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Effects of grape xylem sap and cell wall constituents on *in vitro* growth, biofilm formation and cellular aggregation of *Xylella fastidiosa*

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Abstract Pierce's disease (PD) of grapevines is caused by the xylem-limited bacterium *Xylella fastidiosa* (*Xf*). All *Vitis vinifera*-based cultivars are susceptible to *Xf* infection; however, many grape species from the southern United States (such as *V. arizonica, V. shuttleworthii, V. simpsonii, V. smalliana,* and *Muscadinia rotundifolia*) are resistant. In this study, the effects of xylem sap from PD-resistant and PD-susceptible grapes, as well as several free cell wall constituents, on *in vitro* bacterial growth, biofilm formation, and cellular aggregation were investigated. Media containing xylem sap from 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 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 *Xf* cellular aggregation *in vitro*. This study suggests that differences in xylem sap composition and cell wall properties among PD-resistant and PD-susceptible grapes may affect characteristics of *Xf* growth, biofilm formation and cellular aggregation involved in pathogenesis.

Keywords Xylem sap · Cell wall · Bacterial growth · Biofilm formation · Aggregation · *Xylella fastidiosa*

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Introduction

Xylella fastidiosa (*Xf*) is a Gram-negative, xylem-limited bacterium causing Pierce's disease (PD) of grapevine (Hopkins 1989). This bacterium is transmitted by xylem-feeding insects, including the polyphagous and invasive glassy-winged sharpshooter, *Homalodisca vitripennis* (Purcell 1975; Purcell et al. 1979; Almeida and Purcell 2003). Although PD has been recognised as a serious disease of grape for more than a century, an effective disease management strategy is lacking, due in part to a limited understanding of *Xf*-host plant interactions affecting virulence and pathogenesis.

The currently accepted explanation for development of PD symptoms in grapevine is that they are the result of water stress resulting from occlusion of xylem vessels by bacterial biofilm and/or accumulation of extracellular polysaccharides, and the subse-

quent blockage of xylem vessels with pectins, tyloses and gums produced by the plant host in response to *Xf* infection (Almeida and Purcell 2003). A functional relationship between xylem chemistry and *Xf* planktonic growth, aggregation and biofilm formation within *Vitis* germplasm has been reported in recent years (Leite et al. 2004; Andersen et al. 2007). Bacterial multiplication and movement occurs more rapidly in xylem of PD-susceptible grapevines compared to that in xylem of PD-resistant grapevine species (Hopkins 1989). Interestingly, intact xylem pit membranes in grapevine do not allow passage of bacteria between adjacent vessels as the pore size (5–20 nm) is much smaller than the diameter of *Xf* cells (0.3–0.5 um) (Davis et al. 1978; Labavitch et al. 2004). Pit membranes are composed of primary cell wall and contain pectin, CM-cellulose, hemicellulose, cell membrane proteins and other constituents (Keegstra et al. 1973; Carpita and Gibeaut 1993). The mechanism by which *Xf* overcomes this barrier may involve secretion of enzymes, including polygalacturonase (Roper et al. 2007). We hypothesise 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.

Although xylem sap is relatively dilute, it contains various amino acids, organic acids, inorganic ions and proteins (Buhtz et al. 2004). In a recent study, exposure of *Xf* to xylem fluids collected from different *Vitis* genotypes resulted in significant differences in both planktonic growth and biofilm formation (Andersen et al. 2007). The objective of this study was to compare *Xf* growth, biofilm formation, cell aggregation *in vitro* in response to the amendment of media with xylem sap from different sources (PD-resistant and PD-susceptible grapes) or amendment of media with a variety of cell wall constituents.

Materials and methods

Plant growth and collection of xylem sap

The PD-resistant (9621–67) and –susceptible (9621–94) selections are siblings selected from the 9621 population. This population was derived from a cross D8909-15 (*V. rupestris* 'A. de Serres', x *V. arizonica/girdiana* b42-26) x F8909-17 (*V. rupestris* 'A. de Serres' x *V. arizonica/candicans* b43-17) (Krivanek et al. 2006). Plants were propagated in a greenhouse as previously described (Fritschi et al. 2007). After three weeks of growth, plants were inoculated with *Xf* (strain Temecula-1) or with water as a negative control (Krivanek et al. 2005). Three months post-inoculation, xylem sap from infected and control groups was collected using a pressure chamber apparatus as described previously (Andersen et al. 1989, Lin and Walker 2004). In brief, a 10×50 cm deep pressure chamber (PMS Instrument Co., Corvallis, Oregon, USA) was used to collect xylem sap from actively growing shoots. The chamber was connected to a nitrogen tank. A 6–13 mm diam grape shoot was girdled by removing phloem tissue. The shoot was immediately inserted into the chamber. The chamber's pressure was gradually increased from 15 to 20 bar. The first few drops of xylem sap were discarded. On average, 0.5 to 2.0 ml of xylem sap was collected from each sample. Sap collected from infected and non-infected plants was transferred to 15 ml tubes and immediately stored at -80°C.

Bacterial growth and treatments

Xf strain Temecula-1 was cultured on solid PW medium (Davis et al. 1978) at 28°C for two weeks. Bacteria were transferred from plates to liquid PW medium and cultured at 24°C with rotary shaking (95 rpm) until cell density reached about 1×10^8 cells ml^{-1}. Bacterial cells were concentrated by centrifugation (2,000 *g*, 10 min, 4°C), washed once with liquid PW medium lacking BSA (-BSA), resuspended in 15 ml of the same medium and cultured overnight at 24°C with rotary shaking (95 rpm) to generate pre-conditioned bacterial cultures. Xylem sap from *Xf*- or water-inoculated resistant (9621–67) and susceptible (9621–94) was filter-sterilised (MCE, 0.2 μm pore size, Fisher Scientific) and mixed with 1/10 volume of 10X liquid PW (-BSA) medium. Alternatively, 100 μl of cell wall constituents in water solution (xylan 10%, w/v, Sigma; laminarin 10%, w/v, Sigma; k-carrageenan 1%, w/v, Sigma; CM-cellulose 1%, w/v, Sigma; β-D-glucan 1%, w/v, Sigma; lichenan 0.1%, w/v, Sigma; or cello-oligosaccharide 0.1%, w/v, Sigma) were added to 15 ml of 1X PW (-BSA) liquid medium. The chemical links and residues of these cell wall constituents are listed in Table 1. Each amended medium (three replicates each) was inocu-

Table 1 Cell wall constituents used and referred to in this study

Name	Chemical links and residues	Source
Xylan	β(1→4)-D-xylopyranosyl residues	Plant
CM-cellulose	β(1→4)-glucan	Plant
β-D-glucan	β(1→,3)-, β(1→,4)-, and β(1→6)-glucan	Plant
Lichenan	β(1→3,1→4)-β-glucan	Plant
Cello-oligosaccharide	either an α- or β-glycosidic link	Plant
Laminarin	β(1→3)-glucan with β(1→6)-linkages	Brown algae
k-carrageenan	β(1→ 3)-D-galactopyranose-4-sulphate-α(1→ 4)-3,6-anhydro-D-galactopyranose	Red seaweed
Pectin	α(1→4)-D-galacturonic acid with (1→2)-linked L-rhamnose	Plant
Xyloglucan	β(1→4)- glucose with (1→6) flexibly linked glycosidic sidechains	Plant

lated with 100 µl of pre-conditioned bacterial culture and incubated at 24°C with rotary shaking (95 rpm). Bacteria were subsequently collected after incubation for 1, 3 and 7 days, and then assayed for bacterial cell density (growth), biofilm formation and cellular aggregation.

Measurements of bacterial planktonic growth, cellular aggregation and biofilm formation

Following culture in amended media for various time periods, bacteria were concentrated by centrifugation (2,000 *g*, 10 min, 4°C), washed once with 0.8% NaCl in PBS buffer (pH7.5) followed by centrifugation under the same conditions, and resuspended in 500 µl of 0.8% NaCl in PBS buffer (pH7.5). Planktonic cell growth density was measured spectrophotometrically by optical density at 600 nm (Andersen et al. 2007). The percentage of cell aggregation over total cell culture in suspension was measured spectrophotometrically by optical density at 540 nm. Briefly, the aggregate containing suspension was allowed to stand for 20 min; bacterial cell aggregates settled to the bottom of the tube. The suspenatant was mostly composed of free (dispersed) cells. Turbidity of the suspenatant was measured spectrophotomeically at 540 nm (ODs). The bacterial cell aggregate was then dispersed with a tissue homogeniser for 1 min, and the total turbidity was measured (ODt). Percent aggregation was estimated as (ODt-ODs)/ODt × 100 (%) as described in Burdman et al. (2000). Measurement of biofilm formation was based on a modification of the standard crystal violet staining method (Leite et al. 2004): bacterial culture tubes were rinsed with sterilised water three times, stained with 0.5 ml of 1% crystal violet (Sigma) for 15 min at room temperature, rinsed with sterilised water three times, and dried completely. Remaining crystal violet stain associated with bacterial biofilm was dissolved by adding 2 ml of absolute ethanol and vortexing at the highest setting for 3 s. The amount of crystal violet remaining is directly proportional to biofilm formation and was determined spectrophotometrically at 600 nm.

Statistical analyses

All experiments with various treatments were repeated three times. The statistical significance was calculated using ANOVA with two factors (one within, one between) at confidence levels of 99% ($P<0.01$) and 95% ($P<0.05$).

Results

Effects of xylem sap from PD-resistant and PD-susceptible grapevines and other cell wall constituents on bacterial planktonic growth

After 1 day of culture in liquid PW (-BSA) medium amended with xylem sap from PD-resistant or PD-susceptible plants, no significant difference ($P<0.4873$) in *Xf* growth was observed. However, after 3 or 7 days, growth of *Xf* in liquid PW (-BSA) medium amended with xylem sap from PD-susceptible plants was significantly greater ($P<0.0282$ and $P<0.0177$, respectively) than growth in the same medium amended with xylem sap from PD-resistant plants (Fig. 1a). Inclusion of BSA in the growth medium at half the normal concentration (1/2X

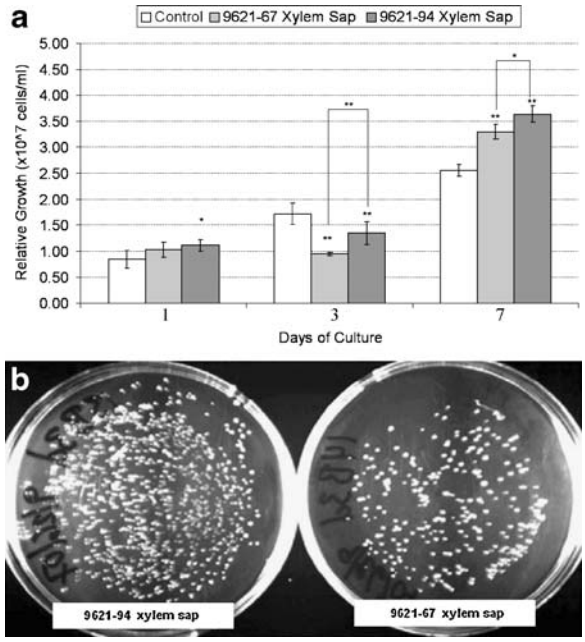

Fig. 1 Effects of the amendment of liquid PW medium with xylem sap from PD-resistant (9621–67) and PD-susceptible (9621–94) grapevines on *Xf* growth *in vitro*. A. *Xf* growth in PW medium containing 1/10 X BSA and amended with xylem sap. Bacterial growth was measured after 1 day, 3 days and 7 days of culture in amended media. Asterisk (*) indicates ANOVA-test results of significance at $P<0.05$; double asterisk (**) indicates significance at $P<0.01$ between treatments and control. Each bracket indicates significance between treatments. B. *Xf* growth on normal PW medium-agar plates after pre-culture for 7 days in PW medium containing 1/10 X BSA and amended with xylem sap from PD-resistant (9621–67) or PD-susceptible (9621–94) grapevines

BSA) resulted in more rapid growth of *Xf* (data not shown) than in PW (-BSA) medium and PW (+1/10 BSA). Nonetheless, *Xf* growth after 3 days was significantly greater ($P<0.0071$) when cultured in PW (1/2X BSA) medium amended with xylem sap from susceptible plants than the growth in the same medium amended with xylem sap from PD-resistant plants (data not shown). Estimates of *Xf* growth under these culture conditions were based on spectrophotometric measurements and did not distinguish between viable and non-viable cells. To address this issue, *Xf* cells grown in liquid PW (-BSA) medium amended with xylem sap for 7 days were plated onto complete solid PW medium (Fig. 1b). 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 compared to 192±89 for xylem sap from PD-resistant plants, with $n=3$, $P<0.01$).

To assess the effects of cell wall constituents on *Xf* growth, we used several commercially available cell wall constituents as substrates for bacterial growth *in vitro*. As shown in Table 2, most cell wall constituents had positive effects on bacterial growth *in vitro* especially after 7 days of culture. CM-cellulose had the most promoting effect for all time-points examined, followed by xylan, cello-oligosaccharide, β-D-glucan, k-carrageenan, and laminarin. In contrast, lichenan inhibited *Xf* bacterial growth. Laminarin and k-carrageenan also had negative effects on *Xf* growth initially after 1 day of culture. Xylan inhibited *Xf* growth after 3 days of culture.

Effects of *in vitro* growth medium amendment with xylem sap from resistant or susceptible grapevines and cell wall constituents on bacterial biofilm formation

As shown in Fig. 2a, xylem sap from PD-susceptible grapevine significantly increased *Xf* biofilm formation *in vitro* (1.48 times higher than the unamended control, $P<0.004$; 1.51 times higher than resistant xylem sap from the PD-resistant grapevine, $P<0.009$). The biofilm formation / growth ratio analysis (Fig. 2b) indicated that xylem sap from the PD-resistant grapevine significantly decreased *Xf* biofilm formation / growth ratio *in vitro* (-1.38 times lower than the unamended control, $P<0.0194$; -1.43 times lower than xylem sap from the PD-susceptible grapevine, $P<0.0223$). Xylem sap from the PD-susceptible grapevine did not show any significant difference from the unmended control in biofilm formation /growth ratio *in vitro* (1.04 times higher than the unamended control, $P<0.3619$), but it significantly promoted *Xf* biofilm formation compared with xylem sap from the PD-resistant grapevines (1.44 times higher, $P=0.0223$).

The effects of some cell wall constituents may be correlated with the effects of xylem sap from both PD-susceptible and PD-resistant grapevines on *Xf* biofilm formation. As shown in Table 3, laminarin, xylan and k-carrageenan significantly enhanced *Xf* biofilm formation. The biofilm formation / growth ratio analysis (Table 3) indicated that lichenan had the most signifi-

Table 2 Effects of cell wall constituents on *Xylella fastidiosa* growth *in vitro*

Treatments[a]	1 Day		3 days		7 days	
	Mean[b]±SD	Factor	Mean[b]±SD	Factor	Mean[b]±SD	Factor
Control	0.85±0.17	1.00	1.72±0.15	1.00	2.56±0.12	1.00
Xylan	1.09±0.12	1.28	1.23±0.61*	-1.40*	3.77±0.53**	1.47**
CM-cellulose	3.22±0.05**	3.79**	4.51±0.05**	2.62**	7.65±0.82**	2.99**
β-D-glucan	0.91±0.49	1.07	2.36±0.21**	1.37**	3.18±0.28*	1.24*
Lichenan	1.11±0.25	1.31	0.57±0.10**	-3.02**	0.46±0.06**	-5.57**
Cello-oligosaccharide	1.31±0.07**	1.54**	1.75±0.13	1.02	3.13±0.27*	1.22*
Laminarin	0.24±0.35**	-3.54**	2.67±0.34**	1.55**	3.12±0.05**	1.22**
k-carrageenan	0.47±0.16**	-1.81**	2.48±0.03**	1.44**	2.58±0.40	1.01

[a] The final concentration of each cell wall component used in this study was: xylan 0.0667%, laminarin 0.0667%, k-carrageenan 0.0067%, CM-cellulose 0.0067%, β-D-glucan 0.0067%, lichenan 0.0007%, and cello-oligosaccharide 0.0007% in w/v. The comparison was taken statistically between control and each treatment in a column, where asterisks * means significant level at $P<0.05$ and ** means significant level at $P<0.01$. [b] Colony forming unit: x 10^7 cells ml^{-1}

cant effect on increasing the *Xf* biofim formation / growth ratio (6.04 times higher than control), followed by laminarin (1.92 times higher than control) and k-carrageenan (1.54 times higher than the control). CM-cellulose and cello-oligosaccharide significantly decreased the biofilm formation / growth ratio (-3.25 times lower than the unamended control, $P=0.00002$; and -1.30 times lower than the unamended control, $P=0.018$, respectively). Xylan and β-D-glucan did not have significant effects on *Xf* biofilm formation/growth ratios ($P<0.3489$ and $P<0.1276$ respectively) when compared with the unamended medium controls.

Effects of *in vitro* growth medium amended with xylem sap from resistant or susceptible grapevines and cell wall constituents on bacterial aggregation

Xylem sap from PD-resistant and PD-susceptible grapevines may have different effects not only on growth, but also on aggregation, especially after infection of host plants by *Xf* due to partial degradation of the xylem cell wall. As shown in Fig. 3, even though xylem sap from both PD-susceptible and PD-resistant grapevines significantly decreased *Xf* cellular aggregation *in vitro* (-3.28 times lower than the unamended control, $P<0.0074$; 2.20 times lower than the unamended control, $P<0.0333$, respectively), xylem sap from PD-susceptible grapevines decreased *Xf* cellular aggregation more than PD-resistant grapevines *in vitro* (-1.49 times lower, $P=0.0352$). Similarly, even though the xylem sap from both PD-susceptible and PD-resistant grapevines significantly decreased *Xf* cellular aggregation / growth ratios *in vitro* (-4.66 times lower than the unamended control, $P=0.0003$ and -2.84 times lower than the unamended control, $P=0.0008$, respectively); xylem sap from PD-susceptible grapevines decreased *Xf* cellular aggregation / growth ratios more than sap from PD-resistant grapevines *in vitro* (-1.64 times lower, $P=0.0079$).

Further analysis of the effects of specific cell wall constituents on *Xf* cellular aggregation showed that lichenan, cello-oligosaccharide, k-carrageenan, laminarin, xylan and β-D-glucan each decreased *Xf* cellular aggregation significantly (3 to 21 times lower than the unamended control, $P<0.01$) (Table 4). Interestingly, effects of these cell wall constituents on *Xf* cellular aggregation were similar to that observed when the growth medium was amended with xylem sap from PD-susceptible plants (Fig. 3a). In addition, CM-cellulose, k-carrageenan, oligosaccharide, laminarin, β-D-glucan, and xylan all decreased *Xf* cellular aggregation /growth ratios significantly (-2.29 to -16 times lower than the unamended control, $P<0.01$).

Discussion

The effects of xylem sap from PD-resistant and PD-susceptible grapevines on Xf culture *in vitro*

Highly PD-resistant and -susceptible *Vitis* selections were used in this study. Differential host responses to

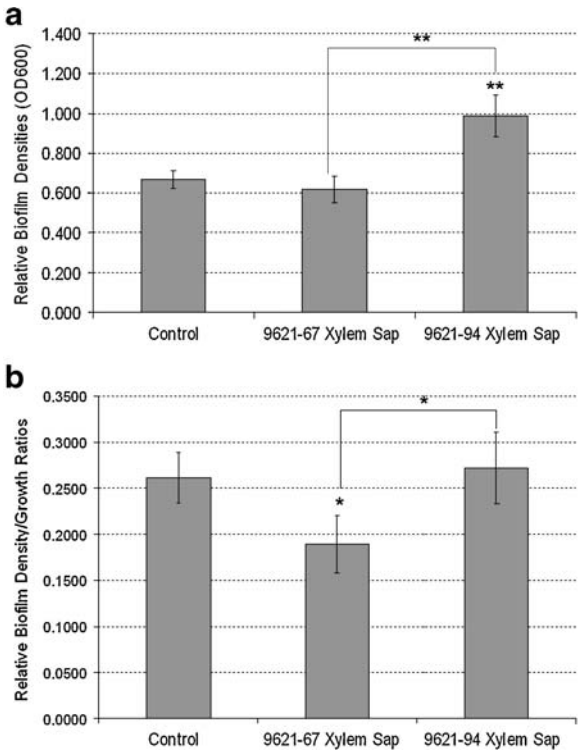

Fig. 2 Effects of amendment of liquid PW medium with xylem sap from PD-resistant (9621–67) and PD-susceptible (9621–94) grapevines on *Xf* biofilm formation. A. *Xf* biofilm formation was measured after 7 days of culture; B. *Xf* biofilm/growth ratio was measured after 7 days of culture Asterisk (*) indicates ANOVA-test results of significance at $P<0.05$; double asterisk (**) indicates significance at $P<0.01$ between treatments and control. Each bracket indicates significance between treatments

Xf infection between the two selections are controlled by a single major locus (the dominant resistance allele is *PdR1*) accounting for 72% of observed phenotypic variation and several modifying loci with minor effects (23%) on observed phenotypic variation; only 5% of phenotypic variance is due to environmental conditions (Krivanek et al. 2006). Host plant responses to *Xf* infection differ between resistant and susceptible genotypes at molecular and physiological levels and also vary with plant organ, as stem and leaf tissues of the same plant responded differently (Lin et al. 2007). Given that *Xf* is limited to xylem vessels, it is logical to conclude that xylem cell wall properties and chemical composition of xylem sap may significantly affect *Xf* pathogenesis. Biochemical analyses of protein composition from several plant species indicated that the most common proteins in xylem sap are peroxidase, lectin-like protein, protease, glycine-rich protein, chitinase, lipid transfer protein-like peptides, and putative apoplastic secretion proteins (Buhtz et al. 2004). Bioinformatics analysis suggests that all of these common xylem sap proteins are secreted. We hypothesised that secretions from metabolically active cells into xylem sap may differ among PD-resistant and PD-susceptible grapevines, and are likely to contribute to host response to *Xf* infection.

Nutrient competition was suggested as a putative mechanism involved in the biocontrol activity of *Ulocladium atrum* on *Botrytis cinerea* sporulation (Kohl et al. 1997). Cell wall-degrading enzymes contribute to nutrient release and thus mediate interactions between host and pathogen (Berto et al. 2001). However, it has not been reported how cell wall constituents may contribute to the response of grapevines to *Xf* infection. Our study provides evidence that xylem sap from different grape cultivars, which are differentially susceptible and resistant to PD, was genetically differentiated and affected *Xf* growth, biofilm formation, and cellular aggregation differently *in vitro*.

Table 3 Effects of cell wall constituents on *Xylella fastidiosa* biofilm formation after 7-day culture *in vitro*

Treatments[a]	Biofilm densities		Biofilm densities/Growth ratios	
	Mean[b]±SD	Factor	Mean[b]±SD	Factor
Control	0.67±0.04	1.00	0.26±0.03	1.00
Xylan	1.06±0.28*	1.58*	0.29±0.12	1.12
CM-cellulose	0.58±0.07	-1.16	0.08±0.01**	-3.25**
beta-D-glucan	0.68±0.11	1.01	0.22±0.05	-1.18
Lichenan	0.72±0.01	1.07	1.57±0.21**	6.04**
Cello-oligosaccharide	0.62±0.09	-1.08	0.20±0.02*	-1.30*
Laminarin	1.56±0.07**	2.33**	0.50±0.03**	1.92**
k-carrageenan	1.00±0.03**	1.49**	0.40±0.08*	1.54*

[a] See note in Table 2

[b] Spectrophotometric absorbance value at 600 nm

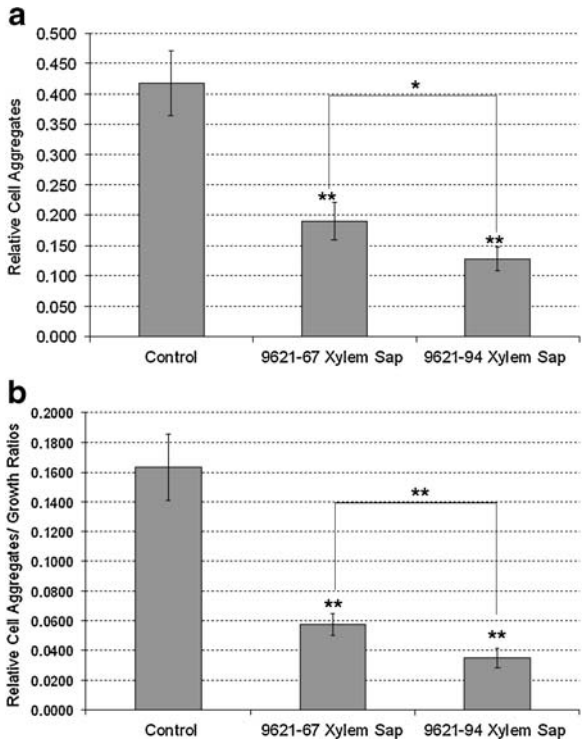

Fig. 3 Effects of amendment of liquid PW medium with xylem sap from PD-resistant (9621–67) and PD-susceptible (9621–94) grapevines on *Xf* cellular aggregation. A. *Xf* cellular aggregation was measured after 7 days of culture; B. *Xf* cellular aggregation/growth ratio was measured after 7 days of culture. Asterisk (*) indicates ANOVA-test results of significance at $P<0.05$; double asterisk (**) indicates significance at $P<0.01$ between treatments and control. Each bracket indicates significance between treatments

Host plant cell wall constituents play important roles in Xf bacterial growth, biofilm formation and cell aggregation *in vitro*

Previous studies showed that different biochemical ingredients in several artificial culture media had significant effects on the *Xf* growth *in vitro* (Almeida and Purcell 2003; Leite et al. 2004). A recent study also showed that xylem sap chemistry influenced *Xf* planktonic growth, biofilm formation and aggregation (Andersen et al. 2007). Within xylem, *Xf* exists in a poor nutritive environment (Andersen et al. 1989). Given the limited supply of nutrients available in xylem sap, *Xf* may respond differently in grapevines that are differentially susceptible or resistant to PD. *Xf* utilises polysaccharide-degrading enzymes to digest cell wall polymers of the xylem pit membranes (Roper et al. 2007). Upon degradation of xylem cell walls, xylem fluid in PD-resistant and PD-susceptible grapevines is likely to differ both qualitatively and quantitatively with respect to chemical composition of cell wall-degradation products.

In this study, we found that most cell wall constituents had positive effects on bacterial growth *in vitro* especially after 7 days of culture; the effective order is CM-cellulose > xylan > β-D-glucan > carrageenan > cello-oligosaccharide > laminarin. In contrast, lichenan inhibited *Xf* growth after 3 days or 7 days of culture. Laminarin and k-carrageenan are found in the cell walls of marine algae. They are analogues to xyloglucan and pectin respectively, the main constituents of dicotyledonous woody plant cell walls. Both had negative effects on *Xf* growth initially. Collectively, these results suggest that uti-

Table 4 Effects of cell wall constituents on *Xylella fastidiosa* cell aggregates after 7-day culture *in vitro*

Treatments[a]	Cell aggregates/Total cells		Cell aggregates/Growth ratios	
	Mean[b]±SD	Factor	Mean[b]±SD	Factor
Control	0.42±0.07	1.00	0.16±0.02	1.00
Xylan	0.02±0.02**	-21.00**	0.01±0.00**	-16.00**
CM-cellulose	0.52±0.04*	1.24*	0.07±0.01**	-2.29**
beta-D-glucan	0.02±0.01**	-21.00**	0.03±0.04**	-5.33**
Lichenan	0.14±0.04**	-3.00**	0.30±0.08*	1.88*
Cello-oligosaccharide	0.12±0.03**	-3.50**	0.03±0.03**	-5.33**
Laminarin	0.04±0.02**	-10.50**	0.03±0.02**	-5.33**
k-carrageenan	0.07±0.04**	-6.00**	0.04±0.04**	-4.00**

[a] See note in Table 2

[b] Spectrophotometric absorbance value at 540 nm

lisation of certain cell wall constituents within xylem fluid may contribute to *Xf* planktonic growth, biofilm formation and aggregation. This contention is supported by the fact that various polymers in plant cell walls, including β-glucans, lignin and oligogalacturonides, are substrates for numerous enzymes secreted by microbial pathogens (Sharp et al. 1984; Bowles 1990; Ham et al. 1991; Walton 1994). Therefore, early release of nutrients upon degradation by *Xf* cell wall-degrading enzymes may be a key step for successful colonisation and subsequent formation of biofilms in xylem vessels. It has been reported that xylem fluid of citrus species (grapefruit, lemon and orange), or asymptomatic hosts generally inhibited biofilm formation by *Xf* compared to that of grapevines or symptomatic hosts (Bi et al. 2007). Our results suggest a potential link among pathogenesis, biofilm formation and some biochemical factors in xylem sap.

Pathogenicity of *Xf* likely requires biofilm formation leading to xylem vessel blockage and subsequent water stress (Newman et al. 2004; de Souza et al. 2005). Bacterial planktonic growth, biofilm formation and cellular aggregation are dependent on the chemistry of xylem sap and can be manipulated by altering xylem chemistry (Leite et al. 2004; Andersen et al. 2007). *Xf* biofilm formation is likely to play a decisive role in vessel occlusion and is a key virulence factor probably required for *Xf* pathogenicity (Marques et al. 2002; Newman et al. 2004; de Souza et al. 2005). We hypothesised that xylem sap from PD-resistant and PD-susceptible grapevines has different effects not only on growth, but also on biofilm formation, especially after infection of host plants by *Xf* due to partial degradation of the xylem cell wall. In this study, we found that xylem sap from PD-susceptible grapevines significantly increased *Xf* biofilm formation *in vitro* when compared with xylem sap from PD-resistant grapevines. Several individual cell wall constituents such as laminarin, xylan, k-carrageenan, lichenan, CM-cellulose and cello-oligosaccharide had different effects on *Xf* biofilm formation. Interestingly, the effects of laminarin, xylan and k-carrageenan on biofilm formation were similar to that observed when the growth medium was amended with xylem sap from PD-susceptible plants (Fig. 2a). The data suggest that artificial manipulation of specific cell wall degradation pathways may lead to a disruption of *Xf* biofilm formation, or blocking of *Xf* growth. *Xf* growth could be enhanced through the manipulation of cell wall constituents in an artificial culture medium. Furthermore, degradation and utilisation of cell wall constituents by *Xf* in xylem vessels of host grapevines would be an important mechanism for *Xf* pathogenicity upon infection.

Cellular aggregation may result from clumping of cells facilitated by extracellular polysaccharides and may be the initial step of biofilm formation (Burdman et al. 2000; Marques et al. 2002; Newman et al. 2004). To identify constituents in the extracellular polysaccharides involved in *Xf* cellular aggregation, we used xylem sap from both PD-resistant and PD-susceptible grapevines and several individual cell wall constituents as additives in culture media. Our study revealed that both *Xf* cellular aggregation and relative cellular aggregation / growth ratio were decreased upon treatment with xylem sap from a PD-susceptible grapevine compared to that from a PD-resistant grapevine. It seems that a potential negative correlation existed between *Xf* cellular aggregation and biofilm formation, i.e. less cellular aggregation, more biofilm formation. In fact, this phenomenon is supported by our observation that more aggregated *Xf* cells were converted into biofilm in the liquid culture medium amended with xylem sap from PD-susceptible grapevines.

The effects of certain specific cell wall constituents on *Xf* cellular aggregation and biofilm formation were very similar to that observed when growth medium was amended with xylem sap from PD-susceptible plants: xylan, laminarin and k-carrageenan inhibited *Xf* cellular aggregation but promoted biofilm formation (Tables 3 and 4). Interactions between proteins and polysaccharides leading to cell aggregation are well established for many microbes (Burdman et al. 2000). It was suggested that media rich in amino acids, rather than carbohydrates, may stimulate *Xf* aggregation and biofim formation (Leite et al. 2004). Nevertheless, actual relationships among planktonic growth, cellular aggregation and biofilm formation could be affected by many other factors, including different buffer systems, inorganic salts, iron sources, carbon sources, organic acids, nucleic acids, chelator agents, growth factors, detoxifiers, antioxidants, or various redox environments, (Leite et al. 2004; Roper et al. 2007; Bi et al. 2007; Marques et al. 2002). Stimuli for cellular aggregation and biofilm formation may involve specific plant-bacterium interactions and

the nutrient status of xylem sap (Andersen et al. 2007). Interestingly, PW medium results in rapid planktonic growth with comparatively little biofilm formation (Davis et al. 1981), whereas, the CHARD2 media provide slower planktonic growth, but high biofilm formation (Leite et al. 2004). However, it is not clear whether the cellular aggregation process *in vitro* is the same as that *in planta* and whether such a relationship between *Xf* cellular aggregation and biofilm formation exists *in planta*. More studies are required to assess relationships between *Xf* growth, cellular aggregation and biofilm formation *in planta*.

The expression of *Xf* adhesion- and adaptation-related genes has been examined in biofilms formed both *in vitro* and *in planta* (de Souza et al. 2003; 2005). Gene expression profiling indicated that a number of genes responsible for metabolic functions, cell division, host cell wall degradation, membrane attachment and virulence-related adaptations are highly expressed during biofilm formation *in vitro* (de Souza et al. 2004). A recent study has shown that *Xf* requires polygalacturonase for colonisation and pathogenicity in grapevines potentially through the digestion of cell wall polymers of xylem pit membranes (Roper et al. 2007). In addition, several regulatory pathways are suggested to be responsible for the transition from planktonic growth to biofilm formation (Davey and O'Toole 2000). However, it remains unclear what cell wall constituents and regulatory genes are involved in *Xf* growth, biofilm formation and aggregation in *planta*. Nevertheless, our current study provides a potential *in vitro* model system to further study the process of transition from planktonic growth to biofilm formation, what genes are involved in this process, and how these genes are potentially networked using cell wall constituents as potential regulators.

In conclusion, our observations support the hypothesis that *Xf*-plant host pathogen interactions are mediated by specific constituents of xylem sap, as opposed to direct interaction between bacteria and metabolically active host cells. Therefore, xylem vessels serve as a confined space for host plants to recognise and interact with *Xf*. Identification of additional xylem sap constituents that differ among PD-resistant and PD-susceptible grapevines may lead to an understanding of the mechanisms through which *Xf*- host plant interactions take place resulting in resistance or susceptibility. Xylem sap composition analysis also may be useful for screening grapevines for PD resistance.

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