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COMPARISON AND OPTIMIZATION OF DIFFERENT METHODS TO ALTER DSF-MEDIATED SIGNALING IN *XYLELLA FASTIDIOSA* IN PLANTS TO ACHIEVE PIERCE'S DISEASE CONTROL

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OBJECTIVES:
1) Compare DSF production and level of disease control conferred by transformation of *Xf* RpfF into several different grape cultivars.
2) Evaluate efficacy of direct applications of palmitoleic acid, C16-cis, and related DSF homologs to grape in various ways to achieve disease control.
3) Evaluate the potential for *Burkholderia phytofirmans* to multiply, move, and produce DSF in grape plants to achieve Pierce's disease control.

ACTIVITIES AND ACCOMPLISHMENTS:

Introduction

Our work has shown that *X. fastdiosa* 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, 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 in various ways to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels – "pathogen confusion". Transgenic, 'Freedom' grape expressing the DSF synthase RpfF from *X. fastdiosa* are much more resistant to disease than the wild type plants in both greenhouse and field trials. Our work has shown however that RpfF is rather promiscuous and that *X. fasti*diosa can both produce and respond to a variety of unsaturated fatty acids - and that the DSF species produced is influenced apparently by the particular substrates available within cells. It is possible that grape varieties might differ in their ability to produce DSF molecules perceived by *X. fastidiosa*. It will be important therefore to determine whether commercial grape cultivars can all produce DSF species capable of altering pathogen behavior in high amounts if transformed with the DSF synthase. Non-transgenic strategies of achieving pathogen confusion might be preferred by the industry. While endophytic bacteria capable of producing DSF species is an attractive strategy, until recently, strains capable of growth and movement within grape could not be found. However, we have now found a *Burkholderia* strain that both colonizes grape and has conferred substantial disease control in preliminary studies. We will investigate the interactions of this endophyte with grape to optimize disease control and determine practical methods of its explication. We have found that *X. fastidiosa* produces additional DSF species including 2-Z-hexadecenoic acid (C16-cis) that are much more active than C14-cis previously found, and that the common, inexpensive,

unsaturated fatty acid - palmitoleic acid is also reasonably active as a signal molecule in *X. fastidiosa*. Using a new *X. fastidiosa* biosensor for DSF in conjunction with such an abundant, inexpensive molecule we can now thoroughly investigate methods by which such a molecule can be directly applied to plants to achieve concentrations sufficiently high in the xylem to alter pathogen behavior and thus achieve disease control.

Objective 1. Production of DSF in a variety of grape cultivars.

While Freedom grape transformed with the *X. fastidiosa rpfF* gene encoding the DSF synthase produced DSF species to which *Xf* was responsive, considerable evidence has been accumulated that RpfF is a rather promiscuous enzyme capable of producing a variety of DSF-like molecules. For example, we detected the production of C14-cis (*Xf*DSF1), C16-cis (*Xf*DSF2) and surprisingly, even DSF (normally produced only by *Xanthomonas* species) in transgenic RpfF-expressing freedom grape. Likewise, introduction of *X. fastidiosa* RpfF into *Erwinia herbicola* yielded the production not only of *Xf*DSF1 and *Xf*DSF2, but other apparently related enoic acids not seen in *X. fastidiosa* itself (data not shown). The enzymatic activity of Bcam0581, a protein highly homologous to *X. fastidiosa* RpfF, that mediates biosynthesis of DSF in *Burkholderia cenocepacia* was recently shown to both catalyze the dehydration of 3-hydroxydodecanoyl-ACP to cis-2-dodecenoyl-ACP as well as to cleave the thioester bond to yield the corresponding free acid. We presume that *X. fastidiosa* RpfF also possesses these same features, although it probably shows a preference for longer chain 3-hydroxyacyl-ACPs since the DSF species produced by of *X. fastidiosa* include 2-*Z*-tetradecenoic acid. The process by which such a compound could be produced by the expression of RpfF in plants remains somewhat unclear. Plant fatty acid synthesis is not prominent within the cytosol, and occurs primarily in the plastid, although some synthesis can also occur in the mitochondria. However, plant tissues are capable of incorporating exogenously provided fatty acids into their endogenous lipids indicating that fatty acids are mobile in the plant. In *B. cenocepacia* it appears that DSF synthesis results from a branch of the more classical fatty acid biosynthesis pathway by diverting 3-hydroxydodecanoyl-ACP. In plants, the majority of such corresponding acyl-ACP substrates for RpfF would be expected to be found within plastids, as a thioesterase is normally involved in converting such compounds to the free acid for release from the plastid. Thus there is either sufficient 3-hydroxyacyl-ACP of either plastid or mitochondrial origin in the cytoplasm of plants to enable RpfF resident in the cytoplasm to produce the DSF observed in the transgenic plants, or the expression of *X. fastidiosa rpfF* in Freedom grape may have allowed some transport of RpfF into the chloroplast. Given that both *Xf*DSF and *Xf*DSF2 were produced in grape harboring RpfF we presume that the corresponding 3-hydroxyacyl-ACP substrates were available in Freedom grape. The production of various DSF species in grape might therefore be somewhat contextual, and different grape cultivars may differ in their ability to provide suitable substrates for RpfF. The various enoic acids that can be produced by RpfF differed substantially in their ability to induce gene expression in *X. fastidiosa* - with those of longer chain lengths such as C16-cis being much more active than those of shorter chain lengths . We have also observed that DSF-mediated signaling in *X. fastidiosa* by active DSF species such as C16-cis can be blocked in the presence of certain other *trans* unsaturated fatty acids. It is therefore possible that in some plants other fatty acid species indigenous to the plant or induced upon transformation of RpfF might interfere with signaling that would otherwise be conferred by the production of C16-cis and other "active" DSF species. To verify that the strategy of production of DSF in RpfF-containing transgenic grape is a robust one, widely applicable in a variety of grape cultivars we propose to compare and contrast the production of DSF species in such a variety of grape cultivars. In addition, it seems likely that targeting RpfF to cellular compartments where the substrates for DSF synthesis may be more abundant could lead to enhanced production of this signal molecule. We thus are comparing the amount and types of DSF produced, and disease susceptibility, in transgenic plants in which RpfF is targeted to plastids and in plants in which it is not targeted.

RpfF was initially introduced only into Freedom grape, because it was the only variety for which transformation was feasible at that time. Continuing work by Dr. Davis Tricoli at the Plant Transformation Facility at UC Davis has now made it possible to transform Thompson seedless as well as the wine grapes Chardonnay and Cabernet Sauvignon and the advanced rootstock varieties 1103 and 101-14. In addition to untargetted expression of RpfF, we have produced constructs which target RpfF to the chloroplast of grape by fusing the small subunit 78 amino acid leader peptide and mature N-terminal sequences for the *Arabidopsis* ribulose bisphosphate carboxylase (which is sufficient to target the protein to the chloroplast) to RpfF. Transformation of the various grape varieties is being conducted at the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis. The following lines are being produced and tested:

Variety	Gene introduced

	Untargeted RpfF	Chloroplast-targetted RpfF
Thompson Seedless	+	+
Chardonnay	+	+
1103	+	+
101-14	+	+
Richter 110	+	+
Freedom	done	+

Transformation of the various varieties is underway and we anticipate that it will take at least an additional six months. There has been little experience in transformation of Richter 110 and Chardonnay, and so their successful transformation may take longer than the other cultivars. Between 5 and 10 individual transformants will be produced for each variety/construct combination. Because the expression of *rpfF* in a given transformant of a given plant line will vary due to the chromosomal location of the randomly inserted DNA, it will be necessary to identify those lines with the highest levels of expression. It is not practical to directly test disease susceptibility in each of the many transformed lines; each line would have to be grown to a sufficiently large size that vegetative clones could be produced (3 months) and then each plant would need to be propagated and assessed for disease susceptibility (5 additional months). Instead, to most rapidly identify those transformants with high levels expression of *rpfF* and production of DSF, three assays that can be rapidly employed on seedling plants will be conducted to identify the most promising transformants. 1) The expression of *rpfF* will be assessed by quantitative RT-PCR of RNA isolated from individual leaves of the transformed plant after they are grown to a height of approximately 40 cm. 2) The distal 20 cm of each 40 cm high plant will be excised, placed in a pressure bomb, and xylem sap extruded under pressure. The approximately 30 µL of xylem sap collected from each plant by this method will be assessed for the presence of DSF species capable of inducing gene expression in *X. fastidiosa* by adding it to micro-cultures (200 µL) of a *phoA* mutant of *X. fastidiosa* harboring a *hxfA:phoA* reporter gene fusion. The alkaline phosphatase activity of the cells of this DSF biosensor, measured as in our other studies, will be proportional to the concentration of various DSF species. This assay will not only identify those transformants within a given variety that maximally express the introduced *rpfF* gene, but will provide early evidence of those species capable of producing DSF species to which *X. fastidiosa* is maximally responsive. 3) A functional "cell release" assay to determine those transformed lines in which *X. fastidiosa* exhibits the highest adhesiveness, (expected of DSF-producing lines) will also be performed on the decapitated plant after extraction of xylem sap. Each excised plantlet will be rooted by placing the excised stem in moist vermiculite in a humid chamber for two weeks. Cells of *X. fastidiosa* (ca. 10^7) will be injected into the petioles of three leaves for each plantlet. The leaves will be excised 3 days after inoculation, the petiole surface sterilized, and the cut end of the petiole introduced into sterile water and gently agitated for 20 min. to release cells from within the xylem vessels. The proportion of cells released from a petiole will be calculated as the ratio of those released from the total number of cells within that petiole (determined by macerating petiole after cell release). The proportion of cells released from plants in such an assay is inversely proportional to the concentration of DSF in those plants (DSF producing plants induce stickiness of *X. fastdiosa* and they are thus not released).

The disease susceptibility of the 2 transformed lines from each treatment combination having highest *rpfF* expression or apparent DSF production will then be assessed. At least 15 vegetative clones each of the lines will be produced from green cuttings of plants developing from the remaining transgenic plant remaining after the essays above. Plants (ca. 30 cm high) will be inoculated with *X. fastdiosa* by needle puncture as in earlier studies. Disease severity will be assessed visually each week. After 14 weeks, when substantial disease will have appeared in untransformed lines, population sizes of *X. fastidiosa* in petioles of leaves collected at 30 cm intervals from the point of inoculation will be assessed as before. We also will assess the efficacy of the best of the two RpfF-expressing rootstock varieties to confer disease control to normal Cabernet Sauvignon scions grafted onto them as in other studies.

The composition of DSF species present in xylem sap and their aggregate signaling activity will be assessed by extracting xylem sap from mature (2 m tall) plants of each of the two best transformed lines of a given variety/construct forwarded for further analysis. Vines will be cut into 40 cm segments and placed in a pressure chamber and subjected to about 20 bar pressure. The xylem sap obtained will be collected into glass containers containing ethyl acetate, mixed vigorously for 5 min and the Etyl acetate (now containing DSF) will be separated from the aqueous phase. The ehyl acetate will be concentrated by evaporation and the dried residue dissolved in methanol. Mass spectrometry analysis of the plant xylem sap-extracts will be performed using an LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization (ESI) source. Mass spectra will

be recorded in the negative ion mode over the range *m/z* = 100-500 using the Orbitrap mass analyzer and spectra processed using Xcalibur software. DSF species will be identified by their m/Z ratio, with *Xf*DSF, XfDSF2, and DSF (having m/Z ratios of 225.18, 253.22, and 211.17, respectively) being readily distinguished in xylem sap of RpfF-expressing Freedom. We will also resolve other chemical species found in RpfF-expressing lines that are not found in control plants by a similar procedure. We expect that more than one enoic acid will be produced in a given line expressing RpfF. Because of this, aggregate DSF signaling activity will be determined in samples of xylem sap collected as above using the *Xf phoA*-based DSF biosensor as described above. Control sap samples in which different concentrations of *Xf*DSF2 (C16-cis) are spiked will enable the DSF signaling activity of xylem sap from a given line to be expressed as that of *Xf*DSF2 equivalents. It is possible that some transgenic lines will exhibit little aggregate DSF signaling activity because of their production of antagonistic fatty acids. Such lines will be identified in two ways: 1) Such a line might contain relatively high concentrations of *Xf*DSF2 and other known inducers of signaling in *X. fastidiosa* (determined by ESI-MS analysis of sap) yet not induce expression of the *Xf* DSF biosensor strain. 2) Direct evidence for such antagonism will be obtained by spiking xylem sap samples from such transgenic lines with *Xf*DSF2 and comparing the alkaline phosphatase activity of the *X. fastidiosa* DSF biosensor in such samples with those of spiked samples of xylem sap from control plants; reduced biosensor activity in the test samples would provide direct evidence for such antagonism.

Objective 2: Direct application of DSF to plants.

Several recent findings in our laboratory of the process of DSF-mediated signaling in *Xf* suggest that Pierce's disease control by direct application of DSF to plant surfaces is both feasible and practical. Studies of the context-dependent production of DSF reveals that DSF species such as *Xf*DSF2 are far more active than *Xf*DSF1 which was originally described (Figure 1). While topical applications of *Xf*DSF1 to grape provided modest reductions in disease severity, applications of *Xf*DSF2 should be far more efficacious. Studies of applications of *Xf*DSF2 were hindered by a limitation of the amount of this material that we could chemically synthesize. Fortunately, our studies of the promiscuity of DSF signaling in *X. fastidiosa* reveal that it is quite responsive to the cheap, commercially available, enoic acid palmitoleic acid (Figure 1).

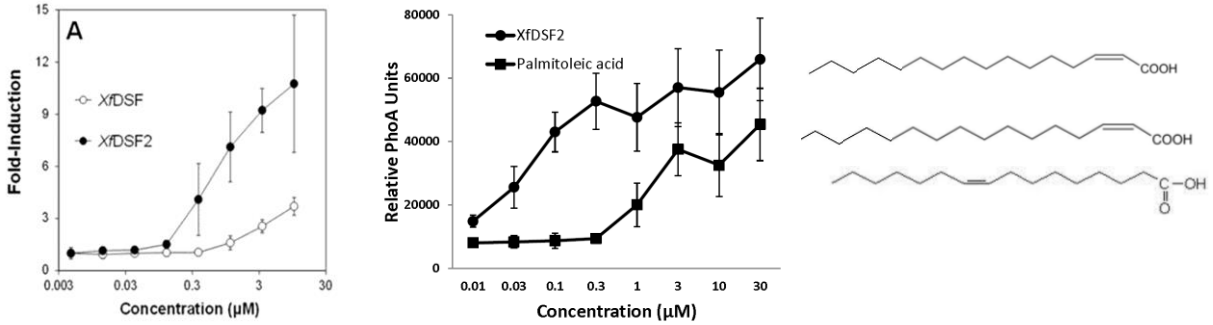

Figure 1. Responsiveness of a PhoA-based *X. fastidiosa* DSF biosensor to different concentrations of *Xf*DSF1 (top molecule), *Xf*DSF2 (middle molecule), and palmitoleic acid (bottom molecule).

While about 8-fold more palmitoleic acid is required to induce gene expression in *Xf* than *Xf*DSF2, it is much more active than *Xf*DSF1 itself. We therefore will conduct a variety of studies to address how such molecules could be introduced into plants in different ways to achieve pathogen confusion. While most studies will use palmitoleic acid, we also will conduct comparative studies using synthetic *Xf*DSF2 and *Xf*DSF1.

We are investigating several strategies by which direct application of DSF molecules can reduce Pierce's disease. While we will determine the effects of application of DSF homologs on disease severity of plants inoculated with *X. fastidiosa* in some studies, direct monitoring of DSF levels in treated plants is a MUCH more rapid and interpretable strategy of assessing this strategy of disease control. As DSF must enter the xylem fluid in order to interact with the xylem-limited *Xf* in plants we have been assessing DSF levels in xylem sap of plants treated in different ways using the PhoA-based *X. fastidiosa* biosensor as described above. We are addressing 4 main issues that we hypothesize to limit the direct introduction of DSF into plants: 1) The penetration of DSF through leaves and other plant tissues may be slow or inefficient, 2) DSF may readily enter plant tissues but only slowly enter the xylem sap, 3) DSF may be degraded after introduction into plants, and 4) DSF may enter plants more readily via certain tissues than others (eg. it may readily be taken up via the roots but more slowly from leaves). We thus are measuring DSF species levels in 1:) xylem sap as well as in 2:) leaf, stem, and root tissue

after removal of xylem sap after applying synthetic DSF to (A) foliage, (B) direct injection into stems, and (C) application to roots as a drench.

As DSF species are somewhat hydrophobic, a variety of adjuvants are being tested for their effects on enhancing their introduction into plants. For example, detergents and solubilizing materials such as Solutol HS15 may greatly increase the penetration and dispersal of DSF and its analogs. We thus are suspending the hydrophobic materials in such carriers prior to foliar sprays or stem injections. We also are assessing the efficacy of applying DSF molecules with surfactants such as Breakthru that have very low surface tension. Solutions in such organo-silicon surfactants have sufficiently low surface tension that they spontaneously infiltrate leaves through stomatal openings. Considerable preliminary results of already been obtained on the ability of such a topically applied palmitoleic acid solutions to enter into the plants. Apparent DSF signaling activity was measured using the biosensors noted above. We inoculated grape with solutions of Palmitoleic acid with different concentrations of the surfactants Breakthru and Triton X-100 as well is the solubilizing agents DMSO and Solutol. Palmitoleic acid was applied at a concentration of 10 mM to plants both as a foliar spray and 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 2). 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 results using penetrating surfactants are very promising and will be a focus of continuing work.

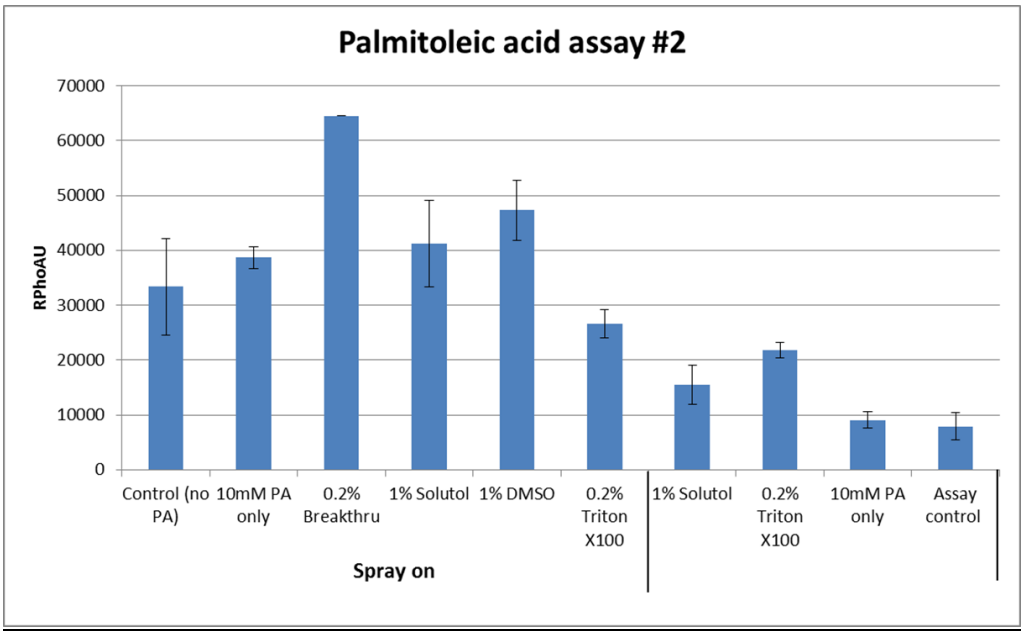

Figure 2. Alkaline phosphatase activity exhibited by 10 µl aliquots of xylem sap extracted under pressure from individual leaves of grape plants treated with 10 mM palmitoleic acid with the various surfactants noted when applied as a foliar spray (left) or a stem injection (right).

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 the irrigation system in the greenhouse caused the plants to severely damage due to desiccation. These experiments will therefore be repeated.

In addition to directly assessing DSF levels within plants as above, the adhesiveness of *X. fastidiosa* cells inoculated into treated plants are also being determined using the cell release assay described above. Since the virulence of various *X. fastidiosa* mutants is inversely related to their release efficiency, and cells are released at a much lower rate from transgenic RpfF-expressing grape that produce DSF that are resistant to disease we expect that treatments with exogenous DSF that reduce the release efficiency of *X. fastidiosa* cells when measured 2 weeks or more after inoculation will also be most resistant to disease. This assay is far quicker than assays in which disease symptoms must be scored after several months of incubation, and will be employed during those times of the year such as the fall and winter when disease symptoms are difficult to produce in the greenhouse.

In addition to the use of purified fatty acids we also are evaluating mixtures of fatty acids for their ability to alter the behavior of *X. fastidiosa*. Macadamia nut oil contains a very high concentration of palmitoleic acid (23%). We have saponified macadamia nut oil by treatment with sodium hydroxide to yield the sodium salts of the constituent fatty acids. In promising preliminary results, we find that this fatty acid mixture has DSF signaling activity. Alkaline phosphatase activity exhibited by the *X. fastidiosa Xf:phoA* biosensor increased with increasing concentrations of the mixture of fatty acids in the soap repaired from the saponified macadamia nut oil (Figure 3). Apparently the other saturated fatty acids that would be found in the lipids of macadamia oil do not strongly interfere with DSF signaling of the palmitoleic acid in this soap. This saponified plant oil is thus very attractive as inexpensive sources of DSF homologs that could be directly applied to grape. We will focus continuing studies on the assessment of saponified plant oils as foliar or soil applied treatments to manage *X. fastidiosa.*

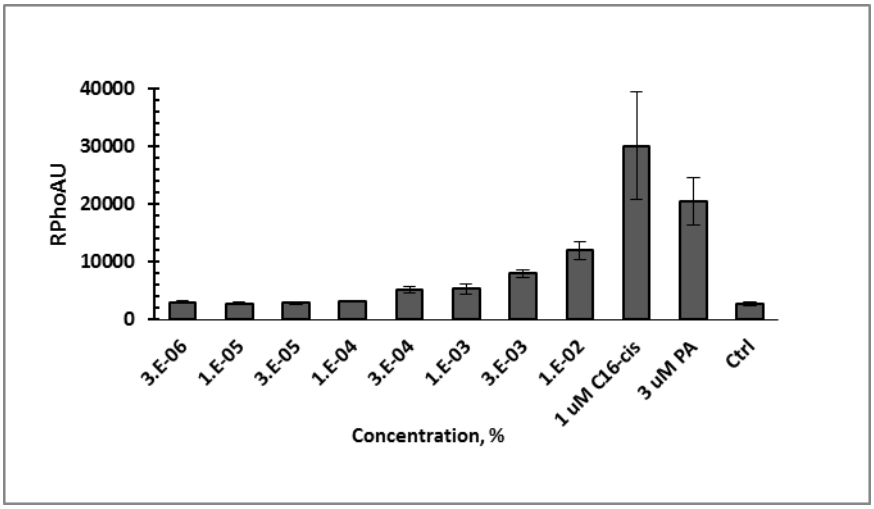

Figure 3. Alkaline phosphatase activity exhibited by the *X. fastidiosa Xf:phoA* biosensor exposed to increasing concentrations of saponified macadamia nut oil as well as 1 uM *Xf*DSF2, 3 uM Palmitoleic acid, or a negative control with no added DSF.

Studies were also conducted to determine the process by which *X. fastidiosa* perceives DSF so as to better understand how to supply DSF molecules to the plants to alter the behavior of this pathogen. The water solubility of most DSF molecules, especially those having relatively long acyl chain lengths such as C16-cis are expected to have relatively low water solubility. It is clear that DSF can be acquired by *X. fastidiosa* from water solutions since changes in gene expression are observed when DSF is applied to cultures. However, because of their low water solubility cells of *X. fastidiosa* may also acquire such extracellular via mechanisms that are not dependent on the solubility of these signal molecules in water. To test this, the responsiveness of the *X. fastidiosa Xf:phoA* biosensor to DSF recovered at various times from cultures of *X. fastidiosa* in different ways was assessed. In preliminary experiments, substantial signaling activity was recovered from entire broth cultures (containing both cells and cell free supernatants) when ethyl acetate extracts were exposed to the *X. fastidiosa Xf:phoA* biosensor (Fig. 4). Much less signaling activity was recovered from ethyl acetate extracts of cell free culture supernatants of these same cultures (Fig. 4). Interestingly, substantial signaling activity was observed when the *X. fastidiosa Xf:phoA* biosensor was exposed to culture supernatants themselves (prior to extraction with ethyl acetate) (Fig. 4). These results suggest that at least a portion, and perhaps a large portion, of the DSF and cultures of *X. fastidiosa* are associated with particulate material, probably either cells themselves or in outer

membrane vesicles produced by *X. fastidiosa*. Furthermore, these results also suggest that *X. fastdiosa* efficiently perceives DSF supplied by particulate material. The most parsimonious explanation for these preliminary results is that DSF occurs both in a water soluble form and also associated with hydrophobic particles such as membrane vesicles, and that membrane vesicles might serve as a conduit by which DSF transits between cells of *X. fastidiosa*. While further studies are underway to better understand the apparent role of particulate material in the cell-cell signaling of X. fastidiosa, these results suggest that delivery of DSF to plants in a manner that would maximize its ability to alter the behavior of *X. fastidiosa* could be facilitated by providing lipophilic carriers. The observation that the maximal detection of DSF when delivered to plants using detergents such as Breakthru and Triton X-100 might be partially explained by the fact that such detergents could form micels capable of transiting DSF into *X. fastidiosa* cells. This important delivery will guide our continued studies to formulate DSF in a way that it can both enter plants and be readily acquired by *X. fastidiosa*. We will explore various detergents and oil emulsions for their ability to maximize perception of DSF by cells of *X. fastidiosa*.

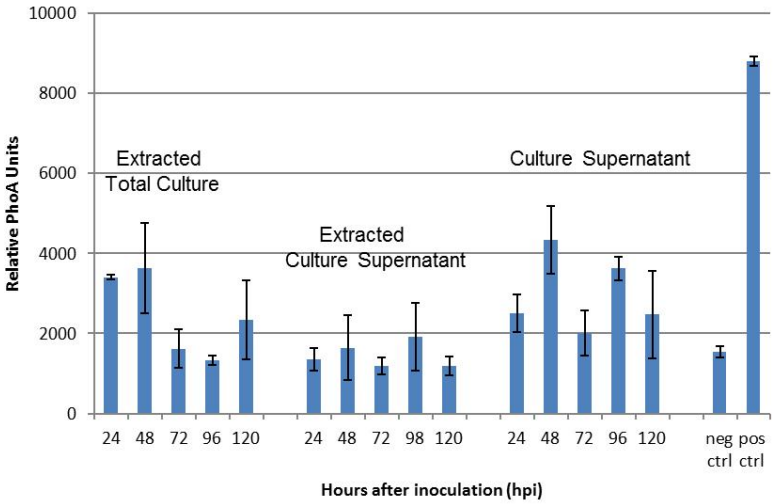

Figure 4. Alkaline phosphatase activity exhibited by the *X. fastidiosa Xf:phoA* biosensor exposed to ethyl acetate extracts of total broth cultures of *X. fastidiosa* harvested at various times (left), of ethyl acetate extracts of cell free culture supernatants from cultures harvested at various times (middle), and of cell free culture supernatants from cultures harvested at various times (but not extracted with ethyl acetate) (right) as well as assays with no added DSF (neg. control) and assay with 3 uM XfDSF2 (Pos. control).

Extensive studies are under way in which various concentrations of Palmitoleic acid as well as different concentrations of sodium salts of saponified macadamia nut oil are being applied to the foliage of Cabernet Sauvignon grape with various concentrations of the penetrating surfactant Breakthru to alter the behavior of *X. fastidiosa*. Individual leaves are being assayed weekly on treated vines and the xylem sap expressed under pressure, and the small volume of xylem sap being assayed for DSF activity using the *X. fastidiosa Xf:phoA* biosensor. In addition, some of these plants were also inoculated with *X. fastidiosa* both before and after application of the fatty acids, and disease severity is being measured weekly. As these studies were initiated in May, disease symptoms are just now starting to appear, and so a full report on the efficacy of these fatty acids in conferring resistance to Pierce's disease will be reported in the next progress report.

Objective 3: Biological control with *Burkholderia phytofirmans* PsJN.
While the biological control of Pierces disease with endophytic bacteria that would grow within grape and produce DSF has been an attractive strategy, until recently we have been unable to find bacteria capable of exploiting the interior of grape. All of hundreds of strains isolated from within grape by our group as well as that of Dr. Kirkpatrick exhibited no ability to grow and move beyond the point of inoculation when re-inoculated. We have recently, however, found that *Burkholderia phytofirmans* stain PsJN which had been suggested to be an endophyte of grape seedlings multiplied and moved extensively in mature grape plants (Figure 5). It's population size and spatial distribution in grape within six weeks of inoculation was similar to that of *X. fastidiosa* itself, suggesting that it is an excellent grape colonist. Furthermore, DSF production has been demonstrated in certain other *Burkholderia* species and the genome sequence of *B. phytofirmans* revealed that it has a homologue of *Xf rpfF*. While we have no evidence for its production of a DSF species to which *X.*

fastdiosa could respond, the promiscuous nature of RpfF in *Xf* and other species suggested that it might make DSF species to which *X. fastidiosa* would respond under some circumstances, such as one growing within plants. Preliminary results suggest that co-inoculation of *X. fastidiosa* and *B. phytofirmans* resulted in greatly reduced disease symptoms compared to plants inoculated with *X. fastdiosa* alone; whereas the number of infected leaders of plants inoculated with X. fastidiosa alone increased rapidly after week 12, very little disease was observed in plants inoculated with X.fastidiosa and *B. phytofirmans* (Figure 5).

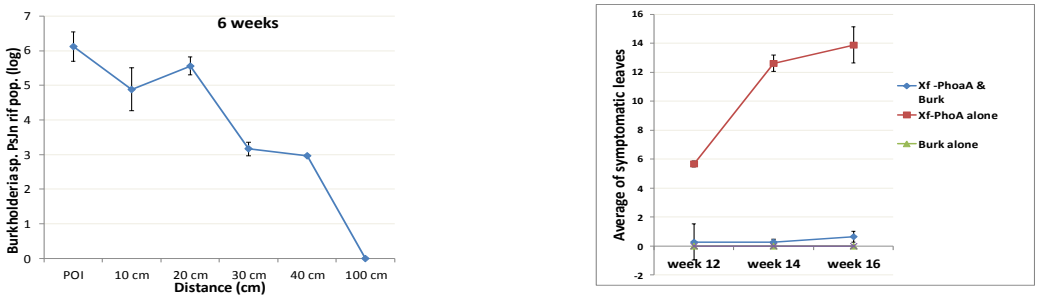

Figure 5. (Left). Population size of *B. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after 6 weeks incubation. (Right). Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *X. fastidiosa* alone (red) or when co-inoculated with *B. phytofirmans* (blue).

Given the promising results of the reduction of severity of Pierces disease in grape treated with *B. phytofirmans* we performed additional experiments in which *X. fastidiosa* was co-inoculated with *B. phytofirmans* as well as when *B. phytofirmans* both preceded or followed inoculation of plants with *X. fastidiosa* by 30 days. As observed before, the severity of Pierces disease of plants co-inoculated with *B. phytofirmans* and *X. fastidiosa* average less than one leaf per vine compared to over 9 leaves per vine on plants inoculated with the pathogen alone (Fig. 6). Importantly, the severity of Pierces disease was also substantially less on plants in which inoculation with *B. phytofirmans* followed inoculation with the pathogen by 30 days then on control plants inoculated only with the pathogen (3.5 leaves per vine compared to nine leaves per vine respectively) (Fig. 6). Almost no disease was observed on plants inoculated with *B. phytofirmans* 30 days prior to inoculation with the pathogen (Fig. 6), although one must recognize that these plants did not have as long a time period in which disease could develop since they were inoculated with the pathogen 30 days later then control plants inoculated only with the pathogen; we are continuing to assess the development of disease in these plants. These results are quite exciting and confirmed that *B. phytofirmans* can confer high levels of disease resistance in grape - both when co-inoculated with the pathogen and also when inoculated into plants already infected with *X. fastidiosa*. It might be anticipated that pre-inoculation of plants with *B. phytofirmans* will yield the largest degree of disease resistance. The initial studies obtained here confirm such an expectation although repeated experiments underway are designed to confirm that disease will not eventually occur in plants pretreated with *B. phytofirmans*.

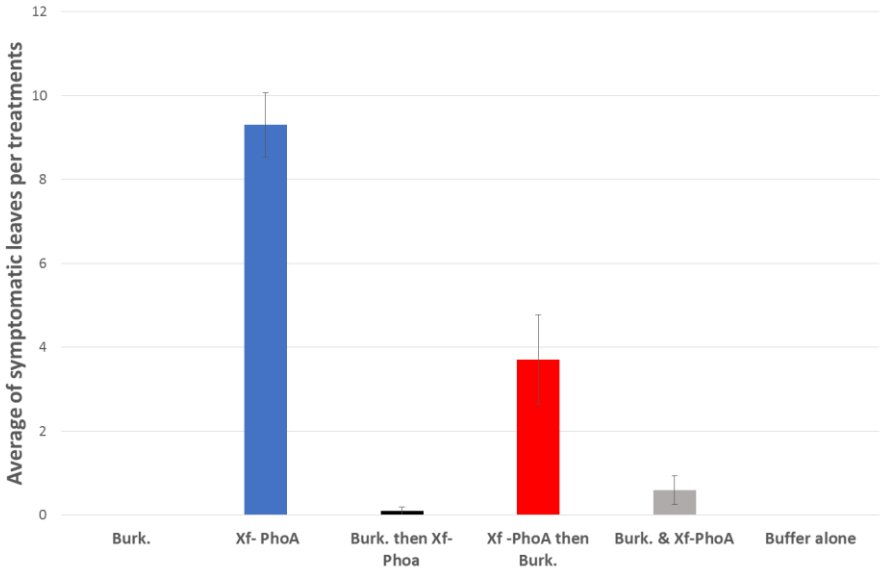

Figure 6. Severity of Pierces disease symptoms (number of symptomatic leaves/vine) on Cabernet Sauvignon plants inoculated only with *B. phytofirmans*, only with *X. fastidiosa* (Blue bar), or co-inoculated with *X. fastidiosa* and *B. phytofirmans* (Grey bar). Also shown is disease severity on plants inoculated with *B. phytofirmans* 30 days before inoculation with *X. fastidiosa* (Black bar) as well as on plants inoculated with *X. fastidiosa* 30 days after inoculation with *B. phytofirmans* (Red bar). The vertical bars represent the standard error of the determination mean disease severity.

While the mechanism by which *B. phytofirmans* reduces the severity of Pierces disease remains somewhat unclear, the biological control activity conferred by this bacterium is associated with its ability to reduce the population size of *X. fastidiosa* in inoculated plants. The population size of *X. fastidiosa* at various locations along the stem from the point of inoculation in plants inoculated only with the pathogen or inoculated both with the pathogen and *B. phytofirmans* in the experiment described in Figure 6 were assessed. Relatively high population sizes of *X. fastidiosa* were recovered from stem segments collected from 30 to 300 cm away from the point of inoculation in plants inoculated only with the pathogen (Fig. 7). As expected, the highest population sizes were seen within the first 120 cm, but population sizes greater than 100 cells per gram were observed as much as 200 cm away from the point of inoculation. In contrast, the population size of *X. fastidiosa* was much lower at a given distance away from the point of inoculation in plants co-inoculated with *X. fastidiosa* and *B. phytofirmans* (Fig. 8). Whereas population sizes of the pathogen were usually in excess of 10^4 cells per gram in stem segments within 120 cm of the point of inoculation in plants inoculated with the pathogen alone, the pathogen population sizes were much lower, decreasing from a high of $10^{2.5}$ to less than 10 cells per gram in plants co-inoculated with *B. phytofirmans* (Fig. 8). Consistent with the somewhat lower ability to produce severity of Pierces disease, the reduction in population sizes of *X. fastidiosa* conferred by inoculation of plants with *B. phytofirmans* 30 days after that of the pathogen were somewhat less than that conferred by co-inoculation (Fig. 9). While population sizes of *X. fastidiosa* were generally in excess of 10^4 cells per gram in stem segments within 120 cm of the point of inoculation in plants inoculated only with the pathogen, population sizes were generally less than about 100 cells/g in plants inoculated with *B. phytofirmans* 30 days after inoculation with the pathogen (Fig. 9). When considered overall treatments, there was a clear relationship between the population size of X. fastidiosa in the stems of plants treated with *B. phytofirmans* or not and the severity of Pierces disease (Fig. 10). Surprisingly, when assessed 12 weeks after inoculation, the population sizes of *B. phytofirmans* in inoculated plants, irrespective of whether *X. fastidiosa* was also inoculated into the grape plants, were quite low – but disease severity was lowest in those plants in which B. phytofirmans populaiotns were the highest (Fig. 11). Given that previous experiments at all shown that *B. phytofirmans* had rapidly colonized grape and had achieved relatively large population sizes and considerable distances away from the point of inoculation within six weeks (Fig. 5), it appears that viable cells did not persist in plants for as much as 12 weeks. These results suggest that the interactions of *B. phytofirmans* with either the plant or *X. fastidiosa* occur early in the infection process. The fact that the effect of inoculation of plants with *B. phytofirmans* reduce population sizes of *X. fastidiosa* most at sites distal to the point of inoculation suggest that it had reduced the motility of the pathogen. Such an effect would be expected if it stimulated DSF-mediated quorum sensing. That

is, the behavior of *X. fastidiosa* in plants treated with *B. phytofirmans* was similar to that seen in transgenic plants harboring *X. fastidiosa rpfF* that produce DSF.

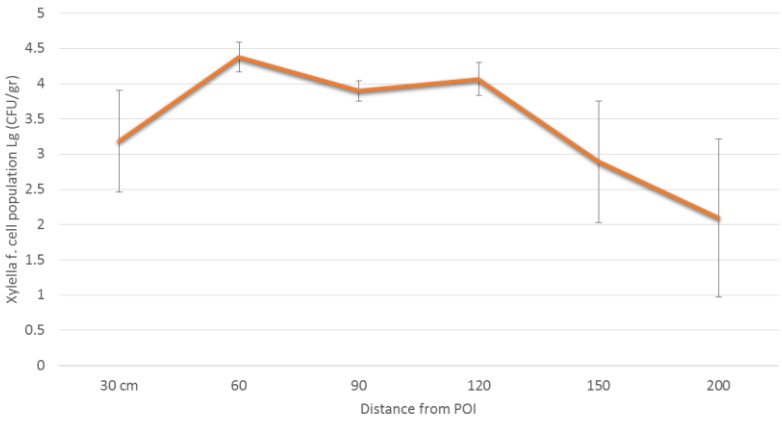

Figure 7. Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation of the pathogen alone when measured 12 weeks after inoculation. The vertical bars represent the standard error of the mean population size/g.

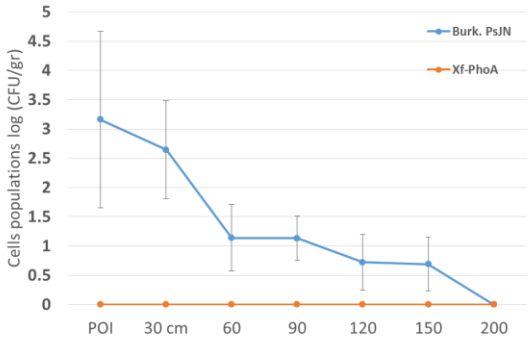

Figure 8. Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation of the pathogen when co-inoculated with *B. phytofirmans* (blue) or populations of *B. phytofirmans* (orange). The vertical bars represent the standard error of the mean population size/g.

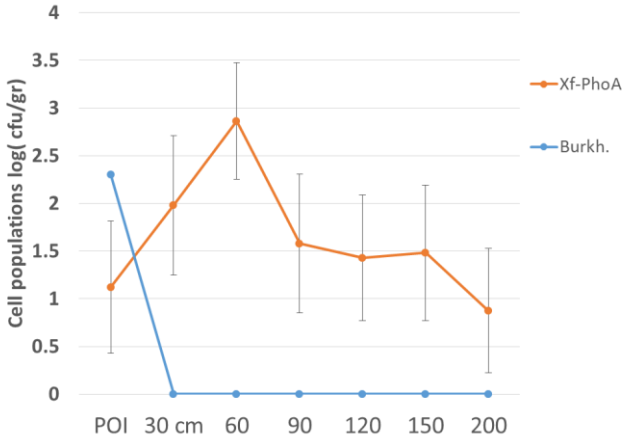

Figure 9. Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation of the pathogen when inoculated with *B. phytofirmans* 30 days after that of the pathogen (orange) or populations of *B. phytofirmans* (blue). The vertical bars represent the standard error of the mean population size/g.

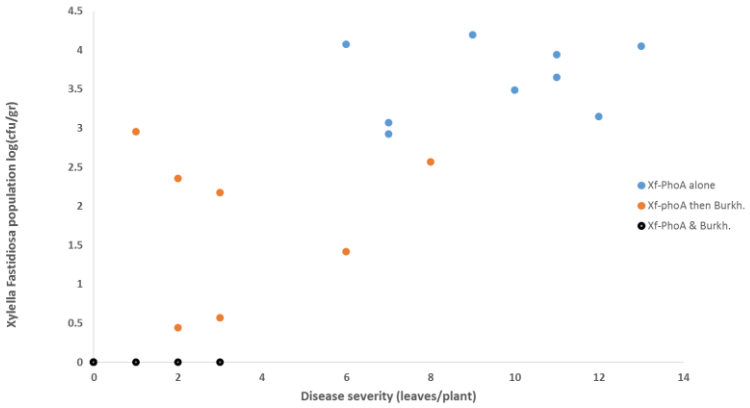

Figure 10. Relationship between mean population size of *X. fastidiosa* recovered at 6 from plants 12 weeks after inoculation of plants with only the pathogen (blue symbols), inoculated with *B. phytofirmans* 30 days after that the pathogen (orange symbols), or co-inoculated with *X. fastidiosa* and *B. phytofirmans* (black symbols). Each symbol represents mean population sizes achieved in a given plant.

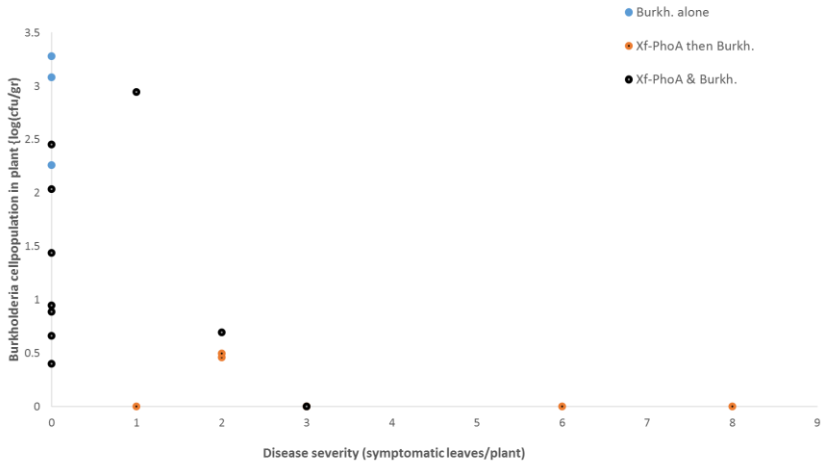

Figure 11. Relationship between mean population size of *B. phytofirmans* recovered from plants 12 weeks after inoculation of plants with *B. phytofirmans* only (blue symbols), inoculated with *B. phytofirmans* 30 days after that the pathogen (orange symbols), or co-inoculated with *X. fastidiosa* and *B. phytofirmans* (black symbols). Each symbol represents mean population sizes achieved in a given plant.

While the droplet puncture method used in Figure 5 to introduce *B. phytofirmans* is an effective way to introduce bacteria into the xylem we have investigated the potential to introduce *B. phytofirmans* into the vascular tissue by topical application to leaves using 0.05% Silwet L77, an organo-silicon surfactant with sufficiently low surface tension that spontaneous invasion of plant tissues can be achieved. The population size of *B. phytofirmans* in the petioles of leaves distal from the leaf on which cell suspensions in L77 (10^8 cells/) have been applied were used as a measure of growth and movement potential from such an inoculation site. Substantial numbers of cells of *B. phytofirmans* could be recovered from petioles within one or two weeks after topical application to leaves in the presence of Silwet L77 or Breakthru (Figure 12). Very few cells were present with and petioles when the bacterium was applied without a penetrating surfactants. Topical application of such an endophyte thus appears to be a very practical means of inoculating plants in the field.

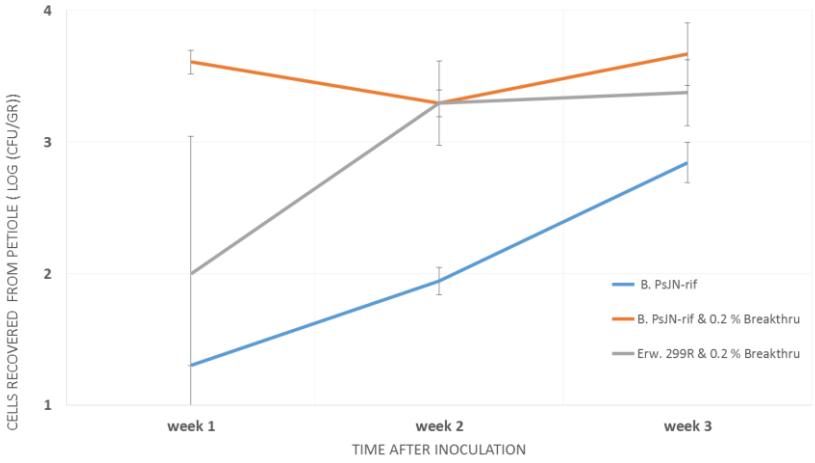

Figure 12. Population size of *Burkholderia phytofirmans* in petioles of Cabernet Sauvignon of plants sprayed with this strain alone (blue line) or this strain applied with 0.2% Breakthru (gray line) or of *Erwinia herbicola* strain 299R applied with 0.2% Breakthru (orange line). Vertical bars represent the mean of log population size at a given sampling time.

The ability of *B. phytofirmans* to confer biological control of Pierce's disease when co-inoculated with *X fastidiosa* and when applied at various times prior to that of the pathogen will be assessed by measuring both *X. fastidiosa* population sizes in petioles distal to the point of pathogen inoculation as well as of disease symptoms at weekly intervals as above. Evidence for any DSF production by *B. phytofirmans* in grape plants are being obtained by assaying xylem fluid collected from plants colonized by the strain using the PhoA-based *X. fastidiosa* DSF biosensor as above.

Considerable effort has been made during this reporting period to better understand the mechanisms by which *B. phytofirmans* alters the behavior of *X. fastidiosa* in plants. DSF production has been described in other *Burkholderia* species including *Burkholderia ceonocepacia*. Furthermore, the genome sequence of *B. phytofirmans* PSJN has been determined, allowing us to putatively identify a gene with some homology to *X. fastidiosa* and *Xanthomonas campestris rpfF*, that thus might be expected to lead to the production of fatty acids capable of conferring signaling activity like that of DSF species. We therefore made a site-directed deletion mutant of the putative *rpfF* gene in *B. phytofirmans*. We subsequently investigated whether ethyl acetate extracts of wild type *B. phytofirmans* culture supernatants or *rpfF* mutants of *B. phytofirmans* could alter the expression of genes in either *Xanthomonas campestris* or *X. fastidiosa* that were known to be regulated by the presence of various DSF species. Interestingly, relatively strong induction of the *eng:gfp* reporter gene fusion in *Xanthomonas campestris* was observed when the biosensor was exposed to extracts of both the wild type and *rpfF* mutant of *B. phytofirmans* (Figures 13 and 14). These results suggest that indeed *B. phytofirmans*

was capable of producing a DSF-like molecule that *Xanthomonas campestris* could respond to. It also suggested however that the putative *rpfF* gene that we had removed was not responsible for producing the putative signal molecule. In contrast to the results that revealed that *Xanthomonas campestris* could respond to way putative signal molecule from *B. phytofirmans*, little or no change in expression of the *phoA* reporter gene was observed when the *X. fastidiosa Xf:phoA* biosensor was exposed to ethyl acetate extracts of either the wild type or *rpfF* mutant of *B. phytofirmans* (data not shown). Given that *X. fastidiosa* and *Xanthomonas campestris* respond to different DSF species, it was not unexpected that they might differentially respond to the signal molecule apparently made by *B. phytofirmans.*

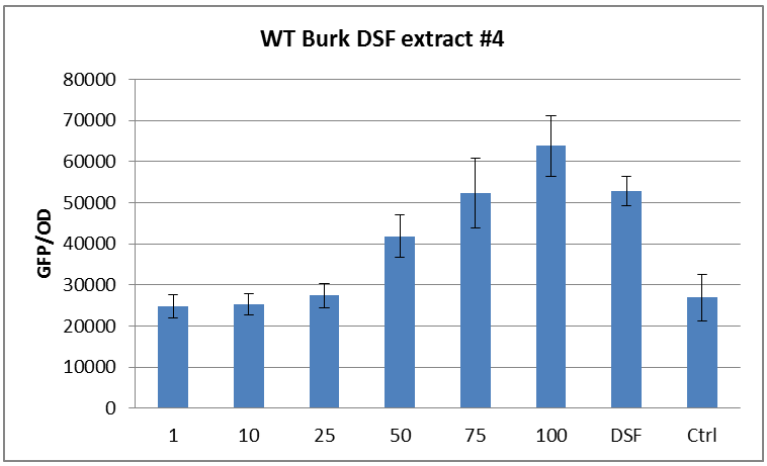

Figure 13. Normalized GFP fluorescense exhibited by the *Xanthomonas campestris* pv. campestris DSF biosensor strain harboring an *eng:gfp* reporter gene when exposed to different concentrations of ethyl acetate extracts (100 ml of supernatant extracted into 1 ml of solvent) from a wild type *B. phytofirmans* strain. Shown on the abscissa are different ul aliquots of the extract added to a 1 mL culture of the biosensor as well as a culture of the biosensor exposed to 1 uM DSF, or to no added material (ctrl).

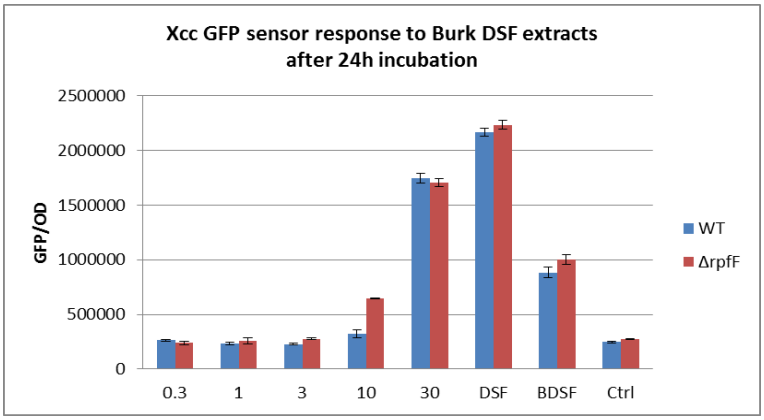

Figure 14. Normalized GFP fluorescense exhibited by the *Xanthomonas campestris* pv. campestris DSF biosensor strain harboring an *eng:gfp* reporter gene when exposed to different concentrations of ethyl acetate extracts (100 ml of supernatant extracted into 1 ml of solvent) from a wild type *B. phytofirmans* (blue bars) or an *rpfF* mutant (red bars). Shown on the abscissa are different ul aliquots of the extract added to a 1 mL culture of the biosensor as well as a culture of the biosensor exposed to 1 uM DSF, 1 uM BDSF,or to no added material (ctrl).

While we did not detect a change in apparent expression of the *hxfA* promoter linked to the *phoA* reporter gene in the *X. fastidiosa Xf:phoA* biosensor when it was exposed to either ethyl acetate extracts of culture supernatants of *B. phytofirmans* or small amounts of culture supernatant themselves, we observed that the biofilm formation (apparent adhesiveness) of *X. fastidiosa* was dramatically higher when either ethyl acetate extracts of culture supernatant or culture supernatant itself from *B. phytofirmans* was added to cultures of either wild type or rpfF* mutants of *X. fastidiosa* (Figure 15). Not only was the amount of bacterial biomass that accumulated in the "ring" which formed at the media/air interface and shake cultures greater, but more

importantly, substantial numbers of cells of *X. fastidiosa* adhered to the walls of class culture flasks below the ring - in the area exposed to turbulent mixing of the culture during shaking (Figure 15). These results suggested that the adhesiveness of *X. fastidiosa* was dramatically higher in the presence of some component of the culture supernatant of *B. phytofirmans.* Furthermore, the fact that biofilm formation was by extracts of both the wild type and putative *rpfF* mutant of *B. phytofirmans*, suggested that the putative *rpfF* gene of *B. phytofirmans* was not involved in production of the signal molecule that induced biofilm formation. The enhanced biofilm formation conferred by culture supernatants of *B. phytofirmans* was readily quantified by determining the biomass of *X. fastidiosa* cells by crystal violet staining (Figure 16). The biomass of *X. fastidiosa* cells in biofilms was over two-fold higher in the presence of culture supernatant of either a wild type or rpfF mutant of *B. phytofirmans* (Figure 16).

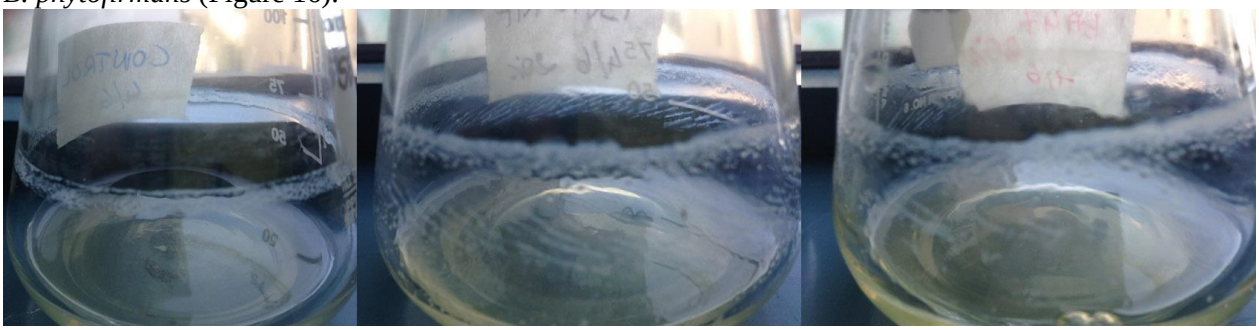

Figure 15. Biofilm formation of wild type *X. fastidiosa* grown in PD three media alone (left), or in media containing 20%v/v of culture supernatant of wild type *B. phytofirmans* (center) or a putative *rpfF* mutant of *B. phytofirmans* (right).

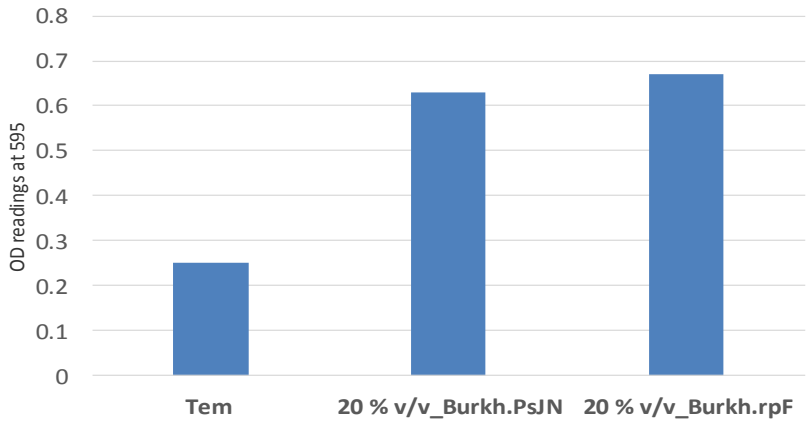

Figure 16. Optical density measured at 595 nm of ethanol extracts of biofilms of *X. fastidiosa* recovered from shake cultures as in Figure 15 after staining with crystal violet.

Interestingly, a large increase in biofilm formation could be conferred by relatively small amounts of extracts of either wild type or the *rpfF* mutant of *B. phytofirmans*, while higher concentrations appeared to lead to some inhibition of *X. fastidiosa* growth, and hence biofilm formation (Figure 17). These results are quite exciting in that it suggests strongly that *B. phytofirmans* produces a signal molecule to which *X. fastidiosa* responds, leading to its increased adhesiveness. It is unclear whether the signal molecule is a fatty acid related to DSF. It is quite possible that *X. fastidiosa* can perceive the putative signal molecule of *B. phytofirmans* using receptors different from those used to detect DSF itself, and that detection of the putative signal molecule of *B. phytofirmans* might lead to expression of somewhat different genes that of DSF itself. Work to determine the identity of the signal molecule is underway. The ability of this putative signal molecule to increase the apparent adhesiveness of *X.fastidiosa* is sufficient to explain the relatively dramatic biological control conferred by co-inoculation or pre-inoculation plants with *B. phytofirmans.* As with DSF itself, increasing the adhesiveness of *X. fastidiosa* would restrict its ability to move within the plant. Given that the putative signal molecule made by *B. phytofirmans* is both a small molecule and active at quite low concentrations, it suggests that it might be readily diffusible throughout the plant, again explaining why biological control conferred by *B. phytofirmans* appears to be so robust.

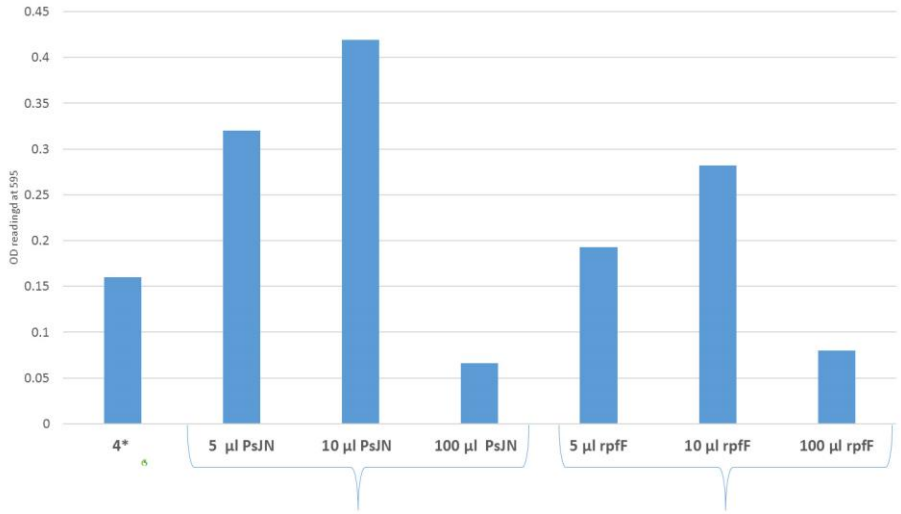

Figure 17. Optical density measured at 595 nm of ethanol extracts of biofilms of an rpfF* mutant of *X. fastidiosa* grown with either no (4*) five, 10, or 100 µL of an ethyl acetate extract ((100 ml of supernatant extracted into 1 ml of solvent) of a wild type *B. phytofirmans* strain (left) or putative rpfF mutant of *B. phytofirmans* (right) added to cultures when cells were recovered from shake cultures as in Figure 15 after staining with crystal violet.

PUBLICATIONS AND PRESENTATIONS

Baccari, C., Killiny, N., Ionescu, M., Almeida, R.P.P., and Lindow, S.E. 2014. DSF repressed extracellular traits enable attachment of *Xylella fastidiosa* to insect vectors and transmission. Phytopathology 104:27-33.

Caserta, R., Picchi, S.C., Takita, M.A., Tomaz, J.P., Pereira, W.E.L., Machado, M.A., Ionescu, M., Lindow, S., and de Souza, A.A. 2014. Expression of *Xylella fastidiosa* RpfF in citrus disrupts signaling in *Xanthomonas citri* subsp. *citri* and thereby its virulence. Molec. Plant-Microbe Interact. 27:1241-1252.

Retchless, A.C., Labroussaa, F., Shapiro, L., Stenger, D.C., Lindow, S.E. and Almeida, R.P.P. 2015. Genomic insights into *Xylella fastidiosa* interactions with plant and insect hosts. In: Genomics of plant-associated bacteria. Ed. Gross, D., Lichens-Park, A. and Kole, C. Springer (in press).

Roper, C and Lindow S.E. 2015. Xylella fastidiosa: Insights into the lifestyle of a xylem-limited bacterium. Pp. XX in (N. Wang, J. Jones, G. Sundin, F. Whie, S. Hogenhout, C. Roper, L. De La Fuente, and J.H. Hams, eds.) Virulence mechanisms of plant pathogenic bacteria. APS Press. St. Paul, MN. (in press)

Ionescu, M., Zaini, P.A., Baccari, C., Tran, S., da Silva, A.M., and Lindow, S.E. 2014. *Xylella fastidiosa* outer membrane vesicles modulate plant colonization by blocking attachment to surfaces. Proc. Natl. Acad. Sci. (USA). 111:E3910-E3918.

Lindow, S.E., Newman, K., Chatterjee, S., Baccari, C., Lavarone, A.T., and Ionescu, M. 2014. Production of *Xylella fastidiosa* diffusible signal factor in transgenic grape causes pathogen confusion and reduction in severity of Pierce's disease. Molec. Plant-Microbe Interact. 27:244-254.

Presentation at the 13th International Conference on Plant Pathogenic Bacteria, Shanghai, China, entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion".

Presentation at the International Symposium on the European Outbreak of *Xylella fastidiosa* in Olive. Gallipoli, Italy, October, 2014.

RESEARCH RELEVANCE STATEMENT

Since we have shown that DSF accumulation within plants is a major signal used by *X. fastidiosa* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this pathogen we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion". Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, and transgenic DSF-producing plants appear particularly promising and studies indicate that such plants provide at least partial protection when serving as a rootstock instead of a scion. While the principle of disease control by altering DSF levels has been demonstrated, this work addresses the feasibility of how achieve this goal, and what are the most practical means to achieve disease control by pathogen confusion. By testing the production of DSF in a variety of different grape varieties in plants transformed with the *rpfF* gene of *X. fastidiosa* we hopefully will be able to demonstrate that this will be a general method of disease control that could be applied to any great cultivar. The tools we have developed to better detect the specific DSF molecules made by *X. fastidiosa* in our previous project have been very useful in our on-going research to test the most efficacious and practical means to alter DSF levels in plants to achieve disease control. We are still optimistic that topically-applied fatty acids that serve as DSF signaling molecules might also ultimately be the most useful strategy for controlling disease. The studies underway to test topically applied palmitoleic acid to plants have already provided encouraging results that hopefully will be verified and continued studies, providing optimism for a spray-on method to achieve pathogen confusion. B. phytofirmans also continues to provide levels of biological control even greater than what we would have anticipated, and the encouraging results of practical means to introduce this strain into plants as well as the fact that it seems to be active even when not co-inoculated with the pathogen is a very promising result that suggests that this method of disease control might also be readily implemented .

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. We are introducing the gene conferring DSF production and to a variety of different grape cultivars to determine if they also will exhibit high levels of disease resistant as did the Freedom cultivar previously constructed. Topical application of commercially available unsaturated fatty acids capable of altering gene expression in *X. fastidiosa* with penetrating surfactants can introduce sufficient amounts of these materials to reduce the virulence of the pathogen. A naturally occurring *Burkholderia* strain reduces the movement of *X. fastidiosa* and thereby its virulence in plants when after or simultaneously with *X. fastidiosa*. This biological control agent produces a small molecule which induces adhesiveness in *X. fastidiosa*; it's impaired movement within the plant appears to account for the reduced symptoms of Pierce's disease in plants inoculated with both *Burkholderia* and *X. fastidiosa*. By comparing disease control by these 3 methods the most efficacious and practical means of control can be identified.

STATUS OF FUNDS

The project as proposed is proceeding on schedule. The funds remaining are sufficient to complete the project as proposed.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY

A patent application (12/422,825) entitled "biological control of pathogenicity of microbes that use alpha, beta unsaturated fatty acid signal molecules" had been submitted March 13, 2009. While many of the claims had been rejected earlier, the University of California patent office has filed on March 13, 2012 a motion requesting reconsideration of the application with clarification of, and justification for, claims related to the production of transgenic plants transformed with the *rpfF* gene from *Xylella fastidiosa*. This petition was approved in June, 2012 and patent US 8,247,648 B2 was issued on August 21, 2012.