

XYLEM CHEMISTRY MEDIATION OF RESISTANCE TO PIERCE'S DISEASE

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ABSTRACT

INTRODUCTION

Xylella fastidiosa (*Xf*) is a Gram-negative xylem-limited bacterium that causes Pierce's disease (PD), plum leaf scald, phony peach disease, almond leaf scorch, citrus variegated chlorosis, and numerous *other* diseases. In susceptible species xylem vessels may get plugged by *Xf* cells and an exopolysaccharide matrix. Vessel plugging results in xylem dysfunction, water stress, and leaf necroses, which are all characteristic of PD. Cell multiplication, formation of aggregates and biofilm are early components of PD that precede visible symptoms. The stimuli for aggregation and biofilm may involve specific plant/bacterium interactions and may involve the nutrient status of xylem fluid. Xylem fluid typically consists of 95 - 98% water; amino acids, organic acids, sugars and inorganic ions are the major components of total osmolality. Recent progress in generating a simple chemically-defined media for *Xf* allows studies of nutritional requirements *in vitro*. We have found that certain chemically-defined media (3G10R and CHARD2) developed in our laboratory promote the development of cell aggregates and biofilm. Aggregation and biofilm formation of *Xf* *in vitro* is dependent on xylem fluid chemistry. For example, xylem fluid of *Vitis vinifera* induced a high degree of aggregation of *Xf* cells, whereas *V. rotundifolia* did not. We have also found that calcium (CaCl_2) also promoted cell aggregation *in vitro*. These results support the calcium bridging hypothetical model that was proposed to explain how *Xf* adheres to xylem vessels (Leite et al. 2002, 2004b), which assumes that the surface of *Xf* cells are negatively charged due to the presence of sulfur in the outer membrane proteins (OMP). Aggregate formation may be facilitated not only by calcium bridging but also by the formation of disulfide bonds in the OMP. The chemistry of xylem fluid may be a function of temperature, fertilization and diurnal/temporal alterations (Andersen and Brodbeck 1989ab, 1991, Andersen et al. 1995, 2004), and pH (Leite et al. 2004b). It is possible that the manipulation of xylem fluid composition, whether it is based on the primary organic compounds, ions or proteins in xylem fluid, is one possible method to affect PD-resistance. The dependence of aggregation and biofilm formation on the nutrient content of xylem fluid and growth media suggests that xylem chemistry is important in the mediation of resistance/susceptibility of PD.

OBJECTIVES

1. Determine the effects of nutrient media and xylem fluid chemistry on *Xf* colony number, bacterial growth, aggregation and biofilm formation of *Xf*.
2. Examine the influence of *Xf* surface chemistry during early stages of *Xf* aggregation and biofilm formation.

RESULTS AND DISCUSSION

Distinct *Xf* aggregation patterns are consistent with modifications in xylem fluid chemistry. Xylem fluid from PD-resistant cultivars (*V. rotundifolia* Noble and Carlos) induced low or no aggregation of *Xf*, whereas susceptible *V. vinifera* cultivars Chardonnay and Chenin Blanc exhibited a high tendency to aggregate (Figure 1A). The number of large aggregates formed after incubation in xylem fluid was highly significant ($P > 0.0001$) as a function of cultivar. X-ray microanalysis showed clearly the difference between calcium and phosphorus concentrations between the most susceptible and the most resistant cultivar. The phosphorus peak was more evident in the PD-resistant cultivar Noble and barely detectable in the PD-susceptible cultivar Chardonnay (Figure 1B). In xylem fluid from California, the ratio of Ca/P for Noble was close to 1, contrasting with a ratio of 14.5 for Chardonnay. Since calcium and magnesium have been implicated as being involved in adhesion and aggregation of *Xf* (Leite et al. 2002), we compared the concentration of calcium, phosphorus and citric acid. The reason for this approach is that phosphorus and citric acid are known to remove calcium and magnesium from a solution by precipitation in the form of complexes or insoluble salts (Van Der Houwen and Valsami 2001). The ranking of resistance of cultivars from California was reflected by the ratios of compounds that either remove divalent cations (phosphorous and citrate) from solution or divalent cations themselves ($[\text{P}]^*[\text{citrate}]/[\text{Ca}]^*[\text{Mg}]$), (Figure 1C). However, the same ratio ($[\text{P}]^*[\text{citrate}]/[\text{Ca}]^*[\text{Mg}]$) did not produce consistent results with xylem fluid from Florida plants (Figure 1C), possibly as a consequence of the presence of citrate and/or phosphate stress (Hoffland et al, 1989; Zhang et al. 1997). Florida xylem fluid much less phosphate than California xylem fluid (data not shown). The concentration of calcium to phosphorus and calcium to citric acid were affected by cultivar and location. The effect of a source of calcium (CaCl_2) on *Xf* aggregation was tested

in vitro. In Figure 2A, high number of large aggregates was observed in concentrations of CaCl₂ above 50 mg/l (Leite et al., 2004a). An exponential curve was obtained by plotting large aggregates versus calcium chloride concentration (Figures 2 A and B).

The pH of xylem fluid samples collected in Florida were consistently acidic, contrary to variable pH readings obtained in California (data not shown). All *V. vinifera* cultivars in California were pH 7.4 and above. Analysis of pectin content showed the most resistant cultivar (Noble) had more pectin than the most susceptible cultivar (Chardonnay) (Figure 2C). Categorized separation of the pectin samples also showed that uronic acids, which are known to bind calcium, were comparatively higher in Noble. A plant with more uronic acids as part of the xylem cell wall could perhaps control levels of free calcium through the so called “egg boxes” (Braccini and Perez 2001). Calcium bridging seems to be critical at the first stages of micro-colony and colony formation. Calcium availability and the number of *Xf* cells within the xylem vessel may influence the amount and size of aggregates formed. Negative surfaces of *Xf* cells (Leite et al 2002) are important for calcium bridging and the number of negative moieties may be associated with strain pathogenicity. Ultimately, cell aggregation by Ca²⁺ may be the trigger for activation of other pathogenicity pathways. Our preliminary results show that the nutrient content of xylem fluid is a significant component for the development of aggregates and biofilm, although the elucidation of the role of specific compounds requires further research.

The minimum xylem fluid-based medium (3G10R) increased the capacity of *Xf* to form biofilm compared to PW+ medium and reduced the number of cells in the planktonic state (Leite et al. 2004a). This knowledge allowed us to design experiments and investigate the role of each component for *Xf* growth and biofilm formation. The approach adopted was the deletion of one component at a time from the original 3G10R, such as: MgSO₄, phenol red, L-glutamine, glucose, ferric pyrophosphate and glutathione. Glutamine is an indispensable medium component for *Xf* (Davis et al. 1981; Chang and Donaldson 1993, Lemos et al 2003, Leite et al. 2004a, Almeida et al. 2004). Glutamine is the most abundant amino acid in *Vitis* xylem fluid (Andersen and Brodbeck 1989, 1991, Andersen et al. 1995, Leite et al. 2004a, Ishida et al. 2004). Glucose is found in lower concentration in xylem fluid of grapevines (Andersen and Brodbeck, 1989, 1991). *Xf* can survive without a glucose source, as demonstrated by Leite et al. (2004a) and this work. *Xf* apparently does not use the pathway to metabolize glucose (Facincani et al. 2003). Iron homeostasis is an important process regulating the expression of genes involved in pathogenicity in bacteria (Vasil and Ochsner 1999, Simpson et al. 2000). In *X. campestris* pv *campestris*, iron-uptake genes are essential for the induction of the hypersensitive response (HR) in non-host plants and disease symptoms in the host plant (Wiggerich and Pühler 2000).

After 4 days of incubation, the ratio of *Xf* biofilm/cells in suspension (planktonic form) was greatly enhanced when glutamine or glucose were withheld from the 3G10-R formulation. (Figure 3A). After 14 days the absence of other elements such as MgSO₄, ferric pyrophosphate and glutathione also seemed to promote biofilm formation. Recently, Ishida et al. (2004) observed that *Xf* in the presence of grapevine xylem fluid (*V. vinifera* cv. Chardonnay) formed biofilm after only 30 min of incubation. Leite and collaborators (2004a) showed that in xylem fluid based-minimum media *Xf* formed more biofilm than in PW⁺ (a complex and nutritional rich medium). These results suggest that *Xf* was able to form biofilm in response to a microenvironment provided by xylem fluid (a mixture of proteins, amino acids, organic acids, sugars and inorganic ions) and *Xf* could use aggregation as survival mechanism in a nutrient poor growth medium. When *Xf* cells were incubated in deionized water or in oxidized glutathione less biofilm was observed (Figure 3B). In contrast, in a reduced environment such as in the presence of the antioxidants 1,4-dithiothreitol (DTT) or reduced glutathione, an increase in biofilm formation was observed (Figure 3B). The details of the mechanism of action of these antioxidants in the *Xf* adhesion process are unknown; however, the calcium bridging hypothetical model supports these findings (Leite et al. 2002). Studies directed toward cell aggregation and biofilm formation may help to understand critical elements of pathogenicity.

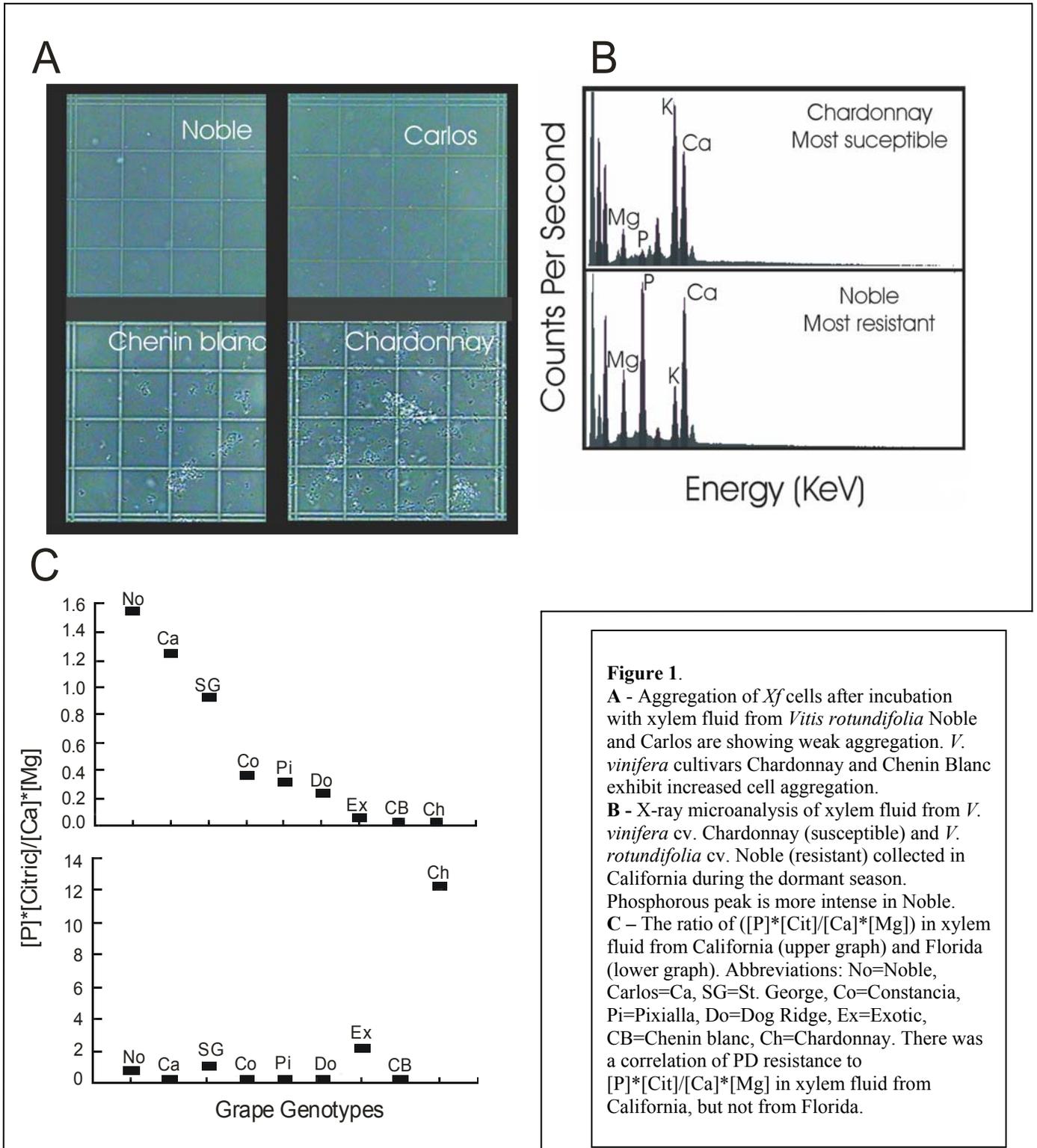
REFERENCES

- Almeida, RP, Mann, R, & Purcell, AH. 2004. *Curr. Microbiol.* 48: 368-72.
- Andersen, P.C. & Brodbeck, B.V. 1989. *Physiol. Plant.* 75: 63-70.
- Andersen, P.C. & Brodbeck, B.V. 1991. *Am. J. Enol. Vitic.* 42: 245-251.
- Andersen, P. C., B. V. Brodbeck, and Mizell, III R. F. 1995. *J. Amer. Soc. Hort. Sci.* 120:36-42.
- Andersen, P.C. et al., 2004. *Vitis* 43: 19-25.
- Braccini, I. & Perez, S. 2001. *Biomacromolecules.* 2: 1089-1096.
- Bradfield, E. G. 1976. *Plant Soil* 44: 495-499.
- Chang, C.J. & Donaldson, R.C. 1993. *Phytopathology* 83: 192-194.
- Davis, M. J. et al., 1981. *Curr. Microbiol.* 6: 309-314.
- Facincani, A.P. et al., 2003. *Gen.Mol. Biol.* 26: 203-211.
- Hoffland, E. et al. 1989. *Plant Soil* 113: 161-165.
- Ishida, M.L., Andersen, P. C. & Leite, B., 2004. *Physiol. Mol. Plant Pathol.* *In press.*
- Leite, B. et al., 2002. *Braz. J. Med. & Biol. Res.* 36: 645-650.
- Leite, B., Andersen, P.C. & Ishida. 2004 a. *FEMS Microbiol. Lett.* 230: 283-290.
- Leite, B., et al., 2004 b. *Phytopathology* 94: S59.
- Lemos, E.G. et al., 2003. *FEMS.* 219: 39-45.

Reisch, B.I. et al., 1993. Information Bulletin 233. Cornell Cooperative Extension.
 Rose, R. K. 2000. Biochimica Biophysica Acta 1475: 76-82.
 Simpson et al., 2000. Nature 13; 406(6792):151-157
 Thar, R & Kuhl, M. 2001. Appl. Environ. Microbiol. 67: 5410–5419.
 Wiggerich H.G. & Pühler A. 2000. Microbiology 146: 1053–1060.
 Zhang, F. S., Ma, J & Cao, Y. P., 1997. Plant and Soil. 196: 261-264.

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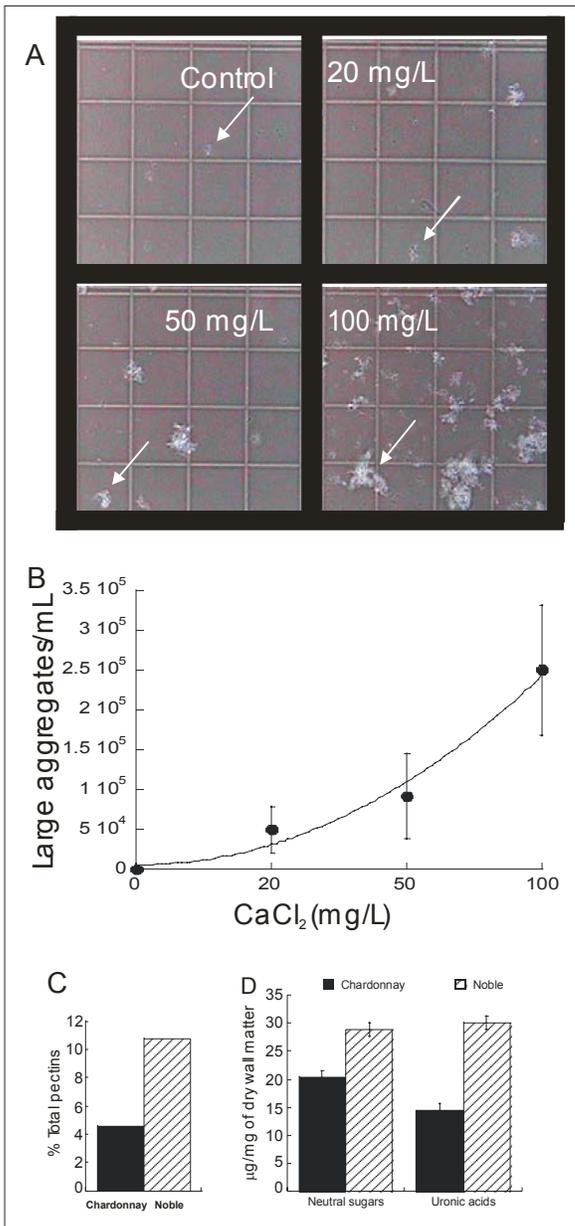


Figure 2.

A - Increasing numbers of *Xf* aggregates in different CaCl₂ concentrations. The number of aggregates was visualized in Neubauer chamber: Highest aggregation is at 100 mg/l.

B - Calcium induces aggregation at same concentration range found within the xylem fluid. Points are mean ± S.D., n = 6.

C - Total pectin content of cultivars Noble and Chardonnay.

D - Concentration of neutral sugars and uronic acids in dry cell wall matter of cultivars Noble and Chardonnay.

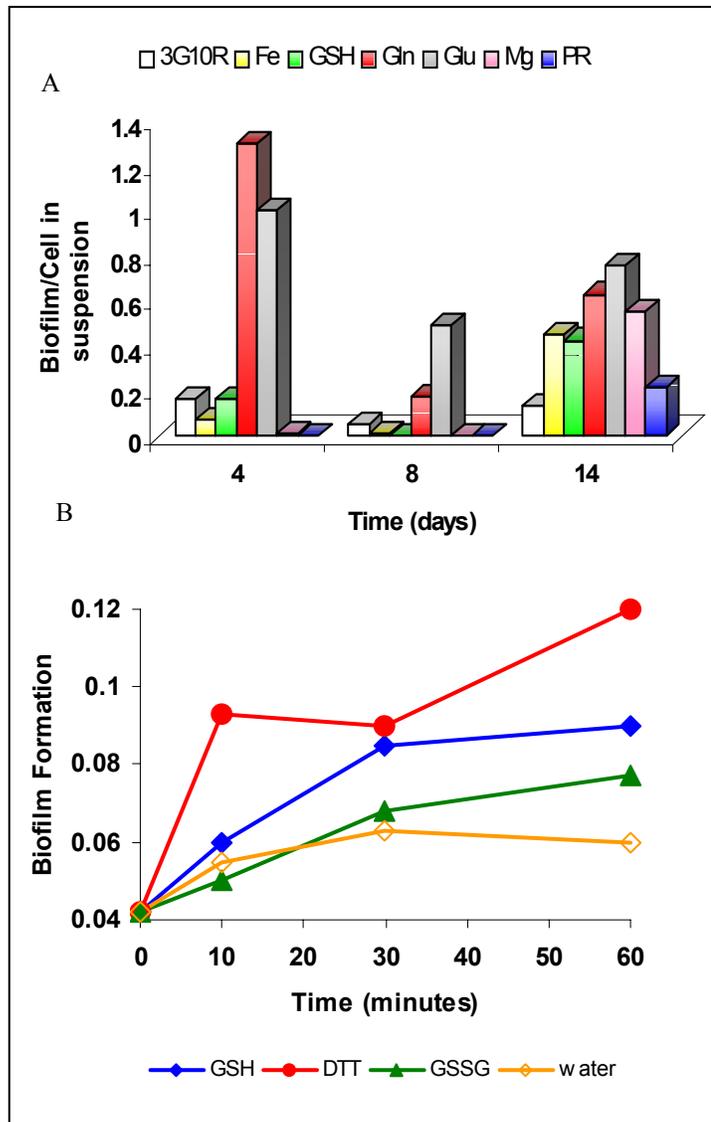


Figure 3.

A - Ratio of Biofilm/Cell in suspension after incubation of *Xf* cells for 4, 8 and 14 days in media generated after deletion of either ferric pyrophosphate (Fe), glutathione (GSH), L- glutamine (Gln), glucose (Glu), MgSO₄ (Mg) or phenol red (PR) from 3G10R.

B - Effect of antioxidants on biofilm formation by *Xf* after variable periods of time. The antioxidants used were 1,4-dithithreitol (DTT) 60 mM and glutathione (20 mM) in the reduced (GSH) and oxidized (GSSG) forms. Treatment with distilled water was used as control.