EXPLOITING PATHOGEN SIGNAL MOLECULES FOR CONTROL OF PIERCE'S DISEASE

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ABSTRACT
The movement of *Xylella fastidiosa (Xf)* in plants as well as insect transmission is controlled by a small diffusible signal factor (DSF) that accumulates when cells are at high cell densities. Pathogen behavior can be 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 most abundant DSF produced by grape strains of *Xf* under the control of the *rpfF* gene as 2-Z-tetradecenoic acid (hereafter called C14-cis). Additional related chemicals forms of DSF with biological activity may exist, and we are currently determining their relative activity and if such molecules cooperate in regulating gene expression in *Xf*. The DSF of *Xf* 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 *Xf*. 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 *Xf*, we are developing *Xf*-based bioassays for C14-cis using an *rpfF* mutant of *Xf* 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. Further bioassays based on immunological detection of cell surface adhesins or EPS as well as by quantifying mRNA associated with these genes in *Xf* are being developed. Initial results suggest that the responsiveness of *Xf* to C14-cis is dependent on the physiological state of cells; young, actively-growing cells appear to respond less than older cells. 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 *Xf* for tests of disease control; modest decreases in disease control with these treatments revealed a need to better assess the efficiency with which DSF was introduced into plants. Substantial decreases in disease severity and incidence of vessel colonization with cells of *Xf* were obtained after application of DSF-containing bacterial extracts, revealing the importance of efficient methods of introduction of this signal molecule for disease control. We have also 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.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) produces an unsaturated fatty acid signal molecule called diffusible signal factor (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 *Xf* has tentatively been characterized as a 14 carbon, unsaturated molecule we will refer to as C14-cis. Both its relatively lower biological activity as assessed in a bioassay in the bacterium *Xanthomonas campestris* pv. *campestris* (*Xcc*) than that of the DSF from *Xcc* itself and lesser activity in an *Xcc* bioassay indicates that there is considerable specificity in the structure-function relationships between different bacterial DSF signal molecules. We have focused on developing a bioassay for the DSF made by *Xf* in *Xf* itself so that we can monitor the fate of DSF applied to grapes for the purpose of pathogen confusion. Preliminary studies conducted in the greenhouse in which synthetic DSF and DSF extracted from bacterial cultures was applied as a topical spray to plants, and by direct injection into the stems of plants before inoculation with *Xf* have shown that some reduction of disease symptoms and extent of colonization of grape vessels is achieved by these treatments.

INTRODUCTION
Research in the Lindow lab has provided considerable evidence for a diffusible signal factor (DSF) encoded by *rpfF*, which we have now shown to be a fatty acid derivative, operates in quorum sensing and biofilm initiation in *Xylella fastidiosa (Xf)*. *Xf rpfF*- mutants, blocked in production of DSF, exhibit increased 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 (PD) is reduced when the levels of DSF are increased in the plant in various ways. For example, the severity of PD 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 PD. In very exciting results, the DSF-expressing grape are MUCH less susceptible to PD. 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 PD by introducing these materials on or into the plant in various ways.

OBJECTIVES

1. Identification and characterization of low molecular weight signaling molecule (DSF) central to behavior of *Xf*
2. Design and synthesize low molecular weight compounds capable of interfering with signal molecule function in *Xf*
3. Evaluate efficacy of signal analogs for control of disease and insect transmission of *Xf*

RESULTS AND DISCUSSION

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 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 $C_{14}H_{26}O_2$. 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. *Xf* 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 *Xf* strain did not produce C14-cis. The putative *Xf* DSF was synthesized using a Still-Gennari olefination followed by saponification. The spectral data for the acid isolated from *E. herbicola* match those obtained for the synthetic 2-*Z*-tetradecenoic acid.

Based on the finding that the DSF from the *E. coli* and *E. herbicola* surrogate hosts harboring *Xf rpfF* , and that isolated from *rpfC* mutants of *Xf* were the same and that all matched that the synthetic material, we tentatively conclude that DSF from *Xf* is C14-cis (**Figure 2**). The putative DSF from *Xf* differs somewhat from the DSF made by *Xcc* in that it has a longer, but unbranched acyl chain.

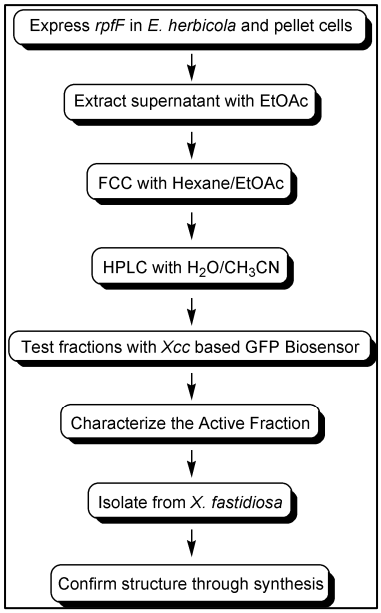

Figure 1. Process by which *Xf* DSF as detected in *Xcc* was isolated and characterized.

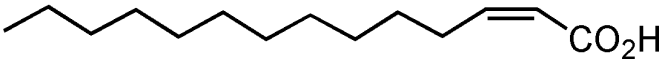

Figure 2. Putative structure of C14-cis, the DSF made by *Xf* that can be detected in *Xcc*.

We are continuing to investigate whether other, related enoic acids are made by *Xf* in addition to the numerically dominant C14-cis. The possibility exists that some of these related enoic acids may be biologically active and perhaps act in synergy with C14-cis to regulate gene expression in *Xf*.

The biological activity of C14 cis was initially 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 3**). 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 *Xf* 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 3**).

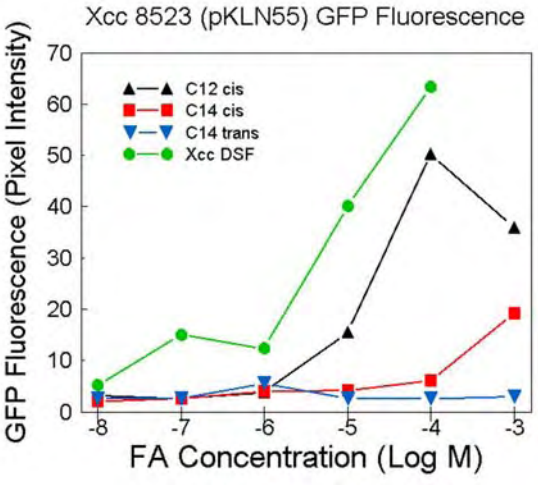

Figure 3. Dose response relationship for DSF from *Xcc* and that from *Xf* as well as other related enoic acids.

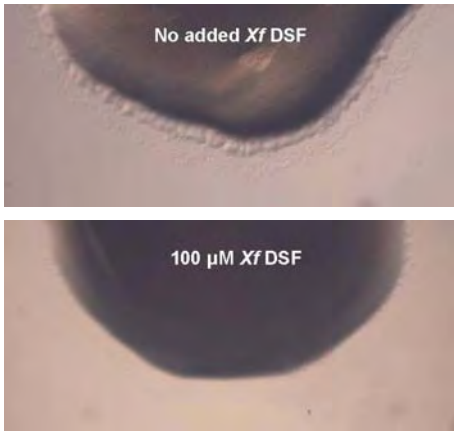

Figure 4. Twitching motility of *Xf* evident as a fringe around the coloniy of an rpfF mutant (top) on PWG medium but not around the colony when grown on medium containing C14-cis.

Biological assays for the activity of C14-cis are also being developed in *Xf* to ensure that the C14-cis molecule detected in *Xcc* is also biologically active in Xf. This is a critical step in also monitoring the translocation and stability of DSF in treated plants (Objective 3). 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 *Xf*, we are developing bioassays for C14-cis using an *rpfF* mutant of *Xf* 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 4**). The twitching assay tends to be highly variable from one assay to another, presumably due to small differences in the physiological state of the *Xf* indicator bacteria or of the agar surface on which twitching is being assayed. 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 *Xf*. Initial results suggest that the responsiveness of *Xf* to C14-cis is dependent on the physiological state of cells; young, actively-growing cells appear to respond much less than older cells. 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 and from Dr. de Souza from Brazil.

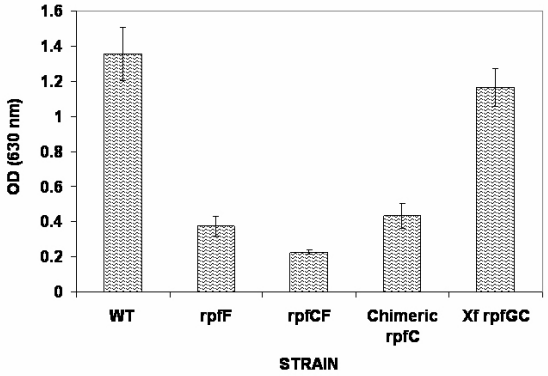

Figure 5. EPS production in various *Xcc* strains as estimated by total sugar content of extracted cells as measured by an anthrone assay

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 endoglucanase 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 have done considerable work on developing an improved DSF biosensor in *Xcc*. Much of our work has focused on producing a chimeric *rpfC* that will recognize DSF from *Xf* but will function in signal transduction in *Xcc*. Our analysis of RpfC from *Xf* indicates that it has a similar cytoplasmic domain as that from RpfC from *Xcc*. In fact, models that predict the 3-dimentional structure of proteins predict that the cytoplasmic domain of these two proteins will have very similar structure.In contrast to the cytoplasmic domain which is predicted to function in signal transduction by phosphorelay, the transmembrane domain of the RpfC of *Xf* is somewhat shorter than that of RpfC from *Xcc*. Given that the transmembrane domain is thought to serve as the DSF binding domain, we hypothesized that an improved DSF biosensor could be made in *Xcc* by replacing its native RpfC with a chimeric RpfC which had the N-terminal transmembrane domain of *Xf* with the cytoplasmic phospho-transfer domain of *Xcc*. This has now been accomplished by forming the hybrid protein with an appropriate fusion point.

The production of EPS in *Xcc* normally increases in response to accumulation of DSF. We therefore assessed the regulation of EPS production wild type and *rpfF* mutants of *Xcc*. There is a large reduction of EPS production as measured by total sugar concentration in extracted cells of an *rpfF* mutant compared to wild type, while EPS content of an rpfC mutant is even lower (**Figure 5**). Introduction of a chimeric *rpfC* into the *rpfFC* double mutant *in trans* restored production of EPS to levels similar to that in an rpfF mutant, suggesting that it functioned in a manner similar to the native *Xcc rpfC*. In contrast, the *rpfC* from *Xf* conferred high levels of EPS production in this mutant background, suggesting that it was inappropriately de-repressed in *Xcc*. Thus the chimeric RpfC appears to be functioning properly in *Xcc*. We are currently determining the levels of EPS production in an *rpfFC* double mutant of *Xcc* harboring the chimeric rpfC in the presence and absence of added DSF; we how to see elevated EPS levels with added DSF.

We have done extensive work to develop alternative reporter genes for use in *Xf*. In our past work we found that gfp and ice nucleation reporter genes were not efficiently expressed, and significant expression of these reporter genes could be detected only when transcription was driven by strong promoters such as the 16S rRNA promoter. We thus have explored the use of two other reporter genes. In one example, we have cloned the gene encoding alkaline phosphatase (*phoA*) from *Xf* and introduced it into a stable plasmid vector suitable for introduction into *Xf* (**Figure 6**). We also have knocked out the expression of the indigenous alkaline phosphatase gene in *Xf* since such a background would be required to measure expression of this gene in response to an environmentally-responsive gene such as those encoding EPS production, adhesins or other genes that are regulated upon increases in DSF concentration. We are in the process of determining if alkaline phosphatase activity can be detected in strains harboring various fusions to this *phoA* reporter gene. We also have obtained variants of a gfp reporter gene that confer much higher levels of expression in *E. coli* than the native gfp reporter gene. We are determining whether fusions to these gfp variants yield sufficient green fluorescence for detection in *Xf*.

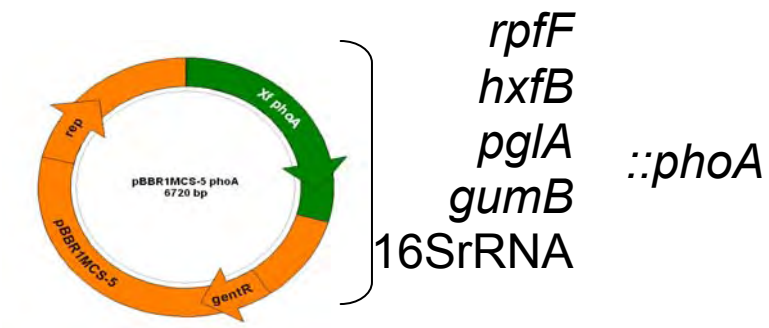

Figure 6 Expression vector harboring *phoA* from Xf being tested for expression in a *phoA* mutant of Xf.

Objective 2. Design and synthesize DSF analogs. We have made several synthetic analogs of C14-cis for testing for biological activity in *Xf* (**Figure 7**). As these materials have only recently been synthesized the biological activity of most have not yet been assayed. We have been waiting to assay them in *Xf* until we have produced a better bioassay for DSF in this pathogen. 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 7**, various halogenated variants will also be synthesized.

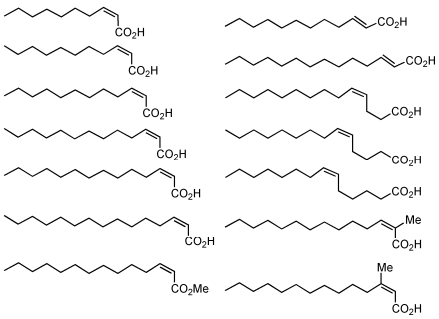

Figure 7 Analogs of the DSF produced by *Xf* that have been synthesized.

Objective 3. Testing of DSF in *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. To understand how best to test these synthetic materials we have used bonafide DSF-containing extracts from both a RpfC mutant of *Xf* as well as from an *E. herbicola* strain harboring the *rpfF* gene from *Xf* and applied them to grape in different ways before inoculating with *Xf*. These materials were injected into stems in initial studies to determine their efficacy for disease control.

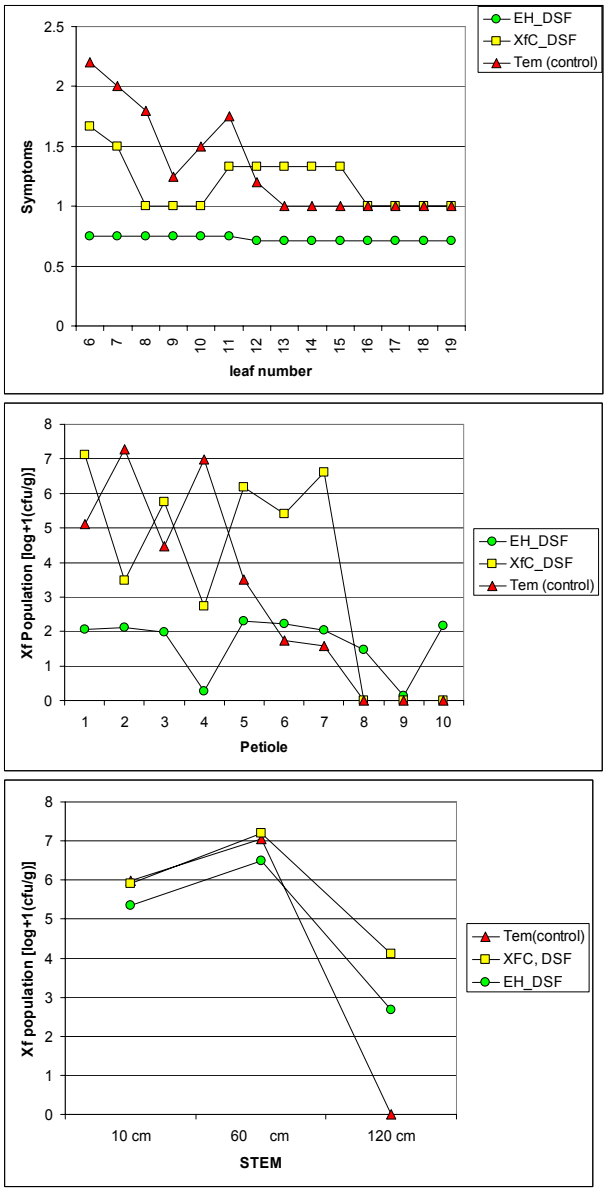

Figure 8. (Top) Severity of disease symptoms on grape injected with a DSF-containing extract from an *E. herbicola* strain harboring *rpfF* from *Xf* (green), from an RpfC mutant of *Xf* (yellow) or with methanol alone (red) before being inoculated with *Xf*. Disease severity of leaves (numbered) at various distances above the point of inoculation is shown. Disease severity was rated as 0= healthy, 1= minor marginal necrosis, 2 = moderate marginal necrosis, and 3= leaves dead. (Middle) Population size of *Xf* in petioles at different distance from the point of inoculation after pre-treatment with DSF as noted above. (Bottom) Population size of *Xf* in 1 cm stem segments collected at different distances from the point of inoculation after pre-treatment of plants with DSF solutions as noted above.

The DSF-containing extracts, particularly those from the *E. herbicola* surrogate harboring *rpfF* from *Xf* substantially reduced both disease severity when injected into stems of grape before *Xf*, as well as reduced the multiplication of *Xf* both in petioles and stems of treated plants (**Figure 8**). The reduction in population size of *Xf* increased with increasing distance from the point of inoculation, suggesting that the DSF reduced the movement of *Xf* within the plant. These results are very promising and have enabled us to initiate further tests to compare different means of introducing synthetic and extracted DSF for disease control.

CONCLUSIONS

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 the abundance of 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 results show promising effects of topical application of DSF in disease control. We have made considerable progress in developing a biological sensor for the DSF produced by *Xf* so that we can better assess methods by which DSF can be introduced into plants and monitor its fate after introduction. Our continuing work will address whether this is a practical means to achieve disease control by pathogen confusion.

FUNDING AGENCIES

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