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ELUCIDATING PROCESS OF CELL-CELL COMMUNICATION IN *XYLELLA FASTIDIOSA* TO ACHIEVE PIERCE'S DISEASE CONTROL BY PATHOGEN CONFUSION

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ABSTRACT:
X. fastidiosa produces an unsaturated fatty acid signal molecule called DSF that modulates gene expression in cells as they reach high numbers in plants. By increasing the expression of a variety of afimbrial adhesins while decreasing the expression of pili involved in twitching motility as well as extracellular enzymes involved in degrading pit membranes and hence movement between vessels, DSF accumulation suppresses virulence of *X. fastidiosa* in grape. We thus are exploring different ways to elevate DSF levels in plants to achieve disease control via "pathogen confusion". As exogenous sources of DSF applied in various ways to grape suppressed pathogen mobility and hence virulence we have further studied the chemical identity of DSF. *X. fastidiosa* can respond to a variety of related unsaturated fatty acids, while it naturally produces at least three different DSF species. While the initial DSF species produced by *X. fastidiosa* was identified as, 2-Z-tetradecenoic acid (hereafter called C14-cis), it can also produce a second compound termed C12-cis, as well as 2-Z-hexadecenoic acid (C16-cis). This latter molecule is the most active molecule. Gene expression in *X. fastidiosa* exposed to various levels of DSF is a sensitive means of assessing DSF levels and *X. fastidiosa* harboring *phoA* reporter gene fusions to hxfA has proven to be an excellent bioreporter. *X. fastidiosa* can respond to *cis* unsaturated fatty acids with the site of unsaturation at the number 2 carbon molecule with chain lengths from 12 to 18 carbon atoms. The corresponding *trans* unsaturated fatty acids not only are not able to induce gene expression, but antagonize gene expression conferred by the corresponding *cis* fatty acid. The commercially available unsaturated fatty acid Palmitoleic acid is also active as a signaling molecule and is being evaluated for its ability to reduce the susceptibility of plants to Pierce's disease when applied topically or introduced into the plant in different ways. The release of extracellular membranous vesicles by *X. fastdiosa* is responsible for the suppression of its adherence to surfaces. These vesicles attach to surfaces such as that of the walls of the xylem vessels. By so attaching, these vesicles prevent the attachment of *X. fastdiosa* cells themselves to such surfaces. Only upon reaching relatively high cell concentrations in a particular vessel would DSF concentrations increase to a level that would suppressed the release of the membranous vesicles, thereby retaining adhesive molecules on the surface of *X. fastidiosa* cells themselves, thus allowing the bacterial cells themselves to attached to surfaces, such as that of insect vectors

LAYPERSON SUMMARY:
X. fastidiosa produces an unsaturated fatty acid signal molecule called DSF. Accumulation of DSF in *X. fastidiosa* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the

plant. We have investigated DSF-mediated cell-cell signaling in *X. fastdiosa* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease. Elevating DSF levels in plants artificially reduces its movement in the plant. In this study we have investigated the variety of different fatty acid molecules that can serve as cell-cell signaling agents in *X. fastdiosa*. Several new DSF species have been found including a 16 carbon unsaturated fatty acid that appears to be far more active than the 14 carbon unsaturated fatty acid that we have previously investigated as well as a commercially available fatty acid (palmitoleic acid). The commercially available fatty acid is sufficiently active as a signal molecule that it is very attractive as an agent to introduce into plans to mediate changes in pathogen behavior. X. fastidiosa is a particularly prolific producer of extracellular vesicles. These vesicles appear to be components of the outer membrane that are shed from the bacterial cells, and the shutting process is suppressed by DSF-mediated cell-cell signaling. The release of extracellular membranous vesicles by *X. fastdiosa* is responsible for the suppression of its adherence to surfaces, thus facilitating its movement through the plant, and is therefore a virulence factor.

INTRODUCTION:

Our work has shown that *Xylella fastidiosa (Xf)* uses DSF perception as a key trigger to change its behavior within plants. Under most conditions DSF levels in plants are low since cells are found in relatively small clusters in most xylem vessels, and hence they do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grape to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels. While we have demonstrated the principles of disease control by so-called "pathogen confusion", our continuing work aims to understand how best to alter DSF levels in plants to achieve even higher levels of disease control. Until now we have suffered from a lack of sensitive methods to detect DSF levels in plants (the *Xanthomonas*-based bioassay we have used previously is relatively insensitive to the chemically distinct forms of DSF produced by *Xf*). That is, while we showed that DSF-producing endophytes, direct application of DSF, and transgenic plants producing DSF all conferred some resistance to disease, we had no way to know why they were not more resistant, nor what would be needed to improve control measures since we could not measure the direct effect of our efforts to increase DSF levels in plants. However, we have now developed several new sensitive biosensors that enable us to measure *Xf* DSF both in culture and within plants. We have recently found that *Xf* apparently produces more than one molecule that can act as a DSF signal molecule and that the molecules made by *Xf* are dependent on its growth environment. We thus need to ascertain which form is most active, whether the various forms all have the same effect on regulating traits in *Xf*, and what are their fates when applied to plants in various ways. Thus the overall goal of our work is to use these new biosensors to examine how DSF levels can best be altered by the various methods we have previously identified. As disease control should be directly proportional to both the concentration of and dispersal of DSF within plants we will quantitatively explore the effectiveness of different strategies to elevate DSF levels throughout plants.

We also have made the discovery that *Xf* produces abundant extracellular membranous vesicles which are shed from the cell. Importantly, the content of outer membrane proteins including the adhesion XadA are controlled by DSF accumulation in cultures of *Xf*, and even more importantly, the shedding of these vesicles from the cell are apparently suppressed by the accumulation of DSF. We therefore are testing the model that DSF signaling in Xf involves two very different processes both of which lead to a rapid, cell density dependent change in its adhesiveness. When cells of *Xf* are found in relatively low numbers within the xylem vessel they have accumulated little DSF, and because of this they do not produce large amounts of the cell surface adhesins including XadA, HxfA, and others, yet such cells shed large numbers of vesicles. However, when cell density, and thus DSF concentrations increase membrane vesicles are not shed by the cell, and the higher concentration of afimbrial adhesins would be retained on the surface of the *Xf* cells rather than being fed into the environment. Such a process would tend to maximize the adhesiveness of *Xf* when DSF levels increased. This increased adhesiveness is apparently needed for their acquisition by insect vectors but would be expected to suppress their ability to move in the plant. In contrast, the shedding of vesicles would tend to prevent access from adhering to surfaces because most cell surface adhesins would no longer be attached to the cell. In this project, we are testing the role of the membranous vesicles, since preliminary data revealed that they may interfere with the adherence of *Xf* to surfaces. That is, by shedding adhesive vesicles which themselves adhere to the surface of plants, access may prevent its own adherence to such surfaces because they are now coded would such vesicles.

OBJECTIVES:

1) Identify additional DSF molecules made by *Xf* that contribute to cell-cell signaling and determine their movement and stability when applied to plants in various ways to improve disease control.
2) Determine the contribution of membrane vesicles shed by *Xf* in the absence of DSF to its virulence and the utility of measurement of vesicular presence within plants as a sensitive means to assess the success of strategies of disease control by pathogen confusion.

RESULTS AND DISCUSSION:

Objective 1: Finding new DSF species. We have optimized methods to use *Xf* itself to detect DSF**.** Among the several genes that we know to be most strongly regulated by DSF are genes such as *hxfA* and *HxfB* which are involved in cell-surface adhesion. We now have successfully used the endogenous *phoA* gene (encoding alkaline phosphatase) as a bioreporter of gene expression in *Xf*. The PhoA-based biosensor in which *phoA* is driven by the *hxfA* promoter is quite responsive to exogenous DSF from extracts of *Xf* cultures as well as C14-cis (hereafter called *Xf*DSF) itself. Assay of *Xf* extracts by *Xf* DSF-specific biosensors provide evidence of more than one *Xf* DSF molecule. Our analysis of the material collected by HPLC from these cultures using electro-spray MS revealed it to be an unsaturated C16 fatty acid. We therefore chemically synthesized this presumptive derivative which we will call C16-cis or *Xf* DSF2 (Figure 2).

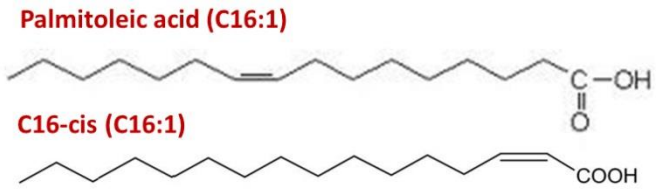

Figure 1. Structure of C16-cis (*Xf* DSF2) and palmitoleic acid

The biological activity of the synthetic *Xf*DSF2 was tested by the addition of this material at various concentrations to an rpfF* mutant strain of *Xf* harboring the hxfA*:phoA* reporter gene fusion and grown in PD3 medium. The rpfF* mutant is unable to synthesize DSF due to two mutations introduced into the catalytic site of the DSF synthase, yet this mutant is still able to respond to exogenous DSF. Importantly, this *Xf:phoA* biosensor exhibited very high alkaline phosphatase activity upon the addition of as little as 100 nM C16-cis (Figure 2). Importantly XfDSF2 also conferred much higher induction of hxfA, as indicated by a higher alkaline phosphatase activity at a given concentration than *Xf*DSF, and also induced other adhesins more highly. Both *Xf*DSF and *Xf*DSF2 strongly induced adhesion of wild type cells of Xf to glass tubes (Figure 2). Thus *Xf*DSF2 seems to be a particularly powerful signal molecule in *Xf*.

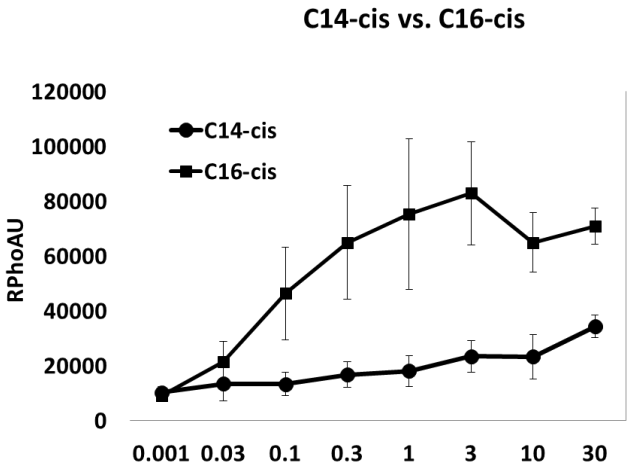

Figure 2. *Xf*DSF and *Xf*DSF2 Dose-dependent induction of the *Xylella fastidiosa*-based DSF-biosensor (*rpfF**-*Xf*HA-biosensor).

To better understand how promiscuous the DSF synthase RpfF from *X. fastidiosa* was, we synthesized a variety of different enoic fatty acids and assayed them with the *Xf:phoA* biosensor. A variety of related fatty acids having the site of unsaturation at the number 2 position but with different carbon chain lengths were assessed. In most cases, the double bond was constructed to be in a *cis* orientation, but a few corresponding *trans* unsaturated fatty acids were synthesized.

Several different patterns of response of the *Xf:phoA* biosensor to these various fatty acids was observed. Some relatively short chain-link fatty acids such as C10-cis were toxic, interfering with gene expression, but not bacterial growth, such that the alkaline phosphatase activity exhibited by the *Xf:phoA* biosensor *Xf:phoA* biosensor decreased with increasing concentration of the fatty acid (Figure 3). In contrast, the alkaline phosphatase activity exhibited by the *Xf:phoA* biosensor increased with increasing concentrations of fatty acid such as for XfDSF itself, thereby indicating a positive response, while there was no response to other fatty acids such as C19-cis (Figure 3).

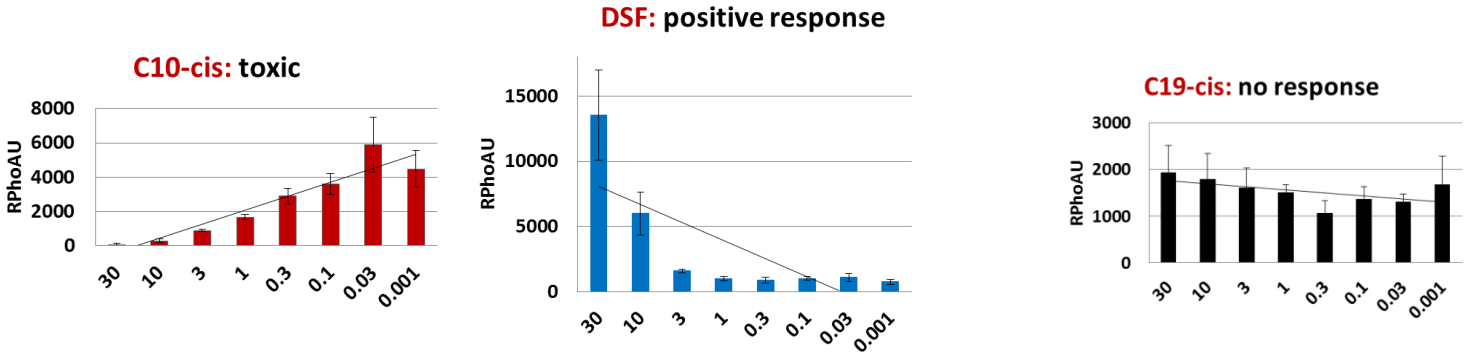

Figure 3: various responses of the *Xf:phoA* biosensor has indicated by alkaline phosphatase activity (ordinate) as a function of the concentration of various synthetic fatty acids noted on the abscissa (uM).

Unsaturated fatty acids differed greatly in their ability to induce quorum sensing in *X. fastidiosa*. While *X. fastidiosa* responded positively to unsaturated fatty acids with chain lengths from 12 to 18 carbons, as long as the site of unsaturation was at the number 2 carbon position, those fatty acids less than 12 carbons in length tended to be toxic while there was no response to those greater than 18 carbons in length (Table 1). While *X. fastidiosa* could respond positively to a wide range of different fatty acids, the lowest concentrations at which some response could be detected was highest for those of carbon lengths of 14-18.

Table 1. Activity of various unsaturated fatty acids as signal molecules in X. fastidiosa

Chain length	Orientation	Location of unsaturation	Molecule name	Xf biosensor		Xcc biosensor	
				Minimum detection concentration (uM)	Fold induction	Minimum detection concentration (uM)	Fold induction
8	*cis*	2	2-z-octanoic acid	Toxic	-	No response	-
9	*cis*	2	2-z-nonanoic acid	Toxic	-	No response	-
10	*cis*	2	2-z-decanoic acid	Toxic	-	10	4.1
11	*cis*	2	2-z-undecanoic acid	Toxic	-	1	12.4
12	*cis*		2-z-dodecenoic acid (BDSF)	3	3.2	0.1	12.4
12	*trans*	2	2-E-dodecanoic acid	Toxic	-	3	7.9
13	*cis*	2	2-z-tridecanoic acid	Toxic	-	0.001	17.9
13	*cis*	2	2-z-11-methyldodecenoic acid (DSF)	3	17.5	0.01	17.9
14	*cis*	2	2-z-tetradecanoic acid (XfDSF)	1	3.3	7	4.8
14	*cis*	5	5-z-tetradecanoic acid	No response	-	No response	-
14	*cis*	6	6-z-tetradecanoic acid	No response	-	No response	-
15	*cis*	2	2-z-pantadecanoic acid	10	4.2	No response	-
15		0	12-methyltetradecanoic acid (CVC-DSF)	1.5	0.32	No response	-
16	*cis*	2	2-z-haxadecanoic acid (XfDSF2)	0.15	8.9	No response	-

17	*cis*	2	2-z-heptadecanoic acid	0.3	8.6	No response	-
18	*cis*	2	2-z-octadecanoic acid	1	6.5	No response	-
19	*cis*	2	2-z-nonadecanoic acid	No response	-	No response	-
20	*cis*	2	2-z-eicosanoic acid	No response	-	No response	-

Given that *X. fastidiosa* appeared to be relatively promiscuous in its perception of a variety of unsaturated fatty acids, a number of different commercially available saturated and unsaturated fatty acids were evaluated for their ability to induce quorum sensing (Table 2). While no saturated fatty acid exhibited the ability to induce DSF-mediated quorum sensing in *X. fastidiosa*, Palmitoleic acid (Figure 1) was quite active as a signaling molecule (Figure 4). While approximately 5 times higher concentrations of Palmitoleic acid was required to induce the *Xf:phoA* biosensor compared to XfDSF2 (C16-cis), it conferred high levels of induction of the biosensor. This is a very exciting finding as it will allow us to proceed with tests to apply such as exogenous sources of DSF as a signal molecule to plants since large amounts of this material can be obtained relatively inexpensively.

Table 2. Commercially available fatty acids evaluated for signing activity in *X. fastidiosa*.

Chain length	Orientation	Location of unsaturation	Chemical name	Common name
14	-	0	tetradecanoic acid	Myristic acid
14	*cis*	9	9-z-tetradecenoic acid	Myristoleic acid
14	*cis*	5	5-z-tetradecenoic acid	Physeteric acid
16	-	0	hexadecenoic acid	Palmitic acid
16	*cis*	9	9-z-hexadecenoic acid	Palmitoleic acid
16	*cis*	6	6-z-hexadecenoic acid	Sapenic acid
16	*trans*	9	9-E-hexadecenoic acid	Palmitelaidic acid

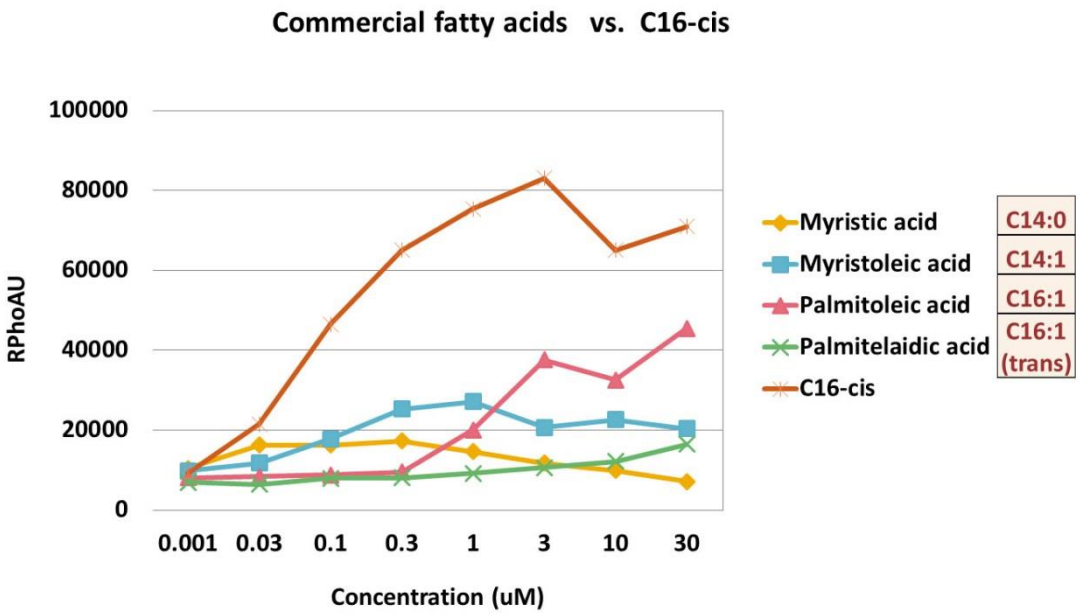

Figure 4: alkaline phosphatase activity exhibited by different commercially available saturated and unsaturated fatty acids tested at various concentrations in the *Xf:phoA* biosensor.

Given that a commercially available unsaturated fatty acid is a potent inducer of quorum sensing in *X. fastidiosa*, we further investigated the extent to which DSF signaling could be interfered with by the presence of other dissimilar saturated and unsaturated fatty acids. Not only does the grape strain of *X. fastidiosa* not respond to the DSF from CVC strains of *X. fastidiosa*, this molecule is a powerful inhibitor of signaling in grape strains of *X.*

fastidiosa in the presence of its own DSF, C16-cis (Figure 5). In the presence of one micromolar C16-cis the induction of the *Xf:phoA* biosensor decreased steadily with increasing concentrations of CVC DSF in the range from 0.1 to 30 micromolar (Figure 5).

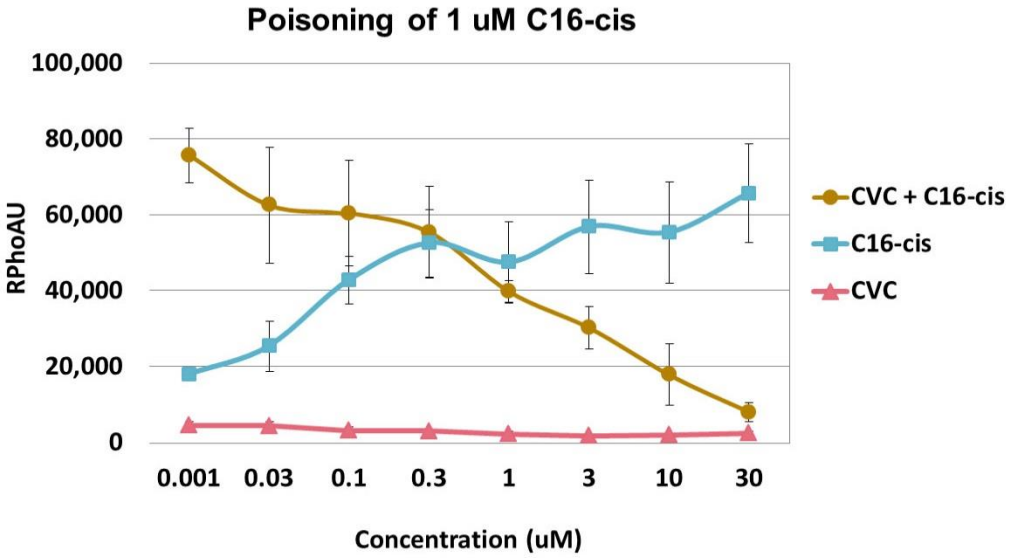

Figure 5: Alkaline phosphatase activity exhibited by cells of the *Xf:phoA* biosensor exposed to different concentrations of CVC DSF (red line), C16-cis (blue line), or to a combination of 1 uM C16-cis and different concentrations of CVC DSF as shown on the abscissa (brown line).

Given that Palmitoleic acid is a promising commercially available fatty acid that can serve as a signaling molecule in *X. fastidiosa*, we tested to what extent its ability to act as a signaling molecule could be blocked in the presence of other fatty acids (Figure 6). Not only did the saturated fatty acids Palmitic acid (C16) and Myristic acid (C14) interfere with signaling induced by C16-cis or C14-cis, but it also interfered with signaling induced by Palmitoleic acid as measured by the *Xf:phoA* biosensor (Figure 6). It thus appears that it might not be possible to use complex mixtures of fatty acids as signaling molecules, although we are continuing to investigate the ubiquity with which various saturated or *trans* fatty acids interfere with signaling in *X. fastidiosa.*

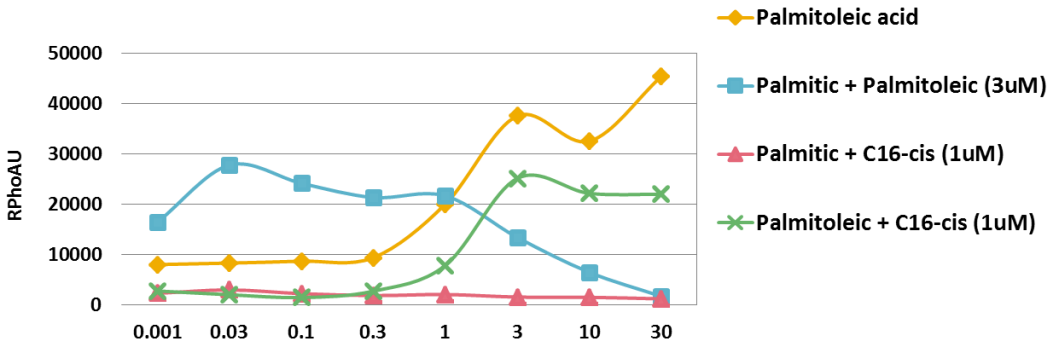

Figure 6: Alkaline phosphatase activity exhibited by cells of the *Xf:phoA* biosensor exposed to different concentrations of palmitoleic acid alone (gold line), various concentrations of palmitic acid and 3 µm palmitoleic acid (blue line), various concentrations of palmitoleic acid and 1 um C16-cis (blue line), or to a combination of 1 µM C16-cis and different concentrations of palmitic acid (red line) as shown on the abscissa.

Given that Palmitoleic acid is a promising commercially available fatty acid that can serve as a signaling molecule in *X. fastidiosa* we have investigated different ways in which it can be introduced into plants. We therefore have initiated large experiments in which we are assessing both the concentration of Palmitoleic acid within the xylem tissue as well as any phytotoxicity of Palmitoleic acid applied either by itself or in conjunction

with various surfactants or solubilizing agents. We thus have inoculated grape with solutions of Palmitoleic acid with different concentrations of the surfactants Breakthru and Triton X-100 as well is he solubilizing agents DMSO and Solutol. Palmitoleic acid was applied at a concentration of 10 mM and to plants both as a foliar spray, as a soil drench, and as a stem injection. While high concentrations of several of these detergents or solubilizing agents caused phytotoxicity no, or limited cytotoxicity was observed at a concentration of less than 0.2% Breakthru, 0.2% Triton X-100, 1% DMSO, or 1% Solutol. The effectiveness of these agents in introducing palmitoleic acid into grape tissue was assessed by assessing the ability of sap extracted from individual leaves using a pressure bomb to induce the expression of alkaline phosphatase activity in the *X. fastidiosa Xf:phoA* biosensor. The initial results of these studies reveal that substantial amounts of palmitoleic acid could be introduced into grape leaves one applied as a foliar spray with 0.2% Breakthru (Figure 7). Lesser amounts could be introduced with foliar sprays including Solutol and DMSO. As a registered surfactant for use in agriculture, Breakthru has the potential to be a practical delivery agent. The efficacy of this material is probably associated with its extraordinarily low surface tension that enables spontaneous stomatal infiltration of leaves with aqueous solutions containing 0.2% of this detergent. Thus, solutions of fatty acid supplied with this concentration of surfactant appear to bypass the cuticular surface as a means to enter the intercellular spaces and presumably also the vascular tissue.

These most promising treatments were also applied to grape plants to evaluate their efficacy in reducing the symptoms of Pierces disease. Initial application of Palmitoleic acid was followed two weeks later by inoculation with *X. fastidiosa*. The Palmitoleic acid treatments were re-applied every three weeks until nine weeks. Just as disease symptoms were appearing, a malfunction of deregulation system in the greenhouse caused the plants to severely damage due to desiccation. These experiments will therefore be repeated.

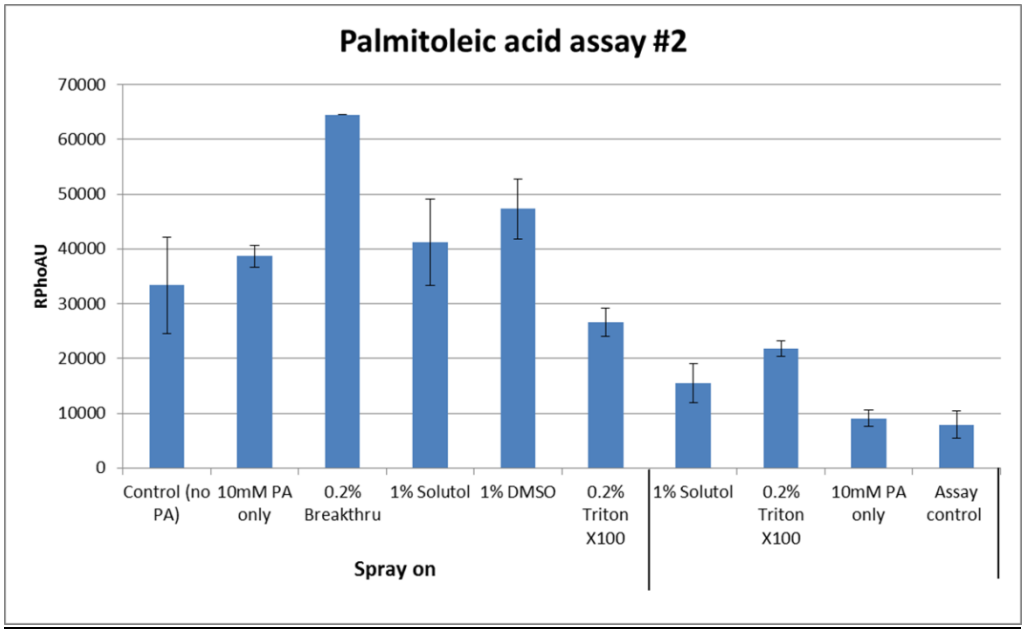

Figure 7. Alkaline phosphatase activity exhibited by 10 µl aliquots of xylem sap extracted under pressure from individual leaves of grape plants treated with 10 mM palmitoleic acid with the various surfactants noted when applied as a foliar spray or a stem injection.

Objective 2: Role of extracellular vesicles

Our continuing work reveals that *Xf* is a very prolific producer of extracellular vesicles. A large numbers of vesicles (>400/cell) can be associated both with the surface of the bacterial cell, as well as a high portion that are shed by the cells to the extracellular environment (Figure 8). The vesicles are generally quite small, ranging in size from about 0.01 to 0.1 µm in diameter. Quantification of vesicles by both deconvolution fluorescence microscopy, flow cytometry, and a Nanovision particle counter reveals that the higher number of vesicles (normalized for the number of *Xf* cells) are present in RpfF mutants, suggesting strongly that DSF accumulation suppresses the release of such vesicles. The *rpfF* mutant produces as much as three times more vesicles (Figure 9). While some vesicles were as large as approximately 1000 nm, the average diameter of vesicles was only approximately 150 nm (Figure 9. We can estimate that each *Xf* cell has shed approximately 100 to 1000 vesicles of different sizes. It is thus clear that vesicles constitute a major extracellular factor produced by *Xf*.

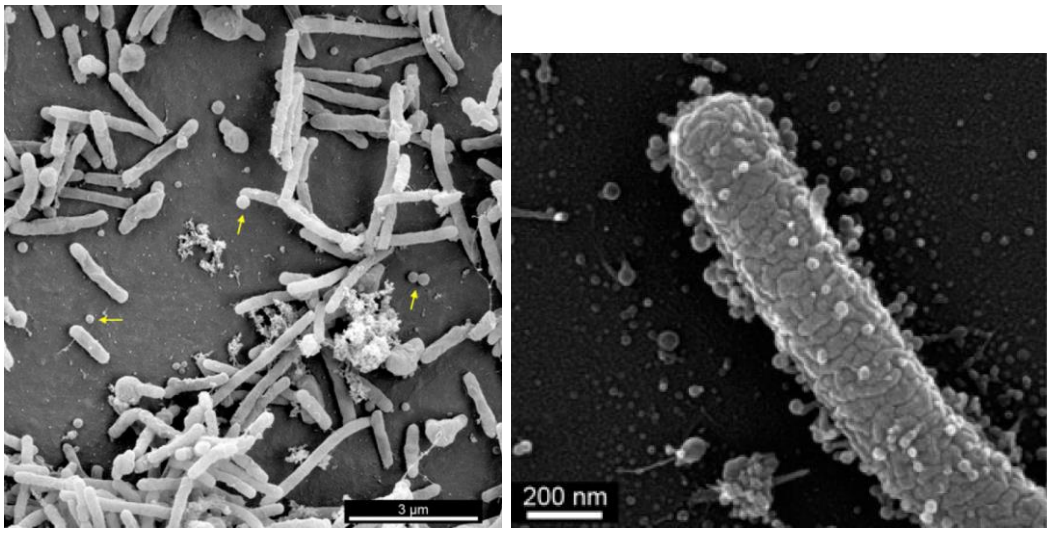

Figure 8. Membranous vesicles forming on the surface of cells of a wild type strain of *Xylella fastidiosa*.

Quantification of vesicles by both deconvolution fluorescence microscopy, flow cytometry, and a Nanovision particle counter reveals that a higher number of vesicles (normalized for the number of *Xf* cells) are present in RpfF mutants, suggesting strongly that DSF accumulation suppresses the release of such vesicles. The *rpfF* mutant produces as much as three times more vesicles (Figure 9). While some vesicles were as large as approximately 1000 nm, the average diameter of vesicles was only approximately 150 nm (Figure 9. We can estimate that each *Xf* cell has shed approximately 100 to 1000 vesicles of different sizes. It is thus clear that vesicles constitute a major extracellular factor produced by *Xf*.

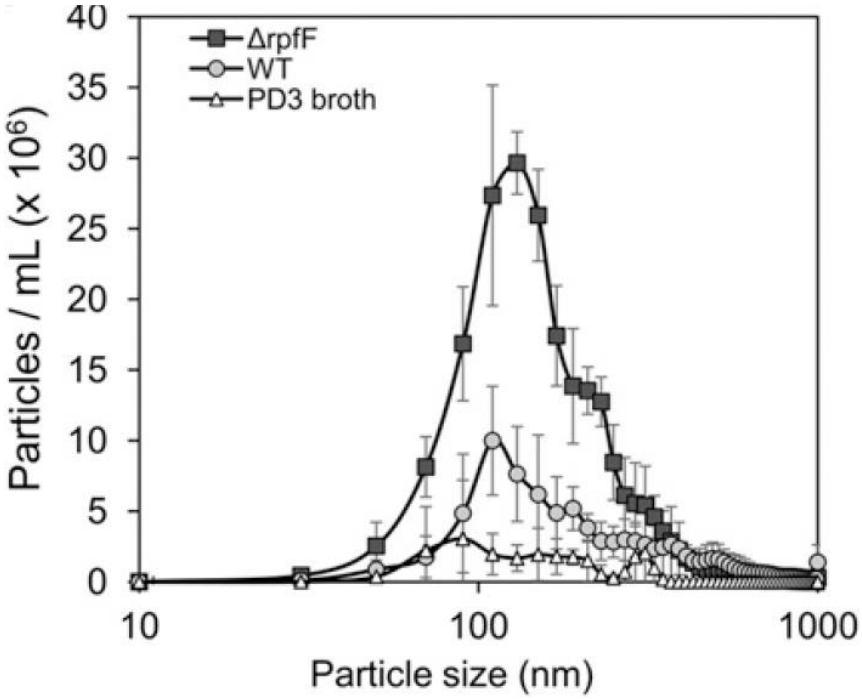

Figure 9. Distribution of sizes and abundance of vesicles of different sizes produced by a wild type and an *rpfF* mutant of *Xf* when grown for two days in PD3 broth when assessed with a Nanovision device. The vertical bars represent the standard error of the estimate of the number of vesicles produced by a given strain.

X. fastidiosa produced abundant vesicles while colonizing plants. Outer membrane vesicles could be readily detected in the xylem fluid of plants infected with the wild type strain, and much higher numbers in plants infected with an RpfF mutant strain. It is thus clear, that the production of outer membrane vesicles by *X. fastidiosa* is not an artifact of their culture in laboratory media, but that it is an intrinsic trait of the pathogen while growing in host plants.

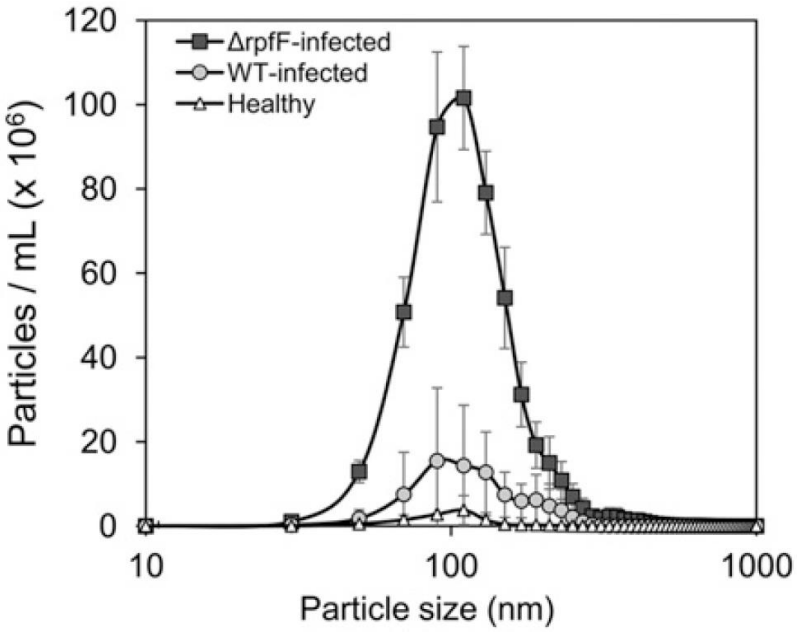

Figure 10. Numbers of particles of various sizes in xylem sap recovered from Cabernet Sauvignon grape infected with the wild type (circles) or an RpfF mutant of *X. fastidiosa* (squares), or from healthy plants. Particles of various sizes were enumerated with a Nanovision device. The vertical bars represent the standard error of the determination of mean particles of a given size.

Xylem sap containing membranous vesicles was shown to reduce the adherence of *X. fastidiosa* to various surfaces. Xylem fluid was collected by pressure bomb from healthy Thompson seedless grape, as well as from plants infected with a wild type strain of *Xf* or with an RpfF mutant of *Xf*. To test the differential adherence of wild type cells of *Xf* to surfaces such as insects in the presence of these different sample collections, we immersed small sections of hind wings of glassy winged sharpshooter in each of these xylem sap samples to which we also added a wild type strain of *Xf*. Many more cells that had been suspended in samples from healthy plants attached them from cells suspended in sap from plants infected with the wild type Xf strain, particularly from sap infected with an RpfF mutant of *Xf* (Figure 11). Over 20-fold more bacterial cells were attached when suspended in sap from the healthy plant compared to that of sap from the plant infected with the RpfF mutant of *Xf* (Figure 11). It is thus clear that the xylem sap environment of plants infected with an RpfF mutant of XF is much less conducive to the adherence of *Xf* to surfaces than that of healthy plants. In fact, the sap environment of plants infected with the wild type strain of *Xf* is also somewhat less conducive to adherence.

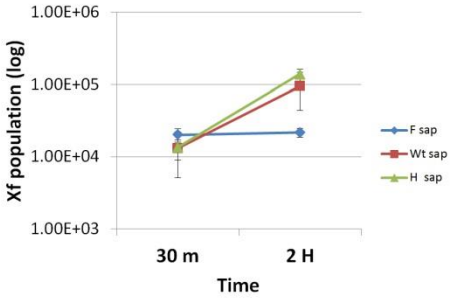

Figure 11. The number of cells of a wild type strain of *Xylella fastidiosa* that had adhered to wings of the glassy winged sharpshooter suspended in xylem sap from plants infected with an RpfF mutant (blue), or a wild type strain of *Xylella fastidiosa* (red), or from healthy plants (green) after incubation for either 30 minutes or 2 hours.

The ability of vesicles to interfere with binding of *X. fastidiosa* to surfaces such as insect wings also suggested that it would interfere with binding to plant surfaces, such as xylem vessels, thereby better enabling the movement of the pathogen through the plant. This was investigated by introducing cells of *X. fastidiosa* to grape stem segments in the presence or absence of purified membrane vesicles. Vesicles were collected by ultracentrifugation of cell free supernatants. Vesicles were than either resuspended in buffer or in culture media and cells of *X. fastidiosa* were then introduced into surface sterilized, 2 cm stem segments in buffer or culture media alone, or in such solutions containing membrane vesicles. After introduction into the stem segments and incubation for one hour, stem segments were flushed with sterile buffer to remove any unattached cells of *X. fastidiosa*. Population size of the attached *X. fastidiosa* cells were then determined by dilution plating. When cells were co-inoculated into stem segments with membrane vesicles suspended in buffer, there was a dramatic reduction (>20-fold) and the fraction of those cells which attached to the xylem vessels compared to that of cells introduced in buffer alone (Figure 12).

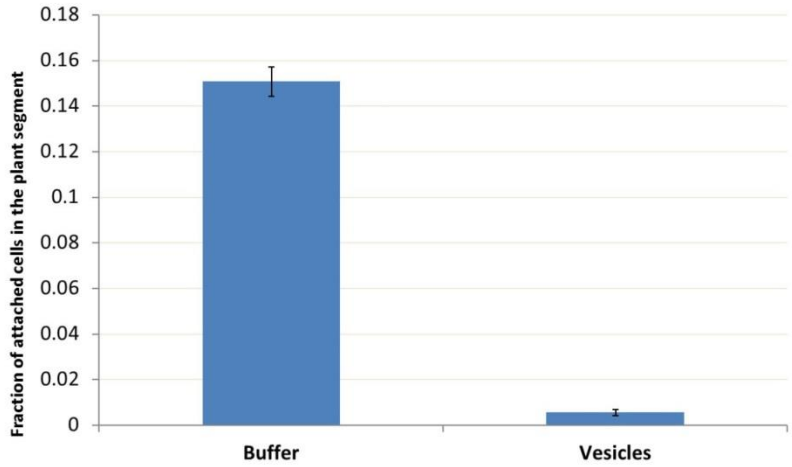

Figure 12: The fraction of total cells introduced into xylem vessels that were retained after one hour incubation when introduced in buffer alone or in phosphate buffer containing membranous vesicles of *X. fastidiosa*. The vertical bars represent the standard error of the mean of the fraction of attached cells.

To better assess the process by which the vesicles were interfering with the attachment of *X. fastidiosa* to plant tissues, we conducted similar experiments as above, but introduced the membrane vesicles in buffer alone, incubated the vesicles with the tissue for one hour before then flushing the vesicles out with buffer, before then introducing bacterial cells in buffer to the same stem segments. In this way, we enabled membrane vesicles to interact with plant tissue before, or instead of, bacterial cells themselves. This design enabled us to determine whether the process of finding of vesicles to plant tissues led to the blockage of finding of *X. fastidiosa* to the plants, or whether binding of the vesicles to the bacterial cells then prevented their binding to the plant tissue. It was clear however that prior treatment of the plant tissue with the vesicles conferred the same dramatic reduction in the ability of *X. fastidiosa* to bind to plants as well as the case when the cells and vesicles were coinoculated into the plant (Figure 13). It thus seems clear that vesicles prevent binding of *X. fastidiosa* to plant by preferential binding to the surfaces to which the bacteria themselves might otherwise have bound.

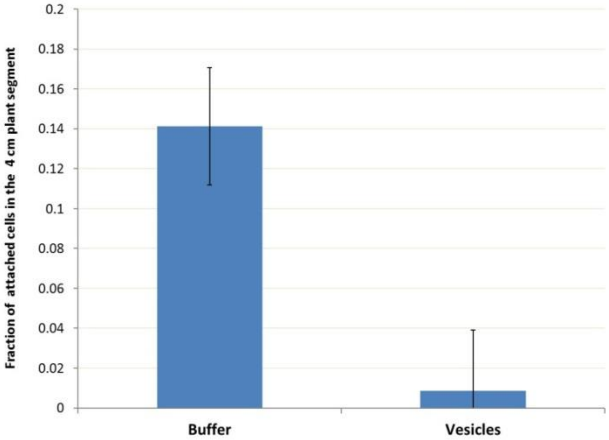

Figure 13: The fraction of total cells introduced into xylem vessels that were retained after one hour incubation when introduced into plants treated one hour earlier with either buffer alone or phosphate buffer containing

membranous vesicles of *X. fastidiosa*. The vertical bars represent the standard error of the mean of the fraction of attached cells.

To better understand the relative ability of outer membrane vesicles to block adhesion of *X. fastidiosa* to various surfaces we performed experiments similar to that above in which hind wings of glassywinged sharpshooters were immersed in cell suspensions of *X. fastidiosa* either in PIM6 medium alone or in PIM6 medium containing membranous vesicles. While the number of *X. fastidiosa* cells that attach to the insect hind wings was lower when suspended in membranous vesicles compared to medium alone (Fig. 14), this effect of vesicles preventing attachment of *Xylella* to surfaces which much smaller than observed in blockage to xylem vessels. That is, while membranous vesicles reduced the proportion of *X. fastidiosa* cells that would attach to xylem vessels by over 20-fold (see figure 13), these vesicles reduced attachment to insect hind wings by only about 3-fold. These results suggest that the membranous vesicles attach more strongly to plant surfaces, and thereby reduce the attachment of *X. fastidiosa* to the same plant surfaces. It is sensible to speculate that this selective blockage of attachment would have been evolutionarily selected in *X. fastidiosa*. Specifically, successful transmission of *X. fastidiosa* from one infected plant to another is dependent on acquisition of the cells by the insect vector. While the membranous vesicles appear to facilitate movement of *X. fastidiosa* throughout the plant by blocking its attachment to the plant , which would be expected to enter its movement, the vesicles are apparently do not strongly affect its acquisition by insect vectors, thereby enabling it to be acquired and thus transmitted.

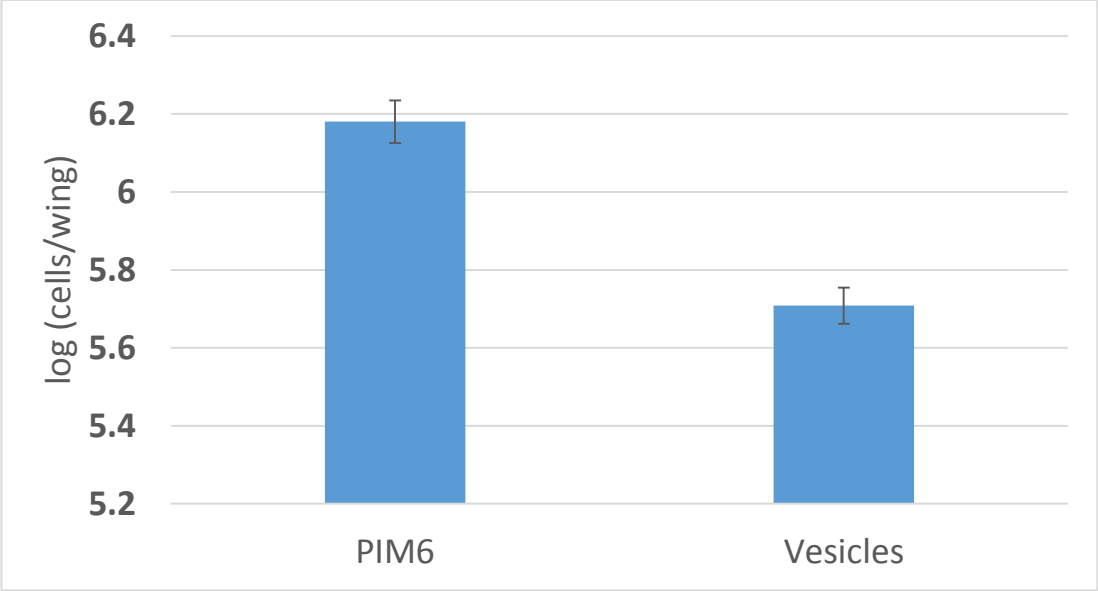

Figure 14. Population size of *Xylella fastidiosa* attached to hind wings of glassy-winged sharpshooters when suspended for two hours in PIM6 culture medium alone or in membranous vesicles of *Xylella fastidiosa* suspended in PIM6 medium. The vertical bars represent the standard error of the determination of mean numbers of cells attached as determined by dilution plating.

CONCLUSIONS:

We are very excited about results to date that show that several means of elevating DSF levels in plants have provided disease control via a strategy of "pathogen confusion". Given the limitations in standard methods of disease control, we are optimistic that DSF interference represents a promising strategy for PD control. Control of Pierce's disease by direct application of DSF is a very attractive disease control strategy since it could be quickly implemented and would utilize commonly used agricultural equipment and methods and would not require the use of transgenic technologies. Our earlier work had shown that C14-cis, a component of *Xf* DSF, conferred some reduction of disease after topical application, but less than might have been expected compared to application of crude DSF-containing extracts of *Xf*. Our recent studies using improved Xf-based DSF biosensors more responsive to the DSF molecules made by *Xf* reveal that at least 2 additional molecules related to C14-cis are biologically active in *Xf*, and DSF2 is much more active than C14-cis. We are very excited to find that a cheap, commercially available molecule palmitoleic acid also is quite active as a DSF signal molecule. We will determine which of these molecules are most biologically active, whether they all have similar effects on gene expression in *Xf*, and which are most abundant within plants infected with *Xf*. Our new sensitive biosensors will be used to document the

absorption and translocation of these molecules by grape after application in various ways. This should enable us to greatly increase disease control by direct application of the most appropriate molecule.

Strong evidence suggests that the release of extracellular membranous vesicles by the RpfF mutant is responsible for the suppression of adherence of *Xf* to surfaces. Since the RpfF mutant of *Xf* does not accumulate DSF, which in turn suppresses the release of extracellular vesicles, a higher concentration of extracellular vesicles would be expected in plants infected with the RpfF mutant. At least some extracellular vesicles would also be expected in the sap of plants infected with the wild type strain as well. A higher concentration of extracellular vesicles, as estimated by the abundance of XadA (which we can use as a marker protein for these membranous vesicles), is found in plants infected with the RpfF mutant of *Xf* compared to that of the wild type strain. These results further support our model of a "Teflon mechanism" of virulence of *Xf* whereby it releases adhesive vesicles, especially one found at relatively low cell densities where DSF would not have accumulated. These vesicles would be expected to attach to surfaces such as that of the walls of the xylem vessels. By so attaching, these vesicles would prevent the attachment of *Xf* cells themselves to such surfaces. Only upon reaching relatively high cell concentrations in a particular vessel would DSF concentrations increase to a level that would suppressed the release of the membranous vesicles, thereby retaining adhesive molecules on the surface of *Xf* cells themselves, thus allowing the bacterial cells themselves to attach to surfaces, such as that of insect vectors. The presumptive anti-adhesive factor apparently plays a major role in facilitating the movement of *Xf* throughout the plant, and further work to elucidate its nature and contributions to this process are warranted.

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