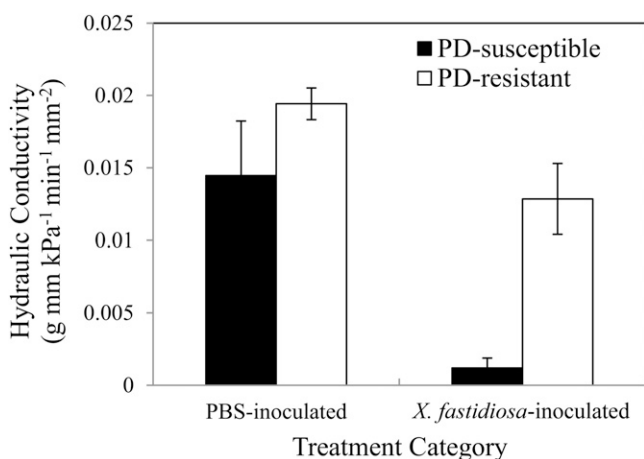


Figure 5. Comparison of specific hydraulic conductivities of stems of PBS- and *X. fastidiosa*-inoculated, PD-susceptible (Chardonnay and U0505-35) and -resistant (89-0917 and U0505-01) grapevine genotypes. Explants used for measurement were 18 to 25 cm and 4 to 5.5 mm in length and diameter, respectively, and were obtained from the shoots containing the internode with the point of inoculation. Each datum is based on six or eight grapevines (three or four grapevines per genotype) and presented as the mean with SD.

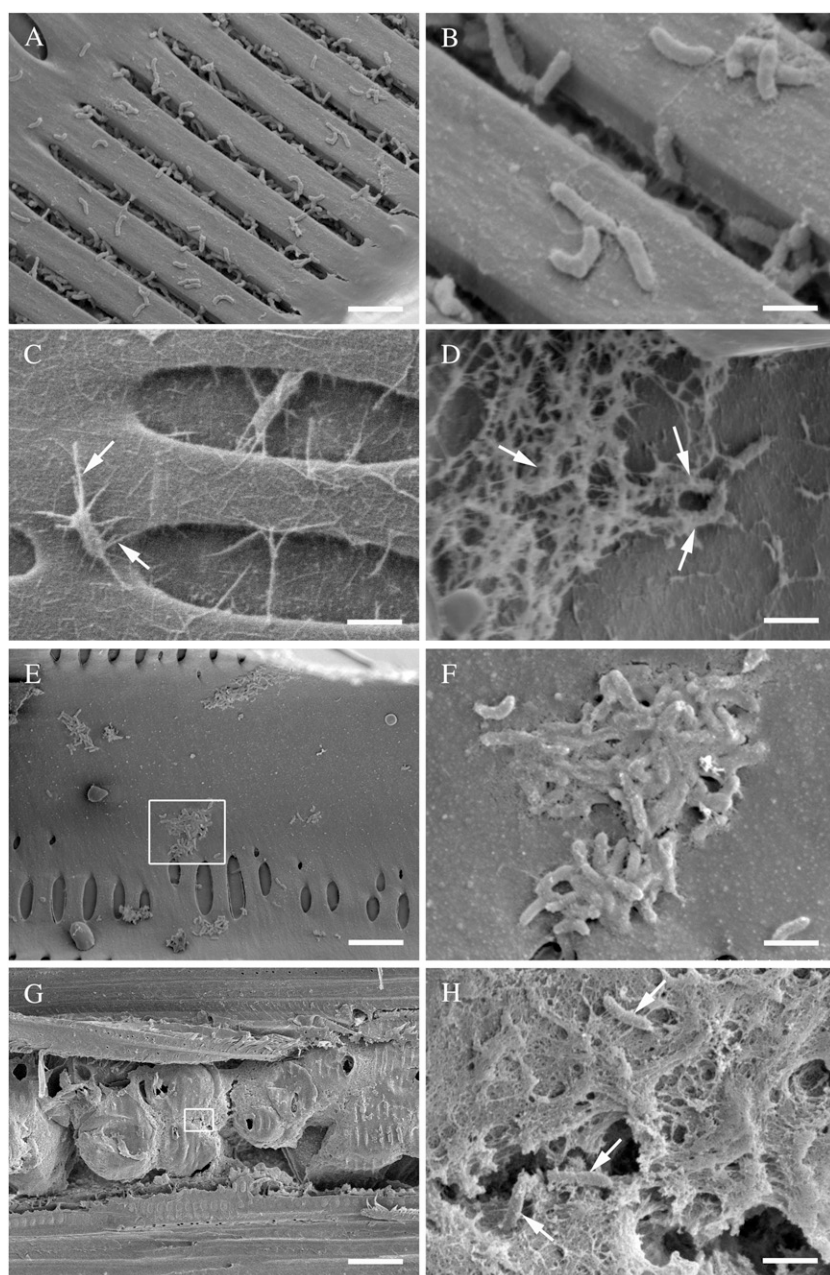


Figure 6. Morphology of *X. fastidiosa* in the vessel system of grapevine stem secondary xylem. A, *X. fastidiosa* cells occur singly in I-V pit cavities (ladder-like slots) and on a vessel's lateral wall. B, Relatively solitary *X. fastidiosa* cells often displayed a relatively smooth surface. C, Individual *X. fastidiosa* cells occasionally bore multiple thread-like surface strings (arrows). D, *X. fastidiosa* cells (arrows) connected to a filamentous network. E, Sparsely distributed aggregates of *X. fastidiosa* cells on a vessel's lateral wall. F, Enlargement of the framed area in E, showing tens of bacterial cells "glued" together. G, A vessel occluded by a few tyloses. H, Enlargement of the framed area in G, showing *X. fastidiosa* cells (arrows) present in the gel layer between compactly arranged tyloses. Bars = 7 μm (A), 1.5 μm (B), 2 μm (C, F, and H), 2.5 μm (D), 12 μm (E), and 40 μm (G).

internodes that were more distant from the inoculation site (Fig. 7).

DISCUSSION

Vascular Occlusions in Response to *X. fastidiosa* Infection in Grapevines

Vascular occlusion is a common occurrence in many plants with vascular diseases and is caused mostly by tyloses and gels, in addition to the pathogen itself (Beckman, 1987; Tyree and Zimmermann, 2002). Plants that form vascular occlusions have been believed to produce only one of these types of inclusions, but not

both (Chattaway, 1949; Bensen and Kucera, 1990). Tyloses are the vascular occlusions in many infected plants, such as oak (*Quercus* spp.) with oak wilt (Struckmeyer et al., 1958; Blaedow and Juzwik, 2010), American elm (*Ulmus americana*) with Dutch elm disease (Ouellette and Rioux, 1992; Rioux et al., 1995), and hop (*Humulus lupulus*) infected with *Verticillium* spp. (Talboys, 1958). Host plant gels are responsible for the vascular occlusion observed in other plant disease situations, such as tomato (*Solanum lycopersicum*) infected with multiple fungal wilt pathogens (Bishop and Cooper, 1984) and carnation (*Dianthus caryophyllus*) infected with *Fusarium* spp. (Baayen et al., 1996). In situations where quite small amounts of gel-like material have been

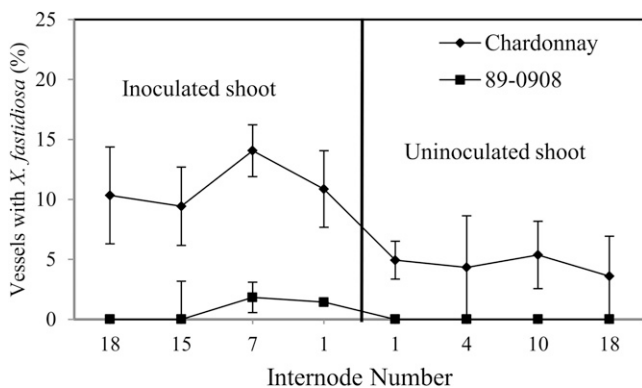


Figure 7. Frequency of vessels with *X. fastidiosa* cells in representative PD-susceptible (Chardonnay) and -resistant (89-0908) vines 12 weeks after *X. fastidiosa* inoculation. The internode number of each shoot is counted from the shoot base upwards, with the lowest internode as number 1. Each datum is based on samples from three or four vines and is presented as the mean with its SD. In each case, the inoculated and uninoculated shoots are parts of the stem system of the same, two-shooted vine.

observed in vessels that also contain tyloses, reports have suggested that the gels were secreted by expanding tyloses and amounted to too little material to occlude a vessel (Bonsen and Kucera, 1990; Rioux et al., 1998). In terms of grapevines infected with PD, several reports have indicated that tyloses are the sole xylem occluding structures in young stems (Stevenson et al., 2004a; Krivanek et al., 2005) and the primary occluding structures in leaf veins (Mollenhauer and Hopkins, 1976). However, some reports have described a vessel-filling accumulation of gels in veins (Fry and Milholland, 1990b) and petioles (Stevenson et al., 2004a) of infected grapevines. These authors suggested that these gels were of bacterial origin (Roper et al., 2007a) due to their close association with *X. fastidiosa* cells. It is important to note that the organs investigated in these studies (i.e. grape leaf petioles, veins, and young stems) contain mostly or only primary xylem tissues.

The current study focuses on grapevine stem secondary xylem and identifies three types of vessel inclusions (tyloses, pectin-rich gels, and crystals) that can contribute to the vessel occlusion that accompanies PD infection. Tyloses are found in most occluded vessels, but gels and crystals are responsible for blockage of a small fraction of the secondary xylem vessels (Fig. 2). Also, the gels in a vessel were not associated with tyloses and often accumulated in large amounts along multiple consecutive vessel elements. The lack of *X. fastidiosa* cells in these gels suggests their host plant origin. Until now, there have been no reports of crystal deposition in the vessels of PD-infected grapevines. Thus, grapevines may be relatively unusual in that their secondary xylem can be occluded by a variety of structures. Sun et al. (2006, 2008) reported that wounding of grapevine stems triggered tylose and gel formation and concluded that these two inclusion types originated from neighboring xylem parenchyma

cells. This raises important questions. What determines whether the parenchyma cells will produce one or the other of these inclusions? Do the two contribute in different ways to disease resistance or susceptibility?

Our observations indicate that both PD-susceptible and -resistant grapevines develop the same kinds of inclusions, suggesting that the differences in disease susceptibility of these genotypes may be due more to the quantity and distributions of xylem obstructions than to the obstruction type (Fig. 4). Extensive secondary xylem occlusion was seen throughout the PD-susceptible grapevines, while the limited amounts of occlusions in the PD-resistant grapevines were restricted to internodes close to the inoculation point. In pruning experiments with these same grapevine genotypes (Sun et al., 2006; Q. Sun, unpublished data), excision of the current year's shoots caused a similar extensive vascular occlusion proximal to the pruning cuts. This suggests the grapevine's comparably strong capacity to form vascular occlusions. The differences in the extent of xylem vascular occlusion following *X. fastidiosa* infection that we saw among the grapevine genotypes should contribute to the differences in the interactions between the pathogen and the xylem tissue of these genotypes, which will be discussed in the following sections.

Vascular Occlusions in Relationship to Disease Resistance/Susceptibility of Host Plants

In vascular diseases caused by xylem-limited pathogens, introduction of the pathogen to a plant's water-conducting system often leads to a progressive systemic spread of the pathogen via the xylem (Hopkins, 1989; Bove and Garnier, 2002) and results in disease symptoms that ultimately kill the host plant (Beckman, 1987; Purcell and Hopkins, 1996). Several studies have reported that the appearance and progression of external disease symptoms is coupled to the pathogens' spread via the vessel system. Because (1) xylem occlusions often form soon after host plant infection by vascular system-localized pathogens and (2) it is not unusual for tyloses and/or gels to completely block vessel lumens, many studies have examined occlusion development vis-à-vis plant resistance or susceptibility to different vascular pathogens. One popular hypothesis suggests that occlusion development contributes to host plant resistance (Elgersma, 1973; Beckman and Talboys, 1981). Two general types of evidence are advanced in support of this perspective. First, tyloses were often seen close to infection points (Reiss et al., 1997; Barry et al., 2000). Second, more tyloses were seen to develop in resistant genotypes than in susceptible genotypes of the same species (Bishop and Cooper, 1984; Grimault et al., 1994). Thus, tylose formation is thought to prevent the pathogen's spread from the point(s) of introduction. It should be pointed out that introduction of pathogens to plants in inoculation tests generally includes local wounding and that

wound induction of tylose formation (Weiner and Liese, 1995; Sun et al., 2006) and gel accumulation in many species (Soukup and Votrubová, 2005; Sun et al., 2008) has been reported. However, the possibility that the wound was responsible for occlusion development following inoculation often has been ignored. In addition, information about the spatial distributions of pathogens and occlusions in resistant plants following inoculation is not available. Another perspective on the role of occlusions in infected plants suggests that they contribute to the host's susceptibility (Dimond, 1955; Aleemullah and Walsh, 1996). Generally, this is based on the observations of (1) more tyloses in susceptible genotypes than in resistant genotypes (Dimond, 1955) and (2) a positive correlation of tylose quantity and disease symptom severity in some plants (Sen and Palodhi, 1984). This viewpoint thus suggests that the major impact of the occlusions is to reduce water conduction in infected plants, exacerbating disease symptoms. However, the extent to which vascular occlusion may affect water transport in infected plants is not clear. The third hypothesis pertaining to the resistance versus susceptibility contribution of occlusions is that they are not relevant to the question (Palodhi and Sen, 1979; Jacobi and MacDonald, 1980). This viewpoint was derived from the findings of a quantitative similarity in tylose development when resistant and susceptible genotypes of some species have been studied (Prior, 1979). The easiest conclusions that can be drawn are that (1) the results of studies depend on the host-pathogen combinations being studied and (2) additional virulence and defense factors not included in many of these studies also play important roles in the interaction.

As demonstrated herein and in prior reports, occlusion of primary and secondary xylem is also the most obvious structural modification in PD-infected grapevines. Furthermore, the three controversial viewpoints related to occlusion roles in vascular disease development (discussed above) also have been used when evaluating the progression of PD symptoms in *X. fastidiosa*-infected grapevines, namely, that occlusions (1) contribute to resistance of host vines (Mollenhauer and Hopkins, 1976), (2) exacerbate PD symptoms (Stevenson et al., 2004a; Krivanek et al., 2005), and (3) are not relevant to PD symptom progression (Fry and Milholland 1990a). The studies that led to these hypotheses clearly helped with an understanding of the host grapevine's response to pathogen invasion. However, they also display some limitations when used to interpret the relationship of occlusions to the host's PD resistance. The studies focused on only a few grapevine anatomical locations (i.e. internodes close to the point of inoculation and/or leaves [petioles and veins] that already displayed visible disease symptoms). Also, they found that only a small portion of vessels (less than 40% and 30% in petioles and stems, respectively [Krivanek et al., 2005], and less than 20% in veins [Fry and Milholland, 1990a]) contained inclusions even in grapevines with maximal xylem occlusion. Furthermore, the actual impact of the

observed occlusions on water transport of the affected grapevines was not evaluated, while its impact on the pathogen's systemic spread was not at all clear.

The current study investigated *X. fastidiosa* infection-induced vascular occlusions and revealed some differences in the spatial distribution and quantity of xylem occlusions among grapevines with different PD resistances. We found that vascular occlusions (primarily, but not exclusively, tyloses) occurred throughout PD-susceptible grapevines and affected a substantial proportion of the secondary xylem vessels (over 60%), but were restricted to several internodes close to the point of inoculation and affected a relatively small proportion of the secondary xylem vessels (around 20%) in PD-resistant grapevine genotypes (Fig. 4). This quantitative difference in xylem occlusion also correlated with the substantial and significant decline in the hydraulic conductivity measured in *X. fastidiosa*-infected, PD-susceptible grapevines (Fig. 5), likely exacerbating disease symptom development.

Our examination of the development of vascular system occlusions included assessments of the distributions of *X. fastidiosa* cells. Even though this was limited to a single time point (12 weeks post inoculation), the analysis did support a limited interpretation of the potential role(s) of occlusions in restricting the pathogen's systemic spread. In PD-susceptible genotypes, *X. fastidiosa*'s extensive movement through inoculated vines was confirmed. Our data (Fig. 7) have indicated that the bacterial population had moved toward the apex of the inoculated shoot and also basally through that shoot, then toward the apex of the other shoot of the vines that had been trained with two shoots only (see "Materials and Methods"). This systemic spread required pathogen movement "with" the transpiration stream (likely facilitated by it) as well as "against" the stream, at least for the few internodes distance to the subtending main stem. That the bacterium can move against the flowing stream has been reported (Meng et al., 2005; De La Fuente et al., 2007), but the rate of movement was low ($5 \mu\text{m min}^{-1}$ against a medium flowing at $20,000 \mu\text{m min}^{-1}$; Meng et al., 2005). However, in the stems where this movement was observed, occlusion development was substantial (Figs. 1–4) and water movement was significantly reduced (Fig. 6). Given that in PD-susceptible genotypes the systemic pathogen spread was seen in the xylem with most vessels fully occluded, it is clear that the timing of the vascular occlusion development is probably more important than the quantity of occlusion formation per se in terms of the potential role of the occlusions in preventing the pathogen from spreading through the vessel system. This has led us to believe that the large amounts of vascular occlusions that form, but presumably not in a timely way, simply reduce water transport (instead of stopping the pathogen's spread) and, therefore, contribute to the PD susceptibility of those vines. This conclusion is also supported by the analysis of pathogen spread relative to occlusion development in the inoculated PD-resistant genotypes

in which pathogen movement away from the inoculation point is limited (Fig. 7). In these vines, occlusion development is reduced and local (Figs. 1, 3, and 4), and the subsequent negative impacts on water conductance (Fig. 6) and, likely, vine survival are greatly reduced.

Some anatomical features of the grapevine vessel system also may have impacts on *X. fastidiosa*'s systemic spread, especially the spread to the other shoot that was not directly inoculated in our experimental protocol. Vessels forming the vessel system in grapevines are relatively short with an average length of 3 to 4 cm (Thorne et al., 2006; Chatelet et al., 2011). Thus, the pathogen's migration through the vessel system requires consecutive vessel-to-vessel movements through the I-V pit pairs and PMs, which make long-distance water flux possible. Our earlier studies (Roper et al., 2007b; Pérez-Donoso et al., 2010) have indicated that, if the PM is intact, its pores are too small (<20 nm) to permit passage of an *X. fastidiosa* cell. We also found that the pathogen may use cell wall-degrading enzymes (CWDEs), including, but perhaps not limited to, polygalacturonase (PG) and endo- β -1,4-glucanase (EGase), to digest the PM pectin and xyloglucan components (Sun et al., 2011) and facilitate its transit from one vessel to the next. Interestingly, our partial immunohistochemical analysis of grapevine PM polysaccharides (Sun et al., 2011) was clear in showing homogalacturonans with a low level of methylesterification and fucosylated xyloglucan in the PMs of PD-susceptible grapevines; however, the images of PMs in PD-resistant vines were less clear, suggesting that these pectic and hemicellulosic PG and EGase substrates were absent or partially masked. This interpretation, combined with the slower movement of the pathogen away from the inoculation points on PD-resistant vines that is implied by the bacteria distribution data (Fig. 7), leads us to suggest that the "altered PM availability" of substrates for its CWDEs could contribute to a slowing of the pathogen's egress from the vessels into which it has been introduced by the natural insect vector, *H. vitripennis*, or a researcher using an artificial inoculation approach.

Pathogen infection has been reported to cause elevated ethylene production of host plants in many species (Pegg, 1976; Pegg and Cronshaw, 1976; Boller, 1991; Abeles et al., 1992), including grapevines infected with *X. fastidiosa* (Pérez-Donoso et al., 2007). Furthermore, ethylene promotes tylose formation in grapevines (Sun et al., 2007) and pectin gel accumulation in other species (VanderMolen et al., 1983). Thus, it is reasonable to suggest that ethylene plays a role in the development of the xylem obstructions that form in PD-infected grapes. Ethylene is generally thought of as a "wound" hormone (Abeles et al., 1992). Even though the specific mechanistic linkage of wounds and enhanced ethylene synthesis is not clear in all plant systems, it is easy to propose that the harm caused by pathogens like *X. fastidiosa* could be perceived as a biochemical/structural wound by the infected grapevine. Furthermore, because disruption of the cell wall PM fabric catalyzed by the bacterium's CWDEs occurs

during PD development (Pérez-Donoso et al., 2007; Sun et al., 2011), it is reasonable to suggest that pectin-derived oligosaccharides, generated by PG cleavage of the sparsely methylesterified homogalacturonans in the PMs, could be a signal intermediate. Such oligosaccharides are known to be generated in ripening tomato fruit and to promote fruit ethylene synthesis (Melotto et al., 1994). However, this sort of interaction would suggest an interplay of counteracting effects, i.e. the pathogen's PG and EGase damaging the PMs so that the bacteria can move through PMs and, eventually, develop a systemic population, an impact that could be negated by the hypothetical oligosaccharide promotion of ethylene synthesis and resulting vascular system occlusion. Again, the counteracting effects depend on the timing of the occlusion development. On the other hand, signal molecules such as ethylene (thus, potentially 1-aminocyclopropane-1-carboxylic acid, the immediate biosynthetic precursor to ethylene; Adams and Yang, 1979) and/or oligosaccharides are small and mobile in the xylem system (Bradford and Yang, 1980; Pérez-Donoso et al., 2010). They could be moved away from where they are produced and trigger occlusion development in vessels not yet associated with the pathogen. This might be responsible for the high percentages of the occluded vessels distant from the xylem sectors where the pathogen is located earlier in infection (Figs. 4, A and B, and 7). Another scenario that might also explain the more localized formation of vascular occlusions is a more direct interaction between pathogen and V-P PMs in vessels containing or close to the pathogen. Cell wall weakening is thought to be a major factor in the promotion of cell elongation, and the elongation of epicotyl and hypocotyl cells in dicot seedlings has long been discussed in terms of the weakening of xyloglucan cross-linkages between cellulose microfibrils (Labavitch and Ray, 1974; Cosgrove, 2005). *X. fastidiosa*'s EGase can digest xyloglucans like those identified in grapevine PMs (Pérez-Donoso et al., 2010). While the weakening of cell walls in the stems of young dicots is often thought of as the result of hormonal impacts on apoplast pH affecting the activities of wall-modifying enzymes (Rayle and Cleland, 1992), could a direct, quite local impact of pathogen enzymes in the vessels on the V-P PMs, which are actually small sectors of the primary wall shared with xylem parenchyma cells, impart a similar wall-weakening effect? If so, could the turgor of the parenchyma cell then exceed the yield threshold of the PM-localized portion of the parenchyma cell's wall (Lockhart, 1965; Ray et al., 1972), causing it to expand into the vessel lumen (i.e. form a tylose) until it contacts other tyloses or the vessel wall, at which point the expanding tylose wall can then resist turgor-driven expansion? Variations in the timing of tylose formation vis-à-vis success or failure of a tylose-based defense against the systemic spread of *X. fastidiosa* in an inoculated grapevine might well depend on subtle variations in the potential tissue mobility and amount of ethylene-related and/or oligosaccharide

signal molecules and the V-P PM polysaccharide susceptibility to the pathogen's CWDEs. Clearly, there is much more that must be learned.

Another interesting finding in the current work is the correlation in quantity and spatial distribution between the vessels with tyloses and the vessels with *X. fastidiosa*. That is, in infected, PD-susceptible grapevines, having a relatively high percentage of tylose-occluded vessels was correlated with systemic pathogen spread, while having a low percentage of occluded vessels that were localized near the point of inoculation was correlated with localized pathogen distribution in PD-resistant grapevines (Figs. 4 and 7). The cause underlying this is not very clear; however, it seems reasonable for us to connect these correlations to differences in the sparsely methylesterified homogalacturonans and xyloglucan presence in I-V PMs (i.e. the PMs that represent the barriers to *X. fastidiosa*'s systemic spread through the xylem system) and in V-P PMs (i.e. the PMs that are potential points of origin for tyloses). We know that the pathogen digests the components of the I-V PMs to facilitate its spread (Sun et al., 2011) and herein we have suggested that the pathogen enzymes weaken the V-P PM structure to initiate the turgor-driven expansion of tyloses. Because the pathogen's systemic spread does not appear to be limited by tylose development in PD-susceptible grapevine genotypes, we conclude that breakdown of the I-V PM structure is more rapid than is the weakening of the V-P PMs. That is, the bacteria move on before tyloses can close off their passageway to the rest of the vine. By contrast, the absence or inaccessibility of sparsely methylesterified homogalacturonans and xyloglucans for the pathogen's CWDEs in I-V and V-P PMs could alter the relative rates of tylose initiation/expansion and pathogen vessel-to-vessel advance. These differences in cell wall polysaccharide digestion could also affect the rates of generation and I-V movement of the proposed ethylene-related signals discussed above. Baccari and Lindow (2011) reported a much higher population of *X. fastidiosa* cells in a single vessel with the pathogen in PD-susceptible grapevines compared with bacteria populations in vessels of PD-resistant grapevines. This quantitative difference in bacterial cells might also lead to the correlations observed in the current study. Thus, a lot of work still is needed to clarify these relationships.

The results discussed above have led us to believe that when analyzing pathogen-host plant interactions at the cellular or tissue levels, it is important to comprehensively analyze the following: (1) xylem anatomical features that may affect the pathogen's initial entry, (2) the structure, composition, and integrity of I-V PMs that have an important impact on a pathogen's subsequent spread through the vessel system, and (3) the dynamics and quantity of vascular occlusions that develop in response to infection and may affect the pathogen's spread and/or water conduction in host plants. These analyses should also include an evaluation of the spatial distribution of the pathogen. It is only through this that meaningful conclusions can be possible.

MATERIALS AND METHODS

Grapevine Genotypes and *Xylella fastidiosa* Inoculation of Experimental Vines

Grapevines used in the current study included three PD-susceptible *Vitis* genotypes, *Vitis vinifera* vars Chardonnay and Riesling, and *V. vinifera* × *Vitis arizonica* var U0505-35, and three PD-resistant genotypes, *V. vinifera* × *V. arizonica* var U0505-01 and *V. arizonica* × *Vitis rupestris* vars 89-0917 and 89-0908. The growth and inoculation of grapevines for each genotype was fully described in Sun et al. (2011). Briefly, each grapevine was grown in a 7.6-L pot in the greenhouse with a daily cycle of 16-h light/8-h dark and trained to retain only two shoots that developed from two robust buds at the scion base. Inoculation of experimental vines was conducted at the sixth internode of one shoot of each vine (the fifth or seventh internode if the sixth internode was too short or surface damaged) when the vine's shoots were about 6 weeks old. For each genotype, some vines were needle inoculated with a 60-μL liquid *X. fastidiosa* inoculum (10^8 colony-forming units mL⁻¹) or with the 60 μL of 0.01 M PBS buffer (0.138 M NaCl, 0.0027 M KCl, pH 7.4, experimental control). The inoculated vines were then grown in the greenhouse for about 12 more weeks until the *X. fastidiosa*-inoculated PD-susceptible vines developed severe external PD symptoms. During this period, each vine was kept to about 2.2 m in height by pruning off the tops of the two shoots. The vine was then used for quantitative and qualitative analyses of vascular occlusions and/or for measurement of stem hydraulic conductivity (as described below). The PD-resistant grapevines did not show obvious symptoms 12 weeks post *X. fastidiosa* inoculation.

SEM and Quantitative Analyses of Vascular Occlusions and *X. fastidiosa* Distribution

SEM was employed to study types of inclusion and quantity and distribution of occlusions in control and *X. fastidiosa*-inoculated grapevines of each genotype. About 12 weeks after inoculation, a 4- to 5-cm-long sample was obtained from each internode of each grapevine's two shoots and immediately fixed in formalin-acetic acid-alcohol (Ruzin, 1999) for at least 48 h. In preparation for SEM examination, three 2-mm-thick stem discs and six longitudinal segments with dimensions of 2 mm (thick) × 10 mm (length) × 8 mm (width) exposing the tangential xylem surface were cut from each formalin-acetic acid-alcohol-fixed internode. Water in the trimmed xylem samples was gradually removed by dehydration via an ethanol series of 50%, 60%, 70%, 80%, 90%, 95%, and 100% (two times) with 30 min at each stop. The samples were then dried with a critical-point drying apparatus (DCP-1, Denton Vacuum, Inc.) and coated with gold/palladium using a cold sputter/etch unit (Desk II, Denton Vacuum, Inc.). The coated samples were examined with the scanning electron microscope (Hitachi S3400N, Hitachi Science Systems) at 3 or 8 kV to analyze inclusion types, morphological features, and distribution of vascular occlusions, as well as distribution and quantity of *X. fastidiosa* cells (to be described below). We used ruthenium red (a dye specific to pectin) with light microscopy to detect the presence/absence and composition of pectin-rich gels as vascular occlusions (if present), according to Sun et al. (2008). Briefly, free-hand sections were made from the same internodes and rehydrated to water via an ethanol series of 50%, 30%, and 10% with 10 min at each stop. The sections were then stained with 0.005% ruthenium red and temporarily mounted with distilled water, followed by examination with a compound light microscope. Pectin-rich gels in a vessel were stained in red and could be distinguished from tyloses and crystals that remain unstained. Some internodes from which sections were made were processed to be observed under SEM to confirm the presence and morphology of pectin-rich gels.

An SEM image with the whole transverse section of each internode was used to quantify vessels with vascular occlusions. The image was enlarged on a computer monitor and overlaid by a transparent overhead project sheet with twelve 30° sectors from a common center. When overlaying the sheet on the image, the common center was placed on the center of the stem pith, and the stem xylem could be correspondingly divided into 12 sectors. Four sectors were selected out of the 12 with a two-sector interval between each selected sector. Xylem tissues located within the four sectors were examined for the total vessel number and the number of vessels with occlusions. With the two numbers, the percentage of occluded vessels was calculated and used to represent that of the specific internode of the specific plant. For each treatment, three to five plants were used to measure the percentage of occluded vessels for each internode.

Quantitative analysis of vessels with tyloses, gels, crystals, and *X. fastidiosa* in each vine was conducted with SEM imaging of a selected set of internodes from each grapevine's two shoots. Every third or fourth internode (inoculated shoot) and every fourth or fifth internode (uninoculated shoot), starting with the basal internode, was chosen. Six tangential longitudinal xylem segments from each of these internodes were prepared and examined with SEM, as described above. All the vessels exposed at the cut surface of each xylem segment (ranging from 103–164 vessels) were screened for the presence/absence of *X. fastidiosa* cells, and the percentage of pathogen-containing vessels was then determined. The same segments were used to identify the kinds of inclusions present and the proportion of vessels with each inclusion type. These evaluations for each internode selected (Figs. 4 and 7) were made using three plants of each genotype/treatment combination, and the values presented are means and the calculated standard deviations.

Measurement of Hydraulic Conductivity of Inoculated Grapevines

The impact of vascular occlusion on the water conduction in inoculated grapevines 12 weeks post inoculation was evaluated with two PD-susceptible (Chardonnay and U0505-35) and two PD-resistant (89-0917 and U0505-01) genotypes. Details of the instrumental setup for these measurements are described in Pérez-Donoso et al. (2010) and as follows. (1) Selected inoculated shoots from greenhouse-grown vines were immersed in degassed distilled water and a sharp razor blade was used to sever the stem at the second internode from the shoot base (the third internode if the second was surface damaged). (2) Without being exposed to air, the cut end of the explant was fitted into plastic tubing, which had been filled with degassed distilled water and connected to a pressurized (33 kPa) water reservoir. This low pressure had been demonstrated to cause no obvious damage to fragile structures, like I-V PMs of water-conducting tissues (Sperry et al., 1988; Pérez-Donoso et al., 2010). (3) An on/off valve positioned between the water reservoir and the explant was set in the off position when fitting the explant to the tubing. All the leaves of the explant were severed at their petioles with a razor blade, and the petioles were immediately sealed with melted gum. (4) After the gum was solidified, another sharp cut was made at the distal end of the explant stem to keep the explant 22 to 28 cm in length. (5) Then the valve was turned to the on position to allow water to flow into the explant's basal end and exit from its distal end for the first 3 min. The weight of the water reservoir was monitored by an electronic balance connected to a computer for data recording, and the weight change in the reservoir during the following 3-min period was obtained to calculate the rate of water flow through the explant. After the water flow experiment, the basal end of each explant was measured for its diameter, from which the transverse-sectional area of the stem was determined. The specific hydraulic conductivity (K) of the explant was calculated based on the formula: $K = FL/PA$ (Sperry et al., 1988), in which F is the water flow rate (g min^{-1}) through the explant, L is the length (cm) of the explant, A is the transverse-sectional area (mm^2) of the explant, and P is the pressure difference (33 kPa in this experiment) applied between the explant's two ends. Stem hydraulic conductivity for each treatment of each genotype was based on three or four vines, and data are presented as means with calculated standard deviations.

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