Progess report for CDFA contract number 07-0323

Control of Pierce's Disease by methods involving pathogen confusion

Principal Investigator
Steven E. Lindow
Department of Plant and Microbial Biology
University of California, Berkeley 94720
Cooperators:
Clelia Baccari and Michael Ionescu
Department of Plant and Microbial Biology
University of California, Berkeley 94720

Reporting period: Results reported here are mostly from work conducted July, 2009 to July, 2010.

Objectives and Activities:

We have found that the virulence of *Xf* 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 *Xf* makes a DSF molecule that is recognized by *Xanthomonas campestris pv. campestris (Xcc*) but slightly different than the DSF of *Xcc* (Figure 1). In striking contrast to that of Xcc, *rpfF*- mutants of *Xf* 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 *Xf* 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 *Xf* are consistent with such a model. We found that *Xf* 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 *Xf*. Importantly, rpfF- mutants of *Xf* plug many more vessels than the wild-type strain. We thus believe that *Xf* 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 Xf 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 *Xf*. In this period we have extensively investigated both the role of DFS-production by *Xf* on its behavior within plants, the patterns of gene regulation mediated by DSF, the extent to which other endophytes can modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, obtained genetic transformation of grape and other hosts of *Xf* to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control.

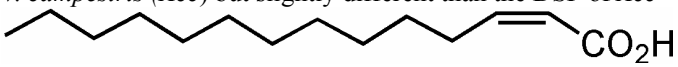

Figure 1

Objectives:
1) Evaluate plants with enhanced production of DSF for disease control
2) Determine if DSF is transferable within plants – eg. whether DSF production in rootstocks can confer resistance to Pierce's Disease in the scion
3) Evaluate enhanced DSF-producing endophytic bacteria for control of Pierce's Disease
4) Investigate DSF-overproducing strains of *X. fastdiosa* as biocontrol agents for Pierce's disease and whether *Xf* strains previously identified with biocontrol potential exhibit an elevated production of DSF
5) Determine if resistance to Pierce's Disease is associated with low rates of degradation of DSF by plants
6) Determine those plant factors that confer induction of virulence genes in *X. fastdiosa* and whether susceptibility to Pierce's Disease is due to differences in induction of virulence factors in the pathogen by the plant

Objective 1. Production of DSF in transgenic plants for disease control.
We have expressed the *rpfF* gene in several different plant species to investigate whether DSF excess can lead to reduced disease caused by *Xf*. In addition to grape, we have transformed genes conferring DSF production into tobacco since this species is colonized by *Xf* and disease symptoms can be produced (Fig. 2). Because transformation of tobacco is much quicker than grape, we have used studies of *Xf* infection of tobacco as a surrogate for studies in grape to speed our assessment of different ways to produce DSF in grape. The various mutants of *Xf* that are hyper and hypo virulence on grape yield similar reactions on SR1 tobacco (Fig. 3).

Figure 2. Symptoms caused by Xf on SR1 tobacco

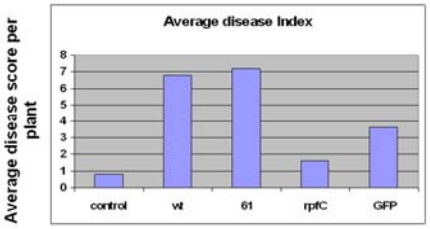

Figure 3. Severity of disease on SR1 tobacco inoculated with WT *Xf* and an *rpfF* mutant (61), an *rpfC* mutant, and a gfp-marked strain.

Table 1 Proportion of leaves of wild-type and DSF-producing SR1 tobacco with marginal leaf scorch after inoculation with *X. fastidiosa*

Treatment	Fraction of leaves
Wild-type SR1	0.52 a
X. fastidiosa rpfF-expressing SR1	0.38 b
Xcc rpfF-expressing SR1	0.27 b
No *X. fastidiosa* control	0.22 c

Further tests of SR1tobacco as a surrogate host to evaluate transgenic expression of *rpfF* as a means to increase DSF abundance in plants were performed. SR1 tobacco which had been transformed with the untargeted *rpfF* genes from either *Xf* or *Xcc* were inoculated with *Xf* ; the incidence of disease was dramatically reduced in *rpfF*-expressing SR1 compared to untransformed tobacco (Table 1). Some of the more mature leaves on the base of the plant had exhibited leaf scorching even on uninoculated plants (Table 1), suggesting that the extent to disease control conferred by expression of *rpfF* was much greater than 50%.

Grape has been transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis with a non-targetted *rpfF* construct. These plants produced only very low levels of DSF but are MUCH less susceptible to Pierce's disease (Fig. 4). While *Xf* spread throughout non-transformed plants causing disease on petioles located great distances from the point of inoculation, disease was observed only very close to the point of inoculation in *rpfF*-expressing plants. We thus expect to find that *Xf* is limited in its movement in plants having even higher levels of DSF due to the expression of *rpfF*, in a manner similar to what we have observed in DFS-overproducing strains of *Xf*.

We have recently transformed tobacco and *Arabidopsis* with an *rpfF* gene that has been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of RpfF in the current transformed plants. Assay of DSF in transgenic SRI tobacco plants-where the RpfF is targeted to the chloroplast, indicates that the DSF levels as well as expression of *rpfF* are much higher as compared to the plants in which the RpfF is expressed in the cytosol. Transcription analysis of the chloroplast targated *rpfF* transformed plants indicates high level expression of the *rpfF* gene (Fig. 5). We have generated seeds from the transgenic SRI tobacco plants and we are conducting pathogenicity assays with *X. fastidiosa* comparing these enhanced producing plants with normal and untargeted RpfF plants.

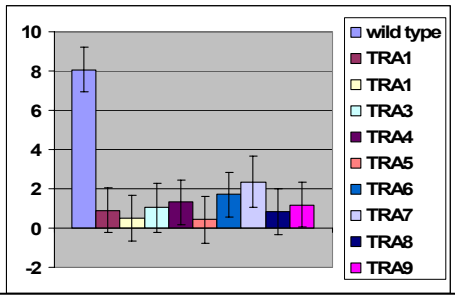

Figure 4. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the r*pfF* gene encoding DSF production and inoculated with *Xf*.

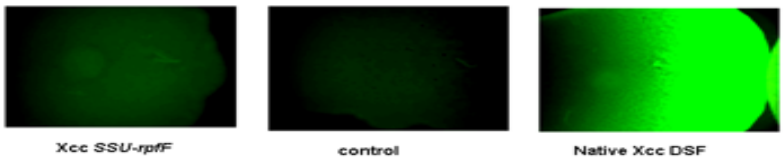

Figure 5. DSF extracted from transgenic tobacco SR1 plants harboring a chloroplast targeted RpfF (left) or from WT tobacco (center) or purified DSF from *Xcc* (right). DSF is spotted on a paper disc on the right side of each image and the *Xcc* DSF bioindicator is to the left. gfp fluorescence is evidence of DSF

Further tests of the efficacy of chloroplast targeting of rpfF implants were preformed by evaluating DSF production in transgenic Moneymaker tomato. Substantial levels of DSF could be detected in the chloroplast-targeted tomato and sufficient amounts of DSF were present to alter the behavior of *Xanthomonas campestris pv. vesicatoria* (*Xcv*) that was inoculated onto leaves. While an average of 323 lesions formed when *Xcv* was inoculated onto normal tomato, 570 lesions formed per leaf on the DSF-producing plants, a finding expected if DSF was present since virulence of *Xcv* is enhanced by DSF. We have also initiated transformation of grapes with a chloroplast targeted *rpfF* construct. Although RpfB is not required for DSF synthesis in *Xf*, 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. We expected 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 can complement the virulence of the non pathogenic *rpfF* mutant of *Xcc* (Table 2). Importantly, transgenic plants expressing both *rpfB* and *rpfF* were more susceptible to the *rpfF* mutant of *Xcc*, indicating enhanced DSF levels. Given this evidence of enhanced DSF production in transgenic *Arabidopsis*, and recent results with similarly-transformed tomato.

Table 2. Disease severity from topical application of bacteria varying in DSF production to *Arabidopsis*. Bacteria were inoculated on different *Arabidopsis* genotypes transformed with *rpfF* or with both-*rpfB* and *rpfF*

Arabidopsis genotype	*Xcc* strains	
	Wild type	*rpfF*-
Col (WT)	++++	-
rpfF transformed	++++	+
rpfF & *rpfB* transformed	++++	++

Transgenic grapes (Thompson seedless) harboring the chloroplast-targetted rpfF from X. fastidiosa were recently completed at UC-Davis and the plants have now been propagated at Berkeley to enable sub-cloning of enough plants for disease assays and for grafting experiments. As of October, 2009 we had generated enough sub-cloned plants of many transformed plants to inoculate them with *X. fastidiosa*. Disease progress was monitored during the wintger months in the greenhouse when symptoms are harder to visualize due to the poor growth of the grape. Initial results revealed that a large difference in apparent disease susceptibility was seen among the different transformed plants (Figure 6). Some plants exhibited as much as 3-fold more disease symptoms than others. The most disease-resistant plants are being re-assessed starting in about April, 2010 dureing the spring growing conditions when more reliable disease assays can be performed.

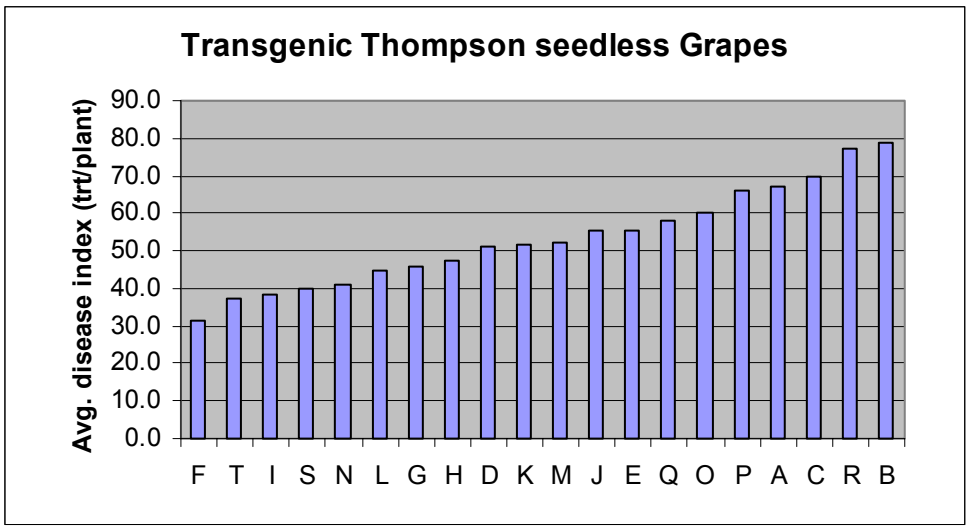

Figure 6. Disease severity of different lines of Thompson seedless grape transformed with the chloroplast-targeted *rpfF* gene from *Xylella fastidiosa*.

We also have inoculated the chloroplast-targeted rpfF plants with a gfp-marked strain of *X. fastidiosa* to assess differences in its movement within the DSF-producing and normal Thompson seedless grape using fluorescence microscopy. Ten Transgenic Chloroplast-targeted rpfF Thompson seedless plants, (which was a mix of several trnasfomred lines) and ten non-transformed Thompson seedless were inoculate with a 10^8 cells/ml of the gfp marked wild type *X. fastidiosa*. Grapes were stem inoculated and inoculation sites were marked with tape. Plants were kept in normal conditions in the greenhouse. At eight weeks after inoculation one cm stem segments were sampled at 10,60,120, cm distal from the point of inoculation, and *X. fastidiosa* populations were determined by culturing and CFU/gr populations were estimated via dilution plating. While the population size of *X. fastidiosa* in the rpfF-transformed lines were similar to that in untransformed lines near the point of inoculation, population sizes were about 10-fold lower in the rpfF-transformed lines at more distal sites on the vine such as 120 cm from the point of inoculation (Figure 7).

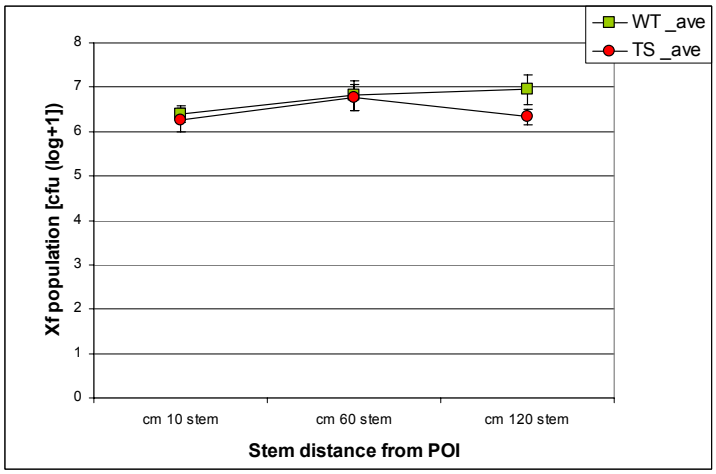

Figure 7. Population size of *X. fastidiosa* in wild type Thompson seedless grape (squares) or transgenic rpfF-expressing grape (circles) at different distances from the point of inoculation.

Microscopy was also carrying out at the same sampling sites to assess the frequency with which xylem vessels were colonized by *X. fastidiosa*. We recorded as positive any vessel harboring *X. fastidiosa* irrespective of whether they harbored few cells or many cells. An average of five stem cross sections were examined for each sampling distance from the point of inoculation on each plant (Figure 8). The proportion of vessels of the *rpfF*-transformed grape that were colonized by *X. fastidiosa* was only about 50% that in non-transgenic lines, suggesting that the movement of the pathogen through the plant was inhibited by expression of *rpfF* and thus production of DSF in the plant. It was also noteworthy that the incidence of vessel colonizaiotn varied greatly between transgenic lines evaluated, with some lines having a similar incidence of colonization as the wild-type line while others having very little colonization.

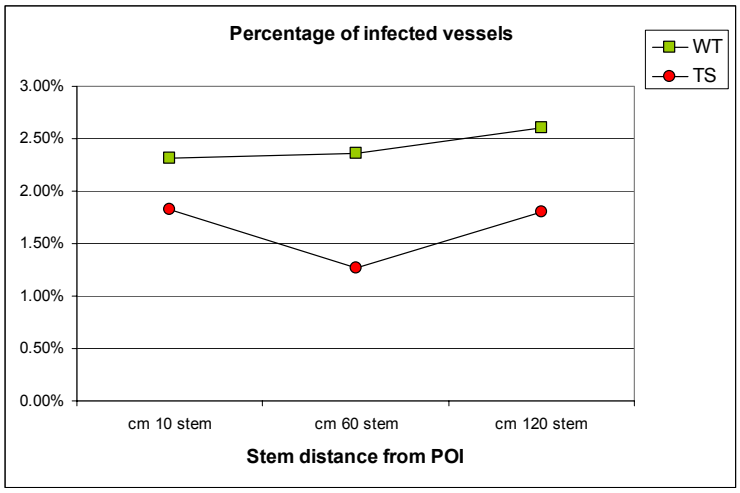

Figure 8: Average of *X. fastidiosa*- infected vessels per grape stem cross section in transgenic rpfF-transformed Thompson grape (red circle) and in non-transformed Thompson seedless in (green square).

To evaluate whether the differences in colonization of *rpfF*-transformed grape was due to differences in DSF production in the different transgenic lines, we estimated DSF production using an improved *Xcc*-based DSF biosensor developed in this project period. Macerates from stems of same plants assessed for vessel colonization were evaluated with the DSF sensor. While no DSF was detected by the biosensor in any wild-type plant, DSF was clearly apparent in plant #3 and plant #5 for the *rpfF*-transformed line. (Figure 9). Interestingly, plant #5 had the lowest incidence of colonization by X. fastidiosa .

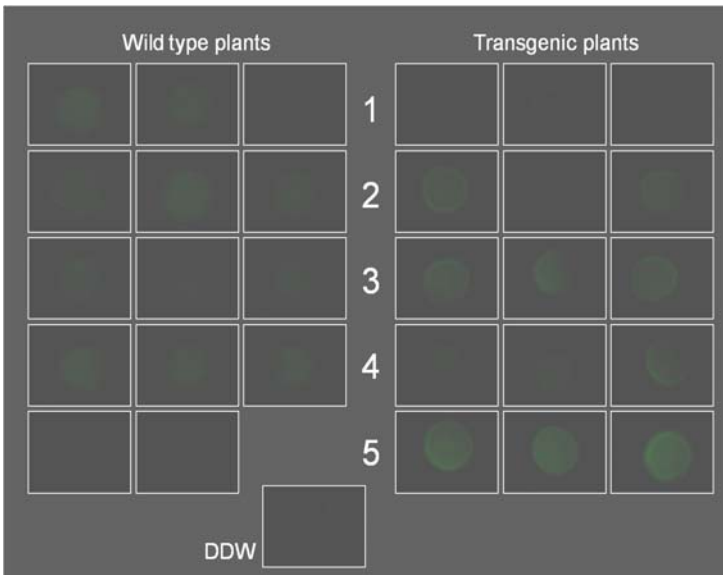

Figure 9. Detection of DSF, visualized as green gfp fluorescence from macerates of leaves from wild-type Thompson seedless (left panel) or different *rpfF*-transformed plants (right panel). In each panel the left-most column is from samples taken near the point of inoculation while the center and right column are from stem segments taken 60 cm and 120 cm from the point of inoculation, respectively.

Direct application of DSF to non-transgenic grape can also confer disease control. While we have very recently tentatively determined the chemical structure and have synthesized the putative DSF of *X. fastidiosa,,* for these studies we used crude ethyl acetate extracts of a DSF-producing *E. herbicola* strain as a source of DSF. The DSF was either topically applied as well as needle inoculated into the stems of grape either once a day before inoculation with *Xf* or weekly. While a single needle application of DSF reduces disease index, a weekly application of DSF into the stem of the plant was much more effective (Fig. 10). These results are exciting in that they suggest that disease control from external applications of DSF might be a practical means of disease control. We are currently repeating these studies using DSF extracted from various surrogate hosts as well as from an rpfC mutant of *X. fastidiosa* to compare the efficacy of these various sources of DSF to determine whether the amount and chemical identity of DSF from these sources are the same. This will be very helpful in our continuing efforts to unambiguously determine the chemical structure of DSF and to justify the synthesis of large amounts of DSF for plant experiments. Grapes have now been treated with these extracts and inoculated with *X. fastidiosa*; initial disease severity measures should be taken in mid-October, 2009.

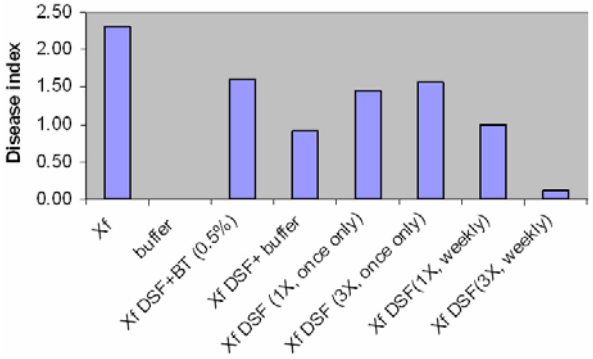

Figure 10. Disease incidence-severity relation (disease index) for grape inoculated with *Xf* and to which DSF was topically applied or introduced into the stem.

Objective 2. Graft transmissibility of DSF. To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing tobacco transformed with the rpfF of *Xf* are used as rootstocks to which normal SR1 tobacco is grafted as a scion (Fig. 7). Over 100 of such grafted plants have now been made, and they have been inoculated with *Xf* to test whether normal SR1 scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization; The average disease severity rating on the normal SR1 tobacco grafted onto the rpfF-expressing rootstock was 0.97 compared to a rating of 0.84 on SR1 grafted onto normal SR1 rootstocks (control); these ratings did not differ significantly. However, the average disease rating on *rpfF*-expressing SR1 tobacco scion grafted onto an *rpfF*-expressing rootstock was only 0.24, which was significantly lower than that of a normal scion grafted onto an *rpfF*-expressing rootstock or onto a normal rootstock. These preliminary results suggest that putative DSF production in the scion is much more effective on reducing the movement and growth of *X. fastidiosa* in the scion than that of the rootstock. This work is being repeated.

Non-chloroplast targeted RpfF-expressing transgenic Freedom grape plants have been propagated in sufficiently large numbers to produce enough plants to serve as rootstocks to test with *Xf* inoculations in larger scale studies. Over 100 such plants have now been propagated and green-grafting of Cabernet Sauvignon has been successfully employed to produce grafted plants with a normal Freedom and a DSF-producing Freedom rootstock (figure 11). Inoculation of the grafted plants was done in late 2008, but the plants suffered injury from pesticide sprays in the greenhouse during the winter, making assessment of disease difficult and the results inconclusive. The studies using grafted plants have now been repeated in 2009. Initial estimates of disease severity indicate that there were about 30% less symptomatic leaves of the normal Cabernet scion when grafted onto a *rpfF*-expressing rootstock compared with plants on a normal Freedom rootstock (Figure 12). 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 are repeating these experiments and will be inoculating the plants in a variety of ways to determine the efficiency of disease control from rootstock modification.

Figure 11. Grafted SR1 tobacco plants (left) and Cabernet sauvignon grape grafted onto DSF producing Freedom rootstocks (right) onto which *Xf* has been inoculated. The plants are as yet asymptomatic.

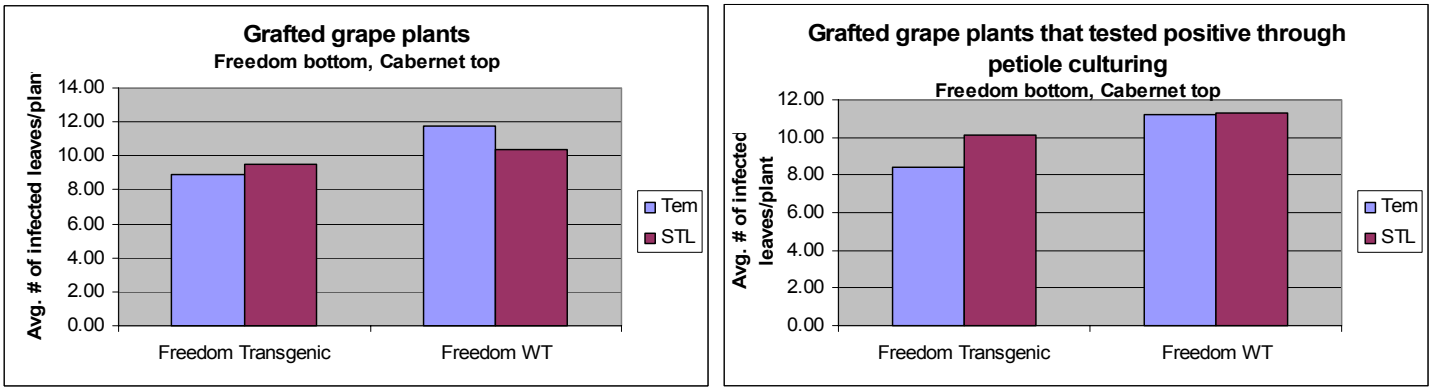

Figure 12. Severity of Pierce's disease (left box) or number of leaves per vine infested with *X. fastidiosa* (right box) of Freedom grape having a Cabernet sauvignon grape scion grafted onto the rootstock noted on the abscissa. Plants were inoculated with either *X. fastidiosa* strain Temecula (blue) or strain STL.

Objectives 3 and 4. Disease control with endophytic bacteria**.** We have been successful in producing large quantities of DSF in endophytes like *Erwinia herbicola* and also in lab strains of *E. coli* (Table 2). We recently were able to transform a putative efficient endophyte of plants, *Rizobium etili* G12 with both the *Xcc* and *Xf rpfF* (DSF biosynthetic gene) and have obtained production of DSF in this strain . This DSF-producing endophyte has been inoculated into grape to determine both its ability to move and multiply within grape as well as its ability to interfere with the disease process. The *R. etli* strain G12 was found to move within grape tissue after inoculation into either the stem or the leaves. When measured 4 weeks after inoculation by puncture inoculation into one site in the stem measurable populations of *R. etli* were seen as far as 50 cm away from the point of inoculation (Fig. 13). While the population size away from the point of inoculation were relatively low in this short time interval since inoculation, this strain clearly has the ability to move within grape; we are excited about this result since no other of several bacterial strains that we have investigated for the ability to move within grape has ever exhibited any ability to move beyond the point of inoculation. Further studies are underway to determine the population sizes to which *R. etli* will grow given more time after inoculation. *R. etli* also has the ability to move within grape leaves and multiply to high population sizes. When applied as a point source to leaves using a penetrating surfactant, cells of *R. etli* could be found up to 3 cm away within 1 week, and population sizes of this strain increased 100-fold within 3 weeks after inoculation (Fig. 14). Studies are continuing to determine the maximum population size that this strain can achieve in grape leaves. The evidence, however, suggests that the bacteria move relatively slowly in grape, and thus such strains would have to be inoculated into grape substantially in advance of the pathogen in order to achieve high levels of disease control. We thus are exploring the possibilities of introducing these bacteria throughout the plant by various means as a way to rapidly increase their population size in the plant, and thus their ability to elevate the DSF levels within the plant. As the use of surfactants to introduce the bacteria into the plant sometimes resulted in some phytotoxicity to leaves, we are exploring an experimental strategy of forcing the bacteria physically into leaves using either pressure or a vacuum in order to achieve leaves that have high levels of bacteria but without any leaf damage that will complicate the interpretation of disease control by such bacteria.

In the most recent reporting period we have greatly increased our efforts to identify naturally-occurring bacterial endophytes of grape that have the potential for producing DSF. For this purpose, we have used the most recently developed DSF-detection strain, an *Xcc* strain in which its own *rpfF, rpfC* and *rpfG* genes have been replaced by their counterparts from *X. fastidiosa*. This biosensor is more responsive to DSF-containing culture extracts form X. fastidiosa than the previously developed Xcc biosensors. In both biosensors DSF sensing is reported due to the induction of an *eng:gfp* reporter gene fusion, and hence over spraying of colonies with the biosensor is a convenient way to detect DSF since its presence is indicated by a green fluorescent halo in the indicator bacteria surrounding the test strain. Using this bioassay we are screening about 800 endophytes that were collected by the lab of Dr. Bruce Kirkpatrick from xylem sap samples from grape. We also are isolating endophytric bacteria from surface-sterilized stems of healthy and PD-infected grape from the field. With the logic that sharpshooters themselves sample the xylem sap of a large number of plants, we have also isolated a variety of bacteria from the mouthparts of sharpshooters collected at various sites in Northern California. These many different bacteria are being assessed for DF production using the improved Xcc DSF biosensor. To date about 1% of the bacterial strain exhibit ability to produce DSF. The factors that control DSF production in these bacteria will be assessed; eg. is DSF production limited to cells as they approach stationary phase. The identify of the DSF producers will be determined by sequence analysis of the 16S rRNA gene, and a collection of the different species of DSF-producing bacteria will be assessed for ability to colonize grape. We also will vacuum infiltrate those strains that are highest producers of DSF into grape in inoculate with *X. fastdiosa* to determine if the DSF producers can confer control of Pierce's disease.

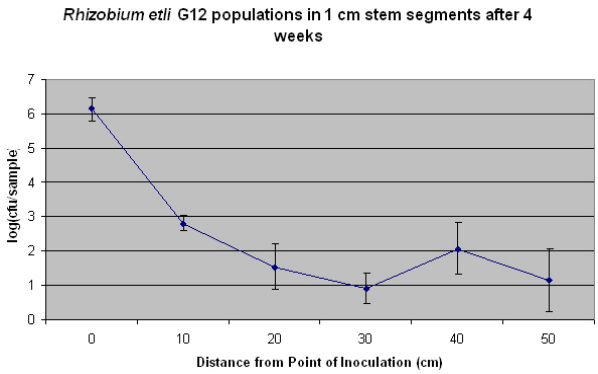

Fig. 13. Population size of *R. etli* in stems 4 weeks after inoculation at one

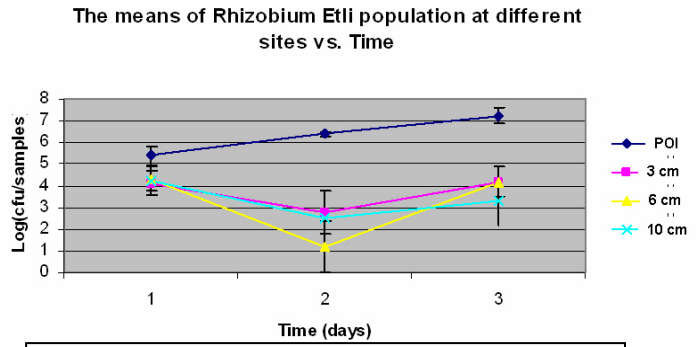

Fig. 14. Population size of *R. etli* in leaves one, two, and three weeks after inoculation at a single point.

Various DSF-producing bacteria were tested for their ability to control Pierce's disease when applied to grape in different ways. DSF-producing *R. etli* were both needle inoculated one or more times at sites near where *Xf* was subsequently inoculated, as well as co-inoculated with *Xf* into grape stems and sprayed onto leaves with 0.5% of the penetrating surfactant Breakthru 1 week before *Xf* was inoculated into stems. The co-inoculation of *R. etli* with *Xf* greatly decreased the incidence of colonization of grape petioles compared to control plants inoculated with *Xf* alone (Fig. 15) while topical application or injection elsewhere in the stem provided little control. We presume that the relatively slow movement of *R. etli* in the stems of plants (Fig. 9) explains why co-inoculation was most effective. *R. etli* was somewhat susceptible to damage from Breakthru and its population sizes were reduced during application with this detergent. We will continue to test different ways in which *R. etli* can be introduced into plants to determine its ability to control PD. We expect that introduction of *R. etli* into stems far in advance of *Xf* will provide much better disease control. RpfC- mutants of *Xf* greatly over-produce DSF so we tested them for their ability to control PD when applied in various ways as discussed above for *R. etli*. The incidence of colonization of grape petioles with *Xf* was greatly reduced when plants were needle inoculated into grape either two weeks before plants were inoculated with *Xf* or when coinoculated with the pathogen (Fig. 16). While the RpfC mutant does not move as well within grape as the wild-type *Xf*, its presence locally in plants can suppress the movement of wild-type *Xf* and thus lead to control of PD. These studies are promising and are being repeated.

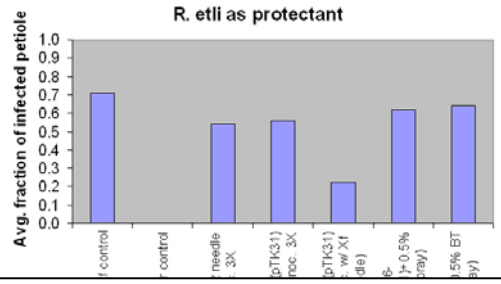

Fig. 15 Incidence of colonization of petiols of grape by *Xf* when plants were treated with DSF-producing *R. etli* in various ways.

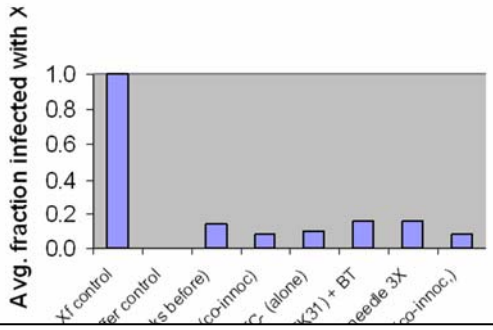

Fig. 16 Incidence of colonization of petioles of grape by *Xf* when plants were treated with RpfC mutants of *Xf* in various ways.

Objective 5. Degradation of DSF by plants.

Development of an *Xcc* biosensor efficient in detecting *Xylella* DSF. For many of the objectives of this project, in addition to the study of DSF degradation in plants, an improved bioindicator for DSF would be very valuable. We are presently using an *Xcc*-based biosensor in which the 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 lower efficiency then the *Xanthomonas* DSF since the two molecules apparently differ slightly. We are investigating a strategy to develop a surrogate *Xcc* biosensor system which will express all the components of DSF signal transduction of *Xf*. This should give rise to a system which is close to DSF signal transduction system in *Xcc*. We have made two different *Xanthomonas* strains in which the endogenous signal synthesis as well as signal recognition system (consisting of the hybrid two component RpfC and RpfG response regulators) has been knocked out. In one of these strains the DSF signal synthase rpfF and the DSF signal sensor RpfC has been knocked out (Fig 17). We have also made an *Xcc* strain in which the

DSF synthase gene *rpfF* has been knocked out in a background of a RpfCHG deletion. These mutants will enable us to express the *Xf* RpfC-RpfG two component system and should serve as a more sensitive surrogate host biosensor. We are also developing a chimeric *rpfC* to introduced into Xcc with the goal of retaining the signal transduction capabilities of the *Xcc* RpfC but to alter the DSF binding domain such that it will more efficiently detect DSF from X. fastidiosa. These studies are described in more detail in the progress report for project 08-0170. We hope to complete the development of this improved *Xcc* biosensor within a couple of months. It then will be applied to the study of *Xf* DSF stability in plant extracts as originally proposed.

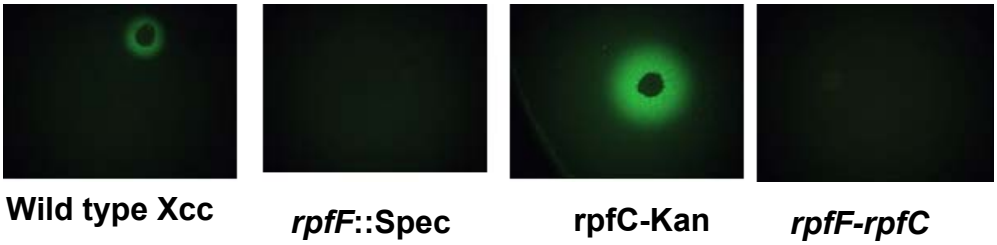

Figure 17. Different *Xcc* mutants constructed to serve as surrogate host for expressing the *Xf* RpfC-RpfG two component DSF signal transduction system. The presently used *Xcc* biosensor 8523/pKLN55 is sprayed over the colonies. Presence of DSF is detected by the GFP fluorescence of the biosensor

We have made much effort in this reporting period to developing methods to use *Xf* itself to detect DSF**.** Among the several genes that we know to be regulated by DSF, those genes most strongly regulated 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. We thus have examined the phenotypes of an *rpfF*- mutant of Xf exposed to different amounts of DSF to determine if it can be used to bioassay for the presence of DSF. Initial results are encouraging. Likewise, cells of the *rpfF*- mutant which are not adherent, 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 18). Thus it appears that we may be able to assess the concentration of DSF in samples using a cell adhesion assay using *Xf* cells, although both assays are time consuming and somewhat qualitative.

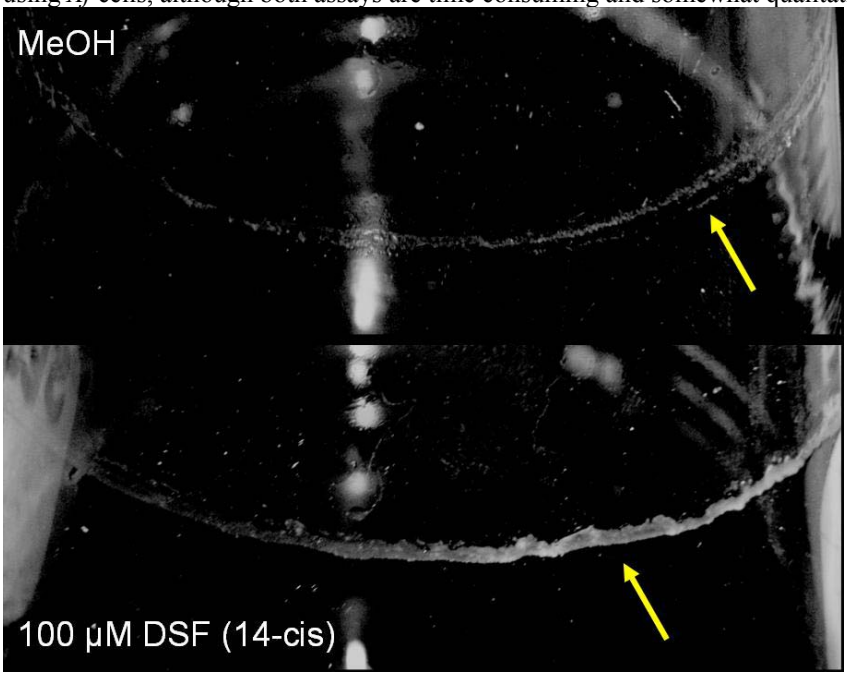

Figure 18. Cellular aggregations that formed at the air-liquid interface in broth cultures of a *rpfF*- mutant of *X. fastidiosa* grown in XFM minimal medium without added DSF (top) or with 100 uM of added C14-cis enoic acid, the presumptive DSF produced by grape strains of *X. fastidiosa*. The yellow arrows note the presence of the ring of adhered cells.

We are also exploring several other methods of assessing the presence of DSF using *X. fastidiosa* itself as a bioindicator. We are taking advantage of the fact that we now know what genes in *Xf* are induced in the presence of DSF. For example, we now know that

gumJ, involved in extracellular polysaccharide (EPS) biosynthesis is strongly induced in the presence of DSF from *Xf* and that DSF-deficient strains produce noticeably less EPS in culture. We are fusing this gene to a *gfp* reporter gene that has been optimized for expression in E. coli (and thus hopefully will also have higher levels of expression in *X. fastidiosa* – see progress report for project 08-0170 for more details) and will introduce it into the genome of *Xf* by homologous recombination to yield cells of *Xf* that will become green fluorescent in the presence of DSF. Likewise, we have cloned the gene encoding alkaline phosphatase from *X. fastidiosa* and are determining if it can be used in in vitro bioassays when fused to DSF-responsive genes in a n alkaline phosphatase-deficient background in *X. fastidiosa*. Such cells should be much more responsive to *Xf* DSF and be useful in assaying biochemical fractions for DSF in the purification processes below and in assaying DSF analogs.

In addition to estimating the transcriptional expression of genes known to be regulated in response to accumulation of DSF, we are also exploring ways of measuring the amounts of gene products (proteins) or EPS that are made in response to the presence of DSF. For example, we are exploring whether we can detect EPS production by *Xf* both in culture and in plants by use of antibodies that recognize the EPS of *Xf*. Such antibodies have recently been described by the group of Bruce Kirkpatrick. Our initial results suggest that DSF-deficient RpfF- mutants of *Xf* exhibit little or no EPS production as monitored by use of fluorescently-labeled antibodies directed against EPS. A gfp-marked RpfF- strain of *Xf* could be used as a DSF detector both in culture and *in planta* by examining co-localization of constitutive GFP fluorescence and red fluorescence when a red-fluorophore-labeled anti-EPS antibody is applied to a sample; GFP fluorescent cells that were not also labeled with the antibody stain would indicate lack of DSF availability while cells that were both GFP and red fluorescent would indicate the presence of DSF.

We are also exploring the use of a simple staining procedure to estimate the abundance of EPS produced by *X. fastidiosa*. For example, our work on the mechanism by which DSF mediates changes in gene expression in *X. fastidiosa* has uncovered the important role of cyclic di-GMP as a so-called second messenger within cells. We have made mutants in gene PD 0279 which encodes a GGDEF domain protein the apparently functions in the synthesis of cyclic di-GMP. The GGDEF mutant produces much more EPS ion culture as apparent from observing colonies, and this difference in EPS can be readily visualized by staining of colony lifts on nitrocellulose filters by staining with Alcian Blue; the GGDEF mutant stains a much darker blue than the wild type, which the over-expression of this GGDEF proteins stains much less intensely (Figure 19). This Alcian blue staining method thus is being pursued as a means to estimate DSF-mediated changes in EPS production in an *rpfF* mutant of *X. fastidiosa* exposed to different amounts of DSF.

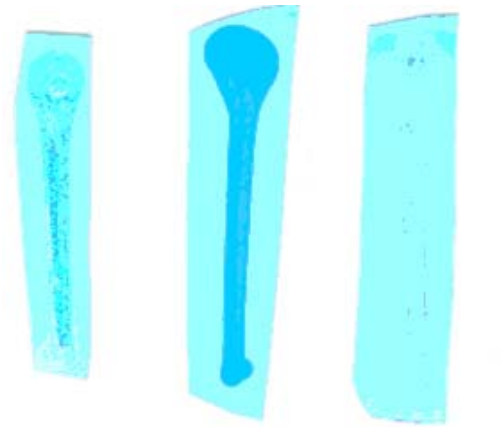

Figure 19. Staining of colony lifts of streaks of WT X. fastidiosa (left), a GGDEF mutant (center) and a GGDEF mutant in which gene PD 0279 has been over-expressed in trans (right) with Alcian Blue.

We have also explored the use of immunofluorescence to detect other DSF-regulated proteins in *X. fastidiosa*. This work is very promising. Antibodies to XadA were provided by Dr. Alessandra Souza who had developed this tool to detect a homologous protein in CVC strains of *X. fastidiosa*. The antibodies cross-reacted strongly to the XadA from grape strains of *X. fastidiosa*. In preliminary experiments we find that cells of an *rpfF* mutant of *X. fastidiosa* harbor very little XadA when grown on XFM minimal medium without added DSF, but that significant amounts of XadA is detected with the antibody when DFS-containing extracts form an rpfC mutant of X. fastidiosa were added (Figure 20). These are very exciting results in that it suggests that such a biosensor would be very useful within plants to monitor the temporal and spatial patterns of DSF production within plants, as well as allow us to monitor the dispersal and stability of DSF that has been applied to plants, or of DSF which has been produced by transgenic plants themselves. We are exploring the use of other antibodies such as those directed against PilC as well for such studies.

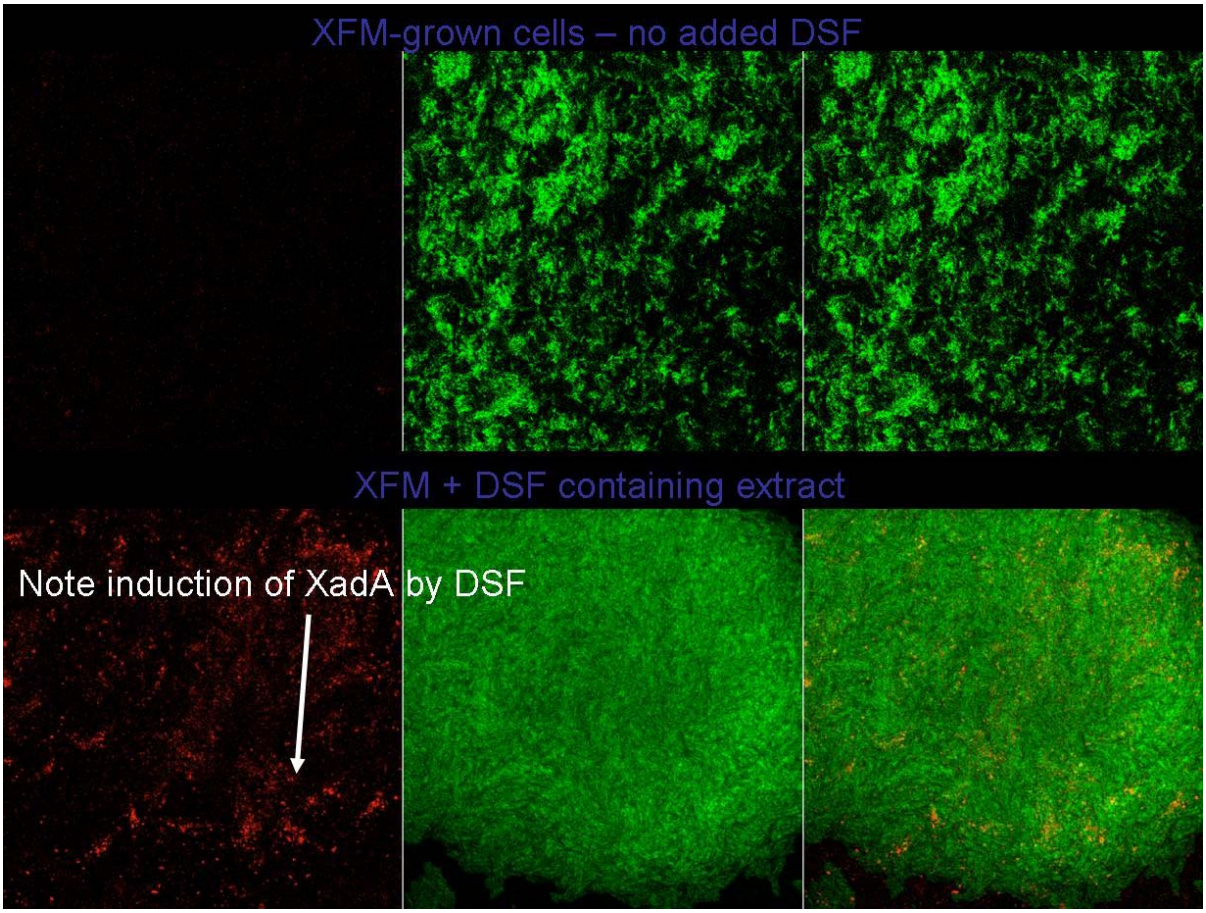

Figure 20 Cells of rpfF- mutant of *X. fastidiosa* grown on a minimal medium and then stained with Syto-9 (green) and probed with rhodamine-labeled anti-XadA antibody (red). Cells were grown in XFM minimal medium without added DSF extract (top) or with added DSF-containing cell culture extract (Bottom).

As noted above, to better assess DSF levels in infected plants and plants treated in different ways to elevate DSF levels, we need a better way to assess DSF levels *in planta*. Furthermore, we need to better understand how DSF levels influence the behavior of *X. fastidiosa*. This information will help us understand how any changes in DSF production or stability that might be mediated by the plant can lead to changes in pathogen behavior. We thus have continued fundamental work to investigate the signal transduction pathways that link DSF production and reception by *X. fastidiosa* with changes in phenotypes. In order to better understand the role the Rpf system and the secondary messenger regulatory nucleotide cyclic-di-GMP in *Xylella fastidiosa* virulence in grape, in addition to the *rpfF* and *rpfC* mutant that have been established before, we constructed an *rpfG*, *rpfGCF* and *clp* (cyclic-di-GMP receptor protein) mutants. These mutants are now being tested for their virulence in grape and for their plant to plant transmission efficiency by sharpshooter insect vectors. It is already clear that the regulator *clp* plays an important role in regulating genes that contribute to the adhesiveness of *X. fastidiosa*, since Clp mutants are extremely sticky in culture. We expect that Clp mutants will be very poorly virulence in plants since the ability to move within plants is inversely related to the adhesiveness of *X. fastidiosa* seen in culture. The effects of DSF perception in *X. fastidiosa* is probably transduced by several downstream regulators such as *clp* in *X. fastidiosa* as it is in *Xanthomonas* species. By identifying clp as a central regulator (downstream from RpfC and RpfG, and one that is likely influenced by a subset of the mixture of DSF species that *X. fastidiosa* likely produces, we can better identify DSF analogs that have a specifically large effect on *X. fastidiosa* adhesiveness, and hence in their ability to mediate control of Pierce's disease.

Since the expression of rpfF from X. fastidiosa in surrogate bacteia and in plants is central to our strategies to alter the behavior of X. fastidiosa by pahotgen confusion, we need to better understand the full function of RpfF in this pathogen. It appears likely that it plays a regulatory role (in conjunction with RpfC and RpfG in addition to its role as a DSF sythase enzyme. To better understand how DSF perception and response occurs in X. fastidiosa we have constructed an *rpfF* allele blocked in DSF production but which should retain its ability to participate in signal transduction. *rpfF*, encoding for DSF synthase, is an enzyme annotated as enoyl-coA hydratase (ECH). The active sites of several ECHs have been characterized with great details allowing us to locate the very same characteristics in *Xylella fastidiosa* RpfF. Its hydratase activity is supposed to be dependent upon two highly conserved glutamate residues (E140 and E160). We constructed an *rpfF* allele in which both glutamates were replaced with alanines and it is blocked in DSF production (Figure 21). This allele will substitute the native *rpfF* allele of Xylella fastidiosa wild type strain and further be tested for role in virulence to grape. The production of an allele of rpfF that can participate in regulatory circuits with RpfC and RpfG but which is not able to act as a DSF synthase will also be extremely useful for use in an improved DSF biosensor in *X.*

fastidiosa or in *Xcc*. A biosensor in which this allele was substituted for the native *rpfF* would eliminate any ability of the bioindicator strain to produce DSF, and hence would greatly increase the sensitivity of the bioindicator. This is being pursued now.

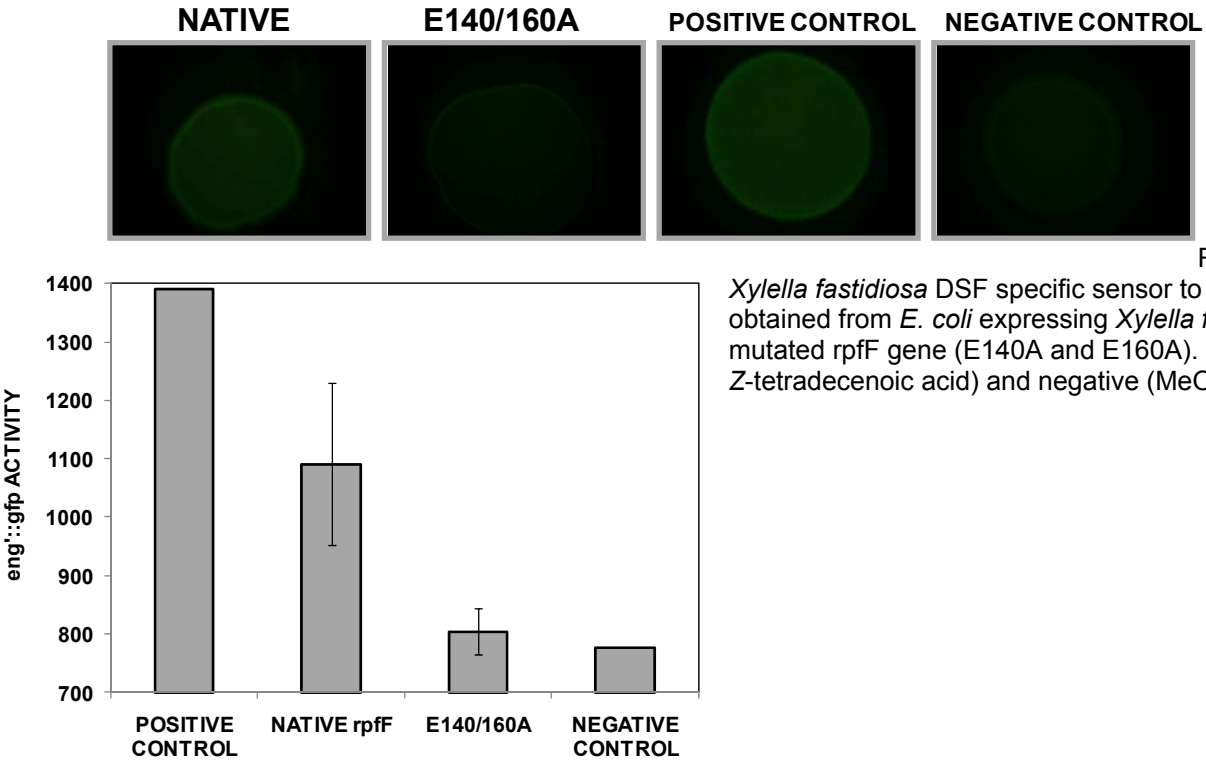

Figure 21: Response of the new *Xylella fastidiosa* DSF specific sensor to DSF extract obtained from *E. coli* expressing *Xylella fastidiosa* native or mutated rpfF gene (E140A and E160A). Positive (100 µM 2-*Z*-tetradecenoic acid) and negative (MeOH) as well.

The proposed mechanism behind our new DSF sensor suggest that RpfF is not only a DSF synthase but also part of the DSF-sensing apparatus. In *Xanthomonas campestris,* RpfC physically interacts with RpfF (He et al., 2006)in *Xcc* and due to that interaction it controls DSF production intensity. Since in our sensor, RpfF seems to repress RpfC interaction with its response regulator RpfG, we hypothesize that RpfF also controls signaling through the RpfCG phosphorelay. In order to address that hypothesis, we first have to show that *Xylella fastidiosa*'s RpfF and RpfC interact with each other as well. For that RpfF, RpfG and the REC domain of RpfC (that contains the proposed RpfF binding interface) were FLAG tagged and are now being purified (Figure 22). Interaction between the purified components will be tested by complex constitution and native gel mobility assay and by far-western analysis using His X 6 tagged RpfF.

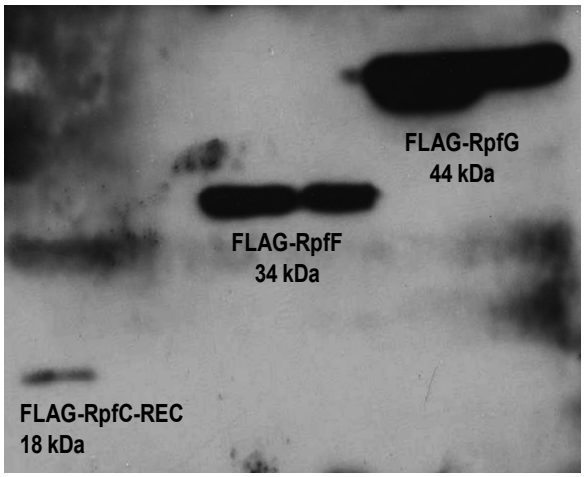

Figure 22: Western blot analysis of FLAG-Rpf components expression in *E. coli* BL21. All gene fusions were cloned into the broad range vector pBBR1MCS-2 under the control of the *lacZ* promoter. Later these vectors will be transformed into *Xylella fastidiosa* in order to detect proteins that interact with Rpf components by co-immunoprecipitation assay.

Objective 6. Plant regulation of *Xf* virulence factors. Before investigating the effects of plant extracts on gene expression in *Xf* we have further examined the complex pattern of gene regulation in *Xf* that is DSF dependent to better understand which virulence genes might be most informative to examine. Analysis of the genome sequence of *Xf* revealed that several genes encoding proteins

potentially involved in intracellular signaling are present. Gene expression of several genes was thus examined in both an *rpfF* and *rpfC* mutant background as well as a double mutant (Table 3). The results have enabled the production of a more complete model of DSF-dependent gene expression in *Xf* (Fig. 21). The several genes identified in Table 3 will be examined by RT-PCR in cultures of *Xf* to which plant extracts have been applied as proposed.

Fig. 23. A proposed model for DFS-mediated cell-cell signaling regulation in *Xylella fastidiosa*.

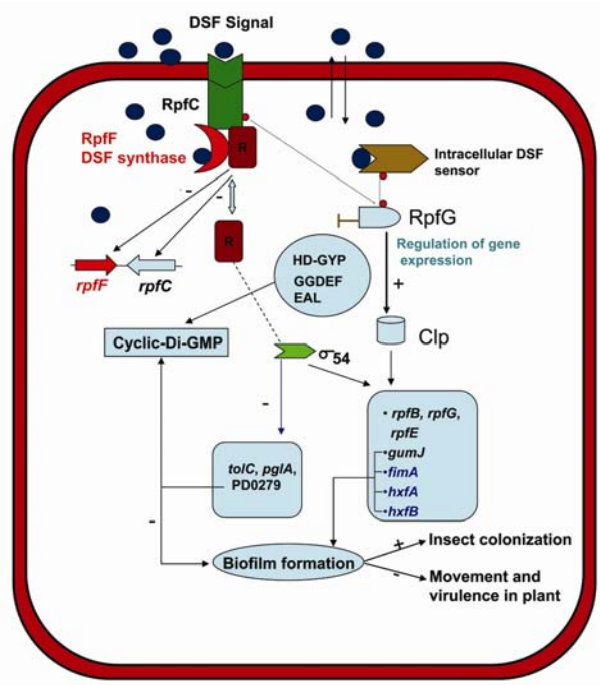

Table 3 Relative quantification of gene expression regulated by *rpfF* and *rpfC* by real-time RT-PCR

Gene name	Fold change ± SE*		
	rpfF⁻	*rpfC*⁻	*rpfF*⁻–*rpfC*⁻
fimA	0.4 ± 0.04	2.15 ± 0.18	0.73 ± 0.19
hxfA (*xadA*)	0.56 + 0.07	3.2 ± 0.1	0.7 ± 0.17
hxfB	0.15 ± 0.05	5.2 ± 0.52	0.49 ± 0.3
gumJ	0.56 ± 0.02	2.6 ± 0.2	0.4 ± 0.04
rpfF	n.d.	6.6 ± 0.71	n.d.
rpfC	4.9 ± 0.4	n.d.	n.d.
rpfE	0.73 ± 0.06	2.2 ± 0.17	0.7 ± 0.12
rpfB	0.6 ± 0.09	2.13 ± 0.07	0.50 ± 0.3
rpfG	0.7 ± 0.06	1.8 ± 0.04	1.13 ± 0.45
(PD0279)	5.3 ± 0.3	3.5 ± 0.23	0.62 ± 0.06
tolC	5.5 ± 0.7	3.8 ± 0.6	0.6 ± 0.09
pglA	1.9 ± 0.17	1.8 ± 0.04	0.7 ± 0.07

*Amount of RNA relative to that in the wild-type *X. fastidiosa* cells is equal to 1.0 and is normalized for cellular abundance by using 16S ribosomal RNA as an endogenous control. n.d. indicates not determined. Standard errors were calculated based on at least two independent experiments.

Intellectual Property issues:
No new intellectual property issues beyond those previously discussed have arisen.

Publications:

Baccari C. and S.E. Lindow 2010. Assessment of the process of movement of *Xylella fastidiosa* within susceptible and resistant grape varieties. Phytopathology (Accepted - in revision).

Chatterjee, S. and S.E. Lindow. 2010. A protein in Xylella fastidiosa having a GGDEF protein involved in cyclic d-GMP levels strongly affects virulence. Molec. Plant-Microbe Interactions (in press).

Research Relevance Statement:
Since we have shown that DSF accumulation within plants is a major signal used by *Xf* 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". This study addresses several ways in which DSF levels can be altered in plants and which method might be most practical. While the principle of disease control by altering DSF levels has been demonstrated, this work addresses the feasibility of how achieve this goal by various means. Our continuing work will address which method is both most practical and efficacious.