CONTINUED ASSESSMENT OF *XYLELLA FASTIDIOSA* FIMBRIAL ADHESINS AS IMPORTANT VIRULENCE FACTORS IN PIERCE'S DISEASE: INFLUENCE OF XYLEM SAP

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ABSTRACT

Specific biological characteristics of *Xylella fastidiosa* (*Xf*) Temecula were investigated in microfluidic flow chambers *in vitro* by examining the effect of xylem sap from Pierce's disease (PD) susceptible *V. vinifera* and resistant *V. smalliana* grapevines on *Xf* cell growth, aggregation, biofilm formation, and motility. Growth of *Xf* was observed in both *V. smalliana and V. vinifera* xylem saps in microfluidic flow chambers. While *Xf* cell density increased in *V. smalliana* sap, the cells exhibited a reduction in aggregation and biofilm formation compared to that observed in *V. vinifera* sap. In addition, motility via pilus twitching activity was reduced in *V. smalliana* sap when compared to similar activities in *V. vinifera* sap, indicating *V. smalliana* sap may inhibit the function of type IV pili. Normal twitching motility of *Xf* was restored once *V. smalliana* sap was exchanged with *V. vinifera* sap, indicating that chemical components of *V. vinifera* sap possibly influence the function of type IV pili of *Xf* cells, that in turn may affect aggregation.

LAYPERSON SUMMARY

Cells of *Xylella fastidiosa* (*Xf*) aggregate, form biofilms, and occlude the host's vascular (xylem) system, resulting in Pierce's disease symptoms in grapevine. Colonization of grapevine xylem by *Xf* involves migration of individual cells through a process of twitching motility by which hair-like type IV pili are repeatedly extended from the cell, attach to the xylem surface, and are retracted, pulling the cell forward. Using microfluidic 'artificial' chambers through which xylem sap from highly susceptible and resistant grapevines is flowing, the biological behavior of *Xf* in these saps was assessed. Toward this we have observed reduced motility in sap from a resistant grapevine, *V. smalliana*. Also reduction in formation of cell aggregates and biofilms.

INTRODUCTION

This project continues efforts toward understanding the biological relationship between *Xylella fastidiosa (Xf)* cells and the xylem environment, and specifically the roles of fimbrial adhesins (type I and type IV pili, and associated proteins) in *Xf* virulence, motility, aggregation and autoaggregation, and biofilm development. The research targets the functional biology of *Xf* in xylem sap. It tests and explores traits of sap and xylem vessels from resistant and susceptible grapevines, and eventually that of citrus, that may inhibit or promote *Xf* cell activities associated with *pil* and *fim* gene products.

Previous observations describing roles for fimbrial adhesins (type I and type IV pili) in *Xf* virulence, motility, aggregation, and biofilm development have provided insight into their genetic mechanisms and regulation (De La Fuente, 2007; 2008). Studies on *Xf* motility and biofilm formation under natural conditions viz., *in planta,* have been hindered in part by the optical inaccessibility of vascular tissue. Recent studies have shown the importance of xylem sap chemistry on growth, aggregation, and attachment of *Xf* cells, highlighting the establishment of stable cultures in 100% xylem sap (Andersen, 2007; Zaini, 2009). Studies with *V. riparia* and *V. vinifera* cv. Chardonnay sap (100%) in either microfluidic chambers or in culture tubes have shown that the pathogen responds to this more natural chemical environment differently than it does in rich artificial media such as PD2 (Zaini, 2009). Aggregation and biofilm development are enhanced (Zaini, 2009), and early indication is that twitching motility is also greater—in both the number of *Xf* cells and in rate of movement. It was reported that xylem sap from Pierce's disease (PD) resistant *V. rotundifolia* maintained *Xf* in a planktonic state, whereas the bacterium was more likely to form aggregates when incubated in xylem sap from susceptible *V. vinifera* cultivars (Liete, 2004). Those directed the attention to a more natural environmental system for *Xf*—one that will greatly enhance the value and significance of information generated in studying *Xf* in an *in vitro* system: the inclusion of xylem sap and xylem vessel tissue.

Previous work that both type I and type IV pili are involved in aggregation and biofilm development (Li, 2007), type IV pili of *Xf* are involved in twitching motility within the xylem vessels of grapevine (Meng, 2005). Citrus is often grown adjacent to vineyards in California and may be considered a potential reservoir for PD *Xf* (Bi, 2007). Xylem sap from commercial citrus plantings in Temecula (grapefruit, orange, lemon) did not support *Xf* biofilm development while at the same time

grapevine xylem sap obtained from adjacent vineyards supported thick biofilms (Shi, 2010). Citrus xylem sap did not support the induction of a number of *pil* and *fim* genes, such as *pilT*, a gene that encodes for type IV pilus retraction (necessary for twitching motility), *pilY1*, a gene encoding a type IV pilus tip adhesion protein, *pilI*, *pilU*, and *fimA* that encodes the type I pilus subunit (Shi, 2010). The significant reduction in *pil* and *fim* gene expression in citrus sap is notable for at least two reasons: i) it may explain, in part, why the PD strain of *Xf* is not symptomatically expressed in citrus i.e. it does not move from the sites of introduction (no twitching motility), nor does it form biofilms, and ii) it may provide valuable clues into what chemical factors from citrus sap may be exploited in grape to reduce or inhibit similar gene product expression. Based on those data, it may be that in xylem sap from Pierce's disease resistant grapevines that pili function is suppressed.

OBJECTIVES

Objectives covered in this report include:

1. Establish a baseline of *Xf* activity *in vitro* for grapevine sap. This will include temporal and spatial activities for pili-associated functions—motility, cell aggregation, and biofilm formation.
2. Assess pili-associated functions in grapevine sap from *Vitis vinifera* cultivars and *Vitis* species expressing distinct PD resistance and susceptibly.

RESULTS

Xf survives in *V. vinifera* and *V. smalliana* sap

We used grapevine xylem sap from bleeding *V. vinifera* and *V. smalliana* vines collected in Geneva, NY during the Spring of 2009 and 2010. *V. vinifera* is known to be susceptible to PD and *V. smalliana* is resistant (Fritschi, 2007; Lu, 2008). To verify whether *V. smalliana* sap was inhibitory to *Xf*, growth of *Xf* was assessed in *V. vinifera* and *V. smalliana* sap as well as in PD2 broth. The *Xf* cell density in *V. smalliana* sap was lower than in *V. vinifera* sap. However, overtime the cell density in *V. smalliana* sap increased (**Figure 1**). At 5, 8, 24, 72 and 120 hours after introducing *Xf* cells into PD2 broth, *V. vinifera*, and *V. smalliana* saps in glass tubes described as above, 100µl xylem sap or PD2 broth containing *Xf* cells was removed from the tubes, serial diluted, and plated onto PD2 agar, and incubated at 28ºC for 7 to 10 days. The resulting bacterial colonies were counted for each dilution, and were described as colony forming unit (CFU)/ml^{-1}. CFU/ml^{-1} in *V. smalliana* sap increased after introducing *Xf* cells into xylem sap. *Xf* cells grew well in *V. vinifera* sap, which is consistent with previous observations for growth of *Xf* in xylem sap (Andersen, 2007; Zaini, 2009; Shi, 2010). The increase in the number of *Xf* cells in *V. smalliana* sap (**Figure 1**) suggest *V. smalliana* sap is not lethal to *Xf* and supports growth, but at a reduced rate.

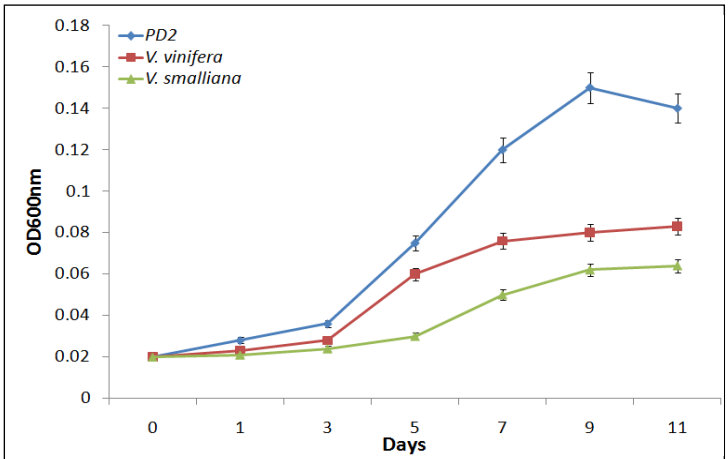

Figure 1. Growth of *X. fastidiosa* in PD2, *V. vinifera* and *V. smalliana* sap. The cell concentration in the xylem sap and PD2 was measured at OD_{600} at every 2 days after inoculation.

Xf aggregation and biofilm formation in *V. vinifera* and *V. smalliana* sap

The relative percentage of *Xf* cell aggregation in PD2 broth, *V. vinifera* and *V. smalliana* sap was measured as previously described (Burdman, 2000). Aggregation of *Xf* cells, overtime, was lower in *V. smalliana* sap than in *V. vinifera* sap and PD2 broth. Biofilm formation of *Xf* in PD2 broth, *V. vinifera* and *V. smalliana* sap was determined by a crystal violet staining method (Leite, 2004). *Xf* had a reduced biofilm development in *V. smalliana* sap compared to *V. vinifera* sap and PD2 broth (**Figure 2**). This indicates that *Xf* cells were able to survive and multiply in *V. smalliana* sap, but with reduced aggregation and biofilm formation.

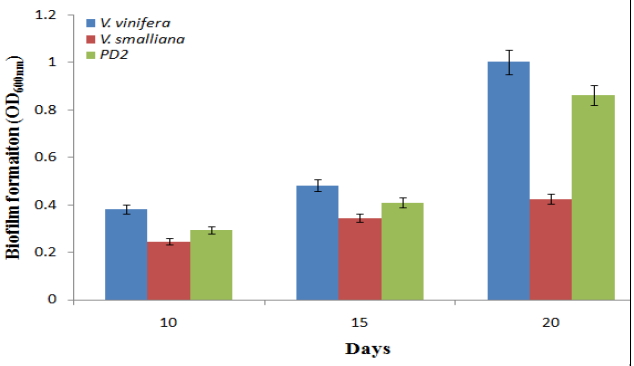

Figure 2. Quantitative assessment of biofilm formation of *Xf* in various media. Data represent three replicates for each experiment.

Aggregation of *Xf* cells in *V. vinifera* and *V. smalliana* sap in microfluidic flow chambers

A new four-channeled chamber was developed and used to facilitate experimental observations of several parameters at the same time (**Figure 3**). The width and depth of the individual channels was designed so that in subsequent experiments thin sections of grapevine xylem wood can be placed in the channels for direct observation of *Xf* on xylem vessel walls—in xylem fluid.

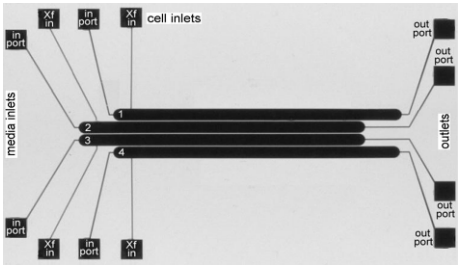

Figure 3. Four-channeled microfluidic device with each channel having media in and out ports, and one access port for introduction of *Xf*.

In PD2, *Xf* cells were observed to aggregate and form large clumps at 2-7 days after introduction of the *Xf* cells into the microfluidic flow chamber (**Figure 4**). The aggregates eventually developed a thick biofilm and slowed flow. *Xf* cells in *V. vinifera* sap formed many small clumps throughout the chamber. However, *Xf* cells in *V. smalliana* sap aggregated to form smaller clumps distributed throughout the chamber. This result indicates *V. smalliana* sap may inhibit aggregation of *Xf* cells.

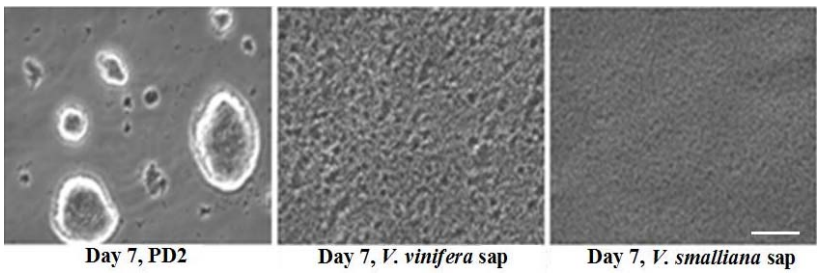

Figure 4. Images taken after 7 days of introducing *Xf* in PD2 and xylem sap. Large aggregates are formed in PD2, small aggregates in *V. vinifera* sap; and smaller clumps in *V. smalliana* sap. Scale bar equals 50 µm.

Twitching of *Xf* in *V. vinifera* and *V. smalliana* sap in microfluidic flow chamber

Microfluidic flow chambers provide a constant supply of 'new' sap and represent a condition more akin to that *in planta* as opposed to culturing in test tubes. *Xf* cells twitched in PD2 broth and *V. vinifera* sap at 2-8 days after introducing of the *Xf* cells into microfluidic flow chamber. The cells aggregated and formed relatively homogenous biofilms after nine days in the chamber. In *V. smalliana* sap, relatively few *Xf* cells attached to the glass surface; furthermore, few *Xf* cells exhibited

twitching motility. Long non-separated cell profiles with clear division points were observed, and confirmed that *Xf* cells were able to grow in *V. smalliana* sap. The fact that *Xf* cells in *V. smalliana* sap exhibit greatly reduced twitching activity may explain the observation that *Xf* spreads faster in xylem vessels of PD-susceptible grapevines compared to PD-resistant or tolerant grapevines (Hopkins, 1984; Fry & Milholland, 1990).

Effect of changing PD2 to *V. smalliana* sap on the twitching motility of *Xf* cells

Xf cells were observed to twitch and to form large aggregates in PD2 broth, but not in *V. smalliana* sap during the 1st-2nd day after introducing the *Xf* cells into the chamber (**Figure 5**). On day three, PD2 was exchanged with *V. smalliana* sap for 3-5 additional days. Interestingly, after about eight hours, the aggregated clumps disaggregated, and *Xf* cells dramatically reduced twitching motility (**Figure 4**). Finally, *Xf* cells became uniformly distributed within the chamber. The data suggest that either the presence or absence of a chemical component of *V. smalliana* sap prevents efficient aggregation of *Xf* cells, and prevents *Xf* cells from twitching.

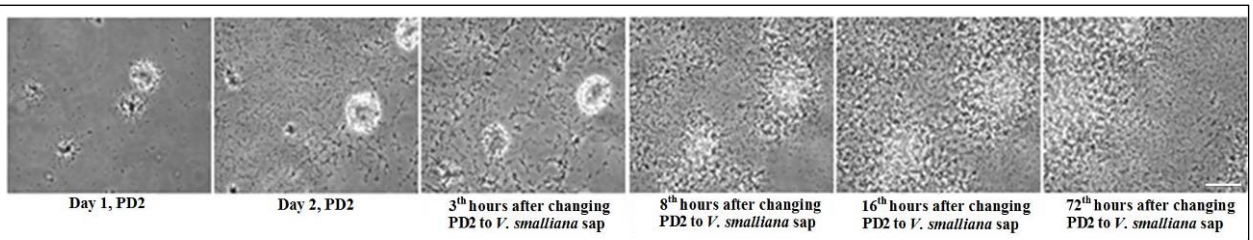

Figure 5. Images taken before and after PD2 was changed to *V. smalliana* sap. Aggregates in PD2 disaggregated in *V. smalliana* sap. Scale bar equals 50 µm.

Effect of changing *V. vinifera* to *V. smalliana* sap on the twitching motility of *Xf* cells

Xf cells were observed to readily twitch and to form aggregates in *V. vinifera* sap during the 1st-2nd day after introducing the *Xf* cells (**Figure 6**). On day three, *V. vinifera* sap was exchanged with *V. smalliana* sap for three additional days. After eight hours, aggregated clumps disaggregated. *Xf* cells dramatically reduced twitching motility and appeared to attach to chamber surface at one cell pole (presumably, the pilus pole). Five days after exchanging sap, the *Xf* cells were uniformly distributed within the chamber and without the formation of large aggregates. As a control, *Xf* cells in *V. vinifera* sap in an adjacent channel continued to twitch and form aggregates, eventually forming a robust biofilm. These results indicate that *V. smalliana* sap affect the function of type IV pili of *Xf* cells, and thus prevents *Xf* cells from twitching and aggregating.

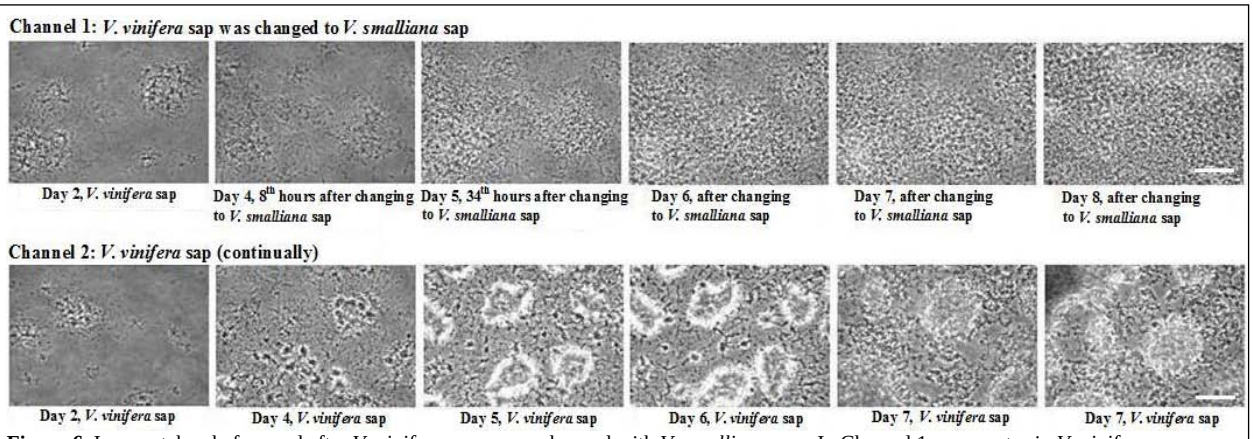

Figure 6. Images taken before and after *V. vinifera* sap was exchanged with *V. smalliana* sap. In Channel 1, aggregates in *V. vinifera* sap were disaggregated in *V. smalliana* sap. In Channel 2, *Xf* aggregated and twitched to form biofilm in continuously *V. vinifera* sap. Scale bar equals 50 µm.

Effect of changing *V. smalliana* to *V. vinifera* sap on the twitching motility of *Xf* cells

In *V. smalliana* sap during the 1st-9th day after introducing the *Xf* cells into the chamber, *Xf* cells were observed to not be twitching, but were attached at one pole to the chamber surface (**Figure 7**). On day nine, *V. smalliana* sap was exchanged with *V. vinifera* sap (**Figure 7**). After one hour, few *Xf* cells were twitching; however, after five hours cells within the aggregates dispersed, and many initiated twitching activity; after twenty-four hours, many more cells were actively twitching. The loss of twitching motility of *Xf* in *V. smallaina* sap was recovered once the sap was exchanged with *V. vinifera* sap (**Figure 7**), suggesting the function of type IV pili was inhibited by a components in *V. smallaina* sap. The chemical components of *V. vinifera* sap may activate/induce the function of type IV pili of *Xf* cells.

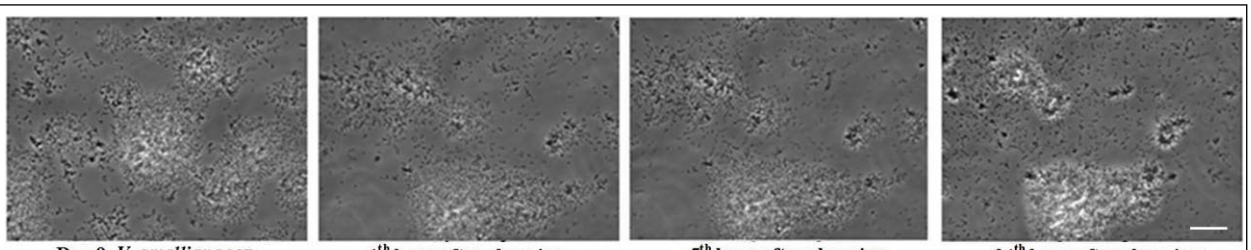

Figure 7. Images taken before and after *V. smalliana* sap was changed *V. vinifera* to sap. Aggregates in *V. smalliana* sap were disaggregated and free *Xf* cells started twitching in continuously *V. vinifera* sap. Scale bar equals 50 µm.

CONCLUSIONS

The symptomatic development of PD in grapevine is related to biological features of the *Xf* pathogen and how it interacts with its host. By establishing more natural features of xylem vessels environment to study the motility and aggregation of *Xf*, we hope to provide a better understanding of the biological features of the *Xf* in natural xylem sap. The long-distance directional upstream migration of *Xf* might enhance intraplant spread of the bacteria and colonize grape xylem vessels from the initial site of infection. The present results might suggest that the inhibition of twitching motility of *Xf* by the chemical components in *V. smallaina* sap may limit the spread of *Xf* in xylem vessels in PD-resistant grapevines, resulting in the restriction of *Xf* to fewer xylem vessels and less proportion of *Xf* colonized vessels, which results in a limitation of systemic infection and no PD development in resistant grapevine.

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