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Enhancing control of Pierce's Disease by augmenting pathogen signal molecules

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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". Plants expressing *rpfF* from *X. fastidiosa* produce low levels of DSF and are highly resistant to Pierce's disease. Chloroplast targeting of RpfF apparently substantially increases DSF production. *X. fastidiosa* moved much less rapidly in *rpfF*-transformed grape, colonized many fewer xylem vessels, and achieved a much lower population size indicating that elevated DSF levels suppressed movement within the plant. 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. Preliminary evidence suggests that DSF is comprised of three closely related fatty acid molecules. One component is 2-Z-tetradecenoic acid (hereafter called C14-cis) while a second compound termed C12-cis is apparently also produced. The chemical identity of a third component is as yet undetermined and is being investigated. We are currently determining the relative activity of these forms of DSF and if such molecules cooperate in regulating gene expression in *X. fastidious.* The various forms of DSF may preferentially affect different behaviors of *X. fastidiosa*. Since some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggests that DSF produced by rootstocks can somewhat move to scions and confer disease control the control of disease, grafted plants are being made that have a relatively large rootstock to test the hypothesis that increased supply of DSF to the scion will be associated with a larger rootstock. Naturally-occurring endophytic bacteria within grape were assessed for DSF production; only about 1% of the endophytic bacteria in grape produce DSF and these were tested for their ability to move within plants after inoculation. Most were not found to move or grow within plants. A *Burkholderia* strain however did exhibit extensive growth and movement, and appears promising as a potential biological control agent. As studies of pathogen confusion will be greatly facilitated by having an improved bioassay for the DSF produced by *X. fastidiosa*, we have been developing several immunological and biochemical means to assay for the presence of DSF using *X. fastidiosa* itself as a bioindicator. Bioassays based on immunological detection of the cell surface adhesin XadA and EPS have been developed. Gene expression in *X. fastidiosa* exposed to various levels of DSF is also being directly assessed using *phoA* reporter gene fusions. *Xanthomons campestris*-based biosensors in which Rpf components have been replaced by those from *X. fastidiosa* also selectively detect the DSF produced by *X. fastidiosa*. An improved *X. Campestris–based biosensor in which a mutant allele of RpfF from X. fastidiosa* that does not confer production of DSF but which apparently still interact with RpfC has been produced; since this biosensor strain does not produce DSF, it has much lower background GFP reporter gene activity as well as a high responsiveness to exogenous DSF. The adhesiveness of wild type strains of *X. fastidiosa* grown in a minimal medium rapidly increases upon addition of DSF. The extent of increase in the adhesiveness of the strain, as measured by binding to the walls of glass tubes, increases with concentration of DSF added. The cell adhesive assay therefore makes a valuable means of detection for DSF. DSF was readily detected and transgenic grape expressing the X. fastidiosa rpfF gene when the *X. campestris*-based biosensor was applied to agar in which intact leaves were detected. The use of intact leaves therefore appears attractive as a method to screen transgenic plants for DSF production. Adherence of mutants of *X. fastidiosa* to grape vessels is predictive of their virulence, indicating that adhesiveness is a major factor affecting the ability of *X. fastidio*sa to cause disease. Such adhesive assays should enable us to more rapidly screen transgenic plants for their resistance to Pierce's disease in future studies as well the efficacy of chemical analogs of DSF to induce resistance. The adherence of WT strains of *X. fastidiosa* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *X. fastidiosa* was much higher than non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced it ability to move within the plant after inoculation.

Introduction:

We have found that the virulence of *X. fastidiosa* is strongly regulated in a cell density-dependent fashion by accumulation of a signal molecule called DSF encoded by *rpfF* and involving signal transduction that requires other *rpf* genes. We now have shown that the pathogen makes at least one DSF molecule that is recognized by *Xanthomonas campestris pv. campestris (Xcc*) but slightly different than the DSF of *Xcc* (Figure 1).

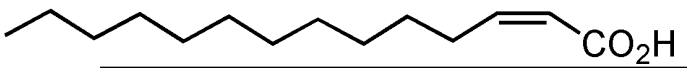


Figure 1 Structure of DSF: C14-cis

Our on-going work suggests that it also makes other, closely related signal molecules as well. In striking contrast to that of Xcc, *rpfF*- mutants of *X. fastdiosa* blocked in production of DSF, exhibit dramatically increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors since they do not form a biofilm within the insect. These observations of increased virulence of DFS-deficient mutants of *X. fastdiosa* are consistent with the role of this density-dependent signaling system as suppressing virulence of *Xf* at high cell densities. Our observations of colonization of grapevines by *gfp*-tagged pathogen are consistent with such a model. We found that *X. fastidiosa* normally colonizes grapevine xylem extensively (many vessels colonized but with only a few cells in each vessel), and only a minority of vessels are blocked by *X. fastidiosa.* Importantly, rpfF- mutants of *Xf* plug many more vessels than the wild-type strain. We thus believe that the pathogen has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply since xylem sap flow would cease and thus the DSF-mediated virulence system in *Xf* constrains virulence. That is, *Xf* would benefit from extensive movement throughout the plant where it would partially colonize xylem vessels but would have evolved not to grow to excessively within a vessel, thereby plugging it and hence blocking the flow of necessary nutrients in the xylem sap. Given that the DSF signal molecule greatly influences the behavior of *X.fastidiosa* we are investigating various ways by which this pathogen can be "confused" by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. We thus are further exploring how DSF-mediated signaling occurs in the bacterium as well as ways to alter DSF levels in the plant. Our work has shown that the targets of Rpf regulation are genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel as well as adhesins that modulate movement. Our earlier work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *X. fastidiosa.* In this period we have extensively investigated both the role of DFS-production by the pathogen on its behavior within plants, the patterns of gene regulation mediated by DSF, the frequency with which other endophytes can produce signal molecules perceived by *X. fastidiosa*, have further characterized the behavior of the pathogen in grape genetically transformed to produce DSF, and explored other means to alter DSF abundance in plants to achieve PD control. We have particularly emphasized the development of various methods by which DSF abundance in plants can be assessed so that we can make more rapid progress in testing various ways to modulate DSF levels in plants, and have also developed more rapid means by which the behavior of *X. fastidiosa* in plants can be assessed that does not require the multi-month Pierce's disease assay. Lastly, we have developed better methods to assess DSF-mediated changes in phenotypes in the pathogen itself.

Objectives:

1) Using novel, improved biosensors for the DSF produced by *Xf*, identify naturally-occurring endophytic bacteria which produce Xf DSF, and evaluate them for biological control of Pierce's disease after inoculation into plants in various ways.
2) Evaluate plants with enhanced production of DSF conferred by co-expressing RpfB, an ancillary protein to DSF biosynthesis, along with the DSF synthase RpfF for disease control as both scions and as rootstocks.
3) Optimize the ability of DSF-producing in rootstocks to confer resistance to Pierce's Disease in the scion.
4) Determine the movement and stability of synthetic DSF and chemical analogs of DSF applied to plants in various ways to improve disease control.

Results and Discussion:

Objective 1.

Disease control with endophytic bacteria**.** The severity of Pierce's disease can be reduced when DSF-producing bacteria such as rpfF-expressing *E. coli* and *E. herbicola* and certain *Xanthomonas* strains are co-inoculated with *X. fastidiosa* into grape. However, these bacteria do not spread well within the plant after inoculation. Presumably to achieve control of Pierce's disease by endophytic bacteria where *X. fastidiosa* might be inoculated at any point in the plant by insect vectors it will be important to utilize endophytic bacteria that can colonize much of the plant in order that DSF be present at all locations within the plant. Naturally-occurring endophytic bacteria that produced the DSF sensed by *X. fastidosa* and which might move extensively within the plant would presumably be particularly effective as such biological control agents. For that reason we initiated a study of naturally-occurring bacterial endophytes for their ability to produce DSF. This objective was possible since much effort devoted in the last two years has resulted in the development of better biosensors for the DSF produced by *X. fastidiosa* (C14-cis and related molecules) (discussed below). We have developed several highly sensitive assays for *X. fastidiosa* DSF. Our new biosensor, however has allowed us to screen large numbers of bacteria recovered from BGSS insect heads and grape plants for *f* DSF production. We are executing this part of the project to address objective 1 using two approaches; the first approach is building our own endophyte library using mainly endophytes isolated from insect head and wild grapes. The second approach involved screening an existing large grape endophyte library which was kindly made available to us by Dr. B. Kirkpatrick. Our results reveal that 0.9% of the endophytes recovered from either insect mouthparts or from grape xylem produce either a DSF detected by the *Xcc*-specific DSF biosensor, or the *Xf* DSF-specific biosensor or both. More than twice as many strain produce a DSF detected by the *Xcc*-specific DSF biosensor, suggesting that the DSF produced by *X. fastdiosa* is not as common as that produced by other bacteria such as environmental *Xanthomonas* strains etc. Many more strains remain to be tested as a relatively large library of DSF-producing bacteria has been assembled. Those strains found to produce the same DSF as *X. fastidiosa* are then being assessed for their ability to grow and move within grape plants as well as their ability to reduce symptoms of Pierces disease when co-inoculated and pre-inoculated into grape before *X. fastidiosa*.

Not only must endophytic bacteria produce DSF could be potentially affected biological control agents of pierces disease of grape, but they also must grow and move within grape plants after inoculation. Unfortunately, virtually all of the bacteria found within grape tissues exhibited little growth or movement when pre-inoculated into grape stems. For this reason, it was not perhaps too

surprising to find that they conferred relatively little reduction in the incidence or severity of pierces disease when coinoculated with *X. fastidiosa*, or when inoculated into plants in advance of the inoculation with the pathogen. For that reason, we emphasize the study of bacteria that could grow well within grape plants as a prelude to the development of successful biological control agents. We were therefore very pleased to find that *Burkholderia* strain PSJN which had been described as an endophyte of roots of grapes, was a quite successful colonist of grape. A rifampicin-resistant variants of the strain was selected, and grape stems were inoculated by the droplet puncture method similar to that used for inoculation with X.fastidiosa itself. Large population sizes of this *Burkholderia* strain was found not only at the point of inoculation, but at distances up to 60 cm from the point of inoculation within six weeks (Figure 2). These results are quite exciting in that this strain, which is not very want to grape, exhibits nearly as much growth and movement capabilities within grape as does *X. fastidiosa* itself. Furthermore, the genome sequence of this strain has been published and it reveals that it contains a gene with very high homology to that of rpfF from *X. fastidiosa*. Future work will evaluate whether the strain is capable of producing DSF species, and the particular unsaturated fatty acids that it might produce. Anticipating that this strain can produce a DSF species that can be perceived by *X. fastidiosa* plants have been coinoculated with this strain and the pathogen to evaluate the potential for biological control. We are highly optimistic that such a strain could be a useful biological control agent, and expect that future work will emphasize more detailed studies of this or related strains.

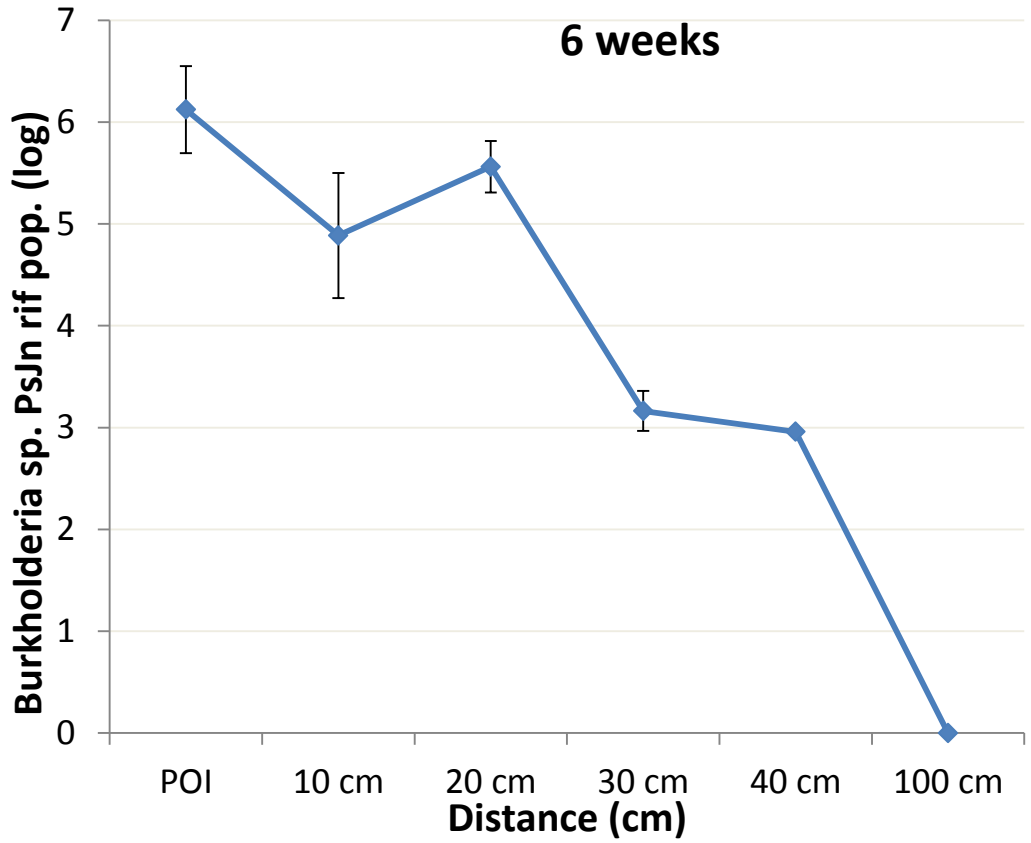


Figure 2. Population size of a rifampicin resistant variant of Burkholderia PSJN at various distances from the point of inoculation when measured six weeks after inoculation.

Objective 2.
Produce plants expressing both *rpfF* and *rpfB* from *X. fastidiosa*.

As a proof of principle that RpfB expression in addition to that of the DSF synthase RpfF could augment the production of appropriate unsaturated fatty acids detectable as DSF by *X. fastidiosa*, transgenic *Arabidopsis* plants were produced in which both enzymes were present. The abundance of DSF was determined indirectly by its effect on cell cell signaling in *Xanthomonas campestris*, a pathogen of *Arabidopsis*. Wild type *Arabidopsis*, as well as transgenic plants harboring either rpfF from X.fastidiosa or both *rpfF* and *rpfB* from *X. fastidiosa* were spray inoculated with Xanthomonas campestris, and the number of lesions which formed within two weeks were enumerated. Significantly more lesions formed on plants harboring both *rpfF* and *rpfB* than on plants harboring only *rpfF*. This result provided evidence that the presence of RpfB facilitated DSF production, since *Xanthomonas campestris* is increased by the presence of DSF (and not suppressed as it is in *X. fastidiosa*). The coexpression of both RpfF and RpfB in Arabidopsis was much easier to achieve than it would be in grape however. Most of the work conducted on this objective therefore was to create constructs which we could efficiently express genes encoding both enzymes simultaneously. Such constructs have now been produced and are available for transformation into grape.

Objective 3.

Optimizing disease control from transgenic rootstocks

To test whether DSF is mobile within the plant we performed grafting experiments in which DSF-producing Freedom grape transformed with the *rpfF* gene of *Xf* were used as rootstocks to which normal Cabernet Sauvignon grape were green-grafted as a scion. As a control, normal Freedom was also used as a rootstock. These plants were inoculated with *Xf* to test whether normal scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization as a rootstock than as a scion. Initial estimates of disease severity indicate that there were about 30% less symptomatic leaves of the normal Cabernet scion when grafted onto an *rpfF*-expressing rootstock compared with plants on a normal Freedom rootstock. Thus, like in the studies of the *rpf*F-expressing tobacco, it appears that DSF production in the scion is more efficacious for disease control than is the expression of *rpfF* in the rootstock. We also have obtained direct evidence that DSF is present in xylem fluid. Xylem fluid was recovered from transgenic rpfF-expressing freedom grape collected from the field trial established in Solano County. DSF was extracted from the xylem fluid and subjected to electrospray ionization mass spectrometry. Fragmentation pattterns with m/Z ratios characteristic of the known DSF species produced by *X. fastidiosa* itself were detected (Figure 3). This result shows clearly that DSF is mobile within the xylem fluid, hence explaining why transgenic DSF-producing rootstocks could confer some alteration in pathogen behavior in the scion, thereby leading to disease control.

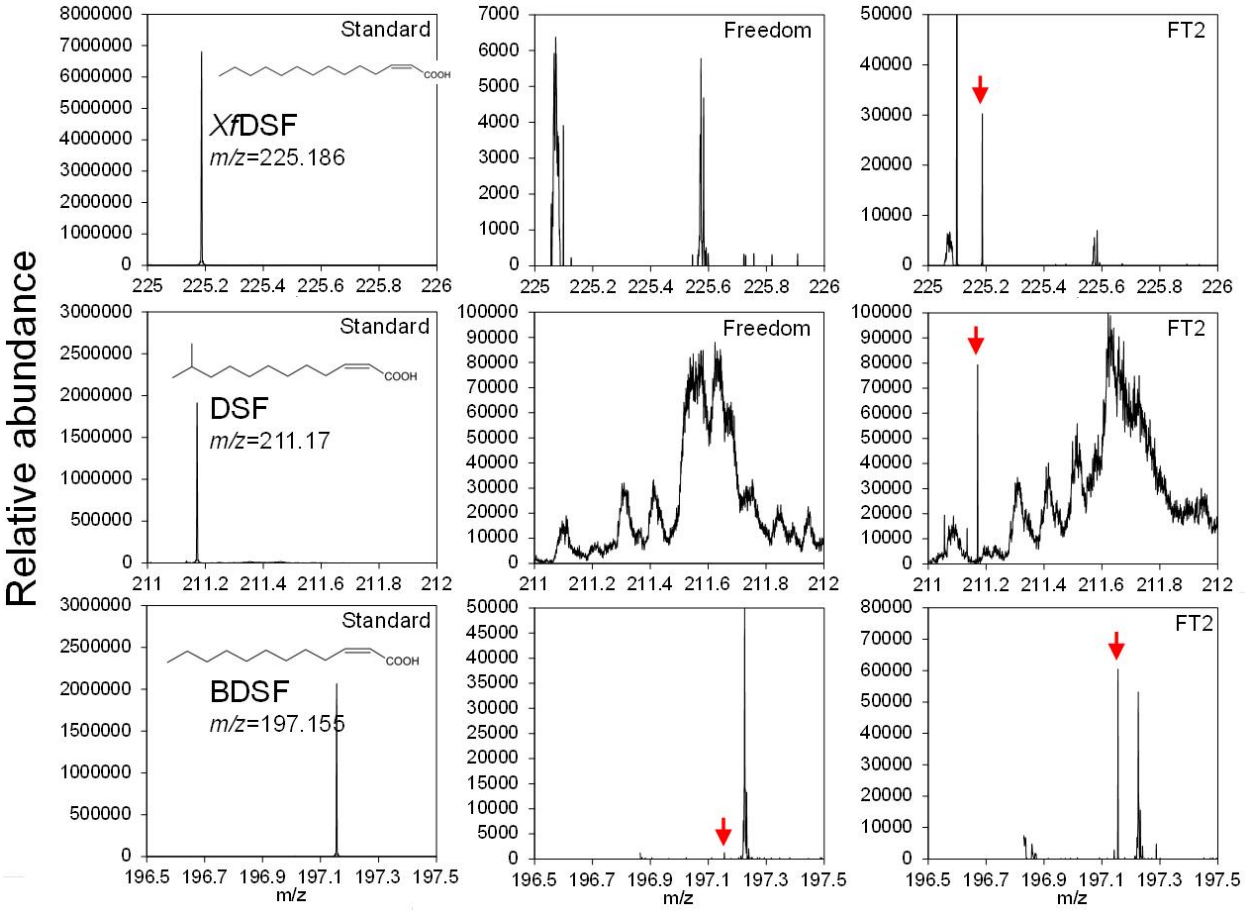


Figure 3. Detection of different DSF species in xylem sap of transgenic grape line FT2 transformed with the *rpfF* gene from *Xylella fastidiosa*. ESI-MS analysis of a standard of *Xf*DSF (top left), DSF (middle left), or BDSF (bottom left) showing the characteristic ionization fragment for each molecule. The presence of the mass ion characteristic of a given DSF species in analyses of xylem sap from FT2 is shown in the right column. Note the complete or near complete absence of these ions in analyses of xylem sap from parental Freedom plants (middle column).

To better estimate the concentration of DSF presence in xylem fluid we subjected DSF biosensor cells of either *Xanthomonas campestris* or *X. fastidiosa* itself to DSF present in leaves or in xylem fluid. Not only could DSF be readily detected in leaf tissue, but in *X. fastidiosa* DSF biosensor could readily detect DSF in xylem sap of transgenic freedom plants harboring *X. fastidiosa rpfF* (Figure 4). Given that the detection limit for DSF by this biosensor is approximately 1 micromolar, it appears clear that at least this concentration of DSF is present in the xylem sap of transgenic *rpfF*-containing freedom when used as a rootstock.

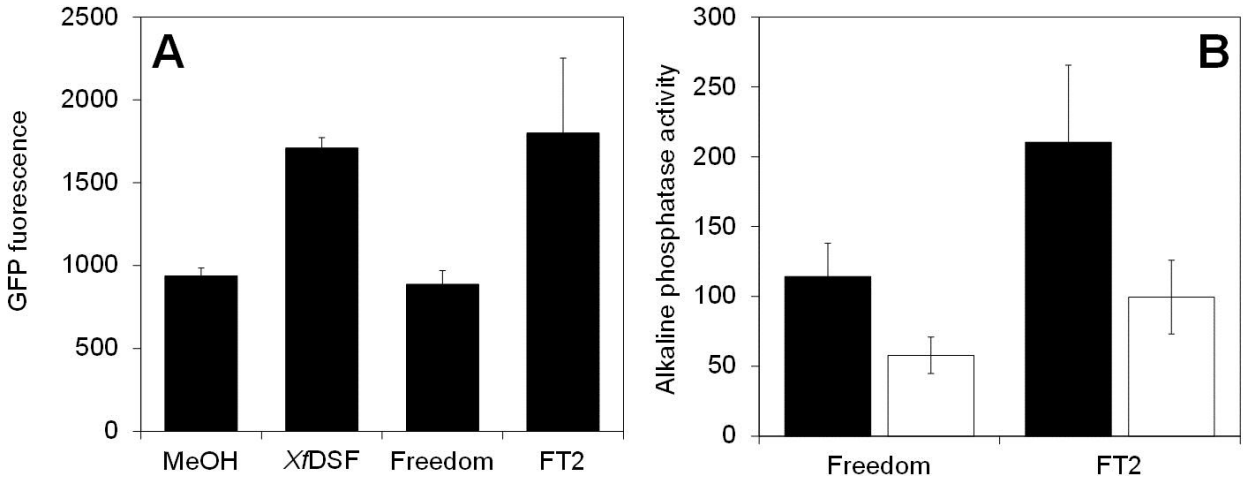


Figure 4. A) GFP fluorescence exhibited by *Xanthomonas campestris*-based GC^{ch}F DSF biosensor cells recovered from colonies grown on agar plates in which leaves of wild-type Freedom grape or rpfF-containing transformed line FT2 harboring the *rpfF* gene from *Xylella fastidiosa* that had been embedded in the agar or on agar containing only methanol or *Xf*DSF (10 µM). B) alkaline phosphatase activity exhibited by cells of *X. fastidiosa Xf*HA DSF biosensors (black bars WT, white bars *rpfF** mutant, both harbor *hxfA'::phoA* reporting fusion, incubation time of 72 h) after suspension and xylem sap recovered from Freedom grape or line FT2. The vertical lines represent the standard error of the mean.

Given that the concentration of DSF in xylem fluid is at least one micromolar, and that it is clearly mobile within the plant, we tested the hypothesis that increasing the size of the rootstock will increase its potential to distribute DSF to the scion. This hypothesis is based on the assumption that a substantial dilution of DSF occurs when a relatively large scion is produced on a relatively small root stock. We produced wild-type Cabernet Sauvignon scions grafted to rootstocks of transgenic Freedom of differing sizes. Green grafting proved difficult because the normal process for green grafting involves grafting the scion onto the rootstock which is been simultaneously rooted. The very large rootstocks we were attempting to both graft and root did not successfully undergo routine. Establishing a large rootstock before grafting has also made establishment of the grafted scion more difficult. Some success was achieved. Unfortunately, and equipment no function in the greenhouse shortly before these plants were to be inoculated cause them to overheat and die. In another strategy to address objective 3, we used dormant transgenic grape from our field trial in Solano County to produce grafted plants with a very large rootstock. The plants that were established in 2010 in the Solano County trial and by early 2011 were quite large. The USDA –APHIS permit was amended to enable us to collect dormant vines from this field trial for terminal used in our greenhouses at UC Berkeley. A large amount of dormant vines from both transgenic Freedom as well as transgenic Thompson seedless plants that had not been inoculated with *X. fastidiosa* during the summer of 2011 were collected. Non-transgenic Cabernet were grafted onto the top of dormant twigs of different lengths, and the lower rootstock sections are being rooted in the greenhouse. In an initial study, another greenhouse malfunction damaged a large number of plants, and we were therefore unable to evaluate disease severity. The study however was repeated from dormant plant material collected during the winter of 2012-2013 and grafted plants with large rootstocks were produced by June, 2013. These plants were inoculated and at the time of this report are still being evaluated for disease susceptibility. We are optimistic that this strategy, more in line with normal grape propagation procedures, will be successful in producing plants with relatively large rootstocks that will exhibit a higher level of disease resistance than those from plants with relatively small transgenic DSF producing rootstocks. Rootstocks of 1 meter in length or more should be readily producible by this strategy.

Objective 4.
Use of DSF for disease control and its effect on pathogen behavior

Characterization of DSF made by *X. fastidiosa*.
While the molecule C14-cis is one component of DSF made by *X. fastidiosa*, further support for the possibility that more than one fatty acid signal molecule is made by RpfF was obtained by the use of a Thin Layer Chromatography (TLC) method to assess the fatty acids produced by *X. fastidiosa*. In this method, acidified ethyl acetate extracts of culture supernatants of a wild-type *X. fastidiosa* strain and an RpfF- mutant and a RpfB mutant were subjected to TLC and fatty acids visualized by iodine vapors. Interestingly, three different fatty acids were visualized in the wild type strain, while these were largely missing in an RpfF- mutant, with only very small amounts of two other putative fatty acids present (Figure 5). It also was of interest to see that the RpfB mutant produced an altered pattern of putative fatty acids, with the major chemical species produced by the WT strain missing, and much larger amounts of one of the other species produced. The top-most spot observed in extracts of a WT strain of *Xf* co-migrates with C14-cis, a chemical form of DSF that we have previously characterized. Interestingly, the middle band found in the WT strain, which migrated similarly to the topmost, and most abundant band seen in the rpfB mutant, co-migrates with C12-cis. It is noteworthy, that a re-examination of the DSF species produced by *Xanthomonas campestris* pv. campestris using different methods have revealed that this species also produces C12-cis. The most prominent, lower, band seen in the WT strain is not observed in the *rpfB* mutant. This supports the

model that RpfB, a putative long-chain fatty acyl CoA ligase, serves to produce suitable substrates for RpfF, the DFS synthase. Although RpfB is not required for DSF synthesis in *X. fastidiosa*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availably of the appropriate substrates for DSF synthesis by RpfF. It is interesting to note that *rpfB* mutants have an altered behavior compared to *rpfF* mutants and WT strains of *Xf*. While *rpfF* mutants are hyper-virulent in grape, *rpfB* mutants were nearly as virulent as WT strain. In contrast, while rpfF mutants are non-transmissible by sharpshooters, the *rpfB* mutants exhibit only a slight decrease in their transmission suggesting that they are retained by sharpshooters more efficiently than the rpfF mutant. Given that the *rpfB* mutant appears to make C12-cis but not C14-cis, this suggests that C12-cis is sufficient to enable signaling that leads to insect transmission but does not greatly affect virulence. We expect that co-expression of RpfB and RpfF in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic *Arabidopsis* plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xcc* indicated that the transgenic plants expressing both *rpfB* and *rpfF* transgenic plants can better complement the virulence of the non-pathogenic *rpfF* mutant of *Xcc*. Based on this and other data, we preparing constructs so that both RpfF and RpfB can be simultaneously expressed in transgenic plants for optimum production of suitable DSF molecules. We thus are preparing genetic constructs to transform grape with these two genes to further enhance DSF production.

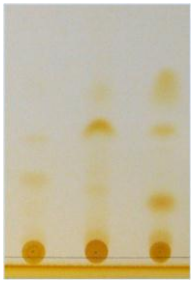


Figure 5. Fatty acids resolved by TLC from a RpfF mutant of *X. fastidiosa* (left lane) a RpfB mutant (center lane) and a wild type strain of *X. fastidiosa* (right lane). Fatty acids were visualized after exposure to iodine vapor.

We have also used improved DSF biosensors (see below) to screen for active fractions in chemically separated culture supernatants of *Xylella fastidiosa*. As noted above, C14-cis is produced by *X. fastidiosa*, but is probably not the only DSF species produced by this bacterium. We therefore have further investigated the conditions under which DSF is produced by X. *fastidiosa*, as well as performed chemical fractionation of the molecules produced by *X. fastidiosa* to determine the identity of additional chemical species having signaling activity in the pathogen. Nearly all of the work we have conducted to date has utilize the growth medium PWG to culture *X. fastidiosa*. While *X. fastidiosa* grows well on PWG medium, our recent results indicate that it either produces relatively little DSF on that medium or that the DSF is bound to medium ingredients, most likely bovine serum albumin which is a major medium ingredients. We therefore have studied the production of DSF in cells of *X. fastidiosa* grown on PD3 medium. Not only does *X. fastidiosa* exhibit abundant growth on this medium, but the yield of DSF seems to be much higher than on PWG medium. Chemical fractionation of PD three medium in which *X. fastidiosa* has been grown yield a variety of fractions, differing in polarity, that have biological activity as measured by the *X. campestris* –based biosensor (Figure 6). NMR and Mass Spectral analysis of these fractions are currently underway to identify the chemical species associated with biological activity. After identification, these chemical species will be synthesized and applied to plants. Continuing work conducted as part of CDFA agreement 12-0224-SA has now identified at least one additional component of *X. fastidiosa* DSF as a 16 carbon unsaturated fatty acids similar to that of C14-cis. Further work on its identity, synthesis, and biological activity will be reported in progress reports that agreement.

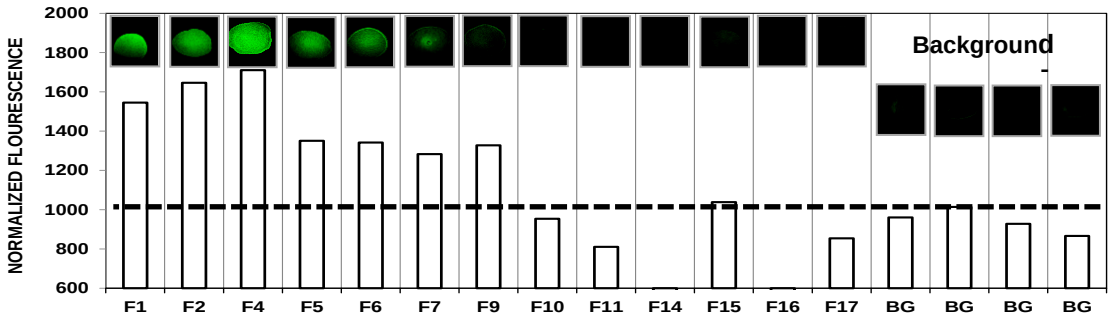


Figure 6. Biological activity of different fraction of a culture extract of *X. fastidiosa* grown in PD3 media as detected by gfp fluorescence of a *X. campestris*-based biosensor

Studies of adhesion of *X. fastidiosa* to grape

Our studies have suggested strongly that adhesion of *X. fastidiosa* to plant tissues inhibits movement of the pathogen through the plant, and hence tends to reduce the virulence of the pathogen. RpfF- mutants of the pathogen that do not produce DSF adhere to glass surfaces and to each other much less effectively than WT strain that produce DSF. This is consistent with the apparent rpfF-

regulation of adhesins such as HxfA, HxfB and XadA etc. To better correlate levels of DSF in the plant and the stickiness of the *X. fastidiosa* cells we have developed a practical assay to measure and compare stickiness of *X. fastidiosa* cells in grapes infected with *X. fastidiosa gfp-Wt* and *X. fastidiosa* mutants. In this assay, the release of cells of *Xf* from stems and petioles tissue from grape infected with *X. fastidiosa* wild type Tem and gfp-rpfF mutant were compared. Tissues from infected Thompson seedless grapes were surface sterilized. From the sterile tissues, 5mm stem or petiole segments were cut and placed individually in sterile buffer and shaken gently for 20 minute. After 20 minutes the number of cells released from the cut end of the segment were estimated by dilution plating on PWG. To determine the total number of cells in a given sample (the number of cells that potentially could have been released by washing) the washed segment was macerated and *X. fastidiosa* populations again evaluated by dilution plating. Total cell populations were calculated by summing the cells removed by washing and those retained in the segment. The ratio of easily released cells to the total cells recovered in the samples was termed the release efficiency. In both stems and petioles the release efficiency of the *rpfF* mutant was much higher than that of the WT strain (Figure 7). There was a very strong inverse relationship between the adhesiveness of the cells to grape and their ability to cause disease one a variety of different mutant strains differing in their production of our response to DSF are considered (Fig. 7). This very striking difference in the adhesiveness of the *X. fastidiosa* cells experiencing different levels of DSF in the plant suggest that this release efficiency assay will be valuable for rapidly assessing the susceptibility of grapes treated in various ways. For example, the adhesion of cells could be measured within a couple of weeks after inoculation of WT *X. fastidiosa* cells into transgenic plants harboring various constructs designed to confer DSF production in plants, or in plants treated with DSF producing bacteria or topical application of chemicals with DSF-like activity. Such an assay would be far quicker than assays in which disease symptoms must be scored after several months of incubation, and could be employed during those times of the year such as the fall and winter when disease symptoms are difficult to produce in the greenhouse.

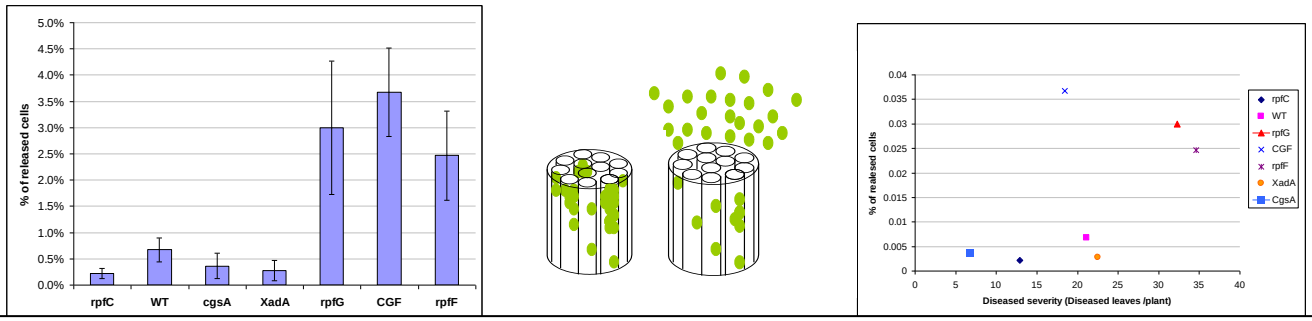


Figure 7. (left) Proportion of total cells of various mutants of *X. fastidiosa* that were released during gentle washing of grape stem segments in buffer as depicted (center). The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment. (right) Relationship between proportions of various *X. fastidiosa* mutants released from tissues and the virulence of those strain in Thompson seedless grape.

To further address the usefulness of the cell release assay to assess treatments designed to limit the movement of *X. fastidiosa* in plants to achieve disease control, we tested the adherence of WT strains of *X. fastidiosa* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *X. fastidiosa* compared with that to non-transformed plants. Plants were inoculated with a gfp-marked wild type strain of *X. fastidiosa* and petioles were removed from plants at a distance of about 20 cm from the point of inoculation, and the percent of the cells removed during a brief washing step measured as above. The adherence of WT strains of *X. fastidiosa* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *X. fastidiosa* was much greater than that of cells in the non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced its ability to move within the plant after inoculation. That is, the percentage of cells that was released from *rpfF*-expressing plants was from 2 to 3-fold less than that of control plants (Figure 8). As seen before, cells of an *rpfF* mutant that does not produce DSF exhibited about 3-fold higher percentage of cells released from a normal Thompson grape compared to the WT strain in the non-transgenic grape (Figure 8). These results suggest strongly that DSF production presumably has occurred in the *rpfF*-expressing plants, and that the enhanced adhesiveness of these cells is associated with their reduced ability to spread through the plant and cause disease. These results suggest that the release efficiency assay should be a useful tool to rapidly assess treatments designed to control Pierce's disease.

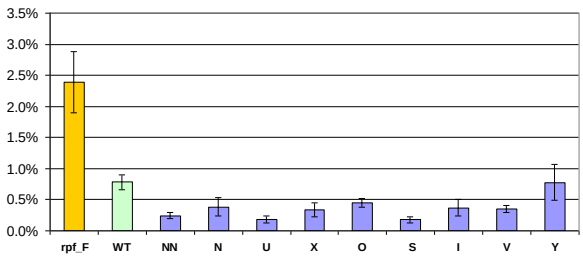


Figure 8. Percentage of total cells of a gfp-marked WT strain of *X. fastidiosa* (blue and green) and a gfp-marked rpfF mutant of *X. fastidiosa* (orange) in petioles of non-transformed Thompson (Orange and Green) or of transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *X. fastidiosa* (blue) that were released during gentle washing of the segments in buffer. The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment.

While the movement of *X. fastidiosa* has been recognized as an important trait necessary for disease, the process is still poorly understood. Other studies we have performed in our lab are strongly supportive of a model of progressive and sequential colonization of a large number of xylem vessels by *X. fastidiosa* after inoculation of a single vessel. Furthermore, we believe that the process of movement of *X. fastidiosa* through plants is a stochastic one which is characterized by growth in a given xylem vessel into which it is introduced followed by "active escape" of at most a few cells into adjacent uncolonized vessels, and then further multiplication of the cells which starts the process anew. We have exploiting the use of mixtures of phenotypically identical strains of *X. fastidiosa* differing by only one or two genes to better understand the process of progressive movement of *X. fastidiosa* through plants. We hypothesize that anatomical features of plants (nature of pit membranes and other barriers to vessel to vessel movement in the stem) limit the number of *X. fastidiosa* cells that can transit from one vessel to another and are major factors conferring resistance in plants. *X. fastidiosa* must move from one xylem vessel to another dozens or hundreds of times to be able to move longitudinally down a vine as well as laterally across the vine to achieve the extensive colonization of the stem that are typical of diseased vines. If, at each step in this movement process only a few cells are transferred then, by chance, xylem cells distal to the inoculation point will receive by chance only one of the two genotypes of the pathogen. Thus for a given plant inoculated with a mixture of cells, the proportion of one strain compared to the other would either increase or decrease along a predictable trajectory given the stringency of the "bottleneck" that it faced while moving from one vessel to another. We now have identified differently marked *phoA* mutants as having identical behavior in grape as the WT strain, enabling us to examine the process of spatial segregation of cells of *X. fastidiosa* during colonization of grape. The population size of the WT strain of *X. fastidiosa* was similar to that of the *phoA* mutant whether inoculated singly or in a mixture. More importantly, the proportion of cells of the two strains that were recovered from different locations within a given plant differed greatly between plants (Figure 9). For example, 8 plants were inoculated with an equal mixture of the WT and *phoA* mutant, yet in some plants all of the cells recovered from locations either 10 cm or 120 cm form the point of inoculation were either one strain or the other; seldom was a mixture of both strain found, and a similar fraction of the plants harbored one strain or the other, suggesting that the two strains had an equal likelihood to move within the plant, but that stochastic processes determined the movement. We hypothesize that resistant grape varieties harbor anatomical differences from susceptible varieties that limit the movement of *X. fastidiosa* from vessel to vessel. Such plant would thus present a more extreme "bottleneck" to *X. fastidiosa* at each movement event and hence we would expect a more rapid segregation of mixtures of *X. fastidiosa* at a given point away from inoculation. We thus are currently further exploring the spatial dependence of this segregation process in different grape varieties that differ in resistance to Pierce's disease. Plants have been inoculated and assessment of the ratios of cells of the two strains in the mixture are currently being made. Not only should this provide considerable insight into the process of movement which, while central to the disease process, remains very poorly understood, but it should also provide new tools for screening grape germplasm for resistance to *X. fastidiosa*.

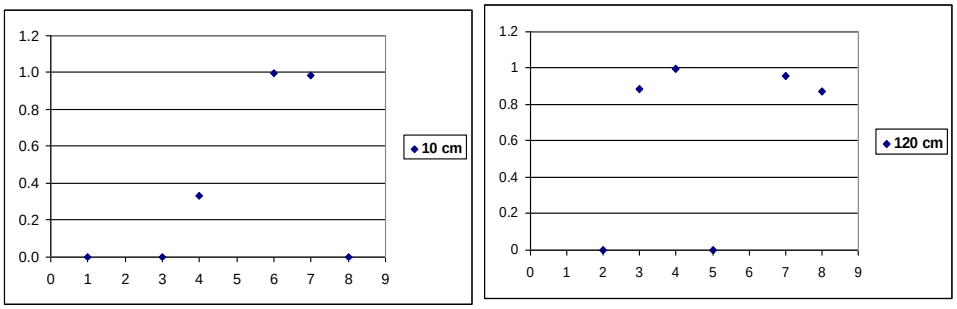


Figure 9. Proportion of cells recovered at 10 cm (left panel) or 120 cm (right panel) from the point of inoculation from individual plants that were inoculated with an equal mixture of a gentamycin-resistant PhoA mutant strain of *X. fastdiosa* and a kanamycin–resistant PhoA mutant (abscissa) that were the kanamycin-resistant strain.

In contrast to the effect of DSF on increasing the adhesiveness of cells of *X. fastidiosa*, particularly as they would reach high population sizes in the plant, *X. fastidiosa* also appears to produce a molecule or to induce the production of a molecule by the plant that reduces its ability to self-aggregate and to adhere to surfaces. Cells of a GFP-marked *X. fastidiosa* wild type strain were suspended in xylem sap recovered from both uninfected plants, as well as plants infected with a wild type strain of *X. fastidiosa* or a plant infected with an RpfF mutant. While many cells of the wild type strain formed relatively large cellular aggregates after one day of suspension in samples from healthy plants, the cells remain dispersed and occurred primarily as single cells when suspended in sap from a plants infected with the RpfF mutant (Figure 10). Cells suspended in sap collected from a plant infected with a wild type strain of *X. fastidiosa* exhibited intermediate levels of cellular aggregation (data not shown). Since most of the sap recovered from infected plants would likely have been from vessels that had relatively low population sizes of *X. fastidiosa* (its more heavily infected vessels would have been plugged in therefore xylem fluid would have been difficult to collect), we presume that the factors present in the sap

of the plants infected with the wild type strain and particularly the RpfF mutant of *X. fastidiosa* had produced one or more materials that interfered with the adhesiveness of the cells. Quantification of the process of cellular aggregation as a function of time after cells were suspended in sap from grape infected with different strains of *X. fastidiosa*, revealed that sap from plants infected with the RpfF mutant was particularly effective in preventing cellular aggregation of *X. fastidiosa* (Figure 11). Such anti-adhesive factors produced by the RpfF mutant might account for its enhanced ability to move within plants that had been previously observed. It thus seems likely that *X. fastidiosa* alters the chemical environment within the xylem vessels to restrict its ability to adhere to plants, thereby maximizing its ability to move along vessels but also between vessels through pit membranes.

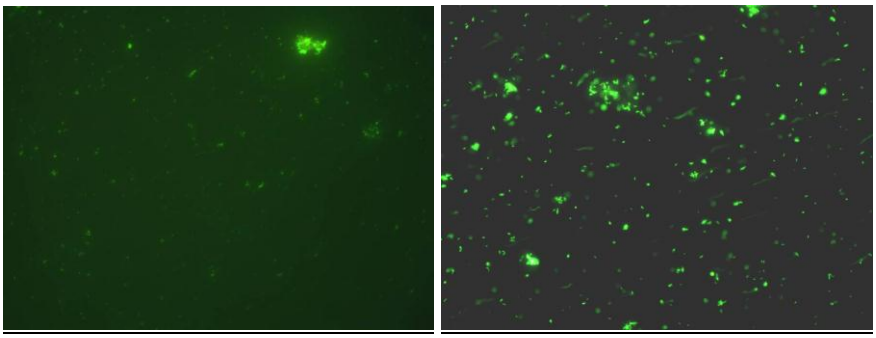


Figure 10. Cellular aggregates of a GFP marked strain of X.fastidiosa suspended in sap from grape infected with an RpfF mutant of X.fastidiosa (left panel) or from staff from an uninfected plant (right panel) when visualized at 20 X magnification. At this magnification only cellular aggregates are visible as relatively large green spots.

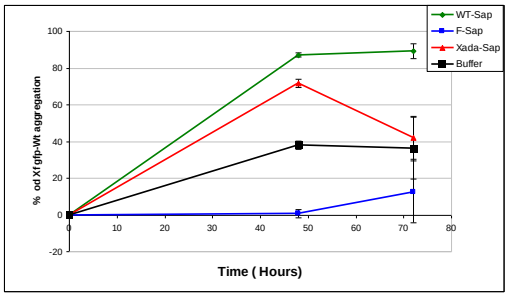


Figure 11. Increases in cellular aggregation of cells of a wild type *X. fastidiosa* strain suspended in xylem sap of grape plants inoculated with different strains of *X. fastidiosa*, as measured by a decrease in turbidity of cells over time. Note the substantially lower level of aggregation of cells suspended in sap from plants infected with an RpfF mutant compared to that in buffer alone (blue versus black line).

The adhesiveness of GFP-marked strains of *X. fastidiosa* to chitin surfaces mimicking that of the mouth parts of a sharpshooter also were strongly influenced by materials found in xylem sap of grape infected with different strains of *X. fastidiosa*. The adhesiveness of *X. fastidiosa* to the wings of glassy winged sharpshooter suspended in sap from grape infected with an RpfF mutant of *X. fastidiosa* was much less than that on to wings suspended in sap from a plant infected with the wild type strain (Figure 12). The attachment to wings was more extensive than cells suspended buffer compared to that of cells suspended in xylem sap from infected grape (data not shown) suggesting that both wild type and RpfF mutants of *X. fastidiosa* release materials into the xylem fluid that inhibits their attachment to surfaces, thereby facilitating their movement through the plant. The lack of attachment of the wild type strain of *X. fastidiosa* to the chitinacious surface in the presence of sap from plants infected with an RpfF mutant offers that explanation for why the RpfF mutant itself is not readily vectored by insects, and why prior infection with an RpfF mutant inhibited the subsequent transmission of a wild type strain of *X. fastidiosa*.

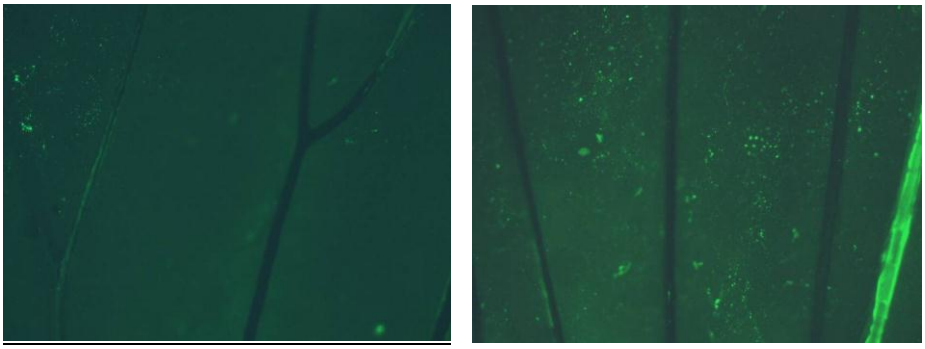


Figure 12. Visualization of GFP marked cells of *X. fastidiosa* suspended in sap from grape infected with an RpfF mutant of *X. fastidiosa* (left panel) or from sap from grape affected with a wild type strain of *X.fastidiosa* (right panel) at 10 X magnification. *X. fastidiosa* cells are apparent as tiny green dots in both images. Note the much larger number of *X. fastidiosa* cells in the right-hand panel.

Development of *Xcc*-based biosensors efficient in detecting *Xylella* DSF. For many of the objectives of this project, an improved bioindicator for DSF would be very valuable. Until recently we have used an *Xcc*-based biosensor in which the *Xcc* endoglucanase gene is linked to a GFP reporter gene. Previous studies have shown that this biosensor is able to detect the DFS made by *Xf* but that it detects *Xf* DSF with a much lower efficiency then the *Xanthomonas* DSF since the two molecules apparently differ slightly. We constructed two independent *Xcc* -based DSF biosensors specific to the DSF produced by *X. fastidiosa* RpfF and which is sensed by *Xf* RpfC. In the first sensor, we replaced the Rpf-DSF detection system of *Xcc* with that of homologous components from *X. fastidiosa*. An *Xcc* mutant in which both *rpfF* and *rpfC* was deleted was transformed with a pBBR1MCS-2 based plasmid harboring *Xf rpfC* and *rpfF* genes. A second *Xcc*-based *Xf* DSF sensor was constructed that is composed of an *Xcc rpfF* and *rpfC* double mutant into which *X. fastidiosa rpfF* and *rpfG* and a hybrid *rpfC* allele composed of the predicted trans-membrane domain of *X. fastidiosa* RpfC and the cytoplasm domain of the *Xcc* RpfC has been added. We named the three different sensors based on their DSF sensing element, *Xcc*-rpfC, *Xf*-RpfC and *Xf-Xcc* chimeric RpfC. The *eng::gfp* transcriptional fusion in *Xcc* was inactive in the absence of DSF but was strongly induced by ≥0.1 μM 2-*Z*- tetradecenoic acid. We compared the activity of these two *Xf*-DSF specific biosensors to the activity of our original *Xcc*-DSF specific biosensor (*Xcc rpfF* mutant harboring an *eng'::gfp* reporting fusion) in response to diluted DSF extracts obtained from *Xcc* and from *X. fastidiosa*. The three *Xcc*-based DSF biosensors (*Xcc*RpfC (also called pKLN55), *Xf*RpfC (also called GCF), and *Xcc*chimericRpfC (also called Chimeric or GFC^{ch})) exhibited similar sensitivity to the DSF containing extract from *Xcc* but in sharp contrast, *Xcc*RpfC exhibited much lower sensitivity to the DSF containing extract from *Xf* than the two *Xf*-DSF specific biosensors (figure 13). These results suggest that *Xf*-RpfC can interact with a wider range of molecules then *Xcc*-RpfC. In addition, these two sensors were found to become activated even in the presence of dilutions of the *X. fastidiosa* extract that contain less than the material contributed by a single PWG plate from which the DSF was originally extracted.

We also improved the *Xcc*- based GCF biosensor by replacing the *Xf rpfF* gene (which encodes the DSF synthase) with a mutant allele in which glutamate codons 141 and 161 which are essential for DSF production activity were replaced by Alanine codons. This mutant form of RpfF is no longer capable of DSF synthesis, but is still apparently capable of interaction with RpfC, and thus proper response to DSF. Fortuitously, the altered RpfF apparently has a more relaxed interaction with RpfC, and cells harboring this mutant allele are more responsive to exogenous DSF. The mutant RpfF protein in this strain not only appears to bind to DSF, but suppresses the activation of the downstream *eng*:gfp reporter gene that would otherwise occur in the absence of Rpf F (Fig. 15). Thus *Xcc* cells harboring the mutant *Xf* RpfF as well as the *Xf* RpfC and RpfG (which we term GCF*-gfp) exhibits very strong gfp fluorescence in response to DSF molecules in extracts of *Xf* cultures and it also has the advantage that it does not produce DSF, leading to low background activity. We have used this optimized biosensor extensively in our continuing studies of the movement of applied DSF in objective 4 and because of the ease of its use.

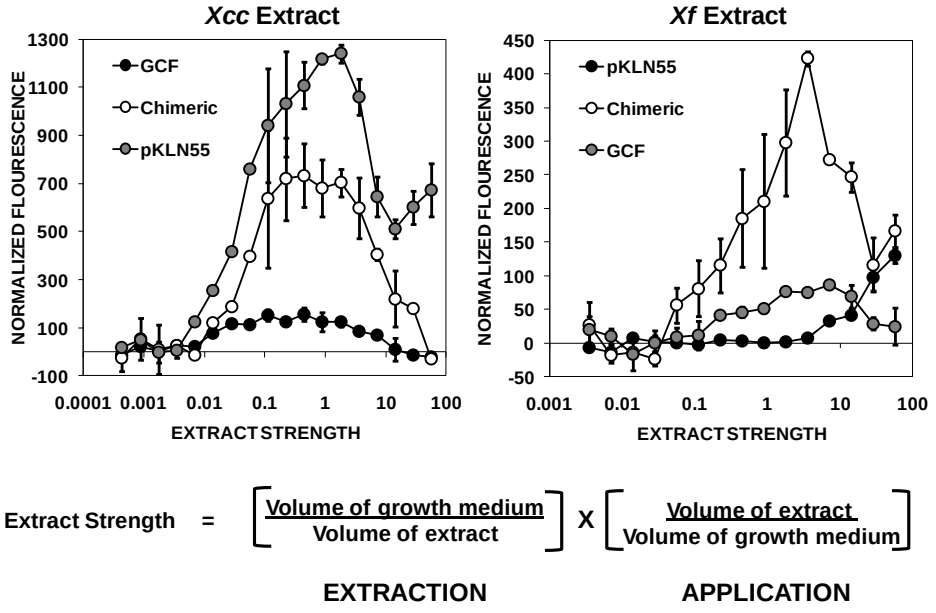


Figure 13. GFP fluorescence of different DSF biosensors to increasing concentrations of DSF from culture extracts of *Xcc* (left panel) or from *X. fastidiosa* (right panel).

To test the specificity of the DSF biosensors we compared the activity of those sensors to a panel of pure DSF analogues. Quantification of the response of the three biosensors to various fatty acid molecules revealed a substantial difference in their selectivity to compounds of different chain length. The Xcc GCF and *Xcc*ChimericRpfC biosensors, based on the use of an *X. fastidiosa* RpfC receptor were clearly more responsive than the XccRpfC biosensor for fatty acid molecules greater than 12 Carbon atoms long (Figure 14). Given that the DSF produced by *Xcc* is 12 Carbons in length, while at least one of the molecules made by *Xf* is apparently 14 Carbons in length, it appears that the RpfC receptor has evolved to bind fatty acid signal molecules of a particular length. This also suggests that while *Xcc* may be relatively unaffected by exposure to DSF made by *X. fastidiosa*. The converse is probably not the case. The two *X. fastidiosa* DSF specific DSF biosensors, particularly the *Xcc* GCF biosensor is much more responsive to C14-cis, and hence will be far more useful that the original *Xcc* RpfC biosensor for assessing DSF levels in plants and bacterial cultures. When compared directly for their ability to detect C14-cis, a DSF species produced by *X. fastidiosa*, it is clear that the *Xcc* rpfGCF biosensor was for more responsive, especially at relatively low concentrations (1 to 10 micromolar) than the original Xcc RpfC biosensor (Figure 16). We would expect that the new *Xcc* rpfDCF* biosensor would be even more responsive to this DSF species.

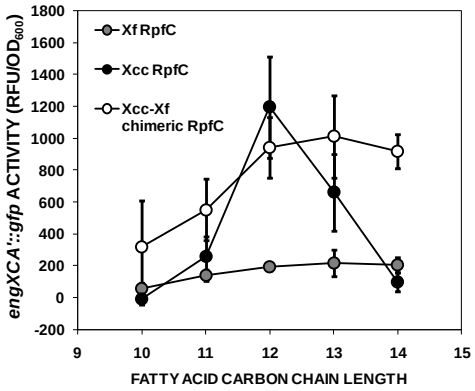


Figure 14. Relative responsiveness of three different DSF biosensors to unsaturated fatty acids of different chain lengths.

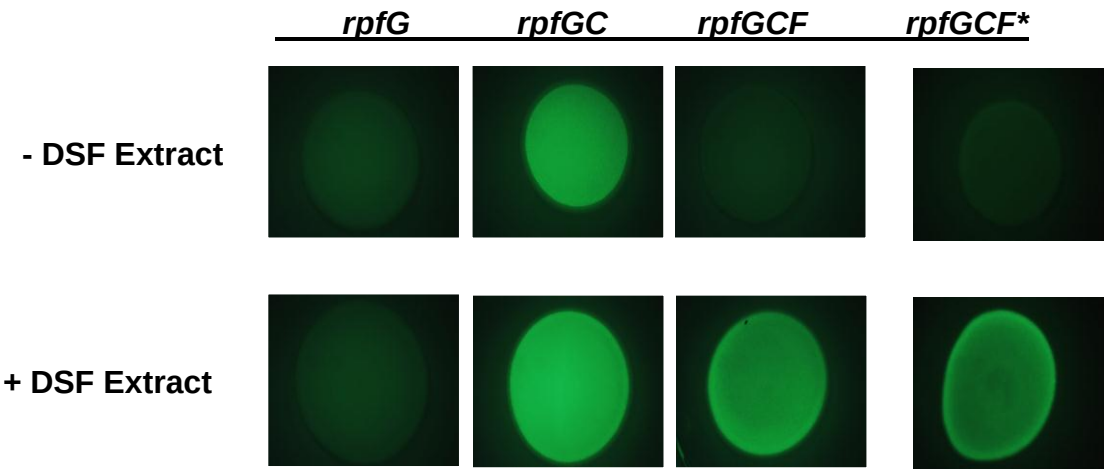


Figure 15. GFP fluorescence exhibited by different Xcc-based biosensor strains in the absence (top row) or presence (bottom row) of DSF recovered from culture extracts of a culture of *X. fastidiosa*. Note that both the rpfGCF and rpfGCF* biosensors exhibit GFP fluorescence only in the presence of DSF.

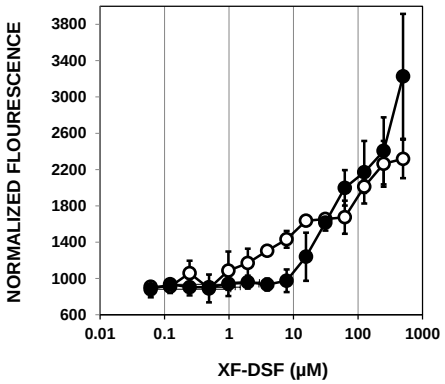


Figure 16. GFP fluorescence exhibited by the original *Xcc* RpfC (filled circles) and the *Xcc* rpfGCF biosensor (open circle) when cells were exposed to different concentrations of C14-cis in culture media.

Since the various DSF sensors appeared to have high specificity and sensitivity for DSF, they are suitable for large-scale testing of DSF molecules and transgenic plants as well as for DSF produced by antagonistic bacteria. Methods were therefore tested to ascertain the most expedient way in which these biosensors could be deployed for the detection of DSF. A broth culture assay in which the biosensors were suspended in xylem sap of transgenic plants expressing RpfF from X. fastidiosa successfully detected DSF. We were pleased to find that GFP fluorescence and hence DSF responsiveness of the biosensor could be easily monitored in such a high throughput manner.

Development of a *X. fastidiosa*-based bioreporter for DSF. We have developed methods to use *X. fastidiosa* itself to detect DSF**.** Among the several genes that we know to be most strongly regulated by DSF include *pil* genes involved in twitching motility, several genes such as *fimA* and *hxfA* and *HxfB* which are involved in cell-surface adhesion, and gum genes involved in production of EPS. Previous attempts to establish *gfp* or *inaZ*-based transcriptional fusions in *Xf* failed, presumably due to its incapability to express foreign genes properly we have successfully use the endogenous *phoA* gene (encoding alkaline phosphatase) as a bioreporter of gene expression in *X. fastidiosa* (Figure 17).

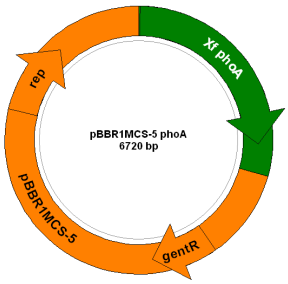


Figure 17. Vector construct which enables the use of alkaline phosphatase as a reporter gene for monitoring gene expression of DSF responsive genes such as *hxfA* in *X. fastidiosa*.

To utilize a PhoA-based biosensor in *X. fastidiosa* it was necessary to create a PhoA mutant, thereby eliminating background alkaline phosphatase activity. The PhoA-based biosensor in which *phoA* is driven by the *hxfA* promoter is quite responsive to exogenous DSF Both hxfA and hxfB expression in *X. fastidiosa* was strongly induced by C14-cis but not by the related molecule myristic acid as assessed using alkaline phosphatase as an assay (Figure 18).

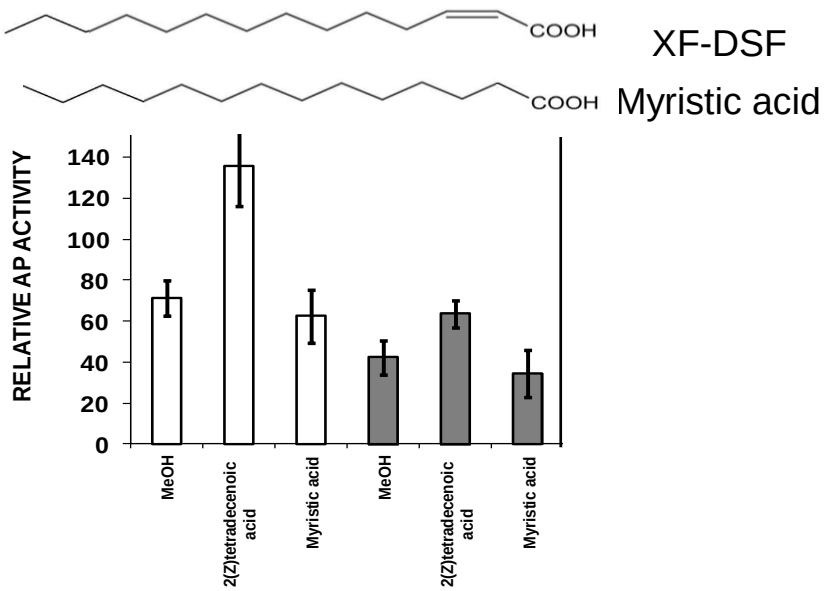


Figure 18. Alkaline phosphatase activity of cells of X. *fastidiosa* harboring a fusion of *phoA* and *hxfA* (open bars) or hxfB (closed bars) and exposed to C14-cis or myrisitic acid.

The PhoA biosensor has proven to be very responsive to even small quantities of DSF. The alkaline phosphatase activity expressed by cells of *X. fastidiosa* harboring a *hxfA:phoA* fusion increased with increasing concentrations of C14-cis above about 500 Nana molar (Figure 19). AThis biosensor therefore appears to be highly responsive to DSF therefore very useful in assays of DSF from culture media as well as from DSF applied to plants.

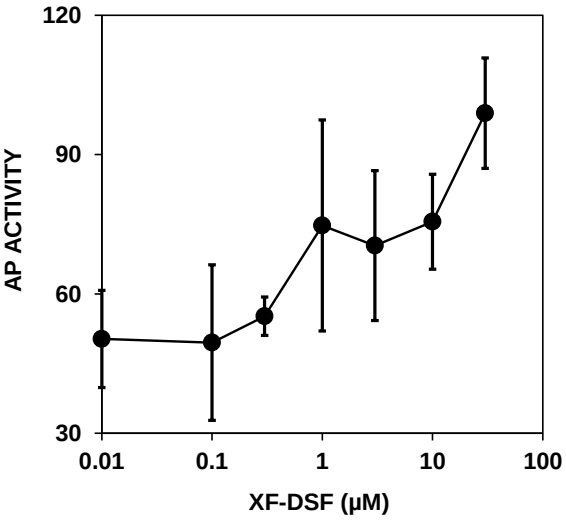


Figure 19. Alkaline phosphatase activity exhibited by cells of *X. fastidiosa* harboring a *hxfA:phoA* fusion when grown on culture medium containing various concentrations of C14-cis, a DSF species produced by *X. fastidiosa*, when measured 24 hours after inoculation.

The alkaline phosphate reporter gene proves to be very useful in monitoring the production of and response to DSF by cells of *X. fastidiosa* in broth cultures. The use of the reporter gene revealed that maximal DSF production apparently occurred at about XD hours growth in PD3 medium (Figure 20). This information will be highly valuable and are further studies to isolate large quantities of DSF for further chemical fractionation to identify the other chemical species present besides C14-cis that are produced by *X. fastidiosa*. It was also apparent from the use of this biosensor that *X. fastidiosa* produces relatively large amounts of DSF chemical species besides C14-cis onPD3 media since no GFP fluorescence was exhibited in assays of this culture media by *Xcc* rpfC, a biosensor that should have detected this chemical species. We therefore will be exploring PD3 cultures of *X. fastidiosa* as a source of novel DSF chemical species in our continuing work.

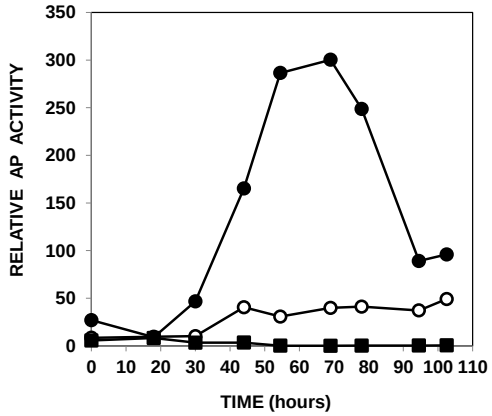


Figure 20. Alkaline phosphatase activity exhibited by cells of a wild type *X. fastidiosa* strain harboring either a *hxfA:phoA* or *hxfB:phoA* reporter gene fusions and grown for various times on PD3 medium.

Alkaline phosphatase activity could also be readily measured in cells of *X. fastidiosa* harboring the E141/161A mutation in RpfF that abolished DSF synthase activity, but retained the ability to respond to DSF. Alkaline phosphatase has been measured in an *X. fastidiosa* strain that harbors the RpfF* mutation (in which no DSF is produced) as well as a mutation in *phoA*. In such a background, alkaline phosphatase activity increased within 72 hours after application of C14-cis to culture media but not in control media to which methanol alone had been added (Figure 21).

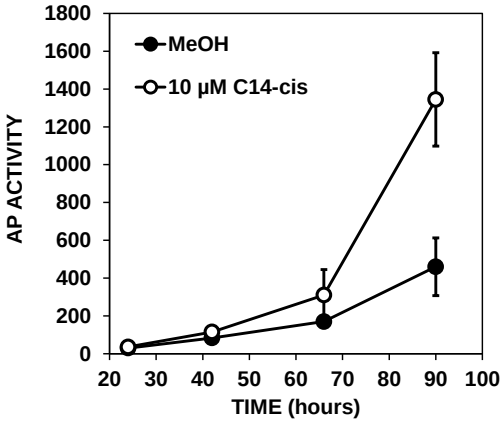


Figure 21. Alkaline phosphatase activity exhibited by cells of a RpfF*, PhoA mutant of *X. fastidiosa* grown for various times on culture media to which 10 micromolar C14-cis (open circles) or methanol alone (closed circles) had been added.

We also examined the phenotypes of an *rpfF*- mutant and WT strain of *X. fastidiosa* exposed to different amounts of DSF to determine if it can be used to bioassay for the presence of DSF. The DSF-induced behaviors of *X. fastidiosa* were found to be strongly dependent on the culture medium that the cells were grown in. All strains of *X. fastidiosa* are much more responsive when cells are grown in a minimal medium such as PIM6 or PDA than in media such as PWG containing BSA. For example, cells of the WT strain which are not adherent in culture, and thus which do not form cell-cell aggregations became much more adherent to each other when DSF was added to shaken broth cultures. The increased adherence is readily visualized as an enhanced ring of cell-cell aggregates that forms at the liquid-air interface of shaken cultures (Figure 22, left). The attachment of *X. fastidiosa* cells to tubes or wells is readily measured by estimating the number of attached cells by their ability to bind crystal violet. The amount of cells bound to the surface of tubes increased with increasing concentration of C14-cis above about 1 uM. The concentrations of C14-cis at which cell binding was increased as estimated by this assay was similar to that as measured in an *Xcc*-based bioassay (Figure 22, right). Cells of *X. fastidiosa* exposed to DSF in PIM6 minimal medium also appear to adhere to each other more than those in the medium with added DSF. The DSF-medated increase in adhesiveness is readily apparent as cells that clump together when visualized microscopically (Figure 23). Clearly, exogenous DSF stimulates an adhesive state in *X. fastidiosa.* The *X. fastidiosa*-based cell binding assay therefore appears to be a very valuable and rapid method by which DSF response in *X. fastidiosa* can be assessed.

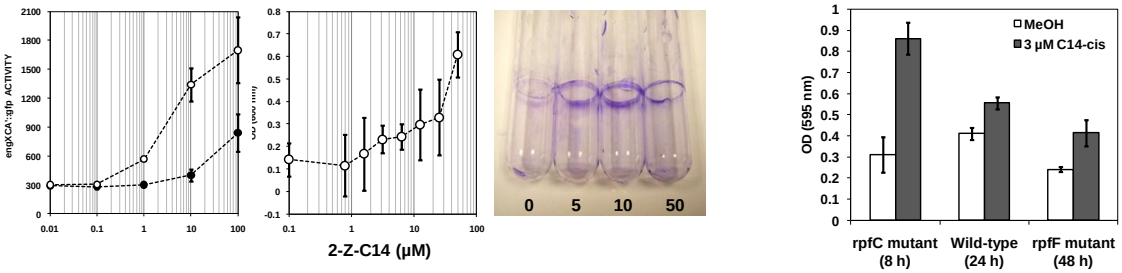


Figure 22. (left 3 panels). Binding of cells of *X. fastidiosa* to the walls of glass tubes at the air-medium interface in cells grown in PIM6 medium containing up to 50 micromolar C14-cis as visualized by crystal violet staining (right panel). The quantification of cell binding as measured by crystal violet absorption is shown in the center panel for the cells. The responsiveness (gfp fluorescence) of an *Xcc*-based biosensor grown in PIM6 medium containing up to 50 micromolar C14-cis is shown in the left panel. (right panel) Quantification of attachment of cells of the RpfC mutant, wild type strain, and RpfF mutant of *X. fastidiosa* to the walls of glass tubes to which 3 uM C14-cis had been added when assayed with a crystal violet binding procedure.

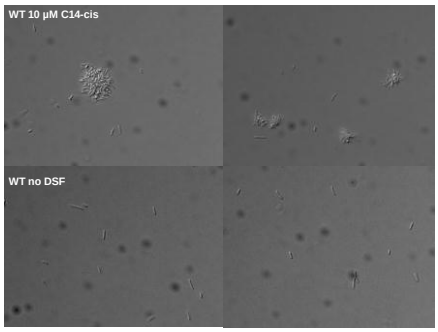


Figure 23. Microscopic depiction of cells of wild type *X. fastidiosa* grown in PIM6 medium for three hours to which 10 uM C14-cis had been added (top panels) and cells grown in PIM6 medium without added DSF (bottom). Note the cellular aggregates found in the top panels in the presence of DSF.

Considerable work was done to address the best use of the various biological sensors for DSF in intact plant tissue. Our work continues to reveal that DSF is either destroyed upon disruption of plant tissue, or that competitor molecules are released upon disruption of plant tissue that makes the detection of DSF impossible. For that reason, we have focused on the use of intact plant tissues for the detection of DSF. DSF can be readily detected in intact tissues. For example, leaf tissue embedded in an agar matrix releases sufficient DSF that the *Xcc*-based rpfCGF biosensor can detect its presence (figure 24). Further work with this DSF sensor and the PhoA-based *X. fastidiosa* proved to be very useful in detecting DSF in both transgenic plants (Figure 4).

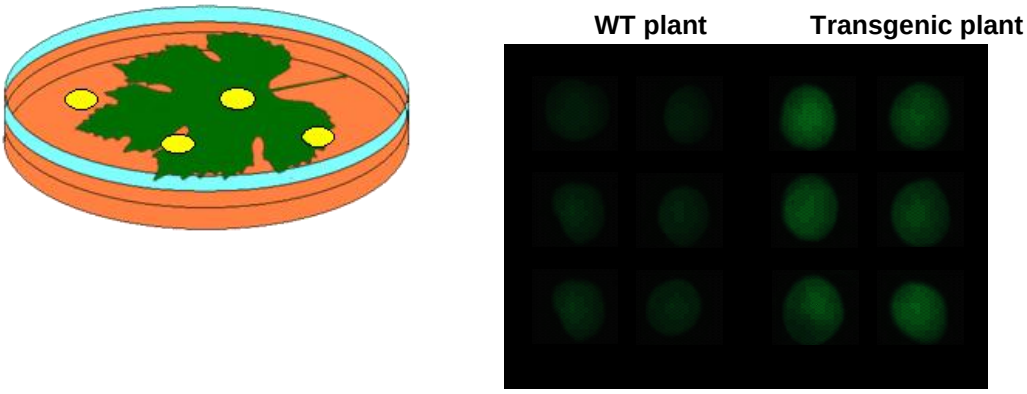


Figure 24. Assay for DSF content of intact plants performed using the *Xcc* rpfCGF DSF biosensor. Contact grape leaves are embedded in agar and cells of the biosensor applied as spots above the leaf tissue (left panel). (Right panel) GFP fluorescence exhibited by this biosensor that had been placed above either wild type plants (left) or above these of transgenic freedom expressing the *X. fastidiosa rpfF* gene (right).

Further evaluation of the behavior of *Xylella fastidiosa* in DSF-producing grapevines. Our studies of DSF-dependent traits in *Xylella fastidiosa* indicate that elevation of DSF in transgenic plants should increase the adhesiveness of the pathogen, thereby reducing its potential to move throughout the plant. To better quantify these parameters, we have made detailed measurements of appearance of disease symptoms in transgenic Freedom grape transformed with the *rpfF* gene from *Xylella fastidiosa*. Previous studies had shown these plants to be more resistant to disease than unmodified Freedom. Temporal measurements of disease severity reveal that initially both wild type and DSF-producing grape had similar incidence of symptomatic leaves (about two leaves per plant)(Figure 25). After approximately 7 weeks after inoculation, however, the incidence of symptomatic leaves increased rapidly in wild type plants to over 12 leaves per plant by week 11, while the number of symptomatic leaves in the DSF producing plants remain low. These results strongly support the model that *X.* fastidiosa could move passively short distances (15 cm or less) and neither plant however, active movement was restricted in the DSF producing plants, limiting infection of only those leaves found within about 15 cm of the point of inoculation. Further work was conducted to determine Continuing work was conducted to determine the adhesiveness of wild type strains of *Xylella fastidiosa* to both wild type and DSF producing Freedom using the cell release assay discussed earlier. A much smaller proportion of cells are released from the DSF producing plants, consistent with the model that cell adhesion is stimulated by elevating DSF levels in transgenic plants.

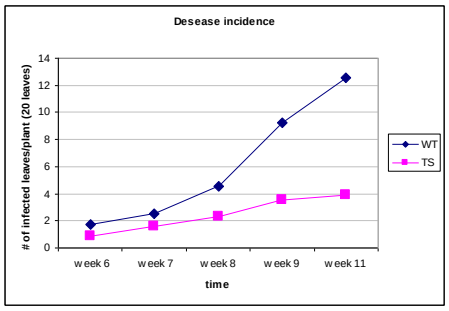
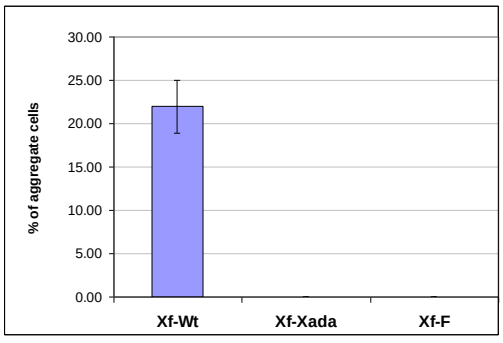


Figure 25. Incidence of leaves exhibiting symptoms of Pierces disease on wild type Freedom grape (blue) or transgenic DSF-producing Freedom (red) assessed at various times after inoculation with *Xylella fastidiosa*.

Investigation of adhesins produced by *Xylella fastidiosa*. Considerable evidence links DSF induction of various membrane-bound proteinaceous adhesins with the attachment of *Xylella fastidiosa* to various surfaces. In this reporting period we have investigated more thoroughly the role of XadA in the behavior of *Xylella fastidiosa*. XadA apparently plays a unique role in *Xylella fastidiosa* because of its propensity to be secreted from cells, apparently in membranous vesicles. Furthermore, while DSF accumulation appears to induce the synthesis of XadA, such accumulation appears to suppress release of membranous vesicles from cells of *Xylella fastidiosa*. We thus have developed a model that XadA, because of its adhesive properties, might adhere to plant surfaces. Cell-free forms of XadA thus might coat pit membranes and other plant surfaces. XadA might also enable cells of *Xylella fastidiosa* to adhere to each other. On the other hand, if XadA was retained in cells, then cells and cells might be more adhesive. We thus assessed the relative adhesiveness of XadA towards various surfaces to better understand its role in the biology of *Xylella fastidiosa*.

To assess the role of XadA in self-adhesion we compared the ability of wild type cells and a xadA mutant of *Xylella fastidiosa* to form cellular aggregates one allowed to sit in either static conditions or and shaken cultures in PIM6 medium. PIM6 minimal medium closely resembles the composition of solutes found in xylem sap. Self-adhesion of *Xylella fastidiosa* was determined by comparing the optical density of cells before and after cells are vigorously suspended by sonication vortexing; aggregated cells D4 act less like than do the same cells dispersed as solitary cells. While a relatively large percentage (10 to 20%) of the cells of a new wild type strain of *Xylella fastidiosa* had formed cellular aggregates in either static or shaken cultures, a much smaller proportion of cells of the *xadA* mutant, or of the *rpfF* mutant had formed aggregates (Figure 26). It thus appears that XadA contributes to the cells to the self-association of *Xylella fastidiosa*.



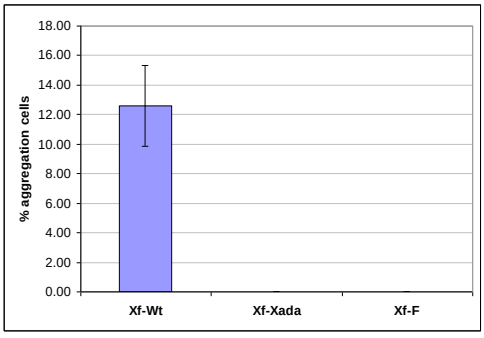


Figure 26: Percent of cells of a wild type *Xylella fastidiosa* strain (left), a *xadA* mutant (center), or an *rpfF* mutant (right) that had aggregated within one week of culturing and a static culture of PIM6 (left panel), or a shaken culture (right panel).

To better isolate the effects of XadA on the adhesiveness of *Xylella fastidiosa* from the contributions of other proteinaceous membrane associated adhesins, the *xadA* gene was cloned and introduced into *Escherichia coli*, a species without abundant adhesins. Cells of the strain were then incubated with wings of the glassy winged sharpshooter, as well as leaves, stems, and petioles of Thompson seedless grape, the and adhering cells washed, and the abundance of the adhering cells quantified by their binding of crystal violet. Substantially more cells of the *E. coli* harboring *Xylella fastidiosa* XadA bound to each of these substrates compared to the parental E. coli strain lacking the adhesion (Figure 27). There did not seem to be a consistently higher binding of the *E. coli* strain harboring XadA to any particular substrate, suggesting that XadA did not show a strong preference for a solid surface. Some preference of E. coli harboring XadA to bind to plastic compared to glass surfaces was however observed (Figure 28). These results indicate therefore, that XadA may preferentially attached to particular surfaces, perhaps plant and insect surfaces. Continuing work is investigating whether the attachment to various solid surfaces is more stringent than to other cells of *Xylella fastidiosa*. This information should support are developing model that the secretion of membranous vesicles harboring XadA (and other adhesins), serve as "decoys" that bind to surfaces such as pit membranes, thereby limiting the adhesion of *Xylella* cells themselves, thus facilitating its movement through the plant.

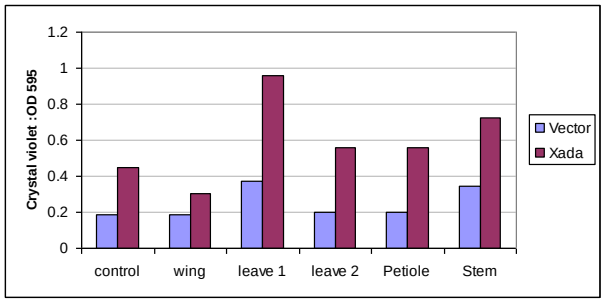


Figure 27: Abundance of crystal violet retained by cells of E. coli harboring XadA from *Xylella fastidiosa* (red bars) or a parental *E. coli* strain (blue bars) that had bound to various substrates. Small pieces (1 cm^2) of various substrates were incubated suspensions of10^9 cells/ml for three hours and then wash before crystal violet staining.

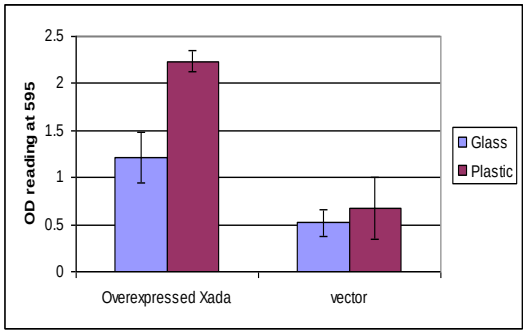


Figure 28: Abundance of crystal violet retained by cells of E. coli harboring XadA from *Xylella fastidiosa* (left) or a parental *E. coli* strain (right) that had bound to either glass surfaces (blue bars) or plastic surfaces (red bars). Small pieces (1 cm^2) of various substrates were incubated suspensions of10^9 cells/ml for three hours and then wash before crystal violet staining.

Publications and presentations:

1. Beaulieu, E., M. Ionescu, S. Chatterjee, K. Yokota, D. Trauner, and S.E. Lindow. 2012. Characterization of a diffusible signaling factor from *Xylella fastidiosa*. mBio 4(1): doi:10.1128/mBio.00539-12
2. Wang, N., Li, J.-L., and Lindow, S.E. 2012. RpfF-dependent regulon of *Xylella fastidiosa*. Phytopathology 102:1045-1053.
3. Almeida, R.P.P., Killiny, N., Newman, K.L., Chatterjee, S., Ionescu, M., and Lindow, S.E. 2012. Contribution of *rpfB* to cell-to-cell signal synthesis, virulence, and vector transmission of *Xylella fastidiosa*. MPMI 25:453-462.
4. Baccari, C., Killiny, N., Ionescu, M., Almeida, R.P.P., and Lindow, S.E. 2013. DSF repressed extracellular traits enable attachment of *Xylella fastidiosa*to insect vectors and transmission. Phytopathology 103: (in press).
5. Presentation at the University of Florida, Department of plant pathology entitled "the complex lifestyles of Xylella fastidiosa coordinated by cell- cell signaling: achieving disease control by pathogen confusion" presented on February 14, 2013.

6. Presentation at the headquarters of the American society for microbiology for "microbes after hours" webcast entitled "the complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion" presented on January 28, 2013.

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. The tools we have developed to better detect the specific DSF molecules made by *X. fastidiosa* will be 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 chemically synthesized DSF molecules might also ultimately be the most useful strategy for controlling disease. The presence of more than one DSF-based signal molecule suggests that perhaps more than one molecule might be needed to achieve changes in pathogen behavior. Our major advances in the development of biosensors to detect the responsiveness of *X. fastidiosa* to signal molecules is a major breakthrough that hopefully will allow us to make rapid progress in ascertaining those transgenic plants most capable of altering pathogen behavior as well as in formulating synthetic molecules suitable for use in disease control. The biological sensors also have proven useful in screening naturally occurring bacteria associated with grape that might also be exploited to produce signal molecules.

Layperson Summary:
X. fastidiosa produces an unsaturated fatty acid signal molecule called DSF. Accumulation of DSF in *X. fastdiosa* 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. We have investigated DSF-mediated cell-cell signaling in *X. fastidiosa* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease. We have investigated both the role of DFS-production by *X. fastdiosa* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *X. fastidiosa* by interfering with cell-cell signaling, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve Pierce's disease control. Elevating DSF levels in plants reduces is movement in the plant. We have found naturally-occurring bacterial endophyte strains that can produce large amounts of DSF but most do not move or grow well inplants. A burkholderia strain does look promsing as a biological control agent as it does grow andmove well in grape. It is being further tested for its ability to move within plants and to alter the abundance of DSF sufficiently to reduce the virulence of *X. fastidiosa*. Given that DSF overabundance appears to mediate an attenuation of virulence in *X. fastdiosa* we have transformed grape with the *rpfF* gene of the pathogen to enable DSF production in plants; such grape plants produce at least some DSF and are much less susceptible to disease. Some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggesting that DSF produced by rootstocks can somewhat move to scions and confer disease control. The chemical composition of DSF itself is being determined so that synthetic forms of this signal molecule can be made and applied to plants in various ways. We have found that the adherence of *X. fastidiosa* to grape tissue is much more tenacious in the presence of DSF, thus restricting its ability to move in the plant, and we thus have developed assays to more rapidly screen transgenic plants for their resistance to Pierce's disease as well the efficacy of chemical analogs of DSF to induce resistance.

Status of funds:

Because some funding from BARD was available to help support one of the postdocs working on this project and thus some of the objectives being pursued here, the funds allocated for this project were used at the rate originally anticipated. In addition, our production of transgenic grape as well as the grafted plants having difference rootstock sizes occurred slower than anticipated, and some of the evaluation of such transgenic plants and grafted plants required work after the projected termination date of this project. A no-cost extension of the project for 1 year beyond the June 30, 2012 end date of this project was granted to enable the completion of the studies of transgenic plants. The funds for the project have now been completely exhausted.

Summary and status of intellectual property associated with the project:
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.