Project Title: Exploiting pathogen signal molecules for control of Pierce's Disease

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Objectives:

1) Identification and characterization of low molecular weight signaling molecule (DSF) central to behavior of *X. fastidiosa*

2) Design and synthesize low molecular weight compounds capable of interfering with signal molecule function in *X. fastidiosa*

3) Evaluate efficacy of signal analogs for control of disease and insect transmission of *X. fastidiosa*

Summary of research accomplishments:
The movement of *Xylella fastidiosa* in plants and insect transmission is controlled by a small diffusible signal factor (DSF) that accumulates when cells are at high cell densities. Pathogen behavior can be dramatically changed and disease reduced by altering the abundance of DSF in plants in a form of "pathogen confusion". To enable new strategies of pathogen confusion we have chemically characterized the DSF produced by grape strains of *X. fastidiosa* under the control of the *rpfF* gene as 2-Z-tetradecenoic acid (hereafter called C14-cis). The DSF is structurally related to, but distinct from, the DSF made by *Xanthomonas campestris pv. campestris (Xcc)*. While an *Xcc eng:gfp* based biosensor for DSF can detect as little as about 1 uM of DSF produced by *Xcc*, more than about 100 uM of C14-cis is required for detection. Biological assays for the presence of C14-cis are being developed in *X. fastidiosa*. As the expression of genes conferring type IV pili and thus twitiching are suppressed while those involved in EPS production and production of various cell adhesins are induced in the presence of DSF in *X. fastidiosa*, we are developing *X. fastidiosa*-based bioassays for C14-cis using an

rpfF mutant of *X. fastidiosa* that cannot produce DSF but which can respond to exogenous C14-cis. Twitching motility of the rpfF mutant was suppressed in the presence of as little as 1 uM exogenous C14 cis while cell-cell adhesiveness and cell-surface adhesiveness was enhanced. Preliminary results indicate that *X. fastidiosa* responds to C14-cis concentrations that are at least 10-fold less than that of the DSF produced by *Xcc* suggesting that indicating that the responsiveness of different DSF-producing bacteria is likely species specific; eg. they respond best to the DSF that they produce. Further bioassays based on immunological detection of cell surface adhesins or EPS as well as by quantifying mRNA associated with these genes in *X. fastidiosa* are being developed. Initial results suggest that the responsiveness of *X. fastidiosa* to C14-cis is dependent on the physiological state of cells; young, actively-growing cells appear to respond much less than older cells. Tests of fractionated cell extracts of wild-type cultures of *X. fastidiosa* suggest that other fatty acids produced by *X. fastidiosa* such as a branched chain, C13 fatty acid may also confer changes in expression of genes such as *hxfA* and *fimA*. We are currently exploring the relative activity of such molecules with C14 cis and also determining if such molecules cooperate in regulating gene expression in *X. fastidiosa*. Sufficiently large amounts of C14-cis, as well as the Sodium salt of this fatty acid which is highly water soluble, have been produced and have been used as topical and injected treatments of grape that have subsequently been challenge inoculated with *X. fastidiosa* for tests of disease control. We have designed and synthesized some DSF-analogs and will soon test them for their ability to alter pathogen gene expression and behavior in culture as well as control disease.

Introduction:
Research in the Lindow lab has provided considerable evidence for a diffusible signal factor (DSF) encoded by *rpfF*, which was considered likely to be a fatty acid derivative, that operates in quorum sensing and biofilm initiation in *Xylella fastidiosa* (*Xf*). *Xf rpfF*- mutants, blocked in production of DSF, exhibit <u>increased</u> virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors. We found that *Xf* colonizes grapevine xylem extensively, with many vessels harboring relatively few *Xf* cells and only a minority blocked by *Xf*.. We thus believe that *Xf* has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply and thus the DSF-mediated virulence system in *Xf* constrains virulence when cell density increases to high levels in the plant. Preliminary data indicate that DSF perception is central to the expression of a large number of genes in Xf, including those that are involved in virulence to plants as well as acquisition by insect vectors. DSF accumulation results in the expression of several fimbrial and afimbrial adhesins, resulting in the cells becoming “sticky” in the plant. DSF accumulation also results in the suppression of expression of extracellular enzymes such as polygalacturonases and endoglucanases that are required for erosion of pit membranes and hence movement through the plant. As the pathogen apparently acquires substantial nutrition from the degradation products of the pit membranes, DSF thus suppresses the multiplication in vessels as cell numbers, and hence DSF, accumulate. *Xf* thus appears to coordinate its behavior in a plant to have both an “exploratory” phase (non-sticky cells highly expressing pit membrane-macerating enzymes) that enable it to spread widely through the plant but not be easily acquired and transmitted by insect vectors, that occurs until cells start to become locally abundant. This phase is followed by an “acquisition phase” (sticky cells that no longer express extracellular enzymes) in a subset of the cells that are maximally transmitted by insects. Thus, because the plant lifestyle (as an endophyte) conflicts with its ability to adhere to insects and be transmitted the pathogen apparently takes on a “bi-polar”

lifestyle of two different physiologies that are adapted for plant invasion and insect transmission, respectively. DSF serves as the switch coordinate the plant lifestyle and convert cells into the insect acquisition phase.

Our earlier work demonstrated that the severity of Pierce's disease is reduced when the levels of DSF are increased inhte plant in various ways. For example, the severity of Pierce's disease is greatly reduced when DSF-producing bacteria are co-inoculated with *Xf* into grape or when DSF expression is enhanced in Xf itself. In a direct approach to altering DSF levels in plants we have transformed grape with the *rpfF* gene from *Xf*. Large numbers of clonal *rpfF*-expressing grapes have been produced and inoculated with *Xf* to test for susceptibility to Pierce's disease. In very exciting results, the DSF-expressing grape are MUCH less susceptible to Pierce's disease. The severity of disease was reduced over 10-fold compared to non-transformed plants. 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. A major goal of this proposal is to determine the structure of *Xf* DSF so that it and analogs can be evaluated in a strategy of control of diseases caused by *Xf* that rely on "pathogen confusion". Synthetic DSF and analogs will be made and tested for efficacy in controlling Pierce's disease by introducing these materials on or into the plant in various ways.

Summary of results:

Objective 1. Characterization of DSF. We determined the conditions that led to optimum production of DSF by *Xf* and surrogate hosts. An *rpfC* mutant of *Xf* that is de-repressed for DSF production was cultured in defined media for the harvest of signal molecules. We found that an RpfC- mutant of *Xf* produces about 11-fold more DSF than a wild type strain and that optimum production is on solidified media after growth for 10 days or more. We also expressed *rpfF* from *Xf* in *E. coli* and *Erwinia herbicola* strain 299R under strong promoters. The yield of DSF as detected in *Xcc* from these surrogate hosts was much larger than even from the *rpfC* mutant of *Xf* because of the much larger number of cells that could be produced in culture. We obtained more than 100-fold more DSF than normally produced by a comparable number of *Xf* cells in such surrogate hosts, and found that that *E. herbicola* is a superior surrogate host compared to *E. coli*.

The scheme depicted in Figure 1 was used to isolate and characterize the DSF from *Xf*. Initial characterization of DSF was made from the large amounts of DSF produced in surrogate hosts. DSF was extracted from culture media using ethyl acetate partitioning. Among several fractions from separations of materials made from these crude extracts made by flash column chromatography, the fraction containing organic acids showed higher activity in an *Xcc* DSF bioassay than other fractions above the background. The *Xf* DSF isolated from reverse phase HPLC of the active fraction showed NMR spectral data

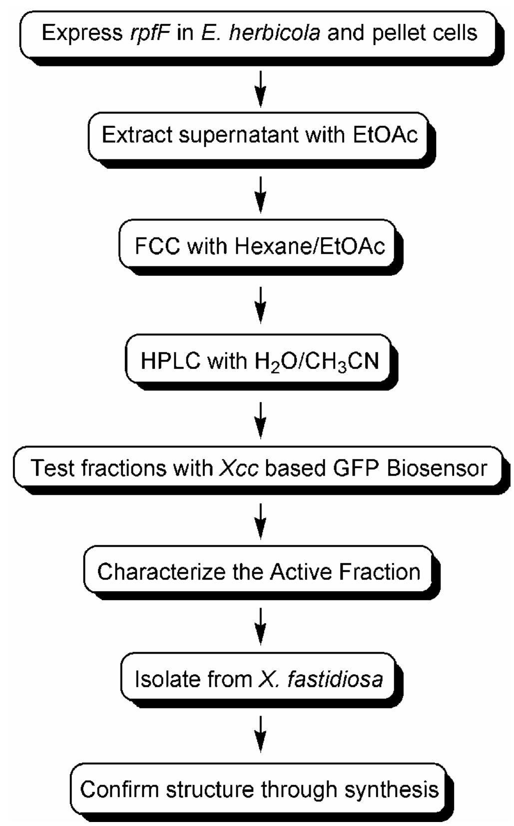

Figure 1. Process by which *X. fastidiosa* DSF as detected in *Xcc* was isolated and characterized.

consistent with a fatty acid containing one site of unsaturation. The DEPT 135 indicates that this is a straight chain acid with no branching. Spectral data suggest the *Xf* DSF has a molecular formula of C14H26O2. The methyl ester was synthesized for GCMS analysis. The methyl ester has a molecular formula of $C_{15}H_{28}O_2$ which means the *Xf* DSF has a formula of $C_{14}H_{26}O_2$. DSF was then extracted from *Xf* and used to verify that the compounds made by *Xf* and the surrogate hosts are the same. *X. fastidiosa* was grown on periwinkle wilt (PW) gel in solid culture. From 200 plates (~4 L volume), we were able to obtain 0.8 mg of the *Xf* DSF. The gel medium was cut into 0.4 x 0.4 cm squares and sonicated with twice the volume of Ethyla acetate. Extracts were purified by flash column chromatography and HPLC as described above. The isolable active compound (DSF) from *Xf* was identified as 2-*Z*-tetradecenoic acid (hereafter called C14-cis). Isolates from an *rpfF* mutant of *X. fastidiosa* strain did not produce C14-cis. The putative *Xf* DSF was synthesized using a Still-Gennari olefination followed by saponification (Figure 2).. The spectral data for the acid isolated from *E. herbicola* match those obtained for the synthetic 2-*Z*-tetradecenoic acid.

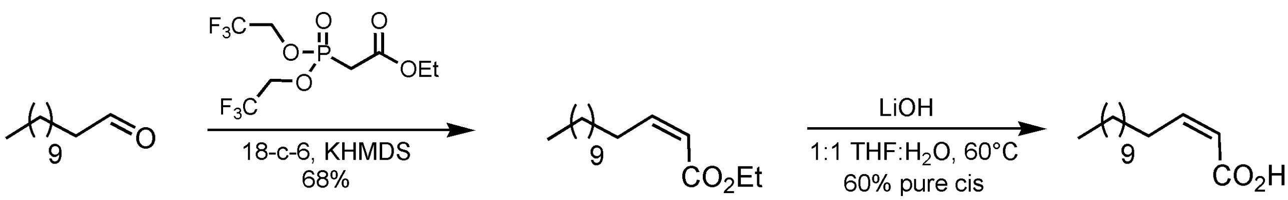

Figure 2. Synthesis of C14-cis

Based on the finding that the DSF from the *E. coli* and *E. herbicola* surrogate hosts harboring *X. fastidiosa rpfF* , and that isolated from *rpfC* mutants of *X. fastidiosa* were the same and that all matched that the synthetic material, we conclude that DSF from *X. fastidiosa* is C14-cis (Figure 3). The putative DSF from *X. fastidiosa* differs somewhat from the DSF made by *Xcc* in that it has a longer, but unbranched acyl chain (Figure 4).

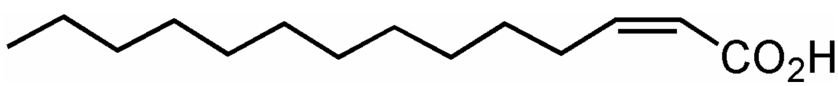

Figure 3. Putative structure of C14-cis, the DSF made by X. fastidiosa that can be detected in *Xcc*.

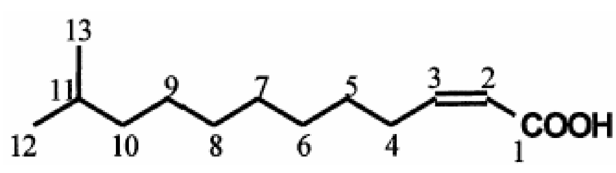

Figure 4. Structure of DSF made by *Xanthomonas campestris*.

The biological activity of C14 cis was assessed using the *Xcc* based biosensor Xcc 8523 (pKLN55). In this biosensor gfp fluorescence conferred by cells harboring an *eng:gfp* reporter gene fusion that is responsive to *Xcc DSF* is measured. While the *Xcc*-based biosensor for DSF can detect as little as about 1 uM of DSF produced by *Xcc*, more than about 100 uM of C14-cis is required for detection. (Figure 5). It is important to note that the biological activity of C14-cis was much less than that of that of Xcc DSF; this was expected as earlier work had revealed that while the *Xcc* biosensor could detect DSF from *X. fastidiosa* the signal was much lower than from a corresponding amount of cells of *Xcc*. It is also clear that the trans form of the C14 enoic acid has no biological activity in this assay in *Xcc* (Figure 5).

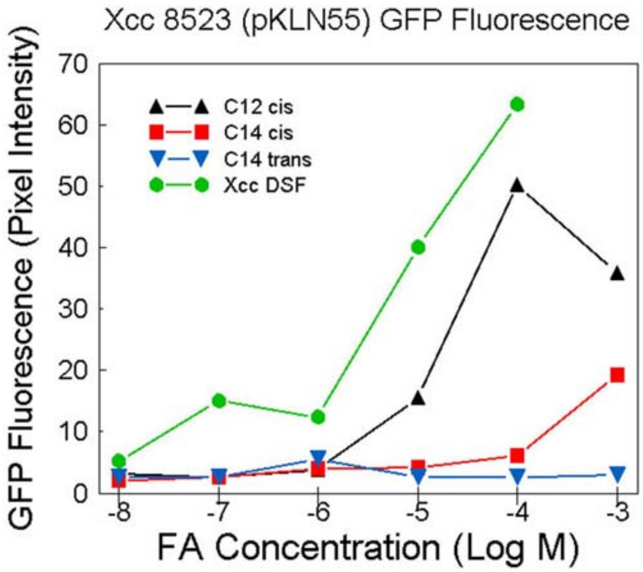

Figure 5. Dose response relationship for DSF from *Xcc* and that from *X. fastidiosa* as well as other related enoic acids.

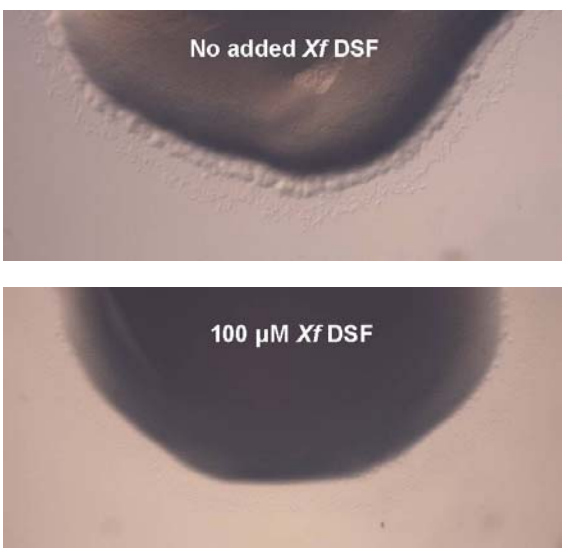

Figure 6. Twitching motility of *X. fastidiosa* evident as a fringe around the coloniy of an rpfF mutant (top) on PWG medium but not around the colony when grown on medium containing C14-cis.

Biological assays for the activity of C14-cis are also being developed in *X. fastidiosa* to ensure that the C14-cis molecule detected in Xcc is also biologically active in *X. fastidiosa*. As the expression of genes conferring type IV pili and thus twitching are suppressed while those involved in EPS production and production of various cell adhesins are induced in the presence of DSF in *X. fastidiosa*, we are developing bioassays for C14-cis using an *rpfF* mutant of *X. fastidiosa* that cannot produce DSF but which should respond to exogenous C14-cis. Twitching motility of the *rpfF* mutant was suppressed in the presence of as little as 1 uM exogenous C14 cis while cell-cell adhesiveness and cell-surface adhesiveness was enhanced (Figure 6). Preliminary results indicate that *X. fastidiosa* responds to C14-cis concentrations that are at least 10-fold less than that of the DSF produced by *Xcc* (Figure 7) suggesting that indicating that the responsiveness of different DSF-producing bacteria is likely species specific; eg. they respond best to the DSF that they produce. More quantitative assays based on expression of the genes involved in twitching motility and in adhesion to surfaces are being developed using quantitative RT-PCR to assess expression of genes such as *hxfA*, *fimA*, and *pilA*. Since it is possible that a functional *rpfF* gene may be needed to properly respond to DSF, the responsiveness of these genes to exogenous DSF is being assessed in both a WT strain as well as an *rpfF* mutant of *X. fastidiosa*. Initial results suggest that the responsiveness of *X. fastidiosa* to C14-cis is dependent on the physiological state of cells; young, actively-growing cells appear to respond much less than older cells. We are continuing to optimize the assay methods for DSF in X. fastidiosa by varying the culture media on which the cells are exposed to DSF and the age of cells that are assayed. Since RT-PCR assays are time consuming and expensive, we are also exploring the use of cell "dot blots" to directly test for expression of EPS and afimbrial adhesins using antibodies obtained from the Kirkpatrick lab.

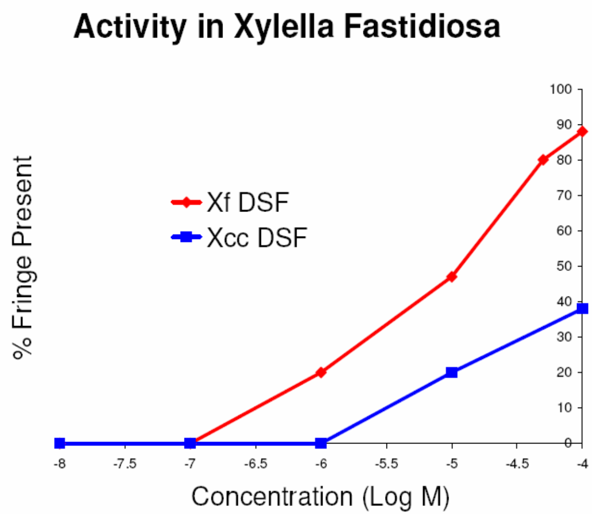

Figure 7. Inhibition of twitching activity of an *rpfF* mutant of *X. fastidiosa* in the presence of different concentrations of DSF from *X. fastidiosa* and *Xcc*.

Further bioassays based on immunological detection of cell surface adhesins or EPS as well as by quantifying mRNA associated with these genes in *X. fastidiosa* are being developed to better assess the activity of DSF and synthetic analogs in future experiments. The current biodetector for DSF that we developed earlier is based on an *eng:gfp* fusion that is expressed in *Xanthomonas campestris* pv. *campestris* (*Xcc*) (it was known that the endoglucanas gene of *Xcc* was induced in the presence of DSF). The *Xcc* DSF biosensor (8523/PKLN55) will detect DSF of *Xf* but we have now shown it to be much less responsive to C14-cis. This may be due to considerable differences in the components involved in DSF sensing like RpfC and RpfG which are hybrid two component sensor and response regulators in Xcc and *Xf*.. We thus are producing improved biosensors by two different means: A) The *rpfC* and *rpfG* genes from *Xf* that are believed to be required for signal transduction in the presence of DSF are being used to replace these homologs in *Xcc*. To increase the sensitivity of Xcc biosensor for Xylella DSF, we will express the whole DSF signal transduction component (RpfC, RpfG and RpfE) of Xf in an *rpfF-* Xcc mutant background. In this strategy we have cloned the entire operon of *rpfC*, *rpfG* and *rpfE* of *Xf* and inserted the operon in a construct containing the flanking sequence of the *Xcc rpf* genomic region. The entire region will be recombined in the *rpfF-* and wild type *Xcc* background. We have already made constructs which can express high levels of *Xf rpfC*. Thus this *Xcc* bioreporter should respond more efficiently to DSF from *Xf*. B) as an alternative, we will take 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 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. We are also exploring the use of other reporter genes that might be expressed better in *X. fastidisoa*. For example, we are developing a variant of gfp that is optimized for translation in *X. fastidiosa*. 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. Alternatively, we 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.

Objective 2. Design and synthesize DSF analogs. We have made several synthetic analogs of C14-cis for testing for biological activity in X. fastidiosa (Figure 8). As these materials have only recently been synthesized the biological activity of most have not yet been assayed. As noted above and as expected, the trans variant of the C14 enoic acid exhibited no activity in any of the biological assays performed today in *Xcc*. In addition to the DSF analogs noted in Figure 8, various halogenated variants will also be synthesized.

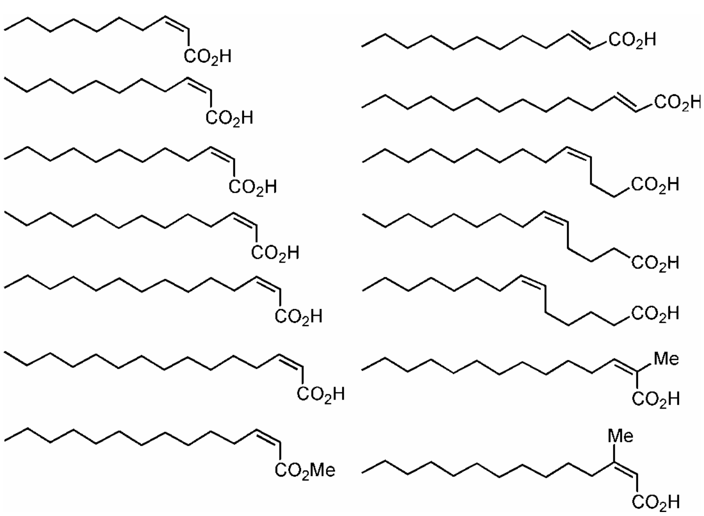

Figure 8. Analogs of the DSF produced by *X. fastidiosa* that have been synthesized to date.

Objective 3. Synthesis of sufficient DSF analogs for *in planta* evaluations. We have synthesized gram quantities of C14 cis as well as the Sodium salt of this fatty acid which is highly water soluble. These quantities are sufficiently large for initial greenhouse studies. These materials are being sprayed onto leaves as well as injected into stems and used as a soil drench in initial studies to determine their efficacy for disease control. The Sodium salt is quite water-soluble and has mild surfactant characteristics which make its topical application to leaves relatively easy. However, the solubility of this material in water is not sufficiently high that milligram quantities could be injected into plants in small(microliter) volumes of water. We thus have injected high concentrations of the acid form dissolved in methanol for such studies. After treatment plants have been challenge inoculated with *X. fastidiosa* and disease incidence is being measured. Initial tests made in Fall, 2008 were inconclusive because of damage to plants that occurred during pest control activities in the greenhouse obscured Pierce's disease symptoms. More extensive studies involving culturing of the pathogen have been initiated.

Publications and reports:

Poster presentation entitled "Exploiting pathogen signal molecules for control of Pierce's Disease" presented by Ellen Beaulieu and Steven Lindow at the Pierces' Disease Symposium held in San Diego, CA, December, 2008.

Poster presentation entitled "Isolation and Characterization of the *Xylella fastidiosa* Diffusible Signaling Factor (*Xf* DSF)" presented by Ellen Beaulieu and Steven Lindow at the Gordon Research Conference on Applied Chemistry, July, 2008.

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 an obvious means of achieving pathogen confusion since direct introduction of DSF via topical application to plants should enable us to alter this signal molecule. While the principle of disease control by altering DSF levels has been demonstrated, this work addresses the feasibility of how achieve this goal using synthetic DSF. Our continuing work will address whether this is a practical means to achieve disease control by pathogen confusion.

Lay Summary

X. fastidiosa produces an unsaturated fatty acid signal molecule called DSF that changes its gene expression in cells as they reach high numbers in plants. Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in *Xf*, but the overall effect is to suppress the virulence of Xf in plants. The DSF produced by grape strains of *X. fastidiosa* has tentatively been characterized as a 14 carbon, unsaturated molecule we will refer to as C14-cis. Both its relatively higher biological activity as assessed in Xcc and *X. fastidiosa* than that of the DSF from *Xcc* and lesser activity in an *Xcc* bioassay is as expected, indicating that there is considerable specificity in the structure-function relationships between different bacterial DSF signal molecules. The production of sufficient *X. fastidiosa* for testing for pathogen confusion has been shown to be possible and we now conducting greenhouse tests in which the synthetic DSF is being applied as a topical spray to plants, as a soil drench, and by direct injection into the stems of plants before inoculation with *X. fastidiosa*. We will be measuring disease severity in treated plants to determine if topical applications of the material can lead to disease control via pathogen confusion.

Status of funds:

There was a slight delay in initiating research on this project due to the delay in arrival of a postdoctoral scientist who has been doing some of the work. Thus some funds will need to be carried forward from year 1 of the project to year 2 to enable us to complete all of the objectives.

Summary and Status of Intellectual Property:

No new intellectual property was developed in this reporting period.