EFFECTS OF GRAPE XYLEM SAP AND CELL WALL CONSTITUENTS ON *IN VITRO* GROWTH AND CELL WALL DEGRADING GENE EXPRESSION OF *XYLELLA FASTIDIOSA*

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ABSTRACT
Purified cell-wall constituents or grape xylem sap added to media affected *Xylella fastidiosa (Xf) in vitro* growth, biofilm formation, cell aggregation and gene expression. Media containing xylem sap from Pierce's disease (PD)-susceptible plants provided better support for bacterial growth and biofilm formation than media supplemented with xylem sap from PD-resistant plants. Culturing *Xf* on media containing various purified cell-wall constituents demonstrated that CM-cellulose, xylan, β-D-glucan, k-carrageenan, cello-oligosaccharide and laminarin promoted bacterial growth whereas lichenan strongly suppressed growth. However, only laminarin, xylan, and k-carrageenan promoted biofilm formation *in vitro*. Lichenan, oligosaccharide, k-carrageenan, laminarin, xylan and β-D-glucan all significantly decreased bacterial cell aggregation in vitro. Quantitative real-time PCR assays revealed that expression of genes encoding extracellular endoglucanase, endo-1,4-beta-glucanase, and periplasm protease were differentially regulated in response to amendment of media with xylem sap from PD-resistant and PD-susceptible grapevines. This study indicates that composition of xylem sap and cell walls may influence the interaction of *Xf* with grape hosts *in planta* and may account for differences in pathogenesis of *Xf* on PD-resistant and –susceptible grapevines.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a Gram negative, xylem-limited bacterium causing Pierce's disease (PD) of grapevine (1). *Xf* is transmitted by xylem-feeding insects, including the polyphagous and invasive glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (2). However, the mechanisms of *Xf* pathogenicity in host plants are not fully understood. The currently accepted explanation for development of PD in grapevine is water stress resulting from occlusion of xylem vessels by bacterial biofilm and/or accumulation of extracellular polysaccharides and subsequent blockage of xylem vessels with pectins, tyloses and gums produced by the plant host in response to *Xf* infection (2). There also is a functional relationship between xylem chemistry and *Xf* planktonic growth, aggregation and biofilm formation within *Vitis* germplasm (3, 4). We hypothesize that cell wall degradation products may affect *Xf* cell growth, aggregation, biofilm formation, and movement within xylem vessels either directly as a source of nutrients and/or indirectly by induction or repression of *Xf* genes.

OBJECTIVES
1. Determine the comparative effects of xylem sap from PD-resistant and PD-susceptible grapes on growth, biofilm formation, and cell aggregation *in vitro*;
2. Determine the effects of a variety of cell-wall constituents on *Xf* growth, biofilm formation, and cell aggregation *in vitro*; and
3. Analyze the effects of xylem saps from PD-resistant and PD-susceptible grapes pre-inoculated with or without *Xf* on the cell-wall degrading enzyme-related gene expression *in vitro*.

RESULTS
Effects of xylem sap from PD-resistant and PD-susceptible grapevines and other cell-wall constituents on bacterial planktonic growth: After one day of culture in liquid PW (-BSA) medium amended with 1X PW medlum (minus BSA) – containing xylem sap from PD-resistant or -susceptible plants, no significant differences ($p<0.4873$) in *Xf* growth was observed. However, after three or seven days, growth of *Xf* in liquid PW (-BSA) medium amended with xylem sap from PD-susceptible plants was significantly greater than growth in the same medium amended with xylem sap from PD-resistant plants ($p<0.0282$ and $p<0.0177$, respectively) (**Figure 1A**). Inclusion of BSA in the growth medium at half the normal concentration (1/2X BSA) resulted in more rapid growth of *Xf* (**Figure 1B**) than in PW (-BSA) medium (**Figure 1A**). Nonetheless, *Xf* growth after three days was significantly greater ($p<0.0071$) when cultured in PW (1/2X BSA) medium amended with xylem sap from susceptible plants relative to growth in the same medium amended with xylem sap from PD-resistant plants (**Figure 1B**). To confirm this result, *Xf* cells grown in liquid PW (-BSA) medium amended with xylem sap

for seven days were plated onto complete solid PW medium (**Figure 1C**). The number of viable cells recovered from growth medium amended with xylem sap from PD-susceptible plants averaged 2.24-fold more than the number of viable cells recovered from medium supplemented with xylem sap from PD-resistant plants (number of *Xf* colonies: 431±7 for xylem sap from PD-susceptible plants versus 192±89 for xylem sap from PD-resistant plants, with $n = 3, p < 0.01$). In addition, we determined the effects of cell-wall components on *Xf* growth. Most cell-wall components had positive effects on bacterial growth *in vitro*, among these, cellulose had the most promoting effect for all time points examined ($p<0.0001$ at Day 1, $p<0.0001$ at Day 3 and $p<0.0014$ at Day 7), followed by laminarin ($p<0.0550$ at Day 1, $p<0.0111$ at Day 3 and $p<0.0014$ at Day 7), xylan ($p<0.1163$ at Day 1, $p<0.0228$ at Day 3 and $p<0.0182$ at Day 7), glucan ($p<0.8569$ at Day 1, $p<0.0126$ at Day 3 and $p<0.0244$ at Day 7), and carragerran ($p<0.0476$ at Day 1, $p<0.0009$ at Day 3 and $p<0.9496$ at Day 7); oligosaccharides promoted *Xf* growth only at one day ($p<0.0120$ at Day 1, $p<0.8030$ at Day 3 and $p<0.1636$ at Day 7). In contrast, lichenan inhibited bacterial growth on the third and seventh days ($p<0.2126$ at Day 1, $p<0.0004$ at Day 3 and $p<0.0001$ at Day 7) of culture (**Figure 1D**).

Effects of in vitro growth medium amendment with xylem sap from resistant or susceptible grapevines and cell wall components on bacterial biofilm formation and aggregation: As shown in **Figure 2A**, xylem sap from PD-susceptible grapevine significantly increased *Xf* biofilm formation *in vitro* (1.57 times higher than in unamended control, $p<0.0162$; 1.60 times higher than resistant xylem sap, $p<0.0082$). In contrast, xylem sap from both PD-susceptible and -resistant grapevines significantly decreased *Xf* cellular aggregation *in vitro* by factors of 3.28 ($p<0.0074$) and 2.20 ($p<0.0333$) times lower than the unamended control (**Figure 2B**). Laminarin and k-carrageenan significantly enhanced *Xf* biofilm formation (2.48 fold greater than control, $p<0.0006$ for laminarin; 1.59 fold greater than unamended control, $p<0.0055$ for k-carrageenan). Laminarin and k-carrageenan also significantly decreased *Xf* cellular aggregation 5.25- fold ($p<0.0397$) and 4.2 fold ($p<0.0178$), respectively, compared to that in unamended control medium (**Figure 2B**). Lichenan and cello-oligosaccharide decreased the *Xf* cellular aggregation very significantly (3.00 fold less than control, $p<0.0090$) and significant (4.67 fold less than unamended control, $p<0.0137$) levels, respectively, but did not affect *Xf* biofilm formation (**Figures 2A and 2B**). In contrast, *Xf* biofilm formation and cellular aggregation were not significantly different in medium supplemented with xylan and β-D-glucan from that in unamended medium controls.

Effects of xylem sap from resistant and susceptible grapevines on bacterial cell-wall degrading-related gene expression: Expression of endo-1,4-β-glucanase gene was significantly increased (2.83 fold greater, $p<0.05$) in PW medium amended with plant xylem sap from PD-resistant grapevine pre-infected with *Xf*, but increased only slightly in medium amended with susceptible plant xylem sap from PD- susceptible grapevine pre-infected with *Xf* (**Figure 3A**). The *Xf* periplasm protease gene was significantly down-regulated (1.67 fold less, $p<0.01$) in medium amended with xylem sap from PD-resistant grapevines pre-infected with *Xf*. In contrast, the periplasm protease gene expression was significantly up-regulated (2.21 fold greater, $p<0.01$) in medium amended with xylem sap from PD-susceptible grapevines pre-infected with *Xf* (**Figure 3B**).

Total protein content and composition changes in PD-resistant and PD-susceptible grape plant xylem sap in response to Xf infection: As shown in **Figure 4**, the majority of *Xf*-induced host proteins in PD-resistant plants are of low molecular weight (15 – 35 Kd) with high pI values (pI 7 – 10), although a small group of proteins with lower pI values (pI 3.5 - 4) were also induced (**Figures 4A and 4B**). In contrast, only a few host proteins with the similar range of molecular weights and pI values were induced by *Xf* in PD- susceptible plants (**Figures 4C and 4D**). Some *Xf*-induced xylem sap proteins were genotype specific, whereas others were specific to *Xf* infection.

DISCUSSION

Genetic differentiation of xylem sap from PD-resistant and PD-susceptible grapevines

Highly PD-resistant and -susceptible *Vitis*' species were used in this study. Differential host responses to *Xf* infection between the two lines are controlled by a single major locus (the dominant resistance allele is *PdR1*) (7). Host plant response to *Xf* infection differs between resistant and susceptible genotypes at both molecular and physiological levels and also varies with plant organ, as stem and leaf tissues of the same plant respond differently (5,10). Given that *Xf* is limited to xylem vessels, we hypothesized that xylem cell wall properties and chemical composition of xylem fluid may significantly affect *Xf* pathogenesis. Although the biochemical properties of xylem sap from these two grapevines have not been determined in detail, our bioassay and protein analysis indicated that xylem sap from PD-resistant and PD-susceptible grapevines differed in protein composition, especially following *Xf* infection (**Figure 4**).

Roles of cell-wall constituents in bacterial growth, biofilm formation and cellular aggregation of Xf

Within xylem, *Xf* is confined to a poor nutritive environment (8, 9). Upon degradation of xylem cell-walls, xylem fluid in PD-resistant and -susceptible grapevines likely differ both qualitatively and quantitatively with respect to the chemical composition of cell wall degradation products. Our results suggest that different cell-wall constituents have different effects on growth, biofilm formation and cellular aggregation of *Xf* at least *in vitro* (**Figures 1 and 2**). Several cell-wall constituents (cellulose, xylan, glucan, laminarin, carragerran and oligosaccharides) enhanced *Xf* growth *in vitro*. Some cell-wall constituents (laminarin, k-carrageenan, cellulose, lichenan and oligosaccharides) inhibited *Xf* cellular aggregation. Only laminarin and k-carrageenan significantly enhanced biofilm formation, and only cellulose significantly enhanced cellular aggregation *in vitro*. It seems clear that different cell-wall constituents are required and actively involved in the different

processes of bacterial growth, biofilm formation and cell aggregation, of which algae and seaweed laminarin- and k-carrageenan-related cell wall components significantly enhanced both bacterial growth and biofilm formation *in vitro*, whereas only cellulose significantly enhanced bacterial growth and aggregation, and most other cell-wall ingredients tested inhibited cell aggregation *in vitro*. Aggregation may result from clumping of cells facilitated by extracellular polysaccharides and may be the initial step of biofilm formation (11, 12). Cellular aggregation of *Xf* in response to xylem sap from PD- resistant and -susceptible grapevine plants, and to different cell-wall constituents, did not mirror responses in planktonic growth and biofilm formation to the same treatment. It is not clear whether cellular aggregation process *in vitro* is different from that *in planta*.

Cell-wall degrading enzymes potentially involved in the early stage of pathogenicity through interaction of Xf with xylem sap of host plants

Pathogenicity of *Xf* likely requires biofilm formation leading to xylem vessel blockage and subsequent water stress (12, 13). Regulatory pathways are responsible for the transition from planktonic growth to biofilm formation (14, 15). Gene expression during the early stage of biofilm formation with planktonic bacteria exposed to plant xylem sap resulted in expression of endo-β-1,4-glucanase and periplasm protease genes in the xylem sap from PD-susceptible grapevines pre-infected was elevated. Increased expression of these genes by *Xf* in PD-susceptible grapevines presumably would result in more efficient degradation of cell-walls and release more free cell-wall constituents available to support bacterial growth (**Figures 3A and 3B**). This conclusion is supported by recent studies showing plant pathogenic bacteria are able to lyse and grow on viable host cells by producing a variety of cell-wall degrading enzymes, including endo-β-1,3-glucanases, proteases, β-1,6-glucanases, mannanases, and chitinases (16).

CONCLUSIONS

Our observations support the hypothesis that *Xf*-grapevine host- pathogen interactions are mediated by xylem sap constituents, as opposed to a direct connection between bacteria and metabolically active host cells. Therefore, xylem vessels may serve as a unique niche for host plants to recognize and interact with *Xf* in xylem sap *in planta*. If this is the case, identification of components of xylem sap that differ among PD-resistant and -susceptible grapevines may facilitate elucidation of mechanisms through which *Xf*- host plant interactions result in resistance or susceptibility. Xylem sap could be used to screen grapevines for PD resistance breeding.

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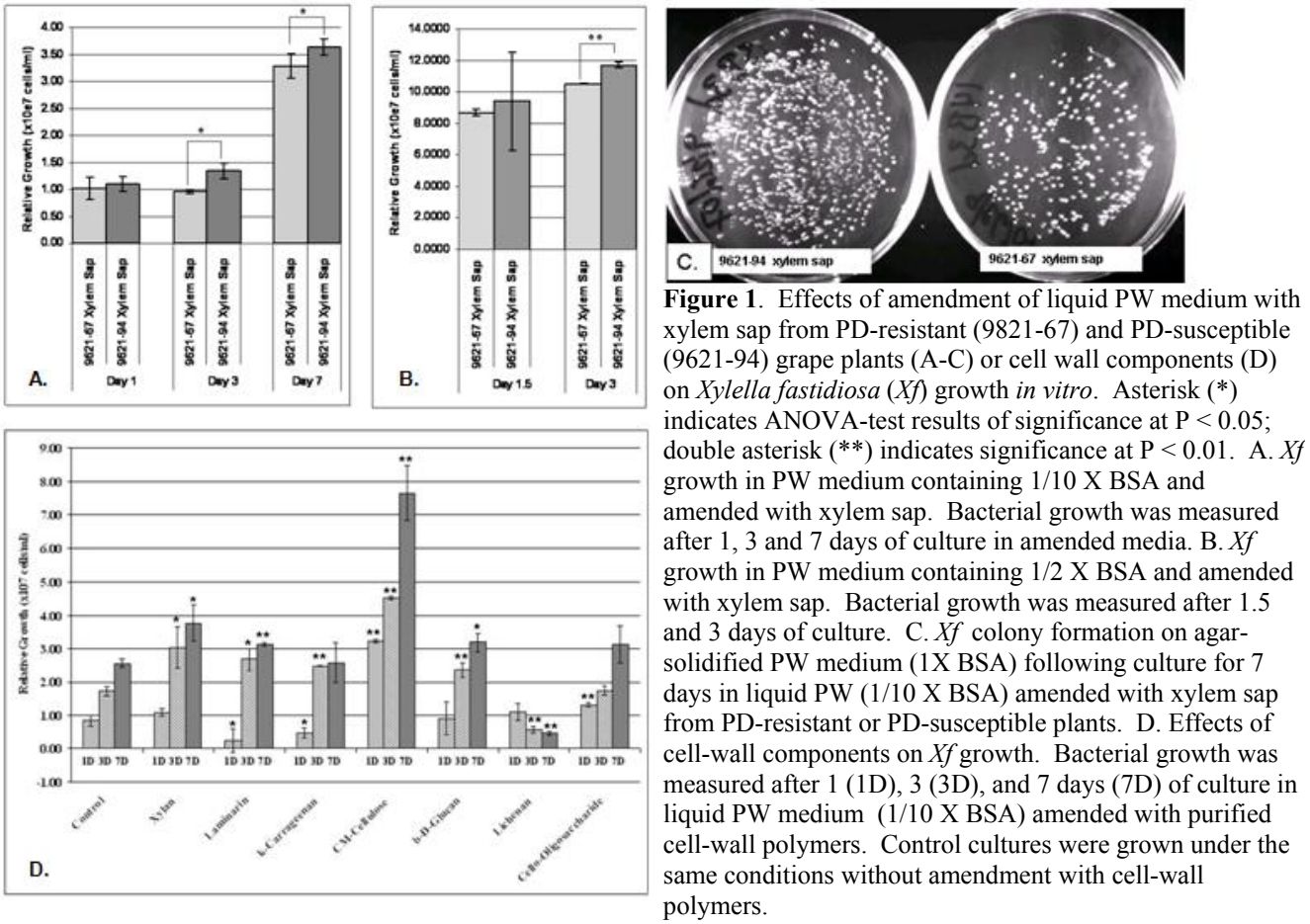

Figure 1. Effects of amendment of liquid PW medium with xylem sap from PD-resistant (9821-67) and PD-susceptible (9621-94) grape plants (A-C) or cell wall components (D) on *Xylella fastidiosa* (*Xf*) growth *in vitro*. Asterisk (*) indicates ANOVA-test results of significance at $P < 0.05$; double asterisk (**) indicates significance at $P < 0.01$. A. *Xf* growth in PW medium containing 1/10 X BSA and amended with xylem sap. Bacterial growth was measured after 1, 3 and 7 days of culture in amended media. B. *Xf* growth in PW medium containing 1/2 X BSA and amended with xylem sap. Bacterial growth was measured after 1.5 and 3 days of culture. C. *Xf* colony formation on agar-solidified PW medium (1X BSA) following culture for 7 days in liquid PW (1/10 X BSA) amended with xylem sap from PD-resistant or PD-susceptible plants. D. Effects of cell-wall components on *Xf* growth. Bacterial growth was measured after 1 (1D), 3 (3D), and 7 days (7D) of culture in liquid PW medium (1/10 X BSA) amended with purified cell-wall polymers. Control cultures were grown under the same conditions without amendment with cell-wall polymers.

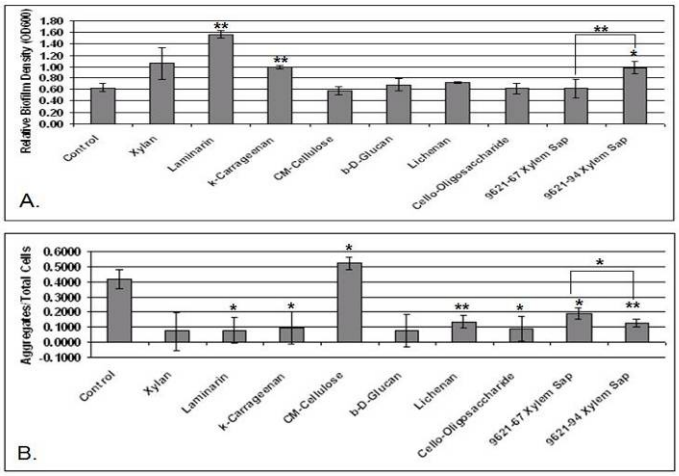

Figure 2. Effects of amendment of liquid PW medium with xylem sap from PD-resistant (9821-67) and PD-susceptible (9621-94) grape plants or cell wall components on *Xf* biofilm formation (A) or cell aggregation (B). Asterisk (*) indicates ANOVA-test results of significance at $P < 0.05$; double asterisk (**) indicates significance at $P < 0.01$.

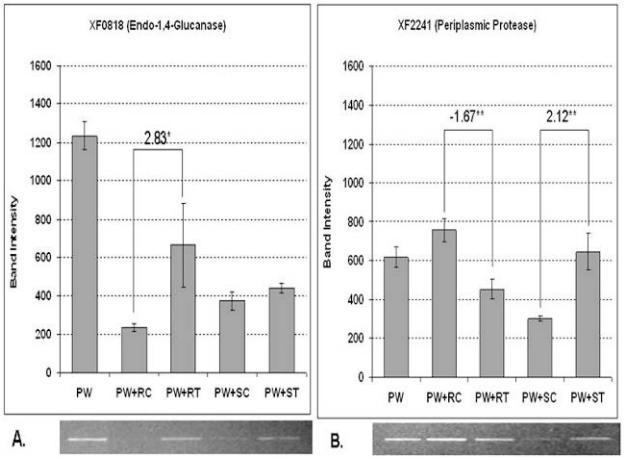

Figure 3. Effect of grape xylem saps on *Xf* gene expression *in vitro*. PW represents PW liquid medium, PW+RC indicates PW liquid medium plus xylem sap from uninoculated PD-resistant plants, PW+RT indicates PW liquid medium plus xylem sap from PD- resistant plants pre-infected with *Xf*, PW+SC indicates PW liquid medium plus xylem sap from uninoculated PD- susceptible plants, and PW+ST indicates PW liquid medium plus xylem sap from PD- susceptible plants pre-infected with *Xf*. Bacterial cultures were subsequently collected for RNA isolation and gene expression analyses.

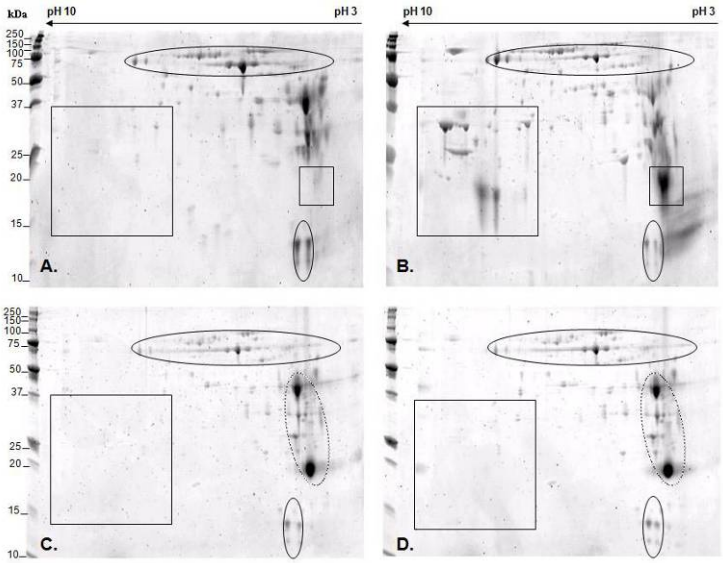

Figure 4. 2D- PAGE analyses of proteins in xylem sap from PD-resistant (9621-67) and PD-susceptible (9621-94) grape plants in response to *Xf* infection. A. 9621-67 uninoculated control; B. 9621-67 *Xf*- infected; C. 9621-94 uninoculated control; D. 9621-94 *Xf*- infected. The pI range is shown on the top, molecular weight standards and sizes are shown on the left. The *Xf*-induced protein zones are boxed, constitutively expressed proteins are circled with solid lines or dashed lines to highlight the different display patterns of protein spots on the gels. Only qualitative analysis was performed to show the presence and variation of major visible protein spots between different xylem sap samples.