XYLEM CHEMISTRY MEDIATION OF RESISTANCE TO PIERCE`S DISEASE

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ABSTRACT
Xylella fastidiosa (*Xf*) is the causal agent of Pierce's disease (PD) of grapevines. The mechanism involved in pathogenecity is largely due to the occlusion of xylem vessels by aggregates of *Xf* cells and biofilm. Increasing concentrations of $CaCl_2$ and $MgCl_2$ consistently induced aggregation of *Xf in vitro* and differences in aggregation patterns occurred when comparing strains of *Xf*. A solution (100 mg/Liter) of divalent cation (calcium or magnesium) increased *Xf* aggregation by about 10 fold. A pre-treatment of *Xf* cells with the reduced form of glutathione (an antioxidant present in the xylem fluid) significantly enhanced the attraction of *Xf* cells for calcium and resulted in more cell aggregation. By contrast, pre-treatment of cells with oxidized glutathione virtually eliminated aggregation. Our data indicate that cell aggregation and biofilm formation in grapevine vessels may be dependent on: a) the presence of free divalent ions and b) the proper redox environment, which in turn modulates surface characteristics (particularly thiol moieties) of *Xf*. These results support the contention that *Xf* pathogenicity mechanisms may involve rapid aggregation in early stages and biofilm formation induced by xylem fluid constituents.

INTRODUCTION
Previous studies have showed that a suspension of *Xf* aggregate when exposed to xylem fluid of grapevine. The formation of cell aggregates is significantly greater in *Vitis vinifera* cv. Chardonnay (PD-susceptible) than in *Vitis rotundifolia* cv Noble (PD-resistant). In addition, cell aggregation was analyzed and compared to the xylem fluid chemistry profiles from nine grape cultivars. The most significant observable fact was the ratio involving calcium, magnesium, phosphate and citric acid. Susceptible varieties were higher in calcium and poor in phosphate. Resistant plants had equivalent levels of calcium and phosphate (Leite et al., 2004; Andersen et al., 2004). It is important to know at this point that, calcium and phosphate are extremely attracted by each other and the formation of calcium phosphates is influenced by pH. Further experimentation demonstrated that the ratio of the concentration of calcium and magnesium and citric acid and phosphate [Ca].[Mg]/[Cit].[P], i.e., aggregation inducers/aggregation inhibitors may be used to separate susceptible and resistant plants growing in California but not in Florida. We have shown that xylem fluids collected in Florida are more likely to be acidic compared to more variable xylem fluid (pH 5.5 to 7.4) from California. These results encourage us to advance the studies of the "Calcium Bridging Hypothesis" (CBH) (Leite et al., 2002) critical to understand its implications and applicability towards management of PD.

OBJECTIVES
1. Determine the effects of calcium chloride and magnesium chloride on aggregation of *Xf* cells.
2. Investigate the influence of oxidized and reduced glutathione on aggregation of *Xf* cells.
3. Search for genomic information that is relevant to "Calcium Bridging Hypothesis".

RESULTS
1. Effect of calcium and magnesium on *Xf* aggregation
Two *Xf* strains were used during the aggregation experiments (UCLA and STL). $CaCl_2$ and $MgCl_2$ were prepared in three different concentrations (0, 20, 50 and 100 mg/L). The SEM images reveled that large aggregates usually congregate 100 cells or more, compared to medium aggregates (± 50 cells), small aggregates (± 25 cells) and free cells (less than 10-cells aggregates or isolated cells) (Figure 1). Highly significant strain by treatment interactions were found for all the aggregate sizes ($p>0.0001$). The number of large sized aggregates was dependent on the concentrations of $CaCl_2$ and $MgCl_2$. The aggregation of *Xf* in both strains was induced at 100 mg/L of $CaCl_2$ (Figures 2A and 2B); however, the number of large aggregates was not consistently proportional to the $MgCl_2$ concentrations. For the UCLA strain the optimum concentration of $MgCl_2$ to induce aggregation was 50 mg/L (Figure 2A). The aggregation response of the STL strain to $CaCl_2$ only occurred for concentrations of 50 and 100 mg/L (Figure 2B); however the concentrations of 20 to 100 mg/L $CaCl_2$ was effective in inducing significant aggregation of the UCLA strain. The same levels of aggregation of the STL strain occurred with 50 mg/L.
Overall, the experiment revealed that the increasing concentrations of $CaCl_2$ and $MgCl_2$ consistently induced *Xf* aggregation. The relationship of calcium in aggregation (calcium bridging) and increased pathogenecity has been described for

streptococcus bacteria and *Pseudomonas aeruginosa* (Rose, 2000, Sarkisova et al., 2005). Similarly, the capacity to form stable and massive amounts of biofilm may help the establishment and colonization by *Xf*. The influence of phosphates and citric acid on calcium availability within the xylem vessel due their high reactivity with this divalent cation has been discussed (Leite et al., 2002, Leite et al., 2004). In addition to other ions or organic acids, the xylem fluid pH and the redox status may affect calcium and magnesium availability (Leite et al., 2003; Leite et al., 2004, Andersen et al., 2004). A fast and vigorous biofilm formation would help to colonize the grapevine xylem vessels. The masses of cells consuming and trapping nutrients may also explain some of the symptomatology of *Xf*-mediated diseases.

Glutathione is a tripeptide that is present in the xylem fluid of many plant species. The presence of reduced glutathione in a chemically-defined medium resulted in an increasing of *Xf* biofilm (Leite et al., 2004). Subsequently, we showed that *Xf* cells aggregated and formed biofilm patterns distinctly according the composition of chemically defined media. The medium CHARD2 promoted biofilm (Leite et. al., 2004; Marques et al, 2005). We also showed that aggregated cells cultivated in different chemically defined media were less affected by the activity of lytic peptide cecropin B (Ishida et al, 2004). Finally, with the optimization of a procedure to evaluate aggregation of *Xf,* based on Rose (2000), we were able to visualize and quantify aggregates. *Xf* aggregation patterns are believed to be a resultant of the cell surface characteristics alteration due the redox status; these differences can influence cell receptivity toward interactions with cations in the xylem fluid.

2. Influence of oxidized and reduced glutathione on aggregation of *Xf*.
As previously demonstrated through X ray microanalysis, sulfur moieties are present on the *Xf* surface. Free cells exhibited sulfur as a surface signal. When treated with calcium solution, aggregated cells were observed and calcium was confirmed as predominant surface signal These observations could indicate that (1) calcium was attracted by cell surface characteristics/properties and (2) this attraction promoted the cell aggregation (Leite et al., 2005).

Previous results suggest that these interactions between cells and calcium are dependent on the redox status of xylem fluid (Leite et al., 2003: Leite et al., 2004; Andersen et al., 2004, Ishida et al., 2004b). Glutathione (GSH) induces *Xf* aggregation. Our working hypothesis assumes in surface proteins containing thiol residues affect how these cells interact with calcium and other cells.

Maximum aggregation was obtained by treating cells with GSH 10 mM for 20 min followed by $CaCl_2$ 50 mg/L (Figure 3, bottom). These results support confirm that a reducing environment facilitates calcium bridging between *Xf* cells. GSH seems to have a profound effect on *Xf* surface chemistry and aggregation. Calcium promotes aggregation by linking negative charges (thiols) on *Xf* cell surface after redox status modification. These observations sustain our previous findings showing that in xylem fluid (PD-resistant) with low calcium concentration, less *Xf* aggregation was observed; in high calcium concentration (PD-susceptible plants), more *Xf* aggregation occurred (Leite et al., 2004). We envision that these results will allow a potential search for environmental conditions (water quality, soil fertility etc) to help reduce the severity of PD symptomatology. Xylem fluid chemistry may mediate the initial steps of cell aggregation and may inhibit or stimulate disease development. Additional research is needed before we can draw definitive conclusions on physiological and genetic basis of PD resistance.

3. Genomic evidence supporting the "calcium bridging hypothesis" (CBH).
Calcium and magnesium are common cation nutrients in the soil and xylem fluid. In 2002, we proposed that calcium and magnesium, when available in the free form within the xylem fluid, may facilitate cell aggregation, vessel plugging and symptom development of PD (Leite et. al., 2002).

SDS PAGE of *Xf* proteins labeled with 5-IAF before the cell lysis, showed that thiol proteins are present on the surface of the *Xf* cells (Andersen et al., 2003). We also showed that thiol proteins varied among strains of *Xf* tested (Ishida et al., 2003; Andersen et al., 2004). These data support the contention that differences in the behavior of *Xf* strains may be attributed to distinct cell surface-protein profiles. In other words, differences in the cell surface proteins of *Xf* may determine the behavior of this bacterium will behave. Thiol moieties present in the outer membrane proteins could possibly carry the residues (cystein or methionine) responsible for the external negatively charge of the *Xf* membrane. We have selected several candidate proteins with these characteristics. Most of them are cell structural surface proteins and adhesins. These proteins have variable numbers of cysteine (thiol) and methionine (negatively charged) residues located at the cell external loop region. These findings confirm the existence of surface proteins that may expose the proper chemical features to maintain the *Xf* surface ready for interaction with other cells and the xylem wall.

We were particularly interested in the hemagglutinin-like secreted proteins due its molecular weight and the number of residues (cysteine and methionine) displayed on the surface of *Xf*. Proteins with these characteristics are strong candidates to be the mediators of adhesion and aggregation of *Xf* cells. Our search suggests that filamentous hemagglutinin-like adhesins are broadly important virulence factors in both plant and animal pathogens. The *Erwinia chrysanthemi* EC16 *hecA* gene predicts a 3,850-aa product which is similar to a member of the *Bordetella pertussis* filamentous hemagglutinin, a family of adhesins. These adhesins are known to be involved in attachment and aggregation processes (Rojas et al., 2002). In addition, recent results obtained with Cowpea Mosaic Virus (CMV), by the Scripps Research Institute, showed that particles with

different patterns of surface proteins, containing cysteine residues, may exhibit distinct attachment properties. It is possible that hemagglutin-like adhesins play a decisive role in PD, by allowing pathogenic cells to aggregate, form biofilm and successfully colonize the xylem vessels. Cysteine residues may be more important in the general context of the CBH. Methionine residues, originally included in the model, may contribute only to charge the cell surface negatively. Type IV pili was recently presented as surface protein on the surface of *Xf* mediating twiching motility (Meng et al., 2005). The type IV pili also exhibits cysteine residues in the external loop sequence. The involvement of type IV pili in aggregation or the behavior of these structures in distinct redox conditions or in the presence of divalent ions has yet to be determined.

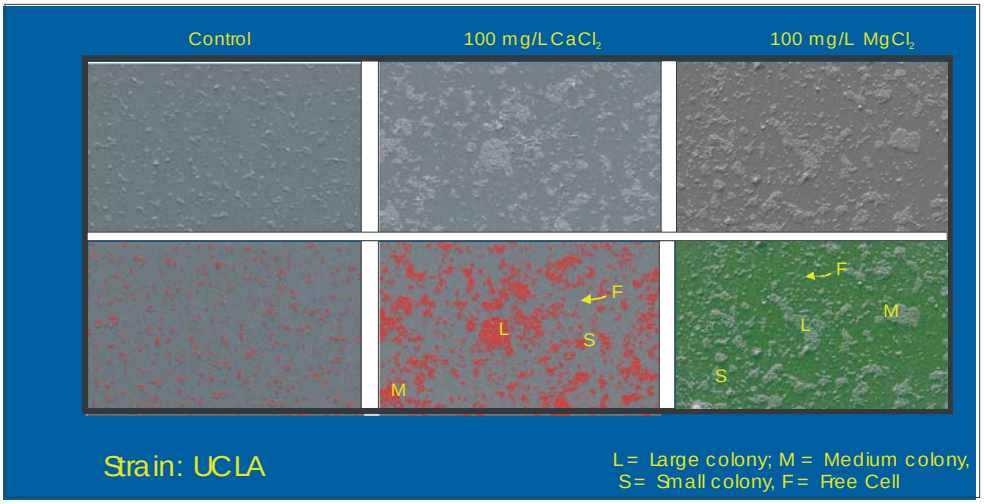

Figure 1: SEM images of *Xylella fastidiosa* cells in different concentration of $CaCl_2$ or $MgCl_2$. Results show the contrast between control and cell suspensions treated with salts that release divalent cations: $CaCl_2$ 100 mg/L and $MgCl_2$ 100 mg/L. Images were software treated (Image J and Corel Draw 10) to facilitate the identification of large, medium, and small aggregates or free cells. The quantification of the number of large colonies is presented in Figure 3 for UCLA and Figure 4 for STL. There is a significant difference observed for aggregate formation when comparing treated and untreated cell suspensions.

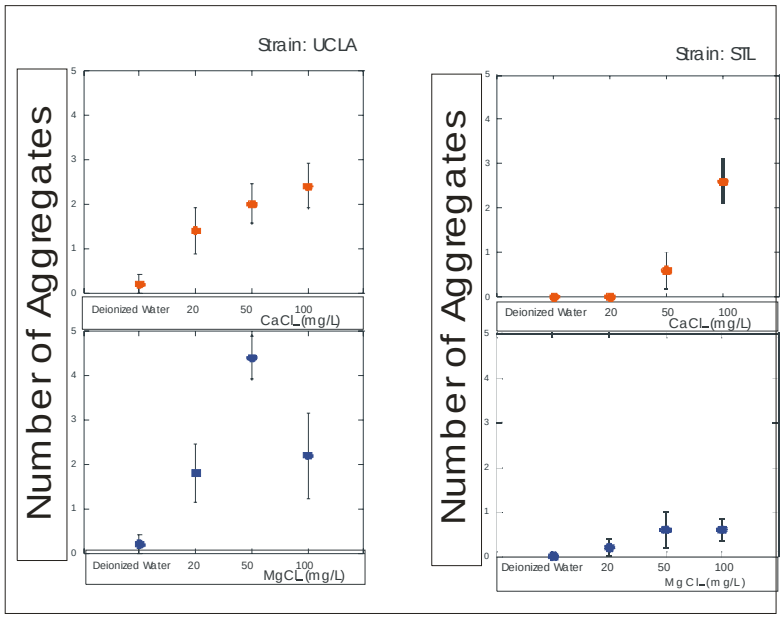

Figure 2A: Number of large aggregates formed by *X. fastidiosa* (strain UCLA) after the treatment with $CaCl_2$ (red) and $MgCl_2$ (blue). The calcium ions were more consistent in terms of inducing the formation of large aggregates as denoted by smaller standard errors. Nevertheless, the aggregation induced by magnesium should not be overlooked. Figure 2B: Number of large aggregates formed by *Xf* (strain STL) after treatment with $CaCl_2$ (red) and $MgCl_2$ (blue). For strain STL, calcium ions produced more aggregates than magnesium ions.

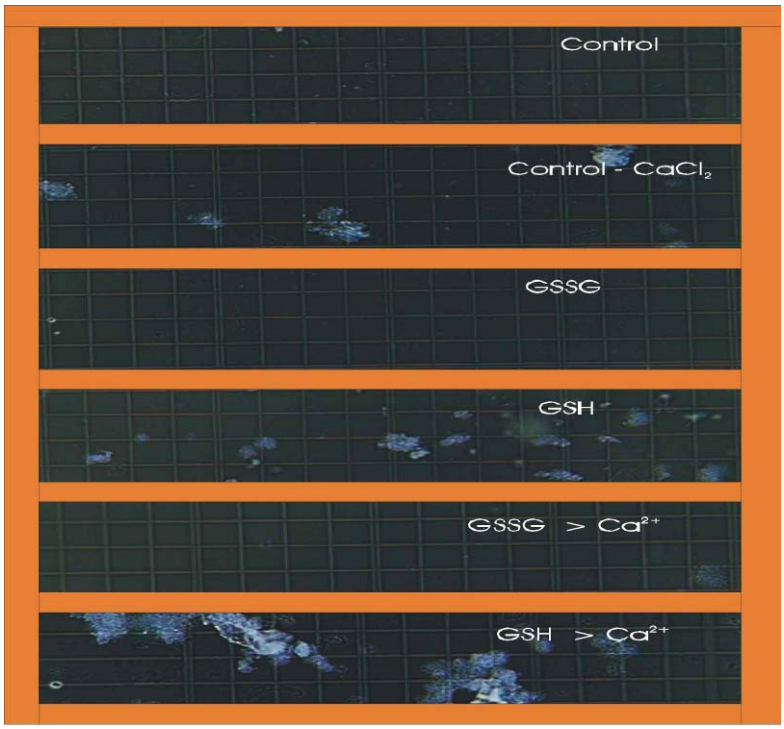

Figure 3: Aggregation of *Xylella fastidiosa* under different conditions (top to bottom). Aggregation was measured with: deionized water (negative control), $CaCl_2$ 50 mg/L (positive control) Reduced Glutathione 10 mM (GSH), Oxidized Glutathione 10 mM (GSSG), GSH 10 µM for 20 min + $CaCl_2$ 50 mg/L and GSSG 10 mM for 20 min + $CaCl_2$ 50 mg/L . Maximum aggregation was obtained with GSH 10 mM for 20 min followed by $CaCl_2$ 50 mg/L. Notice the large sized aggregates formed with the treatment GSH followed by a source of calcium (bottom figure).

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