

# Calcium availability influences the aggregation and adhesion of *Xylella fastidiosa*, the causal agent of Pierce's disease in grapes.

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## Abstract

Xylem fluid collected from the same grapevine cultivars growing in California and Florida showed significant effects of both cultivar and location on the ratio of calcium:phosphate (Ca:P), as well as pH. It has been reported that calcium bridging is critical for the aggregation and adhesion of *X. fastidiosa*. Our results showed that under acidic pH (5.5), calcium induces in solution as free ions and *X. fastidiosa* aggregation is stimulated. In contrast, in basic pH (8.0), calcium phosphate precipitates. In California plants, the Ca:P ratio is close to 1 for *Vitis rotundifolia* cv. Noble (resistant) and 4.5-6.0 for *V. vinifera* cv. Chardonnay (susceptible). Additionally, the content of acidic pectins, which bind calcium, was higher in the xylem fluids of the resistant cultivar Noble. These findings suggest that calcium availability may influence vessel clogging and symptom development in Pierce's disease. Soil type (composition) may alter xylem fluid pH, which in turn may influence calcium availability, thereby impacting aggregation.

## Introduction

*Xylella fastidiosa* is a Gram-negative, rod-shaped, non-flagellate bacterium; it is highly specialized for survival in the xylem of plants and in the foregut of xylem-feeding hemipterans (Pittman and Hopkins, 1990). This bacterium causes diseases in numerous plant species, such as Pierce's disease of grapevines, palm leaf scorch, phony peach disease, almond leaf scorch, and citrus variegated chlorosis (Chang et al., 1993; Pittman and Hopkins, 1990). Pierce's disease, *X. fastidiosa* infection, is the most economically important reason for the growth of *Vitis vinifera* is not well implemented in Florida. Pierce's disease is also responsible for great economic losses in California's grape industry (Bian et al., 2002). Efforts are underway to develop strategies to confer resistance in plants against *X. fastidiosa*. Thompson seedless grapes have been genetically engineered to produce lytic peptides (Schora et al., 1996) and are under field trials. However, at this point, there is no known curative method.

Our research teams have been investigating, respectively, xylem chemistry composition (Pittman et al., 2007) and bacterium attachment by *X. fastidiosa* (Marques et al., 2007). It has been observed that *X. fastidiosa* preferentially forms biofilms in water when grown on wooden sticks (Marques et al., submitted). Recently, chemically defined media have been developed based on the composition of xylem fluid (aggregates and biofilm formation have been altered depending on media formulation (Lefte et al., 2004)). This indicates that some xylem fluid compositions may interfere with the development of *X. fastidiosa* in media composition may interfere with the development or decrease the number of cells in suspension or the rate at which these cells aggregate and bind to biofilms. *X. fastidiosa* aggregation was tested in xylem fluids from susceptible *V. vinifera* genotypes and resistant *V. rotundifolia* cv. Noble. The addition of phosphate and calcium to the simplified chemically defined medium (GDR) was also tested.

## Material & Methods

**Xylem fluid collection.** *X. fastidiosa* originally isolated from infected grapevines in California strain (UC/LAFD) and supplied by Dr. Alexander H. Purcell, UC-Davis was used in this work. *X. fastidiosa* was maintained in PVP medium (Davis et al., 1981) at 28 °C under shaking conditions (150 rpm).

**Xylem fluid.** Our experimental approach involved the xylem fluid extraction of resistant and susceptible *Vitis* genotypes grown in Davis, Napa County, California, and Quincy, Florida. In Napa County, Florida, nine vines were used. Two *Vitis rotundifolia* cv. Noble (resistant), two *V. vinifera* cv. Chardonnay (susceptible), one *V. vinifera* cv. Pinot Noir (susceptible), one *V. vinifera* cv. Cabernet Sauvignon (susceptible), one *V. vinifera* cv. Pinot Noir (susceptible), and three *V. vinifera* cv. Chardonnay (susceptible). In Florida, the most resistant cultivar, Noble, and the most susceptible cultivar, Chardonnay, were the most susceptible. Since the cultivars used were the same in both locations, we were able to compare xylem fluid profiles and correlate them with each genotype's reported resistance to *X. fastidiosa*. Xylem fluid was collected from plants during the dormant season. The profiles of xylem fluid inorganic ions, amino acids, and organic acids were generated at the Analytical Laboratory at the University of Florida and the University of California. The pH of the xylem fluid was also measured for each collected sample.

**Aggregation studies.** We measured the *X. fastidiosa* aggregation in the presence of xylem fluid from ten *Vitis* genotypes using the technique described by Rose (2006). Bacterial cells were inoculated at room temperature for 2 hours at 80 rpm. A 100 µl aliquot of bacterial suspension was placed in a Neubauer chamber, and aggregates were classified and counted. The influence of calcium concentration on *X. fastidiosa* aggregation was tested in vitro as well as tested. Cells were resuspended in CaCl<sub>2</sub> solutions (20, 50, and 100 mg/L). The calcium concentration range was similar to the range found in the xylem fluid collected in California. The cells were also resuspended in distilled water (control).

**Biofilm formation.** Biofilm formation on the surface of control polystyrene tubes was assessed using the crystal violet method (Boussin et al., 2005). After the incubation period, the tubes were rinsed with deionized water, and a 1% (v/v) crystal violet solution was added to each tube. Tubes were incubated at room temperature for 15 min and washed thoroughly. Crystal violet was eluted from the biofilm by adding ethanol to each tube. The absorbance of the solution was measured at 560 nm. For the blank, the crystal violet was eluted from a tube without prior incubation with *X. fastidiosa*.

**Pectin content.** The pectin content was obtained by processing 30 mg of stem tissue from each cultivar analyzed. The material was homogenized and then fractionated. Fractions containing the xylem vessels were prepared for pectin measurement.

## Results & Discussion

Since calcium and magnesium have been implicated in the adhesion and aggregation of *X. fastidiosa* (Lefte et al., 2002), we began comparing the concentrations of calcium, phosphate, and citric acid. The reason for this approach is that phosphate and citric acid are essential for the growth of *X. fastidiosa* (Lefte et al., 2002). In addition, these compounds or insoluble salts (Van Der Horst and Valsami, 2001). We noticed that the calcium:phosphate and calcium:calcium:calcium ratios were affected by cultivar and location. We also noticed that the xylem microanalysis profiles of resistant and susceptible cultivars were distinct (Fig. 1). X-ray microanalysis clearly showed the difference in calcium concentrations between the most susceptible and the most resistant cultivars. The phosphorus peak was more intense in the resistant cultivar (Noble) and barely visible in the susceptible cultivar Chardonnay. In xylem fluid from California, the ratio Ca:P:Na was close to 1, contrasting with a ratio of 4.5 for California.

Different profiles are consistent with distinct aggregation patterns of *X. fastidiosa* (Fig. 2). Resistant cultivars, *V. rotundifolia* cv. Noble and *V. rotundifolia* cv. Chardonnay, showed weak aggregation. In contrast, susceptible *V. vinifera* cultivars Chardonnay and Chénin Blanc exhibit a remarkable tendency to aggregate. Consistently, California data showed the number of large aggregates was highly significant ( $P < 0.0001$ ) as a function of cultivar.

## Results & Discussion (cont.)

The effect of a source of calcium (CaCl<sub>2</sub>) on *X. fastidiosa* aggregation was tested in vitro. In 50 µM CaCl<sub>2</sub>, higher numbers of large aggregates were observed at CaCl<sub>2</sub> concentrations above 50 mg/L. An exponential increasing curve was observed by plotting large aggregates against calcium chloride concentration (Fig. 3B). Similar results were observed in single-cell aggregation studies (Rose, 2006). Since calcium is highly active, we anticipated that its availability might explain due to interactions with other components of the xylem fluid.

*V. vinifera* cultivars are known to perform poorly in soils (Eschmann et al., 1992). Several American varieties unless the soil pH is raised to about 5.5 (Pittman et al., 1992). Several root combinations of the xylem constituents: phosphates, citric acid, and calcium are water-soluble, can be tested with resistance and susceptible cultivars. The timing of resistance of cultivars in California was reflected by the types of components that either remove divalent cations from solution or divalent cations themselves (Pittman et al., 1992) (Fig. 4A). On the other hand, the high concentration of citric acid in Florida samples turned the ratio [P]/[Ca]/[Ca]/[Mg] to be more similar to the ratio seen in California, but not consistently related to resistance for plants in Florida (Fig. 4B). The production of large amounts of citric acid in high-phosphorus is known to be a consequence of phosphate stress (Jeffland et al., 1989; Zhang et al., 1987). Florida xylem fluid presented comparatively less phosphate than California xylem fluid (data not shown).

The effect of adding calcium and phosphate to a chemically defined medium, 3010-R (Lefte et al., 2004), was tested. Results revealed that biofilm formation is stimulated by the addition of calcium. In contrast, biofilm diminishes as the concentration of phosphates is raised (Fig. 5A). The optical density turbidity decreases with phosphate and increases with the addition of CaCl<sub>2</sub> (Fig. 5B). The response is altered whether calcium precipitates, sometimes followed by a change in pH. In fact, the pH of xylem fluid samples collected in Florida is consistently acidic, in contrast to the variable pH readings observed in California (Fig. 6C). All *V. vinifera* cultivars are 7.4 and above. Free cell surface and aggregate surface element profiles were determined using X-ray microanalysis. Cells that aggregate in the presence of calcium were compared to free cells (non-aggregated) that had been washed with deionized water. The typical profile of free cells exhibited a sulfur signal that was not observed in aggregated cells. The typical profile of cells that aggregate in the presence of calcium was similar to the profile of cells that aggregate in the presence of calcium. The presence of sulfur on the surface is in agreement with the hypothetical model that suggests sulfur (thiols) may provide a negatively charged surface for *X. fastidiosa* (Lefte et al., 2002). The presence of calcium in the aggregated cell surface may enhance the possibility of calcium-calcium bridging (Lefte et al., 2002). In Figure 6B, several strains of *X. fastidiosa* were labeled with a specific fluorescent marker (biodegradable fluorescent), (pyrene-labeled), and subsequently disrupted. Some bacterial samples were also treated with 1,4-dithiothreitol (DTT), 10 mM for 15 minutes at room temperature to reduce the cell surface proteins before the thiol labeling procedure. Each sample was loaded in SDS-PAGE (Laemmli, 1970). Different patterns in thiol-labeled band profiles are indicative that *X. fastidiosa* strains differ in thiol content. These findings, in this context, could explain why *X. fastidiosa* strains differ in colonization patterns.

During the preparation of chemically defined media for *X. fastidiosa*, we have observed that calcium and phosphate may precipitate as complexes. The amount of precipitation is dependent on the concentration and pH. The more basic the pH the more precipitation will occur. In phosphate buffer (pH 5.5) no precipitation occurs when CaCl<sub>2</sub> is added to the solution, but precipitation of calcium phosphate progressively increases in pH 7 and 8.5 (Fig. 7A). Light microscopy confirmed that the cell aggregation density is inversely correlated with pH. Most of the aggregation occurred at pH 5.5 (Figs. 7B, C, D).

Analysis of pectin content showed the most resistant cultivar (Noble) has more pectin than the most susceptible cultivar (Chardonnay) (Fig. 7E). Categorized separation of the pectin samples also showed that Noble samples are known to bind calcium, while Chardonnay samples are known to bind calcium. Calcium levels in the resistant cultivar also seem to be controlled by plant biochemical factors (Fig. 8F).

The fact that Florida xylem fluid exhibited more calcium and magnesium levels could indicate that Florida soils have more free calcium for root uptake. It could also indicate that calcium concentration in the xylem fluid is higher in Florida than in California, with a lower CEC is characteristic of sandy acidic soils. Low CEC results from the soil's inability to hold cations such as Ca<sup>2+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup>, and NH<sub>4</sub><sup>+</sup>. Negatively charged particles (clay and humus) are responsible for a high CEC. California soils are known to have CEC levels that keep soil pH within the normal vegetation range.

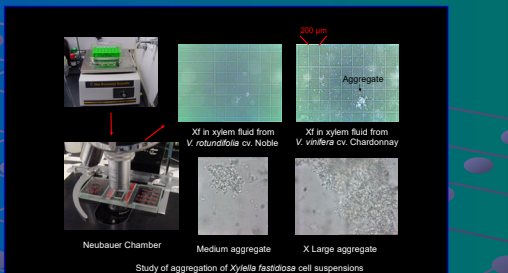
## Concluding Remarks

Our findings place divalent ions, especially calcium, as essential players in the pathogenicity puzzle of *X. fastidiosa*. Calcium bridging seems to be critical in the early stages of microcolony and colony formation. Calcium availability and the number of *X. fastidiosa* cells within the xylem vessel determine the amount and size of aggregates formed. In addition, the host plant itself may help to control the process by removing Ca<sup>2+</sup> from the xylem lumen, which seems to be the case in the resistant cultivars. Calcium has been shown to be a wounding agent in the xylem wall structures. Uronic acid (a type of pectin) is known to withhold calcium. Aggregation may also affect adhesion to the cell wall, since we noticed that high aggregation densities are observed in the distal end of the xylem vessels of California *X. fastidiosa* cells (Lefte et al., 2002) are important for the calcium bridging, and the number of negative moieties may be associated with the strain's pathogenicity. Ultimately, Ca-mediated cell aggregation may trigger the activation of other pathogenicity pathways. Xylem and aggregation have been shown to be sufficient for the development of Pierce's disease. We envision that Pierce's disease management may now take a whole new perspective.

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Study of aggregation of *Xylella fastidiosa* cell suspensions



Figure 3: A - Increasing numbers of *Xylella fastidiosa* aggregates in different CaCl<sub>2</sub> concentrations. The number of aggregates was visualized in the Neubauer chamber. No calcium is present in the control. Arrows pointing to the aggregates represent the average aggregate size for each concentration curve fitted to data shows that calcium induces aggregation of cells in the same concentration range found within the xylem fluid. Points are mean  $\pm$  S.D., n = 6.

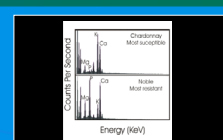


Figure 1: Energy dispersive X-ray microanalysis of lyophilized dormant season xylem fluid samples obtained from *Vitis vinifera* cv. Chardonnay (susceptible) and *V. rotundifolia* cv. Noble (resistant) plants growing in California. Notice difference the phosphorus peak, intense in the resistant sample (bottom), weak in the susceptible (top).

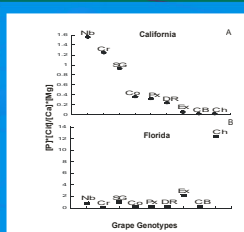


Figure 4: Duncan separation of resistant and susceptible grapevine cultivars according to the index that takes into consideration the concentration of phosphorus (P), citric acid (Ca), calcium (Ca), and magnesium (Mg). A - California cultivars were satisfactorily separated by the index value [P]/[Ca]/[Ca]/[Mg]. Phosphorus and citrate are placed as forces against *Xylella fastidiosa* aggregation, in contrast to divalent ions (Ca<sup>2+</sup> and Mg<sup>2+</sup>), which are believed to induce aggregation. B - The same index [P]/[Ca]/[Ca]/[Mg] was used for the Florida data without success. Florida xylem fluid is chemically distinct, and other components are possibly interacting with *X. fastidiosa* cells. Noticeably, citric acid concentration in xylem fluid from Florida cultivars is considerably higher compared to their California counterparts. Comparatively, *Vitis vinifera* cultivars exhibited higher concentrations of citric acid, with the highest value corresponding to cultivar Chardonnay in California (approximately 250 times higher than the value obtained for Chardonnay in Florida).

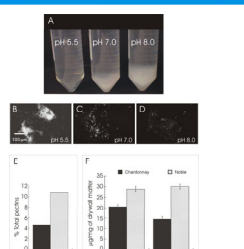


Figure 7: A - Calcium phosphate precipitation in a simple solution composed of phosphate buffer and CaCl<sub>2</sub>. The precipitation was caused by the modification of the pH in the solutions. The pH varied from 5.5 to 8.0. B, C, and D - Suspension of bacterial cells was added to the solutions described above, and the aggregation density was monitored. B - cell aggregation density was the highest at pH 5.5. C and D show aggregation density at pH 7.0 and 8.0, respectively. E - Total pectin content of cultivars Noble and Chardonnay. F - Concentration of neutral sugars and uronic acids in dry cell wall matter of cultivars Noble and Chardonnay.

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