FINAL 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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ABSTRACT:

X. fastdiosa coordinates its behavior in plants in a cell density-dependent fashion using a diffusible signal molecule (DSF) which acts to suppress its virulence in plants. Artificially increasing DSF levels in transgenic grape greatly reduced disease severity in both greenhouse and field trials. We investigated DSF production in additional transgenic grape varieties to determine the robustness of this strategy of disease control. As expected, different transgenic lines of a given grape variety varied in their expression of the introduced *rpfF*-encoded RpfF synthase gene, and in their resistance to Pierce's disease when inoculated with *X. fastidiosa*, but those lines most resistant have been identified and are being prepared for field testing that will initiate in 2018. *fastdiosa* is relatively promiscuous in its production and perception of various unsaturated fatty acids as DSF signal molecules and we have explored ways to introduce the common, inexpensive fatty acid palmitoleic acid and other DSF homologs into plants following direct application. Improved DSF biosensors that we have developed have enabled us to monitor the uptake and redistribution of such molecules in plants. Results suggest that some palmitoleic acid can enter plants after simple topical application, but that the use of penetrating surfactants introduces sufficient amounts of this DSF-like molecule to alter behavior of *X. fastidiosa* in plants. Saponified macadamia nut oil, rich in palmitoleic acid, also appears attractive as an inexpensive source of exogenously applied signal molecule. A naturally occurring *Burkholderia* strain capable of DSF production that is also capable of growth and movement within grape has been found that can confer increased resistance to Pierce's disease. We have explored the biological control of disease using this strain. The movement of *X. fastidiosa* within plants and disease symptoms are greatly reduced in plants in which this *Burkholderia* strain was inoculated either simultaneously with, prior to, or even after that of *X. fastidiosa*. The biological control agent can be applied either by direct introduction into the xylem by droplet puncture or by spray application to foliage using a penetrating surfactant. 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 imploy by various ways of inoculation.

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 introducing the gene conferring DSF production into 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 have generating and tested 4 different DSF-producing wine grape varieties as well as rootstock varieties for their susceptibility to Pierce's disease. The majority of these transgenic grape varieties have now been evaluated under greenhouse conditions at Berkeley to determine those particular transgenic lines that have highest disease resistance. Additional gene constructs were 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. The transformation efficiency of this construct into the various grape varieties was lower than for the untargeted *rpfF* gene. The best transformed line of most of the grape varieties will be available for planting in 2018. 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.

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: 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".

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 compared the production of DSF species in 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 thus 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 compared the expression of the introduced *rpfF* gene 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 was conducted at the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis. Grape varieties Thompson seedless as well as the advanced rootstock varieties 1103, 101-14 and Richter were transformed with the *rpfF* gene from *Xf*. In addition to un-targetted expression of RpfF, we produced 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 was conducted at the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis.

Our goal was to obtain between 5 and 10 individual transformants for each variety/construct combination. As will be summarized below, was 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 was necessary to identify those lines with the highest levels of expression. To determine the disease susceptibility of each line they were grown to a sufficiently large size that vegetative clones could be produced (3 months or more) and then each cloned plant was propagated and assessed for disease susceptibility (5 additional months). A goal wsa to obtain at least 12 vegetative clones each of the lines 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) were inoculated with *Xf* by droplet needle puncture as in earlier studies. Disease severity was assessed visually weekly after inoculation. In this process, we are able to identify the transformant from each variety/construct combination that was most highly resistant to PD, and thus suitable for field evaluation.

While we attempted to obtain at least 10 different transformed lines each variety given genetic construct, several of the grape varieties proved to be difficult to transform, and a lower frequency of transformation was observed for the chloroplast targeted *rpfF* gene compared to the non-targeted construct. For example, Richter 110 and Paulsen 1103 have proven to be somewhat more difficult to transform than other varieties, yielding fewer transformants than other grape varieties. The following table indicates the number of individual independently transformed plants of each combination that were produced. 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

As expected, the different transformed lines of a given variety varied in their expression of the rpfF gene, presumably as a result of the physical location within the chromosome to which transgene was introduced by Agrobacterium mediated gene transfer. As an aid in guiding our evaluation of the plans for disease severity we evaluated gene expression by reverse transcriptase semi-quantitative PCR. Typical results for the variation in gene expression among plants are shown in Figure 1.

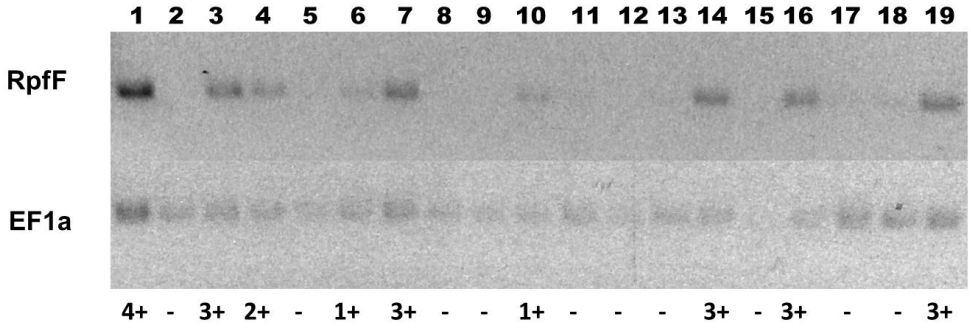

Figure 1. RT-PCR products generated from RNA collected from each of 19 different grape plants transformed with an untargeted rpfF gene from Xylella fastidiosa. For a given column the band in the top panel represents amplicon from the rpfF gene while the band in the lower column is the amplicon from the constitutively expressed EF1a gene from grape as an internal standard to account for the amount of RNA isolated from a given plant. Shown in the bottom of each column is an estimate of the relative expression of rpfF compared to that of the internal control.

The process of evaluating them for disease resistance was slow because the plants obtained from Davis were very small and many were delivered during winter months when they also grew very slowly under our greenhouse conditions. This lengthened the time needed to obtain the vegetative clones required for disease susceptibility testing. We did 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 has this provided us evidence for the relative effectiveness of DSF production as a disease control strategy and the different grape varieties, but it aided us in identifying the most highly resistant variety for a given variety. Typical variation in the susceptibility to Pierce's disease of different transgenic lines of Thompson seedless is shown in Figure 2, in which transgenic line "F" was more resistant another transgenic lines and that of the wild type plant.

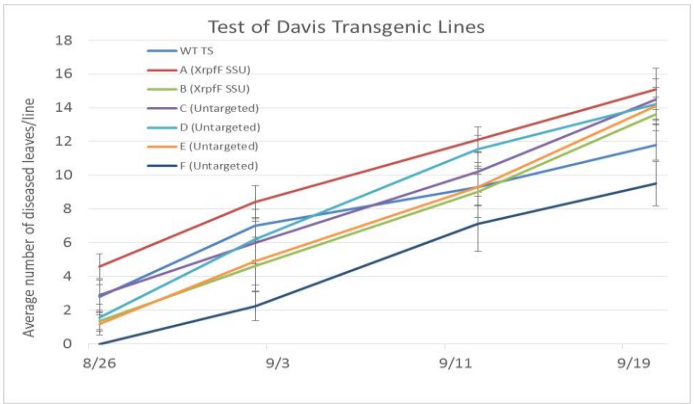

Figure 2. Severity of Pierce's disease of wild type Thompson seedless as well as different transgenic rpfF-expressing lines of this variety measured at different times after inoculation. The vertical lines represent the standard error of the average number of symptomatic leaves on a given plant measured at a given sampling time.

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 3). 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 relatively inexpensive, commercially available, enoic acid palmitoleic acid (Figure 3).

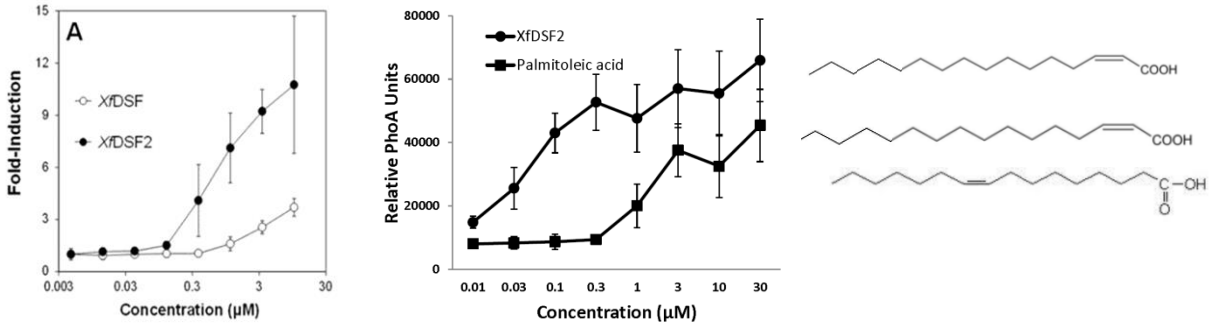

Figure 3. Responsiveness of a PhoA-based *X. fastidiosa* DSF biosensor to different concentrations of *Xf*DSF1 (top molecule), *Xf*DSF2 (middle molecule), and palmitoleic acid (bottom molecule).

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 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 evaluated 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 4). 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.

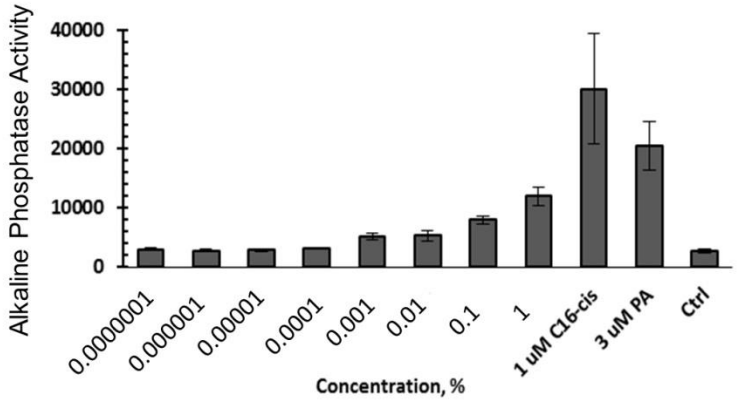

Figure 4. Alkaline phosphatase activity exhibited by the *X. fastidiosa Xf:phoA* biosensor exposed to increasing concentrations of saponified macadamia nut oil as well as 1 uM *Xf*DSF2, 3 uM Palmitoleic acid, or a negative control with no added DSF.

We investigated several strategies by which direct application of DSF molecules could reduce Pierce's disease. While determined 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 was 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 assessed 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 and solvents were 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 increased 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 were 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 biosensor 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 5). 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.

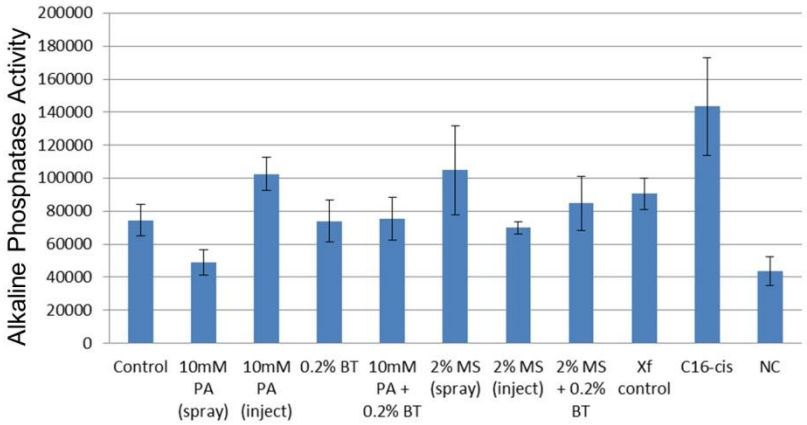

Figure 5. Alkaline phosphatase activity exhibited by 10 µl aliquots of xylem sap extracted under pressure from individual leaves of grape plants treated with 10 mM palmitoleic acid (PA) or 2% macadamia nut oil soap (MS) with 0.2% Breakthru (BT) or without a surfactant as a foliar spray (spray) or a stem 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 seems to be as great as when applied with a surfactant, the cost and convenience of using such treatment seems particularly good.

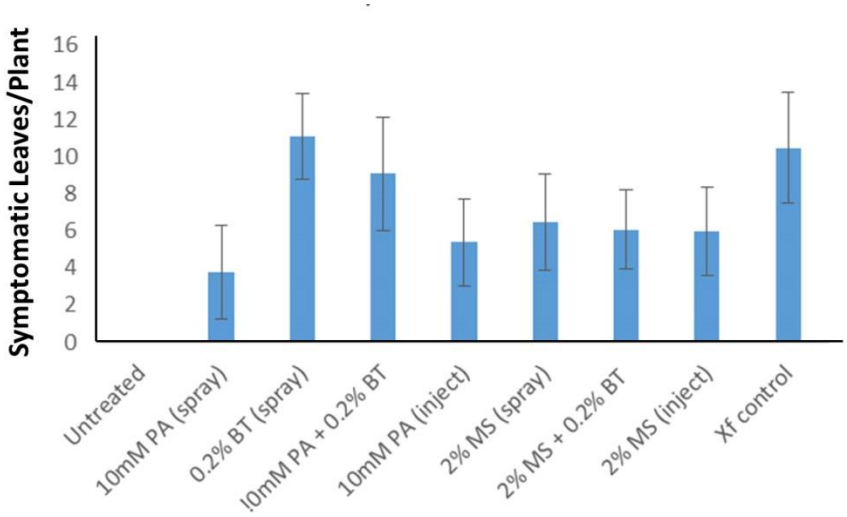

Figure 6. Symptoms of Pierce's disease exhibited by Cabernet Sauvignon seedlings treated with 10 mM palmitoleic acid (PA) or 2% macadamia nut oil soap (MS) with 0.2% Breakthru (BT) or without a surfactant as a foliar spray (spray) or a stem injection (inject).

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 interim progress report prepared for 16-0514-SA contained a portion of the results presented here because of a need to provide context and background for the 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 (recently renamed *Paraburkholderia phytofirmans* due to the recognition that it is genetically unrelated to other *Burkholderia* strains which are potentially human or plant pathogens, and is thus genetically similar to a variety of environmental strains know not to be plant pathogens) that had been suggested to be an endophyte of grape seedlings multiplied and moved extensively in mature grape plants (Figure 7). 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. Our initial intention therefore was to evaluate it as a surrogate host into which the *rpfF* gene from *X. fastidiosa* could be introduced. As will be noted below however, we found that inoculation of plants with strain PsJN itself (without the added *rpfF* gene) could confer disease resistance to inoculated grape. 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 both *X. fastidiosa* and *B. phytofirmans* (Figure 7).

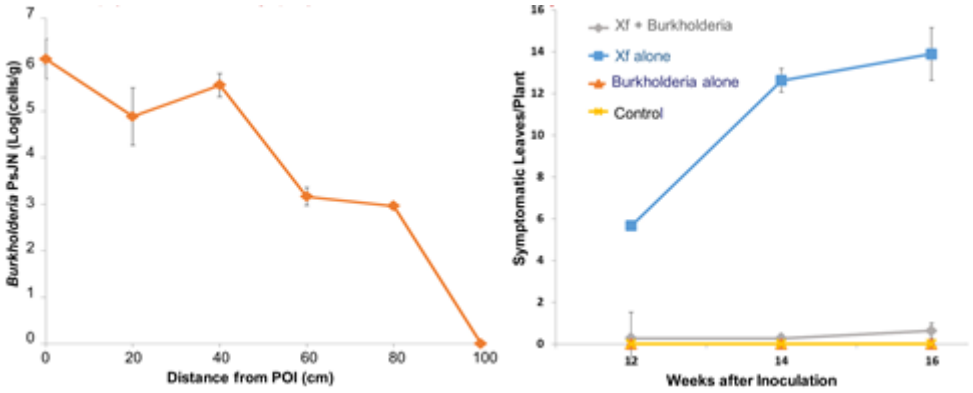

Figure 7. (Left). Population size of *B. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after 6 weeks incubation. (Right). Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *X. fastidiosa* alone (blue) or when co-inoculated with *B. phytofirmans* (grey) or when inoculated with *B. phytofirmans* alone (red).

While the droplet puncture method used in Figure 7 to introduce *B. phytofirmans* is an effective way to introduce bacteria into the xylem we investigated the potential to introduce *B. phytofirmans* into the vascular tissue by topical application to leaves using 0.2% Breakthru, 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 8). 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.

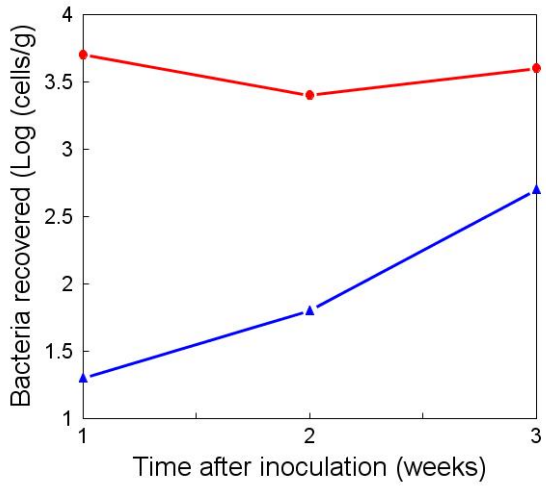

Figure 8. Population size of *Burkholderia phytofirmans* in petioles of Cabernet Sauvignon of plants sprayed with this strain alone (blue line) or this strain applied with 0.2% Breakthru (gray line).

We also perform studies 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 9). 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.*

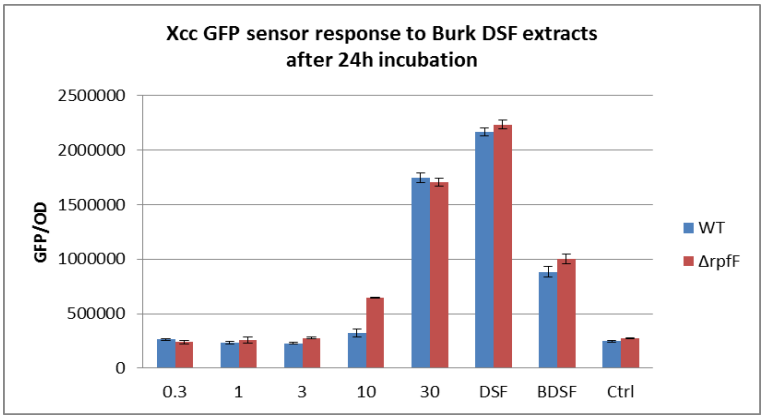

Figure 9. Normalized GFP fluorescence exhibited by the *Xanthomonas campestris* pv. campestris DSF biosensor strain harboring an *eng:gfp* reporter gene when exposed to different concentrations of ethyl acetate extracts (100 ml of supernatant extracted into 1 ml of solvent) from a wild type *B. phytofirmans* (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 10). 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 10). 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 induced 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.

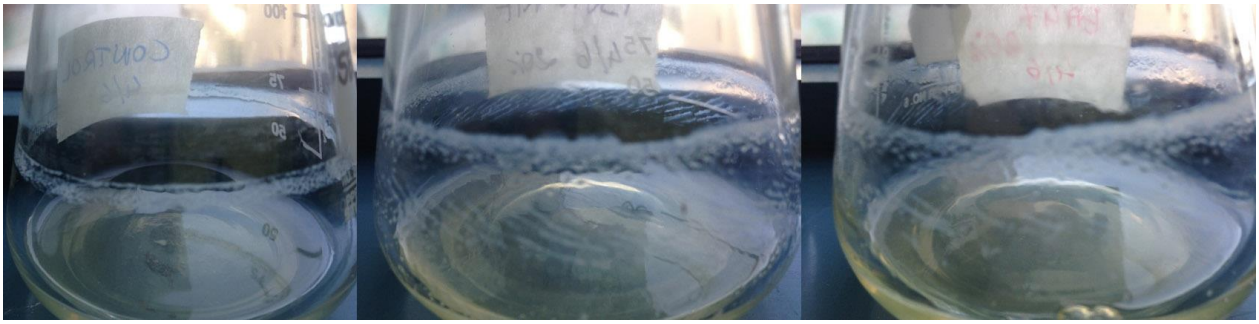

Figure 10. Biofilm formation of wild type *X. fastidiosa* grown in PD three media alone (left), or in media containing 20%v/v of culture supernatant of wild type *B. phytofirmans* (center) or a putative *rpfF* mutant of *B. phytofirmans* (right).

Interestingly, a large increase in biofilm formation was 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 as a component of new project 16-0514-SA. Dramatic ability of the factor produced by *Burkholderia* to increase the biofilm formation of *X. fastidiosa* 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 strongly that the factor that mediates biofilm formation is quite hydrophobic, being released from hydrophobic fractionation columns only at relatively high concentrations of methanol (Figure 11). Further work on its chemical purification is underway as a component of project 16-0514-SA. 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 in new project 16-0514-SA to determine the relative importance of such putative signal molecules and possible host-mediated defenses elicited by *B. phytofirmans* in biological control.

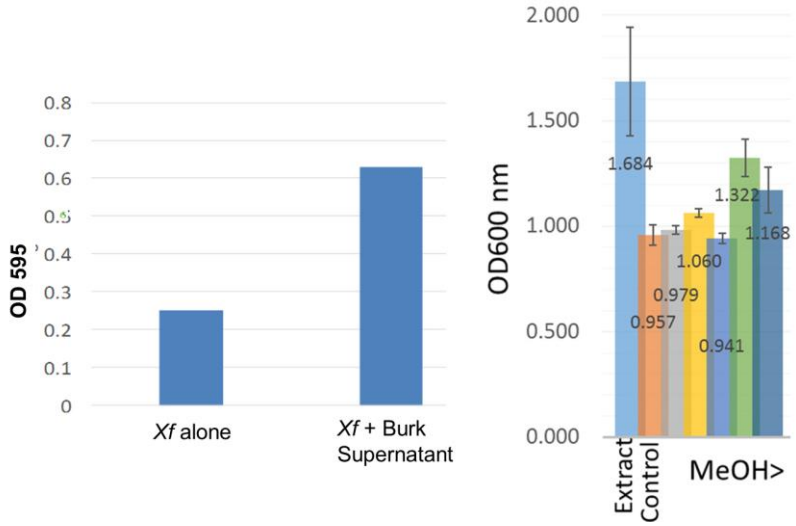

Figure 11. (Left) Quantification of biofilm formation by broth cultures of *X. fastidiosa* to which 1% by volume of a culture supernatant of a *Burkholderia* culture had been added. Biofilm formation was quantified by crystal

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.

CONCLUSIONS:

A variety of additional DSF-producing grape varieties have been produced and evaluated for resistance to Pierce's disease. A number of these plants have higher levels of disease-resistant wild type plants. These are now available for field testing as a component of continuing project 16-0513-SA "Field evaluation of Pierce's disease resistance of various DSF-producing grape varieties as scions and rootstocks". Considerable additional work will be needed as a component of project 16-0513-SA to produce grafted plants with these transgenic plants serving as rootstocks prior to planting. 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. This work demonstrates a proof of principle that direct application of DSF-like signal molecules can be used to achieve disease control. It seems likely however that improve methods of introduction of these materials into plants are available and that further translational work to achieve this goal will be fruitful. 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.

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