CLARIFICATION OF XYLEM FEATURES LIMITING THE ENTRY AND SYSTEMIC SPREAD OF *XYLELLA FASTIDIOSA* IN GRAPEVINES

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ABSTRACT

Grapevine Pierce's disease (PD) caused by the bacterium *Xyllela fastidiosa (Xf)* is a devastating vascular disease and is jeopardizing the grape and wine industries in the United States due to the PD susceptibility of most important commercial grape genotypes. In our recent study, we compared the PD-susceptible commercial grapevine genotypes with some PD-resistant genotypes newly obtained from traditional breeding program. Comparisons included intervessel pit membrane (PM) integrity, bacterial distribution, and tylose development, which are the most important factors determining the vascular disease resistance of a host plant. Our data indicate that *Xf*-infected PD-resistant genotypes could well maintain the integrity of their intervessel PMs and were found to have a very localized distribution of *Xf* cells while infected PD-susceptible genotypes were observed to have very porous or broken intervessel PMs and a systemic distribution of *Xf* cells. This demonstrates the strong correlation of intervessel PM integrity and limited bacterial distribution in infected vines. It is also revealed that grape genotypes with different PD resistances could develop tyloses in response to *Xf* infection. However, tylose development occurred to different spatial and quantitative extents among the genotypes, with an intensive tylose development throughout a vine in PD-susceptible genotypes but only small amounts of tyloses, these close to the inoculation site in PD-resistant genotypes. This also indicates that tylose development in PD-resistant genotypes does not seriously affect the water status of infected vines and suggests that the tylose development in PD-susceptible genotypes should make the PD symptom development worse by blocking vine water transport. These results provide information for identifying the factors affecting grape resistance to PD and, most likely, have identified vine characteristics useful to efforts aimed at the development and evaluation of an efficient approach to control this terrible disease.

LAYPERSON SUMMARY

Efficient approaches in control of Pierce's disease (PD) based on the understanding of the PD-resistance mechanism of grapevine are being sought as strategies for management of PD in vineyards. This work investigated grapevine genotypes with different PD resistances by focusing on three important factors that may affect the pathogen's spread in a host grapevine and water status of an infected grapevine. Our data indicate that pit membranes (PMs; barriers of the bacterial spread through a grapevine's water conducting system) and tyloses (occluding tissues of the water-conducting tissue) may be related to the PD resistance of the host grapevine. In PD-susceptible grapevines, the causal pathogen can become systemic by breaking the PMs in the vine water conducting system and PD disease symptoms may become more extensive due to tylose blockage of the water conducting tissue in infected vines. In PD-resistant grapevines, the PMs were well maintained and restricted bacterial spread and tyloses did not significantly affect the water transport in infected vines. The information is essential for the understanding and evaluation of PD resistance of new grape germplasm obtained from traditional breeding programs.

INTRODUCTION

Pierce's disease (PD) of grapevines has caused tremendous economic losses to the wine and table grape industries in the United States. The causal *Xylella fastidiosa (Xf)* is a xylem-limited bacterium that spreads only through the vessel system of a host grapevine (Purcell and Hopkins, 1996), thus, any factors affecting the systemic expansion of the *Xf* population that has been introduced initially into one or very few vessels should be relevant to the resistance vs. susceptibility of the infected vine.

As the only avenue for the pathogen's spread, the vessel system of grapevine has attracted a lot of research attention (e.g., Chatelet et al., 2006; Sun et al., 2006, 2007; Thorne et al., 2006). Individual vessels in a grapevine's secondary xylem are relatively short (average length of 3-4 cm, Thorne et al., 2006), thus systemic movement of water, minerals or bacteria requires passage through multiple adjacently interconnected vessels. Movement from one vessel to the next requires passage through pit pairs, specialized wall structures that connect a vessel to its neighbors. In grapevines, contact with neighboring vessels occurs at multiple locations along the vessel's length and scalariform (i.e., organized in a ladder-like pattern) pit pairs always occur in the wall regions where two adjacent vessels are in contact (Sun et al., 2006). At each pit pair, two adjacent vessels are separated only by two thin primary cell walls and one middle lamella, which are collectively called an intervessel pit membrane (PM) (Esau, 1977). Intervessel PMs of grapevines have pores with sizes varying between 5 and 20 nm (Choat et al., 2003; Pérez-Donoso et al., 2010) and thus should prevent the passage of *Xf* cells (0.25-0.5 µm x 1-4 µm in size; Mollenhauer and Hopkins, 1974) as long as the PMs remain intact.

It has been proposed that *Xf* cells use cell wall-degrading enzymes (CWDEs) to digest PM polysaccharides and achieve their systemic spread (Newman et al., 2003; Labavitch et al., 2006; Labavitch, 2007). Some microscopic examinations on xylem sections of infected grapevines have shown *Xf* cells traversing intervessel PMs (Newman et al., 2003; Ellis et al., 2010). Furthermore, the introduction of certain CWDEs to explanted grapevine stems caused breaks in the PM polysaccharide network and permitted *Xf* cells to pass through intervessel PMs (Pérez-Donoso et al., 2010). These studies have suggested that the systemic spread of the bacterial cells is achieved by disrupting the integrity of intervessel PMs.

Vascular occlusions were reported in grapevines with external PD symptoms (Stevenson et al., 2004; Lin, 2005). As the major type of vascular occlusion, tyloses (outgrowths of parenchyma cells adjacent to a vessel that expand into the vessel lumen), were found to be abundantly present in secondary xylem of the susceptible genotypes (Labavitch and Sun, 2009). Among the few studies on tylose development, most were done with PD-susceptible genotypes and the rest dealt with some susceptible and resistant grape genotypes but were focused on the vessel systems in leaf blades or petioles (Fry and Milholland,1990a,b). The lack of comprehensive information about tylose development has led to two opposed opinions about the functional roles of tyloses in grape PD; i.e., they either improve the host grapevine's PD resistance or worsen the grapevine's PD symptom development. Our previous study reported intensive tylose development in stem secondary xylem of susceptible genotypes, suggesting a possible role in enhancing the infected grapevine's PD symptoms (Labavitch and Sun, 2009). To further clarify this, studies comparing tylose development among grape genotypes with different PD resistances became important.

Although most commercial genotypes of *Vitis vinifera* are susceptible to PD, many wild *Vitis* genotypes and some hybrid *V. vinifera* crossed with wild *Vitis* genotypes have shown strong PD resistance in greenhouse evaluations (Loomis, 1958; Krivanek and Walker, 2005). In this study we used up to four grape genotypes with different PD resistances: two PD susceptible lines, *V. vinifera* var. Charodanny and *V. vinifera* var. Riesling, and two PD-resistant lines, *V. arizonica* X *rupestris* (89-0908) and *V. vinifera* X *arizonica* (U0505-01). The work in this report examined infected vines of these genotypes from the following three perspectives: integrity of intervessel PMs, distribution of *Xf* cells, and spatial distribution and quantitative character of tyloses. Our aims are to clarify the relationships between these three important factors affecting the systemic spread of the bacterial pathogen and/or plant water status and, in addition, further describe their possible relationship to the PD resistance of grapevines.

OBJECTIVES

1. Determine whether xylem structural features vary among grape genotypes with different PD resistance and clarify what structural features are related to the PD resistance of grapevines.
2. Determine whether PM polysaccharide composition and porosity and the extent of *Xf*'s spread from the inoculation site vary in grape genotypes with different PD resistance, and clarify the extent to which PM polysaccharide structure and integrity are affected by *Xf* inoculation of these genotypes.

RESULTS AND DISCUSSION

<u>Differences in Intervessel PM Integrity among Grape Genotypes with Different PD Resistances</u>

Grapevines of three genotypes, PD-susceptible Chardonnay and PD-resistant U0505-01 and 89-0908, were used to investigate effects of *Xf* infection on grapevine PM integrity. Vines of Chardonnay and 89-0908 were grown each from a grafted root stock in the greenhouse. Buds of each scion were removed with only two robust buds left at the base, which were allowed to develop into two shoots. When the vines of the two genotypes were four weeks-old, some were inoculated with *Xf* while others were inoculated with phosphate buffered saline (PBS) as experimental controls. The bacterial inoculation was carried out only on one shoot of each treated vine, at the 6th internode counting from the shoot base. PBS inoculation for each control vine was done in the same way except that the 0.2M PBS instead of the *Xf* inoculum was used. The two shoots of each control or *Xf*-inoculated vine were limited to about 20-25 internodes in length by pruning the tops off. U0505-01 vines were grown and inoculated in the same way except that each vine was trained to have one shoot by initially leaving only one bud at the base of the scion. That sole shoot was inoculated, either with *Xf* suspension or PBS.

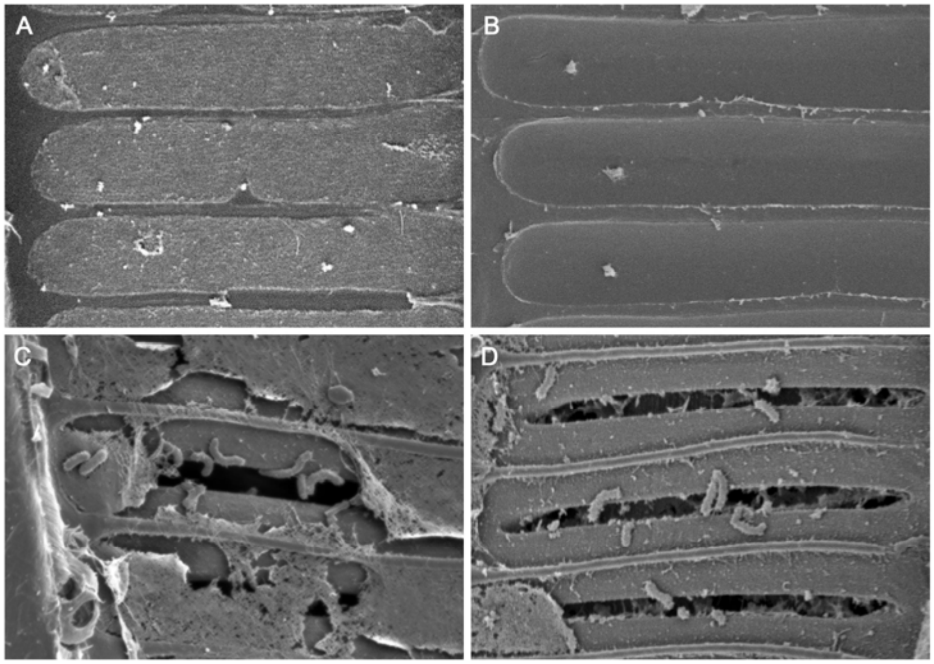

Figure 1. Effect of *Xf* infection on intervessel pit membrane (PM) integrity of grapevines with different PD resistances. Shown are inner surface views of intervessel PMs after the secondary wall borders of each pit were removed to expose the whole intervessel PM. A. Intact intervessel PMs in a vessel not associated with the bacteria in a *Xf*-infected PD-susceptible Chardonnay vine. B. Intact intervessel PMs in a *Xf*-infected PD-resistant U0505-01 vine. C-D. *Xf*-infected Chardonnay vines, showing partial (C) and complete (D) removal of intervessel PMs in vessels that also contain bacterial cells.

The grapevines of the three genotypes were examined for the integrity of their intervessel PMs 12 weeks after *Xf* inoculation, with plants inoculated with PBS used as controls. In all the genotypes, intervessel PMs were intact with no pores detectable under SEM at a magnification of 20,000 in the vessels not associated with *Xylella* cells and those of control vines (**Figure 1A**). In the *Xylella*-infected vines of the two PD resistant genotypes (U0505-01 and 89-0980), intervessel PMs remained intact in all the internodes examined, including the internode with the inoculation site (**Figure 1B**). In infected Chardonnay vines, partially broken intervessel PMs (**Figure 1C**) and/or the complete removal of PMs (**Figure 1D**) were observed in most vessels containing bacterial cells throughout the plants. Thus, *Xylella* infection and intervessel PM disruption are strongly correlated in PD-susceptible genotypes, but not in PD-resistant genotypes.

Table 1. Distributional Comparison of *Xylella fastidiosa* in Some Examplary 12-week-post-inoculation Grapevines with Different PD Resistance

internode[1]	Chardonnay (PD-susceptable)		Riesling (PD-susceptable)		89-0908 (PD-resistant)		U0505-01 (PD-resistant)
	Inoculated shoot[2]	Non-inoculated shoot	Inoculated shoot	Non-inoculated shoot	Inoculated shoot	Non-inoculated shoot	Inoculated shoot
1	+	+	-	-	-	-	-
3	+	-	+	+	+	-	-
5	+	+	+	-	+	-	+
7	+	+	+	+	-	-	-
9	+	+	-	+	-	-	-
11	+	+	+	+	-	-	-
13	+	+	+	+	-	-	-
15	+	-	+	+	-	-	N/A
17	+	+	+	+	-	N/A	N/A
19	+	+	N/A	+	N/A	N/A	N/A
21	+	N/A	N/A	-	N/A	N/A	N/A
23	+	N/A	N/A	N/A	N/A	N/A	N/A

[1]Internodes are numbered from each shoot base upwards with its first internode at the base as internode 1. [2]Inoculation site was at the 6th internode from the shoot base in Chardonnay and 89-0908 vines and at the 4th internode for the U0505-01 vine. [3]+ or - indicate that *Xylella* cells were observed or not observed in a specific internode, and N/A represents inavailability of a specific internode due to the short shoot.

Distributional Differences of *Xf* among Grapevines with Different PD Resistances

Four grape genotypes, including PD-susceptible Chardonnay and Riesling and PD resistant 89-0908 and U0505-01, were used in our experiment. Grapevines of each genotype were grown, trained and inoculated as described above. Samples were collected from both inoculated and non-inoculated shoots (only the sole inoculated shoot for each U0505-01 vine) of each vine at week 12 after inoculation. Every other internode of each shoot was used SEM examination of the distribution of *Xylella* cells in the vines.

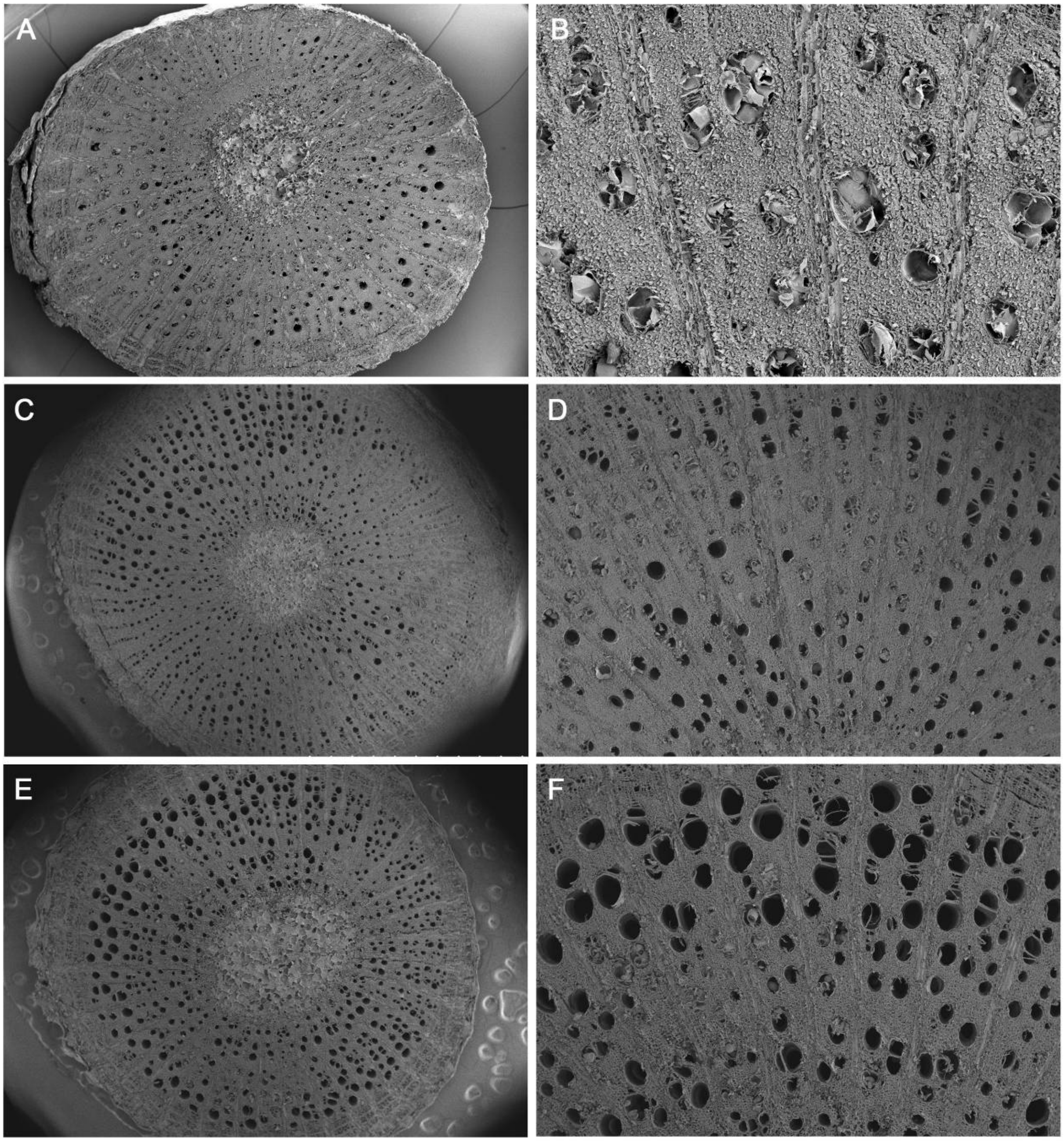

Figure 2. Effect of *Xf* infection on tylose development in grapevines with different PD resistances. Scanning electron micrographs of stem secondary xylem of a PD-susceptible Chardonnay vine (A-B) and a PD-resistant U0505-01 vine (C-F). A. A majority of the vessels were occluded with tyloses and vessels with tyloses were relatively evenly distributed in the 3rd internode above the inoculation site (internodes above the inoculation site were counted upwards with the inoculated internode designated as "zero"). B. Enlargement of part of xylem tissue in A, showing most vessels fully occluded by tyloses. C. The second internode below the inoculation site , showing presence of tyloses in the vessels of some xylem regions. D. Enlargement of part of the xylem with tylose development in C, showing a few vessels filled with tyloses. E. A majority of vessels did not contain tyloses and tylose development occurred in a few vessels in some small xylem regions in the 8th internode above the inoculation site. F. Enlargement of part of the vessels containing tyloses in E, showing a small cluster of vessels associated with tyloses.

Our data have indicated that in Chardonnay and Riesling vines, bacterial cells were observed in most or all of the internodes examined, including those in both the inoculated and non-inoculated shoots of each vine (**Table 1**). This showed not only that the spread of *Xf* cells from the inoculation point occurred in the susceptible vines, and also that the bacterial cells moved downward from the inoculation site on an inoculated shoot to reach the non-inoculated shoot through the common trunk that the two shoots share. In 89-0908 vines, *Xf* cells were not observed in the non-inoculated shoots of all the vines that were examined. Furthermore, *Xf* cells were not always found in the inoculated shoots in some vines examined and, when pathogen cells were detected, they were seen only within a few internodes downward or upward from the inoculation sites (**Table 1**). A similar situation also was seen in the U0505-01 vines examined (**Table 1**). These observations therefore demonstrate the localized distribution of *Xf* cells in the PD-resistant genotypes and the systemic distribution in the PD-susceptible grapevines. When this is considered with the data of intervessel PM integrity, it also provides the evidence for the correlation of the systemic spread of *Xf* cells and the disruption of intervessel PM structure, which has been hypothesized in earlier reports.

Differences in Tylose Development in Grapevines with Differential PD resistance

Two grape genotypes (PD-susceptible Chardonnay and PD-resistant U0505-01) were studied for tylose development in *Xf*-infected vines at week 12 after inoculation. Growth and inoculation of the experimental vines was conducted in the same way as described above. A few internodes along the inoculated shoot, including those above and below the internode inoculated, were examined to clarify both qualitative and quantitative characteristics of tylose development.

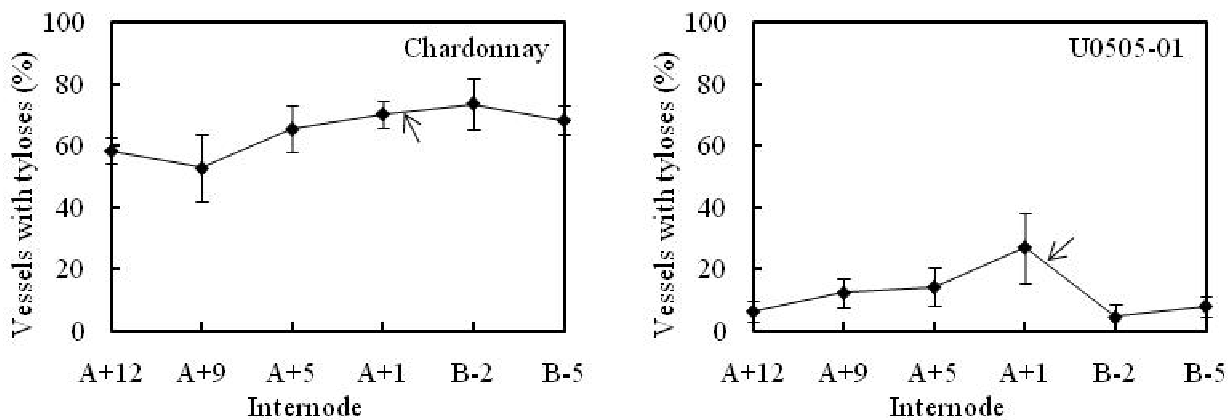

Figure 3. Quantitative comparison of tylose development in PD-susceptible Chardonnay vines and PD-resistant U0505-01 vines. Arrows indicate the internode with inoculation site. Internodes above and below the one with inoculation are indicated by "A+" and "B-", respectively and numbered upward and downward, respectively, with the internode with the inoculation site defined as zero.

Tylose development occurred in all the *Xf*-infected vines but was not observed in the buffer-inoculated control vines for the two genotypes. However, the infected vines of the two genotypes had difference in the spatial distribution of tyloses. In Chardonnay vines intensive tylose development occurred in all the internodes examined from the base to the tip of an infected vine (**Figure 2A**), and vessels with tyloses were more or less evenly distributed in the transverse sections of a stem (**Figure 2B**). In U0505-01 vines, more tyloses were observed in the internodes close to the inoculation site (**Figure 2C-D**) than in the internodes more towards the tip and base (**Figure 2E-F**), and tylose development mostly occurred in one or several secondary xylem regions in which most vessels were associated with tyloses (**Figure 2C, E**). When quantitatively compared for tylose development among the two genotypes, Chardonnay vines generally had over 60% of vessels blocked in the internodes examined throughout the vines (**Figure 3A**), but U0505-01 vines contained 5-27% tylose associated vessels (**Figure 3B**). Our previous study revealed that *Xf* cells themselves did not affect water conduction in infected Chardonnay vines because there were relatively few bacterial cells in only a few vessels in the vines even with severe PD symptoms. This led us to conclude that tyloses were the key factors influencing water transport inside sick vines (Labavitch and Sun, 2009). Furthermore, since tylose development did not always continue along individual vessels in grapevines and open vessels observed in one cross section could be occluded at other places along the vessel lengths (Sun et al., 2006, 2008). This suggests that the percentage of the vessels actually occluded by tyloses should be higher than any value based on the analysis of a single cross section. Therefore, blockage of the majority of vessels in the secondary xylem of a Chardonnay vine should inevitably affect its water supply, consequently contributing to exacerbation of PD symptom development and vine deterioration. As for the resistant genotype, vessels associated with tyloses were much fewer and were restricted to limited xylem regions. Tylose development should have limited effect on the water transport in the infected vines of resistant genotypes.

CONCLUSIONS

1. Grape genotypes with differential PD resistances show differences in intervessel PM integrity and *Xf* distribution. Large amounts of broken intervessel PMs and systemic bacterial distribution are observed in PD-susceptible genotypes while

intact intervessel PMs and restricted bacterial distribution are seen in PD-resistant genotypes. This strongly suggests the positive correlation between intervessel PM integrity and limited *Xf* distribution

2. Tyloses are present in *Xf*-infected grapevines with different PD resistances, but tylose development occurred, both spatially and quantitatively, to much lower extents in PD-resistant and –tolerant genotypes. In these genotypes, tylose development should be considered as a factor that does not lead to PD symptom development.

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