I. PROJECT TITLE: Control of Pierce's Disease by methods involving pathogen confusion

II. PRINCIPAL INVESTIGATOR

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III. OBJECTIVES:

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

IV. RESEARCH ACTIVITIES AND ACCOMPLISHMENTS:

Objective 1. Production of DSF in transgenic plants for disease control.

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

Figure 1. Symptoms caused by Xf on SR1 tobacco

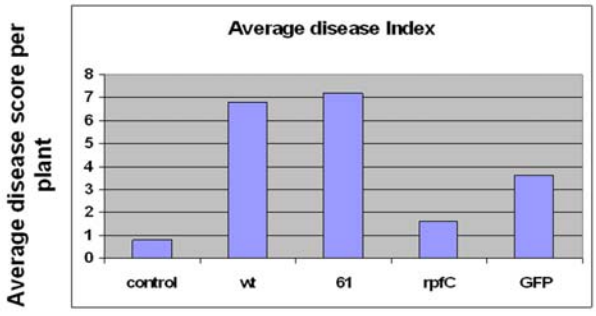

Figure 2 Disease caused by WT Xf, an rpfF- mutant (61), an rpfC- mutant, and a gfp-marked strain on SR1 tobacco

Grape has been transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis. Initially, we submitted a tested but un-optimized *rpfF* construct to the facility. These plants produced only very low levels of DSF. Importantly, the *rpfF*-expressing grape are MUCH less susceptible to Pierce's disease. (Fig. 3). 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. We thus expect to find that *Xf* is limited in its movement in plants having even higher levels of DSF due to the expression of *rpfF*, in a manner similar to what we have observed in DFS-overproducing strains of *Xf*.

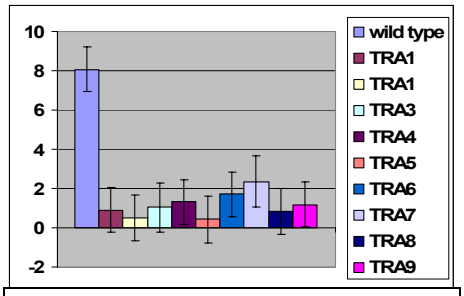

Fig. 3. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the r*pfF* gene encoding DSF production and inoculated with *Xf*.

Enhancing the DSF levels in plants. Given that fatty acid synthesis in plants occurs primarily in the chloroplast and that DSF is presumably a fatty acid derivative, we have recently transformed tobacco and *Arabidopsis* with an *rpfF* gene that has been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of RpfF in the current transformed plants. The *Arabidopsis* ribulose bisphosphate carboxylase small subunit 78 amino acid leader peptide and mature N-terminal which is sufficient to target the protein to the chloroplast, has been fused with the RpfF protein of both *Xylella* and *Xcc*. Assay of DSF in transgenic SRI tobacco plants-where the RpfF is targeted to the chloroplast, indicates that the DSF levels as well as expression of *rpfF* are much higher as compared to the plants in which the RpfF is expressed in the cytosol. Transcription analysis of the chloroplast targated *rpfF* transformed plants indicates high level expression of the *rpfF* gene (Fig. 4). We have generated seeds from the transgenic SRI tobacco plants and we are conducting pathogenicity assays with *X. fastidiosa* comparing these enhanced producing plants with normal and untargeted RpfF plants.

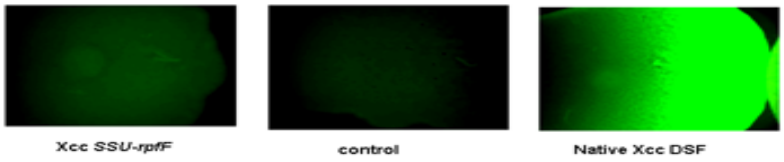

Fig. 4. DSF extracted from transgenic tobacco SR1 plants expressing the chloroplast-targeted RpfF, compare to nontransgenic SR1 and native DSF extracted from Xcc. The DSF is spotted at the right hand side on a filter disc and the *Xcc* DSF bioindicator is streaked on the left side of the spot. The green GFP fluorescence is indicative of DSF production

We have also initiated transformation of grapes with the improved chloroplast targeted *rpfF* constructs at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis. We expect to receive the transformed plants by mid-April, and then will grow them to larger sizes, make green cuttings to produce enough plants for pathogenicity testing by the end of 2008. Although RpfB is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availably of the appropriate substrates for DSF synthesis by RpfF. We expect that co-expression of RpfB and RpfF in the chloroplast will further enhance the DSF levels in plants. We have

produced transgenic *Arabidopsis* plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xanthomonas campestris* pv. *campestris* (Xcc) indicated that the transgenic plants can complement the virulence of the non pathogenic *rpfF* mutant of *Xcc* (Table 2). Importantly, transgenic plants expressing both *rpfB* and *rpfF* were more susceptible to the *rpfF* mutant of *Xcc*, indicating enhanced DSF levels. Given this evidence of enhanced DSF production in transgenic *Arabidopsis*, and recent results with similarly-transformed tomato, we are initiating transformation of grape with similar constructs.

Table 1. Disease severity from topical application of bacteria varying in DSF production to Arabidopsis. Bacteria were inoculated on different *Arabidopsis* genotypes transformed with *rpfF* or with both-*rpfB* and *rpfF*

Arabidopsis Genotype	**Xcc strains**	
	Wild type	*rpfF*⁻
Col (wild type)	++++	-
rpfF transformed	++++	+
rpfF and *rpfB* transformed	++++	++

Direct application of DSF to plants. We have tested whether application of DSF directly to non-transgenic grape can confer control of Pierce's Disease. While we are in the process of identifying the chemical structure and synthesis of DSF, for these studies we used crude ethyl acetate extracts of a DSF-producing *E. herbicola* strain as a source of DSF. The DSF was either topically applied as well as needle inoculated into the stems of grape either once a day before inoculation with *Xf* or weekly. While a single needle application of DSF reduces disease index, a weekly application of DSF into the stem of the plant was much more effective (Fig. 5). These results are exciting in that they suggest that disease control from external applications of DSF might be a practical means of disease control.

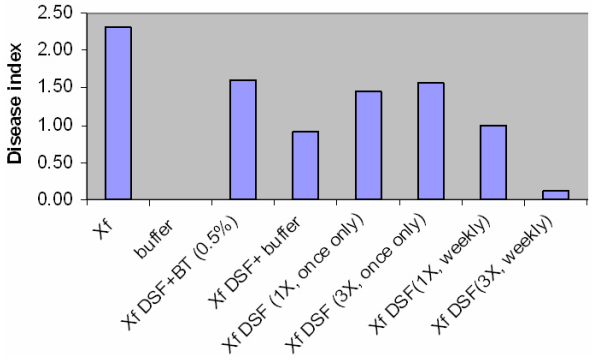

Figure 5. Disease incidence-severity relation (disease index) for grape inoculated with *Xf* and to which DSF was topically applied or introduced into the stem.

Objective 2. Graft transmissibility of DSF. To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing tobacco transformed with the rpfF of *Xf* are used as rootstocks to which normal SR1 tobacco is grafted as a scion (Fig. 6). Over 50 of such grafted plants have now been made, and they will be inoculated with *Xf* in April to test whether normal SR1 scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization. Non-chloroplast targeted RpfF-expressing transgenic Freedom grape plants are currently being propagated to obtain enough plants to serve as rootstocks to test with *Xf* inoculations in larger scale studies. Over 100 such plants have now been propagated and green-grafting of Cabernet Sauvignon is being conducted to produce grafted plants with a normal Freedom and a DSF-producing Freedom rootstock. Initial attempts at green grafting of grape

produced a low frequency of successful grafts, but a new procedure is providing a satisfactory level of graft success; the grafted plants will be inoculated with *Xf* in July to test for graft transmissibility of DFS.

Figure 6. Grafted tobacco plants into which *Xf* has been inoculated. A normal SR1 tobacco scion is grafted onto transgenic DSF-producing tobacco. The graft point is noted with blue tape, and XF has been inoculated above the graft union. The plant is as yet asymptomatic

Objectives 3 and 4. Disease control with endophytic bacteria. Producing DSF in bacterial endophytes. We have been successful in producing large quantities of DSF in endophytes like *Erwinia herbicola* and also in lab strains of *E. coli* (Table 2). We recently were able to transform a putative efficient endophyte of plants, *Rizobium etili* G12 with both the *Xcc* and *Xf rpfF* (DSF biosynthetic gene) and have obtained production of DSF in this strain (Fig. 7). This DSF-producing endophyte has been inoculated into grape to determine both its ability to move and multiply within grape as well as its ability to interfere with the disease process.

Fig.7. Endophytic strains of *R. etili* producing *Xcc and Xf* DSF. The GFP florescence produced by an *Xcc* DSF biosensor which is sprayed on the plates, is indicative of

Table 2. Production of DSF by *E. coli* and *Erwinia herbicola* harboring cloned *rpfF* genes from *Xf* and *Xcc*.

Strains	**Relative DSF production (Units)**
Xcc	100
X. fastidiosa Temecula	4-5
E. coli DH10B (ptrp*XccrpfF*)	3000
E. coli DH10B (ptrp*XfrpfF*)	100
E. herbicola (ptrp*XccrpfF*)	6000
E. herbicola (ptrp*XfrpfF*)	200

The *R. etli* strain G12 was found to move within grape tissue after inoculation into either the stem or the leaves. When measured 4 weeks after inoculation by puncture inoculation into one site in the stem measurable populations of *R. etli* were seen as far as 50 cm away from the point of inoculation (Fig. 8). While the population size away from the point of inoculation were relatively low in this short time interval since inoculation, this strain clearly has the ability to move within grape; we are excited about this result since no other of several bacterial strains that we have investigated for the ability to move within grape has ever exhibited any ability to move beyond the point of inoculation. Further studies are underway to determine the population sizes to which *R. etli* will grow given more time after inoculation. *R. etli* also has the ability to move within grape leaves and multiply to high population sizes. When applied as a point source to leaves using a penetrating surfactant, cells of *R. etli* could be found up to 3 cm away within 1 week, and population sizes of this strain increased 100-fold within 3 weeks after inoculation (Fig. 9). Studies are continuing to determine the maximum population size that this strain can achieve in grape leaves.

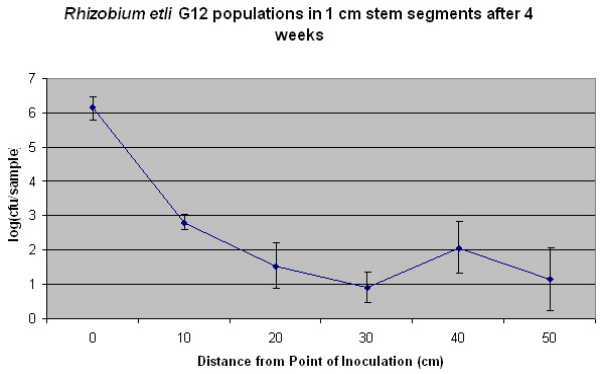

Fig. 8. Population size of *R. etli* in stems 4 weeks after inoculation at one point.

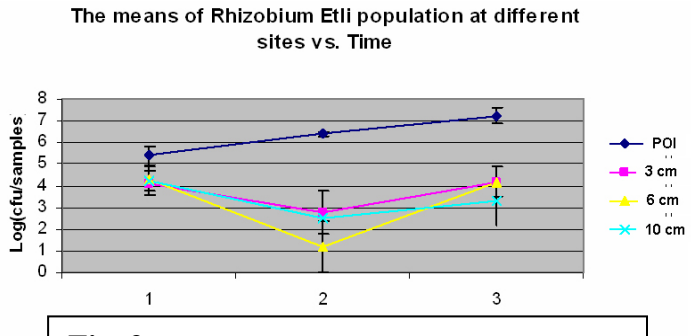

Fig. 9. Population size of *R. etli* in leaves one, two, and three weeks after inoculation at a single point.

Various DSF-producing bacteria were tested for their ability to control Pierce's disease when applied to grape in different ways. DSF-producing *R. etli* were both needle inoculated one or more times at sites near where *Xf* was subsequently inoculated, as well as co-inoculated with *Xf* into grape stems and sprayed onto leaves with 0.5% of the penetrating surfactant Breakthru 1 week before *Xf* was inoculated into stems. The co-inoculation of *R. etli* with *Xf* greatly decreased the incidence of colonization of grape petioles compared to control plants inoculated with *Xf* alone (Fig. 10) while topical application or injection elsewhere in the stem provided little control. We presume that the relatively slow movement of *R. etli* in the stems of plants (Fig. 8) explains why co-inoculation was most effective. *R. etli* was somewhat susceptible to damage from Breakthru and its population sizes were reduced during application with this detergent. We will continue to test different ways in which *R. etli* can be introduced into plants to determine its ability to control PD. We expect that introduction of *R. etli* into stems far in advance of *Xf* will provide much better disease control. RpfC- mutants of *Xf* greatly over-produce DSF so we tested them for their ability to control PD when applied in various ways as discussed above for *R. etli*. The incidence of colonization of grape petioles with *Xf* was greatly reduced when plants were needle inoculated into grape either two weeks before plants were inoculated with *Xf* or when coinoculated with the pathogen (Fig. 11). While the RpfC mutant does not move as well within grape as the wild-type *Xf*, its presence locally in plants can suppress the movement of wild-type *Xf* and thus lead to control of PD. These studies are promising and are being repeated.

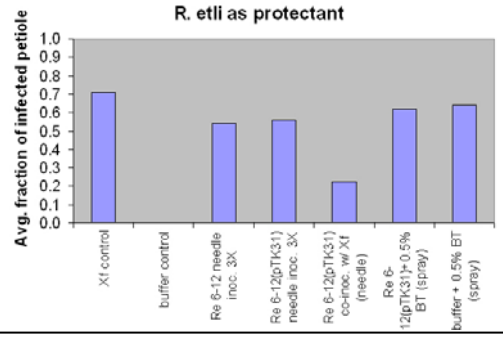

Fig. 10 Incidence of colonization of petiols of grape by *Xf* when plants were treated with DSF-producing *R. etli* in various ways.

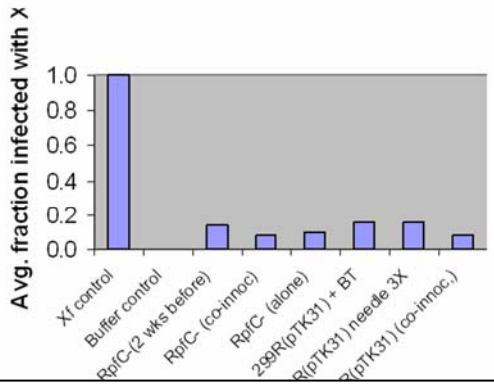

Fig. 11 Incidence of colonization of petiols of grape by *Xf* when plants were treated with RpfC mutants of *Xf* in various ways.

Objective 5. Degradation of DSF by plants. Development of an *Xcc* biosensor efficient in detecting *Xylella* DSF. For many of the objectives of this project, in addition to the study of DSF degradation in plants an improved bioindicator for DSF would be very valuable. We are presently using an *Xcc*-based biosensor in which the endoglucanase gene is linked to a GFP reporter gene. Previous studies have shown that this biosensor is able to detect the DFS made by *Xf* but that it detects *Xf* DSF with a lower efficiency then the *Xanthomonas* DSF since the two molecules apparently differ slightly. We have devised a strategy to develop a surrogate *Xcc* biosensor system which will express all the components of DSF signal transduction of *Xf*. This should give rise to a system which is close to DSF signal transduction system in *Xcc*. We have made two different *Xanthomonas* strains in which the endogenous signal synthesis as well as signal recognition system (consisting of the hybrid two component RpfC and RpfG response regulators) has been knocked out. In one of these strains the DSF signal synthase rpfF and the DSF signal sensor RpfC has been knocked out (Fig 12). We have also made an *Xcc* strain in which the DSF synthase gene *rpfF* has been knocked out in a background of a RpfCHG deletion. These mutants will enable us to express the *Xf* RpfC-RpfG two component system and should serve as a more sensitive surrogate host biosensor. Completion of the biosensor is expected within 3 more months. It then will be applied to the study of *Xf* DSF stability in plant extracts as originally proposed.

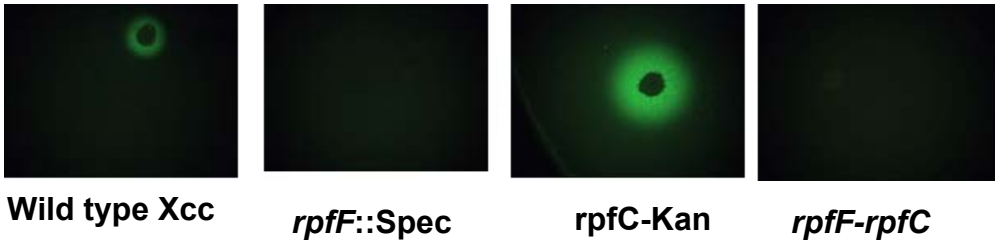

Fig. 12 Different *Xcc* mutants constructed to serve as surrogate host for expressing the *Xf* RpfC-RpfG two component DSF signal transduction system. The presently used *Xcc* biosensor 8523/pKLN55 is sprayed over the colonies. Presence of DSF is detected by the GFP fluorescence of the biosensor

Objective 5. Plant regulation of *Xf* virulence factors. Before investigating the effects of plant extracts on gene expression in *Xf* we have further examined the complex pattern of gene regulation in *Xf* that is DSF dependent to better understand which virulence genes might be most informative to examine. Analysis of the genome sequence of *Xf* revealed that several genes encoding proteins potentially involved in intracellular signaling are present. Gene expression of several genes was thus examined in both an *rpfF* and *rpfC* mutant background as well as a double mutant (Table 3). The results have enabled the production of a more complete model of DSF-dependent gene expression in *Xf* (Fig. 13). The several genes identified in Table 3 will be examined by RT-PCR in cultures of *Xf* to which plant extracts have been applied as proposed.

Fig. 3. A proposed model for DFS-mediated cell-cell signaling regulation in *Xylella fastidiosa*. *rpfF* encodes the DSF synthase. RpfC encodes a hybrid two component sensor which can sense DSF. DSF levels negatively regulate the expression of *rpfF* by feed back which acts as a negative feed back. Expression of other virulence associated functions are regulated by a putative intracellular DSF sensor/response regulator (like-RpfG/Clp). DSF can diffuse inside the cell and is sensed by this putative intracellular DSF sensor/response regulator, which acts as positive regulator of functions associated with attachment-biofilm formation and other components of rpf regulon. The intracellular DSF sensor/response regulator, in the presence of RpfC, can negatively regulate the expression of other virulence associated genes like *pglA*, *tolC* and GGDEF domain encoding gene.

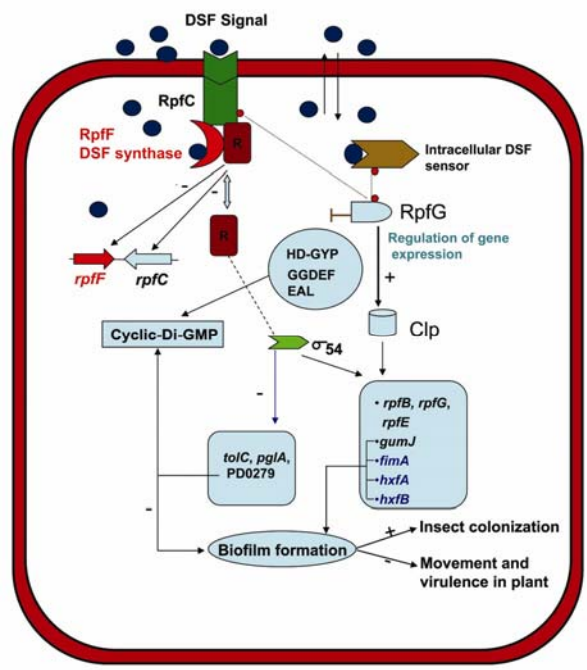

Table 3 Relative quantification of gene expression regulated by *rpfF* and *rpfC* by real-time RT-PCR

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

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

V. PUBLICATIONS:

1. Newman, K.L., Chatterjee, S., Ho, K.A., and Lindow, S.E. 2008. Virulence of plant pathogenic bacteria attenuated by degradation of fatty acid cell-cell signaling factors. MPMI 21: 326-334.
2. Chatterjee, S., C. Wistrom, and S.E. Lindow. 2008. A cell-cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa.* PNAS 105: 2670-2675.
3. Wang, N., W. Feil, and S.E. Lindow. 2007. Identification of traits of *Xylella fastidiosa* conferring virulence to grape and insect transmission by analysis of global gene expression using DNA microarrays. Phytopathology 97:S120.
4. Chatterjee, S., R.P.P. Almeida, and S.E. Lindow. 2008. Living in two worlds: The plant and insect lifestyles of *Xylella fastidosa.* Ann. Rev. Phytopathology 46: (in press).

VI. PRESENTATIONS ON RESEARCH:

1. Invited presentation entitled: "Control of Pierce's Disease of Grape by pathogen confusion" Unified Wine and Grape Symposium, January 29, 2008, Sacramento, CA.
2. Invited presentation at the University of Florida entitled" The plant pathogen *Xylella fastidosa*: A harmless endophyte gone bad?, January 11, 2008.
3. Poster presentation at the Pierce's Disease Research Symposium, entitled: "Management of Pierce's disease of grape by interfering with cell-cell communication in *Xylella fastidiosa",* December 12-14, 2007, San Diego, CA.

VII. RESEARCH RELEVANCE STATEMENT:

Since we have shown that DSF accumulation within plants is a major signal used by *Xf* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this pathogen we have shown proof of principle that disease control can be achieved by a

process of "pathogen confusion". Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, topical application of such bacteria to plants with surfactants, and direct application of DSF itself to plants appear promising as means to reduce Pierce's disease. Transgenic DSF-producing plants appear particularly promising and studies should soon indicate whether they could serve 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.

VIII. LAY SUMMARY:

X. fastidiosa produces an unsaturated fatty acid signal molecule called DSF that changes its gene expression in cells as they reach high numbers in plants. Accumulaiton 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. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease. We have investigated both the role of DFS-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control. *Xf* mutant strains that overproduce DSF cause disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. We have produced bacterial endophyte strains that can produce large amounts of DSF and, by moving within plants apparently they can alter the abundance of DSF sufficiently to reduce the virulence of Xf. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpfF* gene of *Xf* to enable DSF production in plants; such grape plants produce at least some DSF and are much less susceptible to disease. Higher levels of expression of DSF have been obtained in plants by targeting the biosynthetic enzymes to the chloroplast. Studies are underway to determine if DSF produced by rootstocks can move to scions and confer disease control.

IX. STATUS OF FUNDS:

Nearly all of the first-year funds allocated for this project should be encumbered by July 1, 2008.

X. SUMMARY AND STATUS OF INTELLECTUAL PROPERTY:

Based on promising initial results, an application for a patent entitled "Biological control of pathogenicity in microbes that use alpha, beta unsaturated fatty acid signal molecules" was submitted by the UC Berkeley campus in April, 2005. Results from this past year on disease control and successes in producing plants with higher levels of DSF have been provided to the UC Berkeley campus technology transfer office for possible amendment of the application. We have been in contact with PIPRA personnel and as soon as they have developed plant transformation vectors that do not use proprietary methods we will initiate parallel studies of DSF-producing plants transformed using these vectors to maximize flexibility in future commercial adoption of such plants for Pierce's disease control.