IMTERIM PROGRESS REPORT FOR CDFA AGREEMENT NUMBER 14-0143-SA

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

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REPORTING PERIOD: The results reported here are from work conducted July 1, 2015 to February, 2017

INTRODUCTION

Our work has shown that *X. fastidiosa* uses DSF perception as a key trigger to change its behavior within plants. Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence they do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grape in various ways to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels – "pathogen confusion". Transgenic, 'Freedom' grape expressing the DSF synthase RpfF from *X. fastdiosa* are much more resistant to disease than the wild type plants in both greenhouse and field trials. It is possible that grape varieties might differ in their ability to produce DSF molecules perceived by *X. fastidiosa*. It will be important therefore to determine whether commercial grape cultivars can all produce DSF species capable of altering pathogen behavior in high amounts if transformed with the DSF synthase. Non-transgenic strategies of achieving pathogen confusion might be preferred by the industry. Our work has shown that RpfF is rather promiscuous and that *X. fastidiosa* can both produce and respond to a variety of unsaturated fatty acids including the common, inexpensive, unsaturated fatty acid palmitoleic acid. We thus are addressing practical issues about how such molecules might be applied to plants for disease control. Using a new *X. fastidiosa* biosensor for DSF in conjunction with such an abundant, inexpensive molecule we can now thoroughly investigate methods by which such a molecule can be directly applied to plants to achieve concentrations sufficiently high in the xylem to alter pathogen behavior and thus achieve disease control. While endophytic bacteria capable of producing DSF species is an attractive strategy, until recently, strains capable of growth and movement within grape could not be found. However, we have now found a *Burkholderia* strain that both colonizes grape and has conferred substantial disease control in preliminary studies. We are investigating the interactions of this endophyte with grape to understand how it is conferring disease control and determine practical methods for its exploitation.

OBJECTIVES:

1) Compare DSF production and level of disease control conferred by transformation of *Xf* RpfF into several different grape cultivars.
2) Evaluate efficacy of direct applications of palmitoleic acid, C16-cis, and related DSF homologs to grape in various ways to achieve disease control.

3) Evaluate the potential for *Burkholderia phytofirmans* to multiply, move, and produce DSF in grape plants to achieve Pierce's disease control.

RESULTS AND DISCUSSION:

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

Note: Since the transgenic plants developed in this objective will be field tested as part of a new project (16-0513-SA "Field evaluation of Pierce's disease resistance of various DSF producing grape varieties as scions and rootstocks", the information in this objective was also reported as background information in the renewal report prepared for that new project.

While Freedom grape transformed with the *X. fastidiosa rpfF* gene encoding the DSF synthase produced DSF species to which *X. fastdiosa* was responsive, considerable evidence has been accumulated that RpfF is a rather promiscuous enzyme capable of producing a variety of DSF-like molecules. For example, we detected the production of C14-cis (*Xf*DSF1), C16-cis (*Xf*DSF2) and surprisingly, even DSF (normally produced only by *Xanthomonas* species) in transgenic RpfF-expressing freedom grape. The various enoic acids that can be produced by RpfF differed substantially in their ability to induce gene expression in *X. fastidiosa* - with those of longer chain lengths such as C16-cis being much more active than those of shorter chain lengths. We have also observed that DSF-mediated signaling in *X. fastidiosa* by active DSF species such as C16-cis can be blocked in the presence of certain other *trans* unsaturated fatty acids. It is therefore possible that in some plants other fatty acid species indigenous to the plant or induced upon transformation of RpfF might interfere with signaling that would otherwise be conferred by the production of C16-cis and other "active" DSF species. To verify that the strategy of production of DSF in RpfF-containing transgenic grape is a robust one, we are comparing the production of DSF species in such a variety of grape cultivars. In addition, it seems likely that targeting RpfF to cellular compartments where the substrates for DSF synthesis may be more abundant could lead to enhanced production of this signal molecule. we have produced constructs which target RpfF to the chloroplast of grape by fusing the small subunit 78 amino acid leader peptide and mature N-terminal sequences for the *Arabidopsis* ribulose bisphosphate carboxylase (which is sufficient to target the protein to the chloroplast) to RpfF. We thus are comparing the amount and types of DSF produced, and disease susceptibility, in transgenic plants in which RpfF is targeted to plastids and in plants in which it is not targeted. Transformation of the various grape varieties is being conducted at the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis. Grape varieties Chardonnay and Thompson seedless as well as the advanced rootstock varieties 1103, 101-14 and Richter are being transformed with the *rpfF* gene from *Xf*. In addition to un-targetted expression of RpfF, we are producing plants in which RpfF is targeted to the chloroplast of grape by fusing the small subunit 78 amino acid leader peptide and mature N-terminal sequences for the *Arabidopsis* ribulose bisphosphate carboxylase (which is sufficient to target the protein to the chloroplast) to RpfF. This RpfF fusion gene product should be directed to the chloroplast where it presumably has more access to the fatty acid substrates that are required for DSF synthesis (chloroplast-targeted). While the genetic constructs were made at UCB, transformation of the various grape varieties is being conducted at the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis.

Our goal is to obtain between 5 and 10 individual transformants for each variety/construct combination. As will be summarized below, it has been both slow and difficult to obtain sufficient numbers of transformants for certain of these combinations. Because the expression of *rpfF* in a given transformant of a given plant line will vary due to the chromosomal location of the randomly inserted DNA, it is necessary to identify those lines with the highest levels of expression. To determine the disease susceptibility of each line they are being grown to a sufficiently large size that vegetative clones could be produced (3 months) and then each cloned plant is being propagated and assessed for disease susceptibility (5 additional months). A goal of at least 12 vegetative clones each of the lines are being produced from green cuttings of plants developing from each transgenic plant selected in the assays above. These plants as well as an untransformed control plant of a given variety (ca. 30 cm high) are being inoculated with *Xf* by droplet needle puncture as in earlier studies. Disease severity is being assessed visually weekly after inoculation. In this process, we are able to identify the transformant from each variety/construct combination that is most highly resistant to PD, and thus suitable for field evaluation.

Over half of the plants from these variety/construct combinations have now been produced at the UC Davis plant transformation facility and have been delivered to Berkeley where they are being propagated and assayed. The following table indicates the number of individual independently transformed plants of each combination

that have been delivered to Berkeley and are in various stages of disease assessment under greenhouse conditions at Berkeley.

Variety	Gene introduced	
	Untargeted RpfF	Chloroplast-targeted RpfF
Thompson seedless	23	2
Richter 110	6	none
Paulsen 1103	6	none
Milardet et de Grasset 101-14	13	none

Certain of the varieties such as Chardonnay have not been successfully transformed at UC Davis. Furthermore, others such as Richter 110 and Paulsen 1103 have proven to be somewhat more difficult to transform than other varieties, yielding fewer transformants than other grape varieties. Although the reason is unclear, the kanamycin resistance determining construct in which the chloroplast targeted RpfF is being delivered has yielded relatively few transformants, with none being recovered for three of the varieties being investigated. These transformations will again be repeated with a fresh Agrobacterium/vector combination. A modification of this vector is also being developed to determine if it will be more successful. Screening of the non-targeted RpfF plants already delivered is underway and testing of these plans for disease resistance should mostly be done by July 1, 2017. The process of evaluating them for disease resistance has been slow because the plants obtained from Davis have been very small and very slow to grow under our greenhouse condition. This has lengthened the time needed to obtain the vegetative clones required for disease susceptibility testing. We have however now obtain sufficient number of plans from each of the 4 newly transformed grape varieties to evaluate the relative efficacy of expression of RpfF, and thus DSF production to achieve disease resistance in these various varieties. Not only will this provide us evidence for the relative effectiveness of DSF production as a disease control strategy and the different grape varieties, but it will allow us to identify the most highly resistant variety for a given variety. A no-cost extension of project 14-0143-SA will be sought if necessary to enable the completion of the laboratory and greenhouse testing of the full collection of transgenic plants to enable them to be established in field trials beginning in 2017, and more likely extending into 2018. Not only must we identify the transformant for a given grape variety harboring a given rpfF gene construct that confers the highest levels of disease resistance, but we must generate grafted plants with the transformed plants serving as rootstocks were grafted plant having a normal scion. The grafting process will add an additional three months to the process of generating plants for use in field studies.

Objective 2: Direct application of DSF to plants.

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

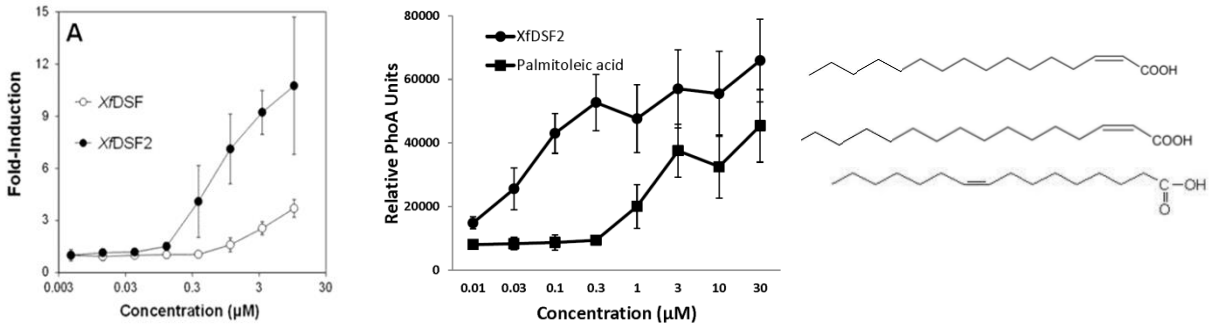

Figure 1. Responsiveness of a PhoA-based *X. fastidiosa* DSF biosensor to different concentrations of *Xf*DSF1 (top molecule), *Xf*DSF2 (middle molecule), and palmitoleic acid (bottom molecule).

While about 8-fold more palmitoleic acid is required to induce gene expression in *Xf* than *Xf*DSF2, it is much more active than *Xf*DSF1 itself. We therefore have conducted a variety of studies to address how such molecules could be introduced into plants in different ways to achieve pathogen confusion. In addition to the use of purified fatty acids we also are evaluating mixtures of fatty acids for their ability to alter the behavior of *X. fastidiosa*. Macadamia nut oil contains a very high concentration of palmitoleic acid (23%). We have saponified macadamia nut oil by treatment with sodium hydroxide to yield the sodium salts of the constituent fatty acids. We find that this fatty acid mixture has DSF signaling activity. Alkaline phosphatase activity exhibited by the *X. fastidiosa Xf:phoA* biosensor increased with increasing concentrations of the mixture of fatty acids in the soap prepared from the saponified macadamia nut oil (Figure 2). Apparently the other saturated fatty acids that would be found in the lipids of macadamia oil do not strongly interfere with DSF signaling of the palmitoleic acid in this soap. This saponified plant oil is thus very attractive as inexpensive sources of DSF homologs that could be directly applied to grape.

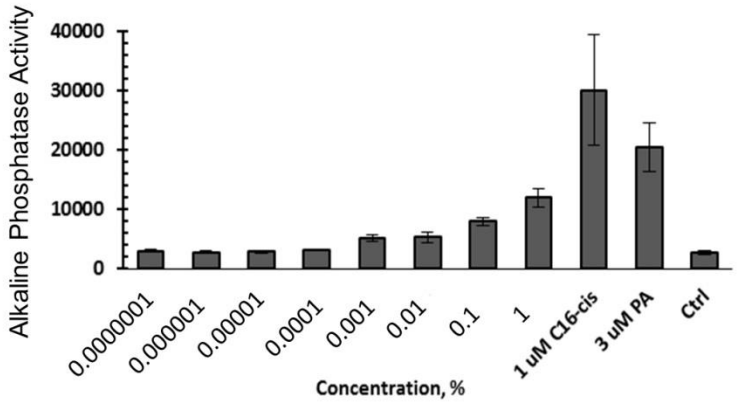

Figure 2. Alkaline phosphatase activity exhibited by the *X. fastidiosa Xf:phoA* biosensor exposed to increasing concentrations of saponified macadamia nut oil as well as 1 uM *Xf*DSF2, 3 uM Palmitoleic acid, or a negative control with no added DSF.

We are investigating several strategies by which direct application of DSF molecules can reduce Pierce's disease. While we will determine the effects of application of DSF homologs on disease severity of plants inoculated with *X. fastidiosa* in some studies, direct monitoring of DSF levels in treated plants is a MUCH more rapid and interpretable strategy of assessing this strategy of disease control. As DSF must enter the xylem fluid in order to interact with the xylem-limited *Xf* in plants we have been assessing DSF levels in xylem sap of plants treated in different ways using a PhoA-based *X. fastidiosa* biosensor. As DSF species are somewhat hydrophobic, a variety of adjuvants have been tested for their effects on enhancing their introduction into plants. For example, detergents and solubilizing materials such as Solutol HS15, Breakthru, Triton X-100, and DMSO and Solutol increase the apparent penetration and dispersal of DSF and its analogs. Solutol and DMSO proved to be rather phytotoxic and therefore were not practical solutions for the production of signaling molecules. The organo-silicon surfactant Breakthru having very low surface tension and allowing spontaneous stomatal infiltration of solutions into leaves not only was not phytotoxic, but it also appeared to be superior to the other agents aiding the entry of signaling molecules. Considerable results have been obtained on the ability of topically applied palmitoleic acid and macadamia nut oil saponification solutions to enter into the plants. Apparent DSF signaling activity was measured using the Xf PhoA-based alkaline phosphatase biosensors noted above. The effectiveness of these agents in introducing palmitoleic acid into grape tissue was assessed by assessing the ability of sap extracted from individual leaves using a pressure bomb to induce the expression of alkaline phosphatase activity in the *X. fastidiosa Xf:phoA* biosensor. These studies reveal that detectable amounts signaling molecules could be introduced into grape leaves one applied as a foliar spray with 0.2% Breakthru (Figure 3). Lesser amounts could be introduced with foliar sprays without this adjuvant. As a registered surfactant for use in agriculture, Breakthru has the potential to be a practical delivery agent. Thus, solutions of fatty acid supplied with this concentration of surfactant appear to bypass the cuticular surface as a means to enter the intercellular spaces and presumably also the vascular tissue. These results using penetrating

surfactants are very promising and will be a focus of continuing work.

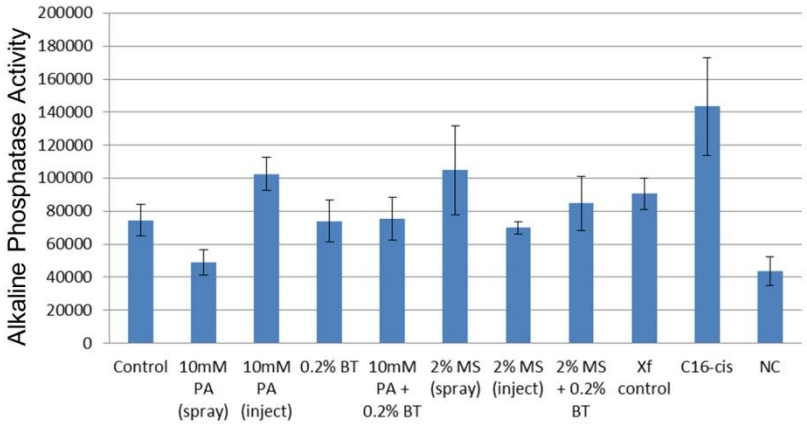

Figure 3. Alkaline phosphatase activity exhibited by 10 µl aliquots of xylem sap extracted under pressure from individual leaves of grape plants treated with 10 mM palmitoleic acid (PA) or 2% macadamia nut oil soap (MS) with 0.2% Breakthru (BT) or without a surfactant as a foliar spray (spray) or a stem injection (inject).

These most promising treatments were also applied to grape plants to evaluate their efficacy in reducing the symptoms of Pierces disease. Palmitoleic acid or macadamia oil soap was applied with various adjuvants two weeks before inoculation with *X. fastidiosa* and at monthly intervals after inoculation with the pathogen. The severity of Pierce's disease was reduced on plants sprayed with a solution of 10 mM Palmitoleic acid as well as on plants in which this fatty acid was injected into the stem. The disease control conferred by a 2% solution of saponified macadamia nut oil was as great as that conferred by purified Palmitoleic acid. The promising results using saponified plant oils are being further pursued as this not only is a very practical but quite inexpensive strategy to achieve disease control. Given that the efficacy of saponified plant oils applied without an adjuvant seem to be as great as when applied with a surfactant, the cost and convenience of using such treatment seems particularly good.

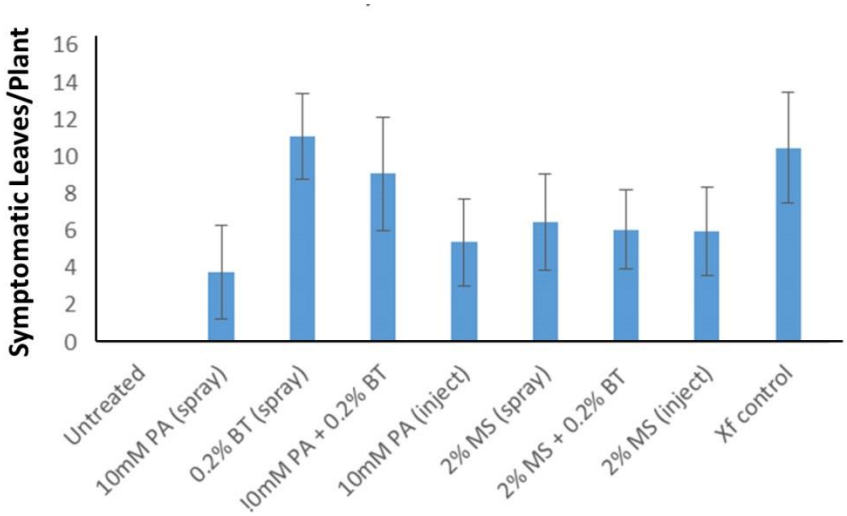

Figure 4. Symptoms of Pierce's disease exhibited by Cabernet Sauvignon seedlings treated with 10 mM palmitoleic acid (PA) or 2% macadamia nut oil soap (MS) with 0.2% Breakthru (BT) or without a surfactant as a foliar spray (spray) or a stem injection (inject).

Objective 3: Biological control with *Burkholderia phytofirmans* PsJN.
Note: As recently approved project 16-0514-SA "Biological control of Pierce's disease of grape with an endophytic bacterium" builds upon the foundational research developed in this project, the renewal report prepared for 16-0514-SA contained a portion of the results presented here because of a need to provide context

and background for new work in that project. All new work on biological control of Pierce's disease is currently being conducted as part of project 16-0514-SA, reported here is the work that was conducted prior to the initiation project 16-0514-SA.

While the biological control of Pierces disease with endophytic bacteria that would grow within grape and produce DSF has been an attractive strategy, until recently we have been unable to find bacteria capable of exploiting the interior of grape. All of hundreds of strains isolated from within grape by our group as well as that of Dr. Kirkpatrick exhibited no ability to grow and move beyond the point of inoculation when re-inoculated. We have recently, however, found that *Burkholderia phytofirmans* stain PsJN which had been suggested to be an endophyte of grape seedlings multiplied and moved extensively in mature grape plants (Figure 1). Its population size and spatial distribution in grape within six weeks of inoculation was similar to that of *X. fastidiosa* itself, suggesting that it is an excellent grape colonist. Furthermore, DSF production has been demonstrated in certain other *Burkholderia* species and the genome sequence of *B. phytofirmans* revealed that it has a homologue of *Xf rpfF*. While we have no evidence for its production of a DSF species to which *X. fastdiosa* could respond, the promiscuous nature of RpfF in *X. fastdiosa* and other species suggested that it might make DSF species to which *X. fastidiosa* would respond under some circumstances, such as when growing within plants. Preliminary results suggest that co-inoculation of *X. fastidiosa* and *B. phytofirmans* resulted in greatly reduced disease symptoms compared to plants inoculated with *X. fastdiosa* alone; whereas the number of infected leaves of plants inoculated with *X. fastidiosa* alone increased rapidly after week 12, very little disease was observed in plants inoculated with *X. fastidiosa* and *B. phytofirmans* (Figure 1).

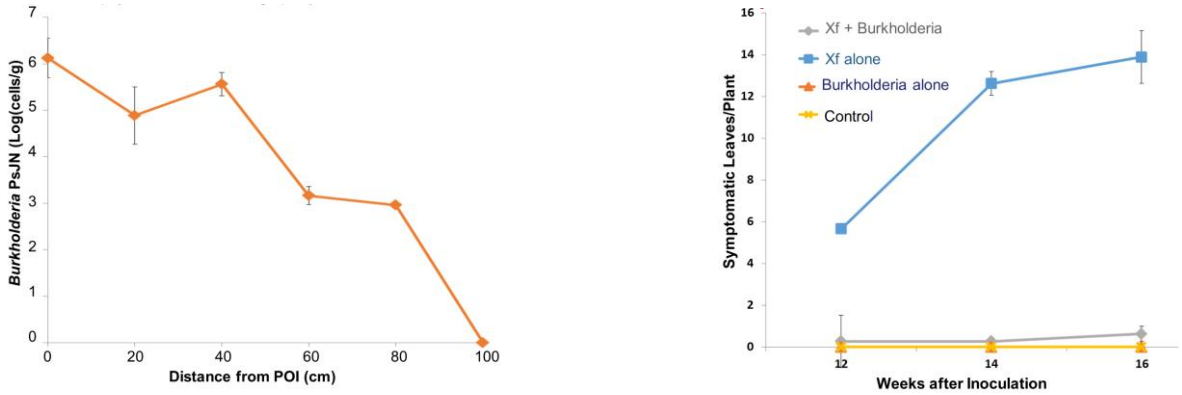

Figure 1. (Left). Population size of *B. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after 6 weeks incubation. (Right). Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *X. fastidiosa* alone (blue) or when co-inoculated with *B. phytofirmans* (grey) or when inoculated with *B. phytofirmans* alone (red).

While the droplet puncture method used in Figure 1 to introduce *B. phytofirmans* is an effective way to introduce bacteria into the xylem we have investigated the potential to introduce *B. phytofirmans* into the vascular tissue by topical application to leaves using 0.2% Brekthru, an organo-silicon surfactant with sufficiently low surface tension that spontaneous invasion of plant tissues can be achieved. The population size of *B. phytofirmans* in the petioles of leaves distal from the leaf on which cell suspensions in Brekthru (10^8 cells/) have been applied were used as a measure of growth and movement potential from such an inoculation site. Substantial numbers of cells of *B. phytofirmans* could be recovered from petioles within one or two weeks after topical application to leaves in the presence of Silwet L77 or Breakthru (Figure 2). Very few cells were present within petioles when the bacterium was applied without a penetrating surfactant. Topical application of such an endophyte thus appears to be a very practical means of inoculating plants in the field.

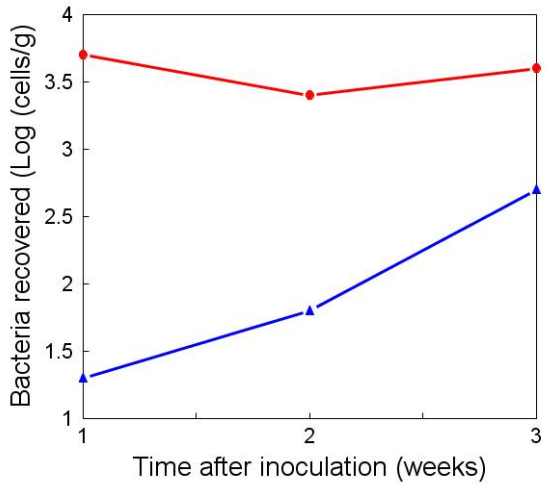

Figure 2. Population size of *Burkholderia phytofirmans* in petioles of Cabernet Sauvignon of plants sprayed with this strain alone (blue line) or this strain applied with 0.2% Breakthru (gray line) or of *Erwinia herbicola* strain 299R applied with 0.2% Breakthru (orange line). Vertical bars represent the mean of log population size at a given sampling time.

Continuing effort has been made during this reporting period to better understand the mechanisms by which *B. phytofirmans* alters the behavior of *X. fastidiosa* in plants. Burkholderia appears to produce compounds that might directly affect pathogen behavior. DSF production has been described in other *Burkholderia* species including *Burkholderia ceonocepacia*. Furthermore, the genome sequence of *B. phytofirmans* PSJN has been determined, allowing us to putatively identify a gene with some homology to *X. fastidiosa* and *Xanthomonas campestris rpfF*, that thus might be expected to lead to the production of fatty acids capable of conferring signaling activity like that of DSF species. We therefore made a site-directed deletion mutant of the putative *rpfF* gene in *B. phytofirmans*. We subsequently investigated whether ethyl acetate extracts of wild type *B. phytofirmans* culture supernatants or *rpfF* mutants of *B. phytofirmans* could alter the expression of genes in either *Xanthomonas campestris* or *X. fastidiosa* that were known to be regulated by the presence of various DSF species. Interestingly, relatively strong induction of the *eng:gfp* reporter gene fusion in *Xanthomonas campestris* was observed when the biosensor was exposed to extracts of both the wild type and *rpfF* mutant of *B. phytofirmans* (Figure 3). These results suggest that indeed *B. phytofirmans* was capable of producing a DSF-like molecule that *Xanthomonas campestris* could respond to. It also suggested however that the putative *rpfF* gene that we had removed was not responsible for producing the putative signal molecule. In contrast to the results that revealed that *Xanthomonas campestris* could respond to way putative signal molecule from *B. phytofirmans*, little or no change in expression of the *phoA* reporter gene was observed when the *X. fastidiosa Xf:phoA* biosensor was exposed to ethyl acetate extracts of either the wild type or *rpfF* mutant of *B. phytofirmans* (data not shown). Given that *X. fastidiosa* and *Xanthomonas campestris* respond to different DSF species, it was not unexpected that they might differentially respond to the signal molecule apparently made by *B. phytofirmans.*

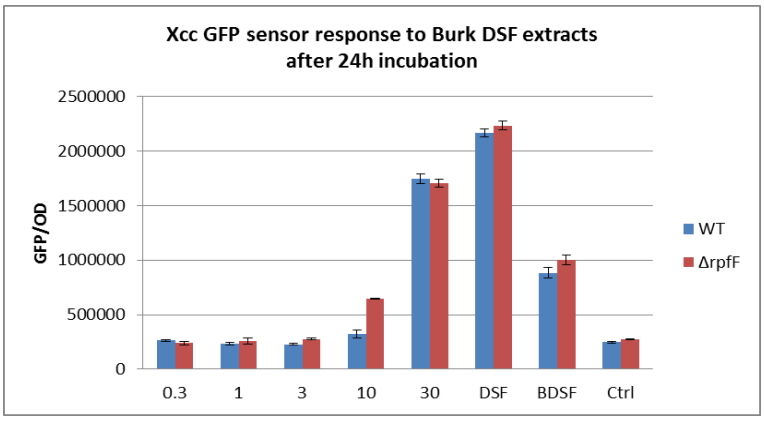

Figure 3. Normalized GFP fluorescence exhibited by the *Xanthomonas campestris* pv. campestris DSF biosensor strain harboring an *eng:gfp* reporter gene when exposed to different concentrations of ethyl acetate extracts (100 ml of supernatant extracted into 1 ml of solvent) from a wild type *B. phytofirmans* (blue bars) or an *rpfF* mutant (red bars). Shown on the abscissa are different ul aliquots of the extract added to a 1 mL culture of the biosensor as well as a culture of the biosensor exposed to 1 uM DSF, 1 uM BDSF,or to no added material (ctrl).

While we did not detect a change in apparent expression of the *hxfA* promoter linked to the *phoA* reporter gene in the *X. fastidiosa Xf:phoA* biosensor when it was exposed to either ethyl acetate extracts of culture supernatants of *B. phytofirmans* or small amounts of culture supernatant themselves, we observed that the biofilm formation (apparent adhesiveness) of *X. fastidiosa* was dramatically higher when either ethyl acetate extracts of culture supernatant or culture supernatant itself from *B. phytofirmans* was added to cultures of either wild type or rpfF* mutants of *X. fastidiosa* (Figure 4). Not only was the amount of bacterial biomass that accumulated in the "ring" which formed at the media/air interface and shake cultures greater, but more importantly, substantial numbers of cells of *X. fastidiosa* adhered to the walls of class culture flasks below the ring - in the area exposed to turbulent mixing of the culture during shaking (Figure 4). These results suggested that the adhesiveness of *X. fastidiosa* was dramatically higher in the presence of some component of the culture supernatant of *B. phytofirmans.* Furthermore, the fact that biofilm formation was by extracts of both the wild type and putative *rpfF* mutant of *B. phytofirmans*, suggested that the putative *rpfF* gene of *B. phytofirmans* was not involved in production of the signal molecule that induced biofilm formation.

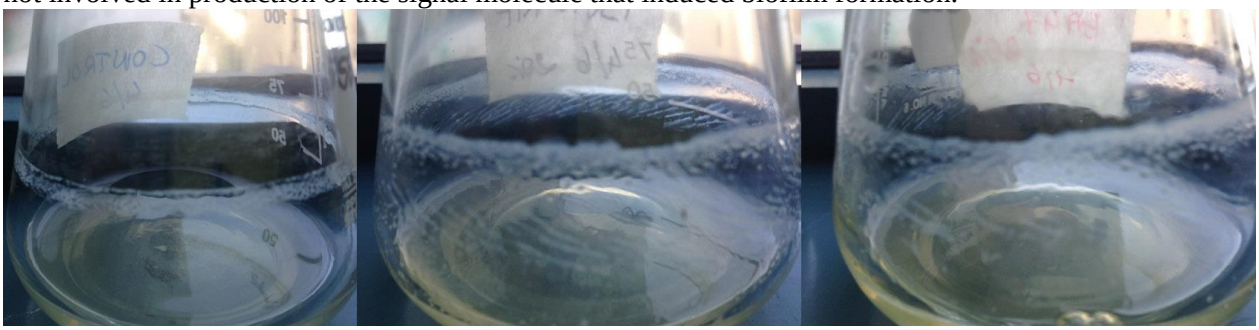

Figure 4. Biofilm formation of wild type *X. fastidiosa* grown in PD three media alone (left), or in media containing 20%v/v of culture supernatant of wild type *B. phytofirmans* (center) or a putative *rpfF* mutant of *B. phytofirmans* (right).

Interestingly, a large increase in biofilm formation could be conferred by relatively small amounts of extracts of either wild type or the *rpfF* mutant of *B. phytofirmans*, while higher concentrations appeared to lead to some inhibition of *X. fastidiosa* growth, and hence biofilm formation. These results are quite interesting in that it suggests strongly that *B. phytofirmans* produces a signal molecule to which *X. fastidiosa* responds, leading to its increased adhesiveness. It is unclear whether the signal molecule is a fatty acid related to DSF. It is quite possible that *X. fastidiosa* can perceive the putative signal molecule of *B. phytofirmans* using receptors different from those used to detect DSF itself, and that detection of the putative signal molecule of *B. phytofirmans* might lead to expression of somewhat different genes that of DSF itself. Work to determine the identity of the signal molecule is underway. Dramatic ability of the factor produced by Burkholderia to increase the biofilm formation of *X. fastidiosa* has facilitated our preliminary purification of the molecules involved. Compounds found in cell free culture extracts of *Burkholderia* were subjected to partitioning into different concentrations of methanol. These preliminary results suggest wrongly that the factor that mediates biofilm formation is quite hydrophobic, being released from hydrophobic fractionation columns only at relatively high concentrations of methanol (Figure 5). Further work on its chemical purification is underway. The ability of this putative signal molecule to increase the apparent adhesiveness of *X. fastidiosa* is likely contributing to the biological control of disease conferred by co-inoculation or pre- or post-inoculation plants with *B. phytofirmans.* As with DSF itself, increasing the adhesiveness of *X. fastidiosa* would restrict its ability to move within the plant. Given that the putative signal molecule made by *B. phytofirmans* is both a small molecule and active at quite low concentrations, it suggests that it might be readily diffusible throughout the plant, again explaining why biological control conferred by *B. phytofirmans* appears to be so robust. Experiments are underway to determine

the relative importance of such putative signal molecules and possible host-mediated defenses elicited by *B. phytofirmans* in biological control.

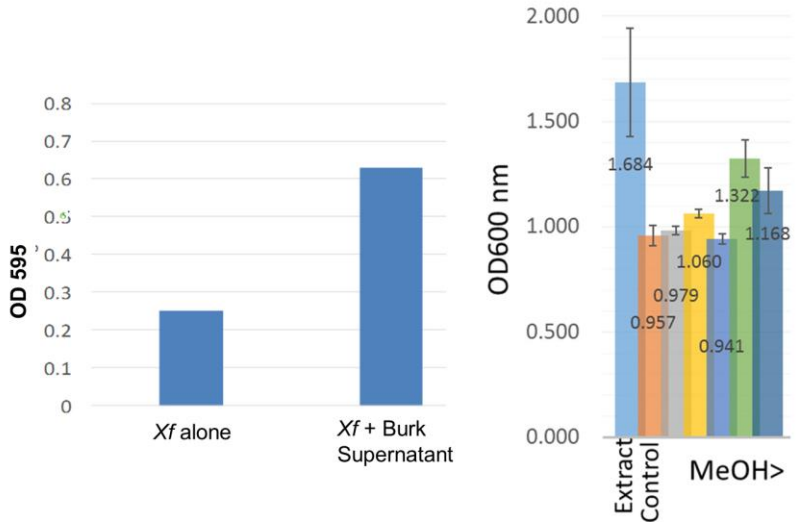

Figure 5. (Left) Quantification of biofilm formation by broth cultures of *X. fastidiosa* to which 1% by volume of a culture supernatant of a *Burkholderia* culture had been added. Biofilm formation was quantified by crystal violet staining and measured at 595 nm. (Right) Characteristics of a compound found in culture supernatants of a *Burkholderia* culture that induced biofilm formation in *X. fastidiosa*. A methanol extract of a *Burkholderia* culture supernatant was absorbed onto a hydrophobic carbon which was then subject to increasing concentrations of methanol and water. The eluates were then evaluated for their ability to induce biofilm formation in *X. fastidiosa*. Increasing ability of eluates to induce biofilm formation was observed with increasing concentrations of methanol used to the compound from the hydrophobic column.

PUBLICATIONS AND PRESENTATIONS:

Presentation entitled "The many density -dependent traits of *Xylella fastidiosa*: achieving disease control via pathogen confusion" presented at the University of Arizona, 2016.

Presentation at the 3rd International Conference on Biological Control of Plant Pathogenic Bacteria, Belgrade, Serbia, entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion". 2016.

Presentation at University of Barcelona entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion". 2016.

Presentation at Microbe 2016 - the Annual Meeting of the American Society for Microbiology, Boston, entitled "The biology of *Xylella fastidiosa* in plants and insects", 2016.

Presentation at the 17th international Congress on Molecular Plant-Microbe Interactions, Portland Oregon, entitled "the many cell density -dependent behaviors of *Xylella fastidiosa*: achieving plant disease control by pathogen confusion". July, 2016.

Presentation at the University of California, Davis entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion". October, 2016.

Presentation at the 2016 Pierce's disease research symposium entitled "Biological control of Pierce's disease with an endophytic bacterium" presented December 14, 2016, San Diego California.

Presentation at the University of Iowa entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion". February, 2017.

RESEARCH RELEVANT STATEMENT:

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

LAYPERSON SUMMARY:

X. fastidiosa produces an unsaturated fatty acid signal molecule called DSF. Accumulation of DSF in *X. fastidiosa* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant. We have investigated DSF-mediated cell-cell signaling in *X. fastdiosa* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease. Elevating DSF levels in plants artificially reduces its movement in the plant. We are introducing the gene conferring DSF production and to a variety of different grape cultivars to determine if they also will exhibit high levels of disease resistant as did the Freedom cultivar previously constructed. We are generating and testing 5 different DSF-producing grape varieties both as own-rooted plants as well as rootstocks for susceptibility to Pierce's disease. The majority of these transgenic grape varieties have now been produced at the plant transformation facility at UC Davis and are under evaluation under greenhouse conditions at Berkeley to determine those particular transgenic lines that have highest disease resistance. Additional gene constructs will be made to generate transgenic plants in which the DSF synthase is directed to a cellular environment in which higher levels of DSF production can be expected in those few grape varieties in which such expression has not yet been successful. While some of the transgenic varieties will be available for establishment in the field plot as own rooted plants or as rootstocks of plants with a normal Cabernet Sauvignon scion in 2017, most of the remaining plants for the field trial will not be available for planting until 2018. While many of the plants have already been produced, the remainder should be delivered within the next few months. Considerable additional work will be needed to assess their production of DSF and disease resistance, but we are optimistic that they also will show at least as high a level of disease resistance as seen in earlier studies in Freedom. Preliminary results using penetrating surfactants to introduce commercially available fatty acids and saponified plant oils capable of inducing signaling in *X. fastidiosa* and achieving disease control are quite promising, and we feel that this strategy of conferring disease resistance by direct introduction of the signal molecule can be better optimized by further attention on different formulations and delivery mechanisms. We are particularly excited about the opportunities for biological control of Pierce's disease using the endophytic bacterium *B. phytofirmans*. Not only is this strain the first that we have ever found that readily colonizes grape, but we continue to see very dramatically lower disease severity on different grape varieties treated with this bacterium both before or after that of *X. fastidiosa*. These results are quite exciting in that they reveal that biological control of Pierce's disease using *B. phytofirmans* is both robust and may be relatively easy to imply by various ways of inoculation.

STATUS OF FUNDS:

The project as proposed is proceeding on schedule. Most of the funds have now been expended, and most of the continuing work being conducted during this no-cost extension has been on the production and testing of transgenic plants expressing DSF. The funds remaining are sufficient to complete the project as proposed.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY:

A US patent 8,247,648 B2 entitled "Biological control of pathogenicity of microbes that use alpha, beta unsaturated fatty acid signal molecules" was approved in June, 2012 and was issued on August 21, 2012. While this patent is not specifically address biological control, depending on the outcome of our studies investigating the mechanisms of biological control, it is possible that some of the practices leading to control of Pierce's disease to be demonstrated here could be covered by this patent if signaling molecules produced by *Burkholderia* are involved in the biological control effect.