ABSTRACT

The xylem-limited bacterium Xylella fastidiosa causes Pierce’s disease (PD), whose disease symptoms are primarily the result of xylem vessel blockage in susceptible grapevines. Stem internode and petiole tissues from infected and uninfected control plants of four grape genotypes (Vitis vinifera, V. rufofomentosa, V. smalliana, and V. arizonica/candicans) differing in PD susceptibility were examined using scanning electron microscopy (SEM). Tyloses, fibrillar networks, and gum plugs were observed in lumens of tracheary elements in petioles and internodes of both water-inoculated control plants and X. fastidiosa–inoculated plants of all genotypes. Bacteria were not observed in control plants. In both petiole and internode tissues, the greatest number of occluded xylem vessels were observed in V. vinifera and the smallest number in V. arizonica/candicans. The number of xylem vessels infested with X. fastidiosa was greatest in V. vinifera and did not differ among the other three genotypes. Systemic infection was found in all genotypes. The frequency with which X. fastidiosa infected vessels were observed using SEM corresponded well with bacterial levels estimated by enzyme-linked immunosorbent assay. Among infected plants, tylose formation in internodes was lowest in V. arizonica/candicans and did not differ among the other three genotypes. Infection with X. fastidiosa strongly induced tylose formation in V. vinifera and V. smalliana but not in V. arizonica/candicans. Analysis across tissues and genotypes indicated an induction of fibrillar networks and gum occlusions in response to X. fastidiosa infection, whereas treatment comparisons within genotypes were not significant except for V. vinifera petioles. Limiting the spread of X. fastidiosa infection by xylem conduit occlusions does not appear to be the mechanism conferring PD resistance or tolerance to V. arizonica/candicans, V. smalliana, or V. rufofomentosa. In contrast, the strong induction of tyloses may be detrimental rather than beneficial for V. vinifera survival after X. fastidiosa infection.

Pierce’s disease (PD) is a bacterial disease that affects grapevines and is capable of devastating entire vineyards (21,33). The causal agent, Xylella fastidiosa, is a gram-negative bacterium transmitted by xylem-feeding insects vectoring the pathogen among a broad range of host plant species (6,21). Infection of susceptible grapevines with X. fastidiosa results in xylem vessel occlusions by bacterial aggregates, tyloses, and gums (18,20,28,30,46). As the disease develops, increasing vessel occlusion progressively impairs water movement, which commonly is thought to result in the typical PD symptoms (15,16,26,27). However, to date, a definitive proof that water stress is the cause for symptom development and eventual death of susceptible plants has not been established. In contrast, a recent publication by Thorne et al. (49) compared the symptomology of PD-infected plants under well-watered and water-deficit conditions as well as various water-deficit treatments. None of the water-deficit treatments resulted in symptoms matching those of PD-infected plants; therefore, the authors suggest that factors other than water deficit may cause symptom development, bringing renewed attention to other possible mechanisms of pathogenesis such as phytoxins, generation of growth regulator imbalances, and programmed cell death which were suggested previously (10,19,25,29). Typical symptoms of PD include low vigor, marginal leaf necrosis, abnormal stem maturation manifested as “green islands,” as well as “matchsticks” formed by the retention of petioles after leaf lamina have separated (46,47,49).

PD is endemic to the southeastern states of the United States and has been implicated as the primary factor limiting the grape industry in those areas (19). Numerous studies have identified a range of wild grape species that exhibit various degrees of resistance to PD (11,12,20,23,31,43,48). However, most of these studies were based on examination of vine longevity, disease symptomology, and levels of X. fastidiosa in the plant. More detailed investigations into the nature of the pathogen–host interaction area necessary to identify possible reasons for the differential sensitivity of various genotypes. The apparently xylem-limited nature and spread of X. fastidiosa through the xylem vessels make this vascular tissue a prime target for further investigations.

General grapevine anatomy, including that of the vascular tissue, has been investigated by various researchers (14,32,34,35). Studies also have investigated various anatomical aspects related to PD development (8,17,28,30,45,47,49,50,52). Generally, these studies were conducted with one susceptible (usually Vitis vinifera) or one resistant (usually Muscadina rotundifolia) grape genotype. Information available for other genotypes characterized in respect to their PD susceptibility is very limited. Krivanek et al. (24) reported on xylem vessel occlusion by tyloses for six field-resistant and two V. vinifera genotypes, and Mollehanauer and Hopkins (30) compared M. munsoniana to V. vinifera and M. rotundifolia in respect to the presence of bacteria, tyloses, and gums. The main objectives of this study were to examine the type and extent of xylem vessel occlusion as well as the percentage of vessel colonization by X. fastidiosa in stems and petioles of four Vitis genotypes previously identified to support a range of X. fastidiosa levels.

MATERIALS AND METHODS
Plant material. Four grape genotypes (V. arizonica/candicans b43-17, V. rufofomentosa DVIT 1416, V. smalliana B028G, and V. vinifera Chardonnay) were propagated from herbaceous cuttings obtained from grapevines in the vineyards of the University of California, Davis. The cuttings were rooted in cellulose sponges on intermittent mist beds with 27°C bottom heat. Rooted cuttings first were transplanted into small 15.6-cm³ plastic pots with soil consisting of a 1:1:1 mixture of Yolo sandy loam, perlite, and peat, and 4 weeks later into 1-liter plastic pots. Vigorously growing plants were pruned to two

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buds to normalize shoot growth. After cut-back, plants were grown for about 6 weeks prior to inoculation. The plants were grown in a greenhouse with supplemental lighting to maintain an average day-and-night cycle of 18 and 6 h, respectively, and temperatures between 20 and 32°C. Plants were irrigated automatically twice per day using a system with 1.9-liter/hr emitters set for 2 min and adjusted as necessary to accommodate increasing requirements of the growing plants. Every 3 weeks, plants were fertilized with 50 ml of diluted Miracle-Gro (N:P:K = 15:30:15; Scotts, Marysville, OH). As plants reached about 1 m, lateral and apical shoot tips were pruned every 4 weeks to increase light penetration into the canopy as well as air circulation.

**Bacterial culture and plant inoculation.** About 6 weeks after cut-back, plants were inoculated with either a strain of *X. fastidiosa* originally isolated from the Stag’s Leap area of Napa Valley, California or double-distilled (dd)H2O in the case of control plants. The *X. fastidiosa* inoculum was prepared by isolating the bacteria from symptomatic Chardonnay plants maintained in the greenhouse. The bacteria were grown on solid periwinkle wilt medium at 29°C (6,7) and washed from the culture plates with ddH2O after about 10 days. Stems were inoculated as described by Krivanek et al. (23). A 10-µl droplet of bacterial suspension, adjusted to 6 × 10^8 CFU/ml (absorbance at 600 nm = 0.25), was placed 10 cm above the base of the stem, and a needle was pushed through the droplet and into the stem. To ensure success, each plant was inoculated twice. The second inoculation was followed immediately by the first around the same location of stem. Piercing of the stem resulted in the uptake of the inoculum into the transpiration stream.

**Enzyme-linked immunosorbent assay.** Leaf blades excised from the petioles used for scanning electron microscopy (SEM) analysis and stem internode tissue harvested near the location of the section utilized for microscopy were used to quantify *X. fastidiosa* levels. Determination of *X. fastidiosa* concentrations were accomplished by enzyme-linked immunosorbent assay (ELISA) as described by Krivanek and Walker (23) and are only outlined here. For each genotype and tissue, a separate standard curve was generated by diluting *X. fastidiosa* in plant tissue extracts from healthy plants to concentrations of 6.5 × 10^6, 3.25 × 10^6, 6.5 × 10^5, 3.25 × 10^5, 6.5 × 10^4, and 3.25 × 10^4 CFU/ml. Extracts from *X. fastidiosa*-free tissue served as negative controls. All samples, negative controls, and standard curves were run in duplicate. Concentrations per gram of plant tissue were established by conversion of the predicted concentrations per milliliter of extract.

**Sample collection, preparation, and SEM.** Stem and petiole samples were collected 16 weeks post inoculation from three *X. fastidiosa*-inoculated plants and two water-inoculated plants of each species. Both tissues were sampled from the bottom, middle, and top positions of each plant. The bottom position comprised the 25-cm segment of the plant immediately above the point of inoculation, and the middle and top positions consisted of the following two 25-cm segments. Multiple cross sections were cut from each petiole and stem position and were fixed in formalin-alcohol-acetic acid (44) for a minimum of 48 h. Prior to cryofracturing, the specimens were infiltrated for at least 30 min with an aqueous solution of 30% glycerol serving as cryoprotectant. A steel plate, serving as working surface, and a razor blade were precooled in liquid N and used to prepare cross sections by fracturing the specimens immediately after immersion in liquid N. Sections were dehydrated in a series of ethanol solutions (30, 50, 70, 90, and 100%) and critical-point dried in carbon dioxide (Autosamdrí 815B; Tousimis Research Corporation, Rockville, MD). Dried specimens were mounted on aluminum stubs with colloidal graphite, sputter coated with gold (SPI-Module Sputter Coater, Structure Probe, Inc. West Chester, PA), and examined in a Hitachi S-3500N SEM (Hitachi High-Technologies America, Schaumburg, IL). The standard operating condition of the SEM was 10 kV; occasionally, however, conditions were adjusted in the range of 5 and 20 kV to optimize observations. Energy dispersive X-ray analysis (EDAX) was conducted using a detector with a QDD Violin Detector (8-µm beryllium window) and VIDX Scan Active Digital Imaging software (EVEX Analytical, Princeton, NJ). For

![Fig. 1.](Image) Concentration of *Xylella fastidiosa*, as determined by enzyme-linked immunosorbent assay, in stems and leaves from the bottom, middle, and top positions of the three plants examined for each genotype. Error bars represent standard error of the mean. Different letters between genotypes indicate significant differences in *X. fastidiosa* levels across positions within tissue.

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EDAX, the microscope was operated at an acceleration voltage of 20 kV, a working distance of 17.8 mm, an elevation angle of 40°, and a magnification of x2,500. The spectra were acquired for 120 s (live time) and a dead time of 2%.

Vessel occlusion analysis. From each sample, three cross sections were analyzed as follows. In all, 50 randomly selected xylem vessels from a minimum of five vascular bundles in petioles and numerous ray-delimited sectors in stem internodes were examined for occlusion to determine the degree and type of occlusion. Thus, a total of 150 xylem vessels was scrutinized and used to calculate an average for each sample, resulting in a total of 450 vessels examined each for internode and petiole tissues of one plant. For each genotype, 2,700 vessels were examined for the infected plants and 1,800 vessels for the control plants. Because individual X. fastidiosa cells and small colonies as well as initials of other occlusions can be difficult to observe, every vessel was examined systematically at magnifications between x800 and 3,000, focusing up and down the vessel interior. The type of occlusion present was determined and the extent of occlusion was categorized into three groups: (i) <25%, (ii) 25 to 50%, and (iii) >75% occlusion of a particular vessel.

Experimental design and statistical analyses. The plants were arranged in a completely randomized design, with each individual plant representing an experimental unit. Three X. fastidiosa-inoculated plants and two control plants were maintained for each genotype. For statistical analyses of ELISA results, bacterial concentrations were natural log-transformed. The SAS software package (version 9.1; SAS Institute, Cary, NC) was used to analyze the data using the June 2006 release of PROC GLIMMIX which employs a generalized, linear, mixed-model approach. Overall data were analyzed as a split-split plot model, with genotype and infection status as main plots, section as the subplot, and tissue as the sub-subplot. Bonferroni adjustment was used for treatment comparisons. Regression analyses were conducted using PROC REG.

RESULTS

Symptomology. The onset of early PD symptoms such as marginal chlorosis as well as chlorotic areas away from the margins occurred 6 weeks post inoculation in V. vinifera plants. Follow-up observations 2 months post inoculation revealed typical symptoms for PD, including marginal leaf necrosis and chlorotic transition zones on leaves from the bottom and middle sections of V. vinifera plants. At the time of sample collection (16 weeks post inoculation), advanced symptoms, including entirely necrotic leaf blades, green islands, and matchsticks, were observed on the V. vinifera plants. In contrast, green islands and matchstick symptoms were not observed in the other three genotypes. Some leaf necrosis symptoms were observed in V. smalliana and V. arizonica/candicans; however, they occurred to a similar extent in water- and X. fastidiosa-inoculated plants. In V. rafotomentosa, leaf necrosis was observed about three times more frequently in X. fastidiosa- compared with water-inoculated plants but did not reach the extent observed in V. vinifera.

ELISA. The concentration of X. fastidiosa was significantly affected by genotype in both stem and leaf tissues (P < 0.01; Fig. 1). In contrast, X. fastidiosa concentrations were not significantly affected by the plant section examined. For stem tissues, the differences were significant between V. vinifera and the other genotypes. Among the other genotypes, no differences were observed between V. rafotomentosa and V. smalliana; however, tendencies toward greater levels of X. fastidiosa in V. rafotomentosa (P = 0.052) and V. smalliana (P = 0.072) than V. arizonica/candicans were found. For leaf blades, X. fastidiosa levels in V. vinifera were significantly greater than in V. smalliana (P = 0.01) and V. arizonica/candicans (P < 0.01). However, large variation in the X. fastidiosa concentrations in V. rafotomentosa leaf blades re-

![Fig. 2. Comparison of the percentage of xylem vessels containing occlusions among the four grape genotypes examined. Error bars indicate the standard error of the means. Different letters between genotypes indicate significant differences in percentage of xylem vessel occlusion within tissue. Comparisons were made within infection status, capital letters corresponding to infected plants and non-capital letters to control plants. Absence of letters indicates that the differences were not statistically significant.](image-url)
sulted in the absence of significant differences between it and the other three genotypes. The average concentration in V. vinifera leaves was $7.2 \times 10^7$ CFU g$^{-1}$ and, in stems, $1.17 \times 10^9$ CFU g$^{-1}$. $X$. fastidiosa levels in the leaves and stems of V. arizonica/candicans did not exceed the positive threshold (mean of control + three standard deviations) employed in this study. In contrast, the $X$. fastidiosa concentrations in V. smalliana and V. rufotomentosa exceeded the positive threshold but were much lower than in V. vinifera. The average concentrations of $X$. fastidiosa in V. smalliana were $7.0 \times 10^6$ CFU g$^{-1}$ in leaves and $5.8 \times 10^6$ CFU g$^{-1}$ in stems. Those in V. rufotomentosa were $3.0 \times 10^6$ CFU g$^{-1}$ in leaves and $6.1 \times 10^6$ CFU g$^{-1}$ in stems.

**SEM.** Examination of xylem vessels using SEM revealed the presence of various types of occlusions in both water- and $X$. fastidiosa-inoculated plants of all genotypes. Tyloses, fibrillar networks, gums, and, on very rare occasions, crystals were observed in both control and $X$. fastidiosa-infected plants and contributed to xylem vessel occlusions. Statistical analysis of

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**Fig. 3.** Scanning electron micrographs of *Xylella fastidiosa*-infested xylem vessels of internodes and petioles of infested *Vitis vinifera* plants. **A,** Loosely arranged small aggregates of *X. fastidiosa* lining the cell wall next to and in pits of a xylem vessel. Bar = 10 µm. **B,** Small aggregate of bacteria attached to the cell wall and connected to each other by fibrillar strands. Bar = 2 µm. **C,** Individual bacterial cells embedded in a thin layer of gum around a pit. Bar = 5 µm. **D,** Large numbers of *X. fastidiosa* in and around pits of adjacent xylem vessel elements. Through the open pit membranes, one can see what appears to be a large aggregate of bacteria in the lumen of the xylem vessel. Bar = 10 µm. **E,** Large aggregate of bacteria blocking a simple perforation plate. Bar = 30 µm. **F** and **G,** Large numbers of bacteria entirely occluding xylem vessels. Note the loose arrangement of the cells in F (bar = 10 µm) and the more tightly embedded bacteria in G (bar = 10 µm).
the sum of all occlusions indicated significant differences for genotype–infection status–tissue (P = 0.0397), genotype (P = 0.0399), infection status (P < 0.0001), and tissue (P = 0.0134). No effect of sampling position on the plant was observed for the sum of all occlusions. In water-inoculated plants, significant effects of the genotype–tissue (P = 0.0154) interaction and the tissue (P = 0.0015) main effect but not the genotype main effect were observed. Although the differences in vessel occlusions of petioles were not significant among the genotypes of control plants, those in stem internodes were significantly greater for V. vinifera and V. rufotomentosa than V. smalliana and V. arizonica/candicans (Fig. 2). In X. fastidiosa-infected plants, xylem vessel occlusion differed between genotypes (P = 0.01) and tissues (P < 0.0001). The average percentage of vessels occluded ranged from 2.1% in stem internodes and 9.0% in petioles of X. fastidiosa-infected V. arizonica/candicans to 34.7% in stem internodes and 55.9% in petioles of V. vinifera (Fig. 2). For both stem internodes and petioles, intermediate values were observed for V. rufotomentosa and V. smalliana. Within genotype, X. fastidiosa infection strongly increased xylem vessel occlusion in V. vinifera (P = 0.0033). However, only marginal increases in the percentage of occluded xylem vessels were observed for X. fastidiosa-infected V. smalliana (P = 0.0635), V. arizonica/candicans (P = 0.0572), and V. rufotomentosa (P = 0.1103). In order to investigate xylem vessel occlusions in more detail, separate statistical analyses were conducted for X. fastidiosa, tyloses, and fibrillar networks and gums, but not for crystals, because they were observed on very rare occasions only.

No X. fastidiosa was found in water-inoculated control plants; consequently, those plants were excluded from the analysis for X. fastidiosa presence in xylem vessels. X. fastidiosa in xylem vessels were observed as individual bacteria and in groups ranging from small clusters to very large aggregates (Fig. 3). In some instances, the bacteria appeared tightly embedded in a matrix whereas, in other instances, bacterial aggregates were loosely arranged. The presence of fibrillar strands connecting neighboring bacterial cells among each other and anchoring bacterial cells to vessel walls were observed frequently. Bacteria often were found on pit membranes or in pits with breached membranes that allowed bacterial movement from one vessel to another. In addition, they were found frequently in crevasses at perforation plates as solitary cells, small clusters, or in larger aggregates.

The presence of X. fastidiosa was influenced by genotype (P < 0.0001) and tissue (P = 0.0002), with a significant interaction effect of genotype–tissue (P < 0.0001). The percentage of vessels containing X. fastidiosa was greatest in V. vinifera for both stem internodes (4.1 ± 1.3%) and petioles (30.9 ± 5.6%) and averaged less than 1% in both stem internodes and petioles of each of the other three genotypes (Fig. 4). Among the V. vinifera samples examined, the maximum percentage of vessels containing X. fastidiosa was 11.3% in stem internodes and 56.7% in petioles. In the other three genotypes, no more than 2.7% of the vessels contained X. fastidiosa. The majority of X. fastidiosa observed were in the form of individual cells or small aggregates that occluded the vessels to less than 25%. In fact, in V. aestivalis var. smalliana, V. arizonica/candicans, and V. rufotomentosa stem internodes, no X. fastidiosa aggregates occluding vessels to more than 25% were found. This also was true for V. arizonica/candicans petioles. In V. smalliana petioles, some vessels (0.1%) with medium-size aggregates were observed and, in V. rufotomentosa petioles, some vessels with medium (0.2%) and large (0.2%) aggregates were found (Fig. 4). The percentage of X. fastidiosa-infested vessels was significantly (P < 0.05) greater in V. vinifera petioles than stem internodes. However, in the other three genotypes, the differences between stem internodes and petioles were not significant.

Tyloses. Tyloses were observed in both water- and X. fastidiosa-inoculated plants of all genotypes (Fig. 5). A large range in tylose occlusions of xylem vessels was

![Fig. 4. Comparison of the percentage of Xylella fastidiosa containing xylem vessels among the four grape genotypes examined. The severity of X. fastidiosa presence was rated based on the extent of blockage caused in a particular xylem vessel. Error bars indicate the standard error of the means for the total percentage of infected xylem vessels. Different letters between genotypes indicate significant differences in percentage of xylem vessel occlusion within tissue.](image-url)
observed. Tyloses were observed as initials barely pushing through pits and as individual or multiple tyloses completely occluding xylem vessels (Fig. 6). Some of the tylose occlusions filled xylem vessels over a considerable length. In some cases, the tyloses originated from single xylem parenchyma cells whereas, in others, multiple xylem parenchyma cells surrounding the xylem vessel formed tyloses.

Tylose formation was significantly influenced by genotype ($P = 0.0374$) and infection status ($P = 0.0001$), and a marginal effect of tissue type ($P = 0.0565$) also was observed. Separate analysis by infection status revealed that tylose formation in the water-inoculated control plants was not significantly affected by the genotype main effect, but that a significant ($P = 0.049$) genotype–tissue interaction and a significant tissue ($P = 0.0294$) effect existed. In contrast, tylose formation was influenced by genotype ($P = 0.0142$) in the X. fastidiosa-infected plants. In addition, a significant ($P = 0.0008$) genotype–tissue interaction effect was observed in the X. fastidiosa-infected plants. Overall analyses found no significant effect of plant position on tylose formation. Tylose formation was increased in response to X. fastidiosa infection in both stem internodes and petioles. Within genotypes, differences in tylose formation between water-inoculated control and X. fastidiosa-infected plants were significant for V. vinifera ($P = 0.0009$) and V. smalliana ($P = 0.0375$), but only marginally significant for V. rufotomentosa ($P = 0.0794$) and not significant for V. arizonica/candicans. In V. arizonica/candicans, the mean percentage of vessels occluded by tyloses was 1.25% (average across tissues and infection status) and the maximum observed among all samples was 6.7%. The percentage of vessels occluded by tyloses was not significantly different between stem internodes and petioles for V. smalliana and V. rufotomentosa. The mean vessel occlusion by tyloses in X. fastidiosa-infected plants was 7.9% in V. smalliana and 17.4% in V. rufotomentosa, whereas it was 0.9 and 2.1% in water-inoculated control plants, respectively. In V. vinifera, the mean percentage of vessel occlusion by tyloses in stem internodes (25.0%) was greater ($P < 0.05$) than in petioles (8.5%) and reached a maximum of 42.7%. With the exception of V. arizonica/candicans and petioles from water-inoculated V. rufotomentosa, for which small tyloses were observed, the majority of tyloses observed occluded the xylem vessels to >75%. In fact, those tyloses accounted for 61% of the total found.

Fibrillar networks and gum. The presence of fibrillar networks in xylem vessels was observed in water-inoculated and X. fastidiosa-infected plants of all genotypes (Fig. 7). These networks typically were attached to vessel walls, sometimes attached around the entire circumference of the vessel or only in some sectors. Aside from the fibrillar nets, vessel occlusions by gum-like material also were observed (Fig. 7). At low magnification, vessel occlusions by gum-like material (Fig. 7A) appeared very similar to occlusions by large aggregates of tightly embedded bacteria (Fig. 3G). However, the occlusions could be differentiated readily after close examination due to different surface appearances and the absence of bacteria in the gum-like material. Fibrillar nets and gum-like occlusions were observed more frequently in X. fastidiosa- than in water-inoculated plants ($P = 0.0034$) and in petioles (3.3%) than in stem internodes (<1%) ($P < 0.0001$). However, because of the low abundance of gum-like occlusions, further statistical analyses were conducted on data pooled with those from the fibrillar networks. Because it was difficult to assess the extent of occlusion of the fibrillar networks, differentiation of the extent of blockage as conducted for the X. fastidiosa aggregates and tyloses was not attempted for fibrillar nets or gum. Overall analysis revealed significant effects of infection status ($P =
0.0109), tissue (P < 0.0001), genotype–tissue (P = 0.0003), and infection status–tissue (P = 0.0033). A significant genotype effect (P = 0.0275) was observed for stem internode results but not for petiole results. Significantly (P < 0.05) greater occurrences of nets or gum occlusions were found in V. vinifera than in V. smalliana for both water- and X. fastidiosa-inoculated stem internodes (Fig. 8). In V. smalliana, V. arizonica/candicans, and V. rufotomentosa, the occurrence of nets or gum occlusions in petiole tissue was significantly greater (P < 0.001) than in stem internodes; however, the difference was not significant in V. vinifera tissues. Although the overall analysis indicated a significant effect of infection status on net or gum occurrence, individual comparisons within genotypes for each tissue revealed a significant effect only in V. vinifera petioles (P = 0.0482). However, across all genotypes, 2-fold (stem internodes) and 5.7-fold (petioles) greater occurrences of net or gum occlusions were observed in X. fastidiosa-over water-inoculated plants. As indicated by genotype comparison of net or gum occurrence in stem internodes across water- and X. fastidiosa-inoculated plants, their presence was observed more often in V. vinifera (3.7%) than in the other three species (0.6%; average across species and infection status). However, the differences were not significant in petiole tissues.

**Crystals.** Intralumenal crystals (Fig. 9) were observed only in three V. vinifera cross sections of infected plants. The crystals were identified as such using X-ray microanalysis which showed high concentrations of Ca (Fig. 10). None of the crystals observed were associated with X. fastidiosa.

**DISCUSSION**

**Symptomology and X. fastidiosa levels.** The symptoms observed on V. vinifera were typical for PD, and marginal leaf necrosis such as that observed on V. rufotomentosa was consistent with previous reports on PD-resistant genotypes (18,20,24,28,38,50). The severity of the symptoms appeared to be correlated with the number of X. fastidiosa in that the symptoms were severe in V. vinifera, mild in V. aestivalis var. rufotomentosa, and inconclusive in V. arizonica/candicans and V. aestivalis var. smalliana.

In this study, we investigated the influence of X. fastidiosa inoculation on xylem vessel colonization and occlusion in petioles and stem internodes among four grape genotypes that differ in the extent to which they support X. fastidiosa growth. Krivanek and Walker (24) have shown a high correlation between the field resistance of grape genotypes and ELISA estimates of the number of X. fastidiosa in stem tissue. We used neighboring stem internode segments for X. fastidiosa quantification by ELISA and SEM analysis of

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Fig. 6. Scanning electron micrographs of grape internode and petiole xylem. A, Internode from a *Xylella fastidiosa*-infected *Vitis vinifera* plant. Longitudinal section through a xylem vessel completely occluded by tyloses. Bar = 100 μm. B and C, Low-magnification views of B, an internode (bar = 500 μm) and C, a petiolar vascular bundle (bar = 100 μm) from a *V. aestivalis* var. *rufotomentosa* control plant showing predominantly empty lumens of xylem vessels. D and E, Transverse section of stem internodes of *X. fastidiosa*-inoculated *V. aestivalis* var. *rufotomentosa* plants. D, Xylem vessel elements completely occluded by advanced stages of tyloses next to vessel elements free of any blockage. Bar = 300 μm. E, Tyloses at various stages of development penetrating through the pits from numerous xylem parenchyma cells. Bar = 20 μm.
by the inoculation of control plants with water and to periodic pruning conducted to increase light penetration and air circulation in the canopy may have contributed to these differences in the control plants.

In internodes of X. fastidiosa-infected plants, significant genotypic differences were observed in respect to total percentage of occluded vessels, X. fastidiosa-infested vessels, tyloses, and fibrillar networks and gums. In petioles, significant differences among genotypes were found for X. fastidiosa and the sum of all occlusions (Figs. 4, 5, 6, and 8). In general, when significant differences among genotypes were observed, V. vinifera exhibited the greatest and either V. smalliana or V. arizonica the smallest number of occluded vessels. Within a genotype, X. fastidiosa inoculation had a very strong effect on the sum of all vessel occlusions in V. vinifera internodes and petioles and in V. smalliana internodes. Tendencies for a greater number of occluded vessels in X. fastidiosa-infected than in control plants also were present in the petioles of V. smalliana and internodes and petioles of V. arizonica/candicans and V. rufotomentosa. The absence of a significant response or only limited response observed in V. arizonica/candicans, V. smalliana, and V. rufotomentosa compared with V. vinifera contrasts with results obtained by Mollenhauer and Hopkins (30) for M. rotundifolia and M. munsoniana. They reported that X. fastidiosa-infected plants of those two species exhibited greater frequency of tyloses and gums than the V. vinifera cv. Thompson Seedless. They suggested that gums and tyloses may be involved in the resistance mechanism of these genotypes by limiting the spread of the infection. Similarly, Huang et al. (22) reported fewer electron-dense occlusions in the susceptible V. vinifera ‘French Colombard’ than M. rotundifolia ‘Carlos’ and also proposed that these vascular occlusions halt X. fastidiosa infection, thus playing a key role in limiting PD expression in M. rotundifolia. Recently, Krivanek et al. (24) compared six Vitis genotypes considered to be field resistant with two susceptible V. vinifera cultivars. Although they observed significantly lower tyloses occlusion in internodes of resistant than susceptible genotypes, this was not the case for gum occlusions, and they did not find a strong correlation between vascular occlusions and the PD resistance status of the investigated genotypes. However, in this study, we observed strong positive relationships of both tyloses and fibrillar net or gum vessel occlusions with X. fastidiosa concentrations. Tylose formation in internodes of infected plants was significantly lower in the resistant V. arizonica/candicans than in the susceptible V. vinifera. The frequency of fibrillar networks and gums was significantly lower in the internodes of resistant V. smalliana than in the susceptible V. vinifera. The results observed for V. smalliana and V. arizonica/candicans contrasted with those documented for M. rotundifolia (22,30), which indicates differ-

Fig. 7. Scanning electron micrographs of xylem vessel occlusions by gum and fibrillar networks. A, Plug presumed to be gum completely blocks a xylem vessel. Bar = 10 µm. B, Fibrillar net spanning across the lumen of a xylem vessel. No bacterial cells can be identified in the net. C, Fibrillar net associated with Xylella fastidiosa attached to the xylem wall. Bar = 2 µm. D, Large fibrillar network filling the lumen of a xylem vessel. Note the sheet-like forms and clumps making up part of this network. Bar = 5 µm. Micrographs A and C, D were obtained from lyophilized rather than critical-point-dried specimens.
ent responses to *X. fastidiosa* infection and possibly different roles and contributions of vascular occlusions in respect to PD resistance. Similarly, the variations in responses observed among *V. arizonica/candicans*, *V. smalliana*, and *V. rufotomentosa* may indicate different levels of tolerance or alternative resistance mechanisms.

In this study, temporal dynamics of the progression of vascular occlusions were not investigated. The time frames involved in the formation of vascular occlusions could have important implications with respect to disease development. For example, in a time-course study on *V. vinifera* Chardonnay, Stevenson et al. (46) collected samples 10, 14, and 18 weeks post inoculation and found that *X. fastidiosa* infestation and spread was followed by increased accumulation of gum in leaves and petioles, which preceded tylose formation in the stem. Tylose formation still was in the initial stages 10 weeks after inoculation whereas advanced stages of tyloses occlusions were observed by 14 weeks. In our study, the majority of the tyloses observed in petioles and stems 16 weeks post inoculation completely occluded the xylem vessels in *V. vinifera*, *V. smalliana*, and *V. rufotomentosa* (Fig. 6; >75% blocked). Although the large number of advanced tyloses suggests that the onset of tylose formation likely was weeks earlier, the occurrence of tylose initials and medium-sized tyloses indicates continued initiation in stems and petioles. However, the rate of tylose initiation likely changes over time. If tylose formation had a role in limiting the spread of the bacteria throughout the plant, one would expect the induction of tylose formation to occur soon after and in the vicinity of the inoculation event. However, in this study, we did not observe any differences in tylose frequency between the bottom third (includes the site of inoculation) and the middle and top thirds of the plants. Similarly, the frequency with which *X. fastidiosa*-infected vessels were observed did not differ among plant positions, indicating that the spread of the bacteria was not limited by tyloses or other forms of occlusions. *X. fastidiosa* bacteria were observed in the bottom, middle, and top sections of all infected plants, suggesting systemic infection in all genotypes. The absence of differences in tyloses among the plant positions could be a result of the advanced infection status. It may be that differences could have been detected if analyses had been conducted earlier. On the other hand, recent evidence indicates open connections among xylem vessels over long distances, providing unimpeded pathways for the spread of *X. fastidiosa* across multiple internodes and into leaves throughout *V. vinifera* and *M. rotundifolia* plants (3,50). Thus, the distribution of bacteria at the time of inoculation could range across multiple nodes and result in tylose formation in an area far removed from the site of inoculation. However, although passive movement over long distances is possible and likely, observation of *X. fastidiosa* in plant tissues that developed post inoculation and the appearance of *X. fastidiosa* colonies and their sizes suggest successful systemic infection in all genotypes, which could not be avoided by tyloses, fibrillar nets, or gel occlusions of the xylem conduits. *X. fastidiosa*-infested vessels were observed much more frequently in *V. vinifera* than in the other three genotypes (Fig. 4). However, in internodes of *V. smalliana*, *V. arizonica/candicans*, and *V. rufotomentosa*, *X. fastidiosa* aggregates completely occluding the xylem vessels were never observed. In most cases, the bacterial cells were observed either as individual cells or in aggregates attached to the cell walls, rather than blocking entire vessels. Thus, the direct effect of *X. fastidiosa* aggregate vessel occlusion appears to be of minor importance in limiting the water conduit. The effect of *X. fastidiosa*-generated signals resulting in the induction of tylose formation or fibrillar nets or gel is of greater consequence to water transport in the xylem conduit.

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**Fig. 8.** Comparison of fibrillar net and gel formation among four grape genotypes inoculated either with water (C) or *Xylella fastidiosa* (I). Error bars indicate the standard error of the means. Different letters between genotypes indicate significant differences in percentage of xylem vessel occlusion within tissue. Comparisons were made within infection status, capital letters corresponding to infected plants and noncapital letters to control plants. Absence of letters indicates that the differences were not statistically significant among genotypes.
Fibrillar networks were observed more frequently in petioles than in internodes. The chemical nature of these networks is unclear. At least those present in water-inoculated control plants should be of plant origin. In X. fastidiosa-inoculated plants, the networks often were observed together with bacteria, but bacteria were not always present. Thus, it cannot be excluded that some or parts of the networks observed in X. fastidiosa-inoculated plants may be of bacterial origin. Similar fibrillar occlusions have been observed in X. fastidiosa-infected plants by others using SEM (1,2,13). Although of unknown origin and nature, it is likely that these fibrillar networks are dehydrated gels. The pattern of occurrence is consistent with those observed for gels and gums by others (8,22,24,30,44). In addition, transmission and cryoscanning electron micrographs of xylem vessel mucilage by Crews et al. (4) show fibrils and mucilage freezing pattern similar to the fibrillar networks we observed. Rioux et al. (40) found evidence that gel formation in trees is accompanied by the secretion of pectin, which exhibits fibrillar pattern. Pectic polysaccharides are known to form gels (5) and may be the main component of the fibrillar networks observed in this study. Fibrillar networks such as those illustrated in Figures 3B and 7C may or may not be of different origin. Figure 3B illustrates an example where the network appears to be localized to a small aggregate and the fibrils connect individual bacterial cells. In contrast, the network shown in Figure 7C spans the lumen of the xylem vessel and is not as closely associated with the bacterial cells. Due to the different sample preparation protocols employed, it is unclear whether this variation in appearance is related to differences in origin or chemical composition or both.

It is unlikely that the crystals observed occluding xylem vessels in infected V. vinifera plants are related to the disease. Although none of them were observed in control plants, they were very rare and never associated with any X. fastidiosa. Tyson et al. (52) previously reported the rare occurrence of Ca-containing crystals in X. fastidiosa-infected plants and also considered it unlikely that they were of importance in respect to PD. The observed crystals may be calcium-oxalate based because calcium-oxalate crystals are the most common crystals found in plants (9).

The four grape genotypes differed in their response to X. fastidiosa infection. The percentage of occluded vessels per stem internode cross section averaged 34.7, 15.7, 11.5, and 2.1% for V. vinifera, V. rufotomentosa, V. smalliana, and V. arizonica/candicans, respectively. However, the significance of those occlusions in respect to water transport is unclear. For instance, Hopkins (18) showed that analysis of serial sections revealed a 4- to 12-fold greater number of occluded vessels than single cross-section analysis. In V. vinifera, massive tyloses were not uncommon and some of them extended for more than 1 mm in length in a single vessel (Fig. 6). Thus, it can be argued that the effective number of vessels occluded likely is considerably greater than indicated by single cross-section counts. However, a threshold level at which the occlusion percentage becomes relevant for the water supply of the tissue apical to the occlusion has not been established. Further investigations into the effect of vessel occlusions on water transport in the stems and the relationship of symptom expression would be of interest. Although the positive relationship of symptom development and xylem vessel occlusion could suggest a causative relationship, so could bacterial factors because symptom development was positively related to the number of X. fastidiosa-infected vessels.

Based on the results obtained in this study, it does not appear that the greater resistance or tolerance of V. arizonica/candicans, V. smalliana, and V. rufotomentosa are related to an ability to limit the spread of the infection. It has been suggested by others that this resistance mechanism may occur in M. rotundifolia and M. munsoniana (22,30); therefore, it is likely that the three species examined here have different mechanisms of resistance. In addition, based on the number and distribution of X. fastidiosa in V. arizonica/candicans, V. smalliana, and V. rufotomentosa, it possible that the mechanisms of resistance are different or at least expressed to a different extent. Because only the frequency and quantity but not the location of X. fastidiosa observations differed among V. vinifera and the other three genotypes, it seems more likely that factors influencing X. fastidiosa reproductive success or virulence confer greater tolerance or resistance to V. arizonica/candicans, V. smalliana, and V. rufotomentosa.

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LITERATURE CITED

2. Alves, E., Marucci, C. R., Lopes, J. R. S., and Leite, B. 2004. Leaf symptoms on plums, coffee and citrus and the relationship with the extent of xylem vessels colonized by Xylella fas-

Goodwin, P. H., DeVay, J. E., and Meredith, C. Pathol. 32:17-32.


G. 2001. Primary vascular patterns in the Vita-


Fry, S. M., and Milholland, R. D. 1990. Multi-


Purcell, A. H. 1986. Pierce’s disease. Pages 62-

96 in: Grape Pest Management. D. L. Fiherty, ed. Cooperative Extension University of California, Division of Natural Resources, Oak-


