Interim Progress Report for CDFA Agreement Number 12-0441-SA

CONTINUED FIELD EVALUATION OF DSF-PRODUCING GRAPE FOR CONTROL OF PIERCE'S DISEASE

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REPORTING PERIOD: The results reported here are from work conducted July, 2011 to March, 2015

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 cells 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 to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels. While we have demonstrated the principles of disease control by so-called "pathogen confusion" in the greenhouse, more work is needed to understand how well this will translate into disease control under field conditions. That is, the methods of inoculation of plants in the greenhouse may be considered quite aggressive compared to the low levels of inoculum that might be delivered by insect vectors. Likewise, plants in the greenhouse have undetermined levels of stress that might contribute to Pierce's disease symptoms compared to that in the field. Thus we need to test the relative susceptibility of DSF-producing plants in the field both under conditions where they will be inoculated with the pathogen as well as received "natural" inoculation with infested sharpshooter vectors.

OBJECTIVES:

1) Determine the susceptibility of DSF-producing grape as own-rooted plants as well as rootstocks for susceptible grape varieties for Pierce's disease.
2) Determine population size of the pathogen in DSF-producing plants under field conditions.
3) Determine the levels of DSF in transgenic *rpfF*-expressing grape under field conditions as a means of determining their susceptibility to Pierce's disease.

RESULTS AND DISCUSSION:

<u>Disease susceptibility of transgenic DSF-producing grape in field trials.</u>

Field tests are being performed with two different genetic constructs of the *rpfF* gene in grape and assessed in two different plant contexts. The *rpfF* has been introduced into Freedom (a rootstock variety) in a way that does not cause it to be directed to any subcellular location (non-targeted). The *rpfF* gene has also been modified to harbor a 5' sequence encoding the leader peptide introduced into grape (Thompson seedless) as a translational fusion protein with a small peptide sequence from RUBISCO that presumably causes this RpfF fusion gene product to be directed to the chloroplast where it presumably has more access to the fatty acid substrates that are required for DSF synthesis (chloroplast-targeted). These two transgenic grape varieties are thus being tested as both own-rooted plants as well as rootstocks to which susceptible grape varieties will be grafted. The following treatments are thus being examined in field trials:

Treatment 1	FT	Non-targeted RpfF Freedom
Treatment 2	TT	Chloroplast-targeted RpfF Thompson
Treatment 3	FW	Non-targeted RpfF Freedom as rootstock with normal Thompson scion
Treatment 4	TTG	Chloroplast-targeted RpfF Thompson as rootstock with normal Thompson scion
Treatment 5	FWG	Normal Freedom rootstock with normal Thompson scion
Treatment 6	TWG	Normal Thompson rootstock with normal Thompson scion
Treatment 7	FW	Normal Freedom
Treatment 8	TW	Normal Thompson

Treatments 5-8 serve as appropriate controls to allow direct assessment of the effect of DSF expression on disease in own rooted plants as well as to account for the effects of grafting per se on disease susceptibility of the scions grafted onto DSF-producing rootstocks.

One field trial was established in Solano County on August 2, 2010. Twelve plants of each treatment were established in a randomized complete block design. Self-rooted plants were produced by rooting of cuttings (about 3 cm long) from mature vines of plants grown in the greenhouse at UC Berkeley. The plants were inoculated in May, 2012 (no natural inoculum of *X. fastidiosa* occurs in this plot area and so manual inoculation of the vines with the pathogen was performed by needle-inoculated with a suspension of *X. fastidiosa*. At least 4 vines per plant were inoculated. Each inoculation site received a 20 ul droplet of *X. fastidiosa* containing about 10^6 cells of *X. fastidiosa*.

The incidence of infection of the inoculated vines at the Solano County trial was reduced about 3-fold in assessments made in August and September (Figure 1). Disease was observed only near the point of inoculation in transgenic Freedom, but had spread extensively in wild-type Freedom grape. Because of the shading of the inoculated vines by subsequent growth of uninoculated vines of the same plant many of the older leaves had died or had fallen from the plant, especially by the September rating, making it difficult to quantify the number of infected leaves per vine. In August, howoever, we found that there were about 3 times as many symptomatic leaves on each inoculated vine of wild-type Freedom than on DSF-producing transgenic Freedom (Figure 2). Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom. The severity of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson, while the incidence and severity of Pierce's disease on Thompson seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plant grafted onto wild-type Thompson Seedless rootstocks (Figure 3).

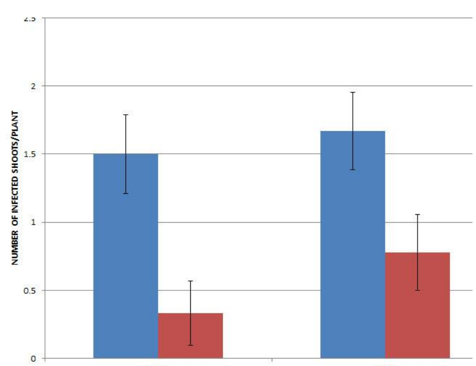

Figure 1. Incidence of vines of DSF-producing transgenic Freedom grape (red) or wild type Freedom having any symptoms of Pierce's disease when rated in August or September, 2012. A total of 3 vines per plant were assessed. The vertical bars represent the standard error of the mean.

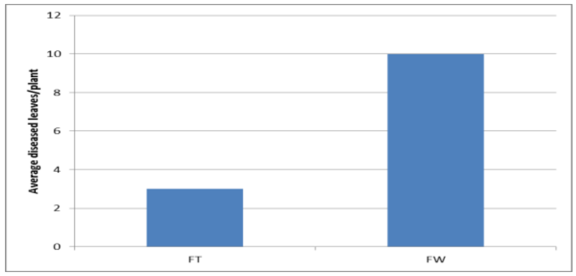

Figure 2. Severity of Pierce's disease on transgenic Freedom grape (FT) and on wild type Freedom grape assessed in August 2012 in the Solano country trial.

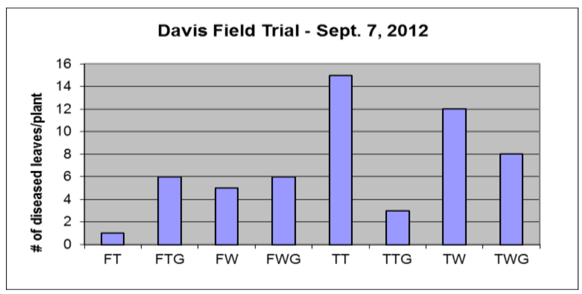

Figure 3. Severity of Pierce's disease on grape assessed in September 2012 in the Solano country trial. See treatment codes above for treatment comparisons.

The plants for the Riverside County trial were planted on April 26, 2011 (Figure 5) and have exhibited much less growth than those at the Solano Country trial (Figure 4). The plants at the Riverside County were subjected to natural infection from infested sharpshooter vectors having access to *X. fastidiosa* from surrounding infected grape vines. Very high levels of Pierce's disease were seen in the summer of 2012, although much less symptoms were seen on the transgenic DSF-producing Freedom grape compared to other plants (Figure 5).

Figure 4. Establishment of grape trial in Riverside County in April, 2010 (left) and image of plot in October, 2012 (right).

Figure 5. Pierce's disease symptoms on transgenic DSF-producing Freedom grape (left) and wild type Freedom grape (right) on October 4, 2012.

The incidence of infection of transgenic DSF-producing Freedom was about 3-fold less than that of wild-type Freedom grape (Figure 6), while the number of infected leaves per vine was about 5-fold less (Figure 9), suggesting that the pathogen had spread less in the DSF-producing plants after insect inoculation. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom (Figure 7). The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson (Figure 8), while the incidence and severity of Pierce's disease on Thompson seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plant grafted onto wild-type Thompson Seedless rootstocks (Figure 9). The effectiveness of transgenic Thompson seedless rootstocks in reducing Pierce's disease was surprising, given that the transgenic Thompson scions were similar in susceptibility to that of the normal Thompson scions. We have seen evidence that in addition to DSF chemical species that serve as agonists of cell-cell signaling in *X. fastidiosa* that transgenic Thompson seedless may also produce chemical antagonists of cell-cell signaling. It is possible that the DSF agonist is more readily transported into the scion than any antagonists, and thus that DSF-mediated inhibition of pathogen mobility can be conferred by grafted DSF-producing rootstocks.

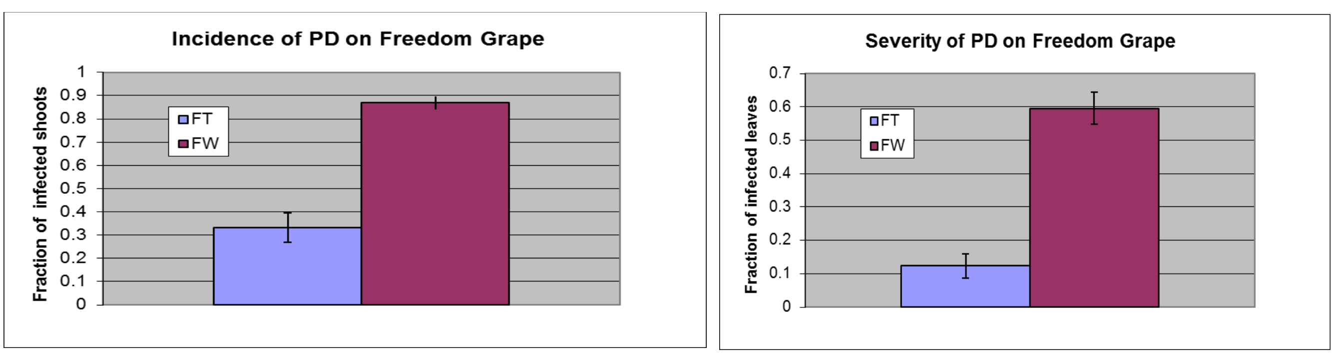

Figure 6. Incidence of Pierce's disease of transgenic DSF-producing Freedom grape (blue bars) or wild type Freedom (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

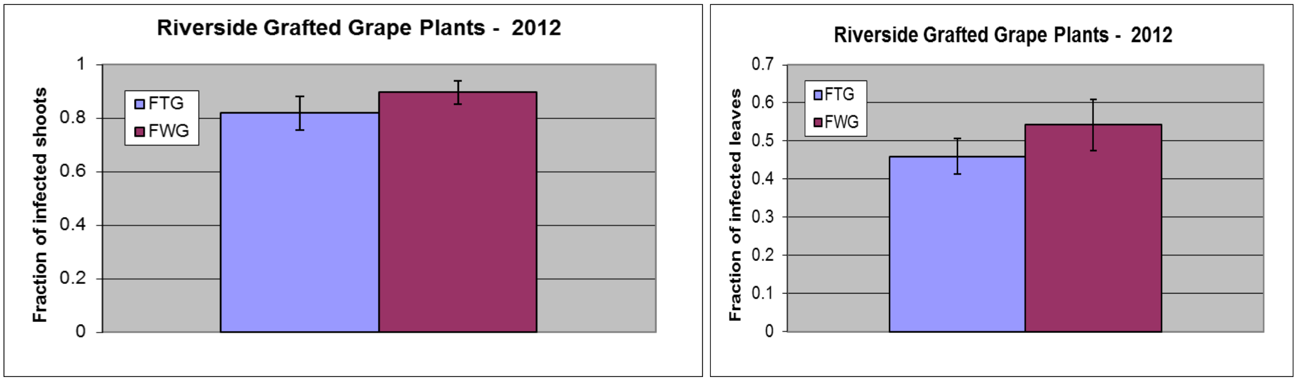

Figure 7. Incidence of Pierce's disease of normal Thompson seedless grape grafted onto transgenic DSF-producing Freedom grape rootstocks (blue bars) or wild type Freedom rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

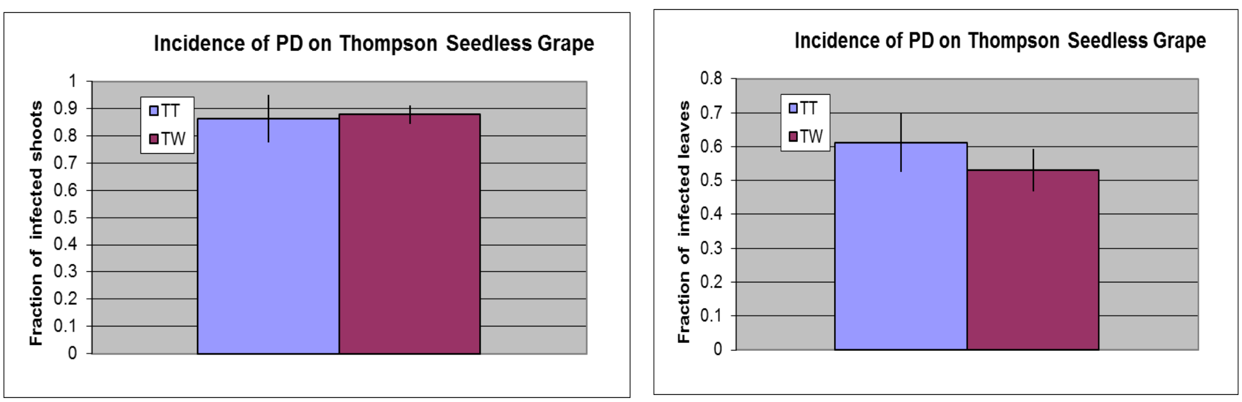

Figure 8. Incidence of Pierce's disease of transgenic DSF-producing Thomson seedless grape (blue bars) or wild type Thompson seedless (red Bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

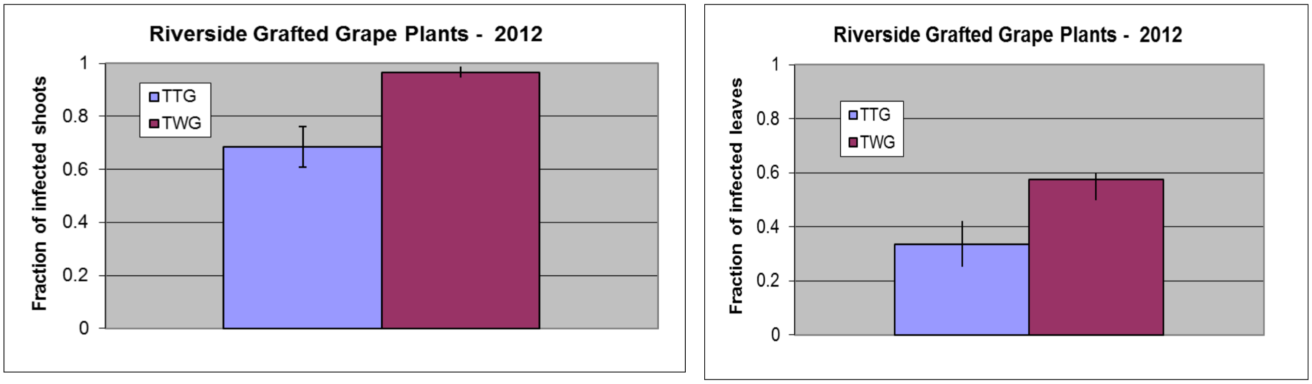

Figure 9. Incidence of Pierce's disease of normal Thompson seedless grape grafted onto transgenic DSF-producing Thompson seedless grape rootstocks (blue bars) or wild type Thompson seedless rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

On May 15, 2013 plants that the Solano County field trial were evaluated for both the incidence of survival over winter, as well as any symptoms of Pierce's disease that was apparent at this early date. Vines that had been inoculated in 2012 had been marked with a plastic tie. The vines were pruned during the winter of 2012/2013 in a way that retained the inoculation site and the plastic marker for each of the inoculated vines 2012. Thus, in May, 2013 the return growth on those inoculated, but pruned, vines was assessed. One or more new shoots had emerged from such vines, and the incidence as to whether at least one new shoot had emerged was assessed (Figure 10). Nearly all of the inoculated vines from both Freedom and transgenic DSF producing Freedom gave rise to new shoots as of May, 2013 (Figure 10). In contrast, many vines of Thompson seedless inoculated in 2012 were dead, and no shoots emerged in 2013. While most new shoots emerging in 2013 appeared asymptomatic at the time of assessment in May, a few exhibited discoloration, possibly indicating early stages of Pierce's disease. A separate assessment of such possibly symptomatic shoots from that of completely asymptomatic shoots was made (Figure 11). It is noteworthy that no symptomatic new shoots were observed on transgenic Freedom, while about 10% of the new shoots emerging from vines of wild type Freedom exhibited some symptoms (Figure 11). It was also noteworthy that a much higher proportion of the vines from Thompson seedless scions grafted onto a transgenic Freedom rootstock gave rise to new shoots in 2013 compared to that on Freedom rootstocks (Figures 10 and 11). Likewise, a higher proportion of vines from Thompson seedless scions grafted onto transgenic DSF-producing Thompson seedless rootstocks gave rise to new shoots in 2013 compared to that of scions grafted onto normal Thompson seedless rootstocks (Figures 10 and 11. Thus, infection of Thompson seedless vines by inoculation in 2012 had led to some morbidity of those vines (and even of the cordon on which they were attached in some cases), but Thompson seedless when grafted onto either transgenic DSF producing Freedom or transgenic DSF producing Thompson seedless rootstocks had a higher likelihood of surviving inoculation in 2012. Continued assessments of disease severity of those new shoots emerging on vines inoculated in 2012 were made in early October, 2013, but the data was not fully analyzed at the time of preparation of this report.

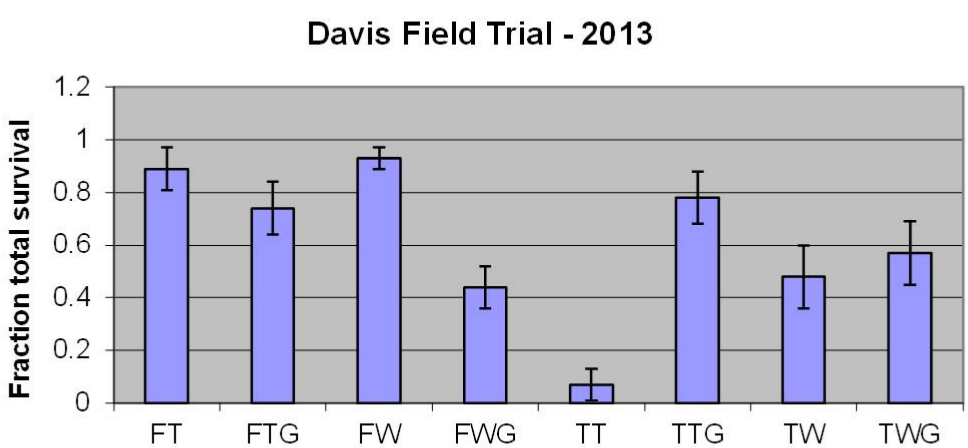

Figure 10. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xylella fastidiosa* that gave rise to at least one new shoot by May, 2013. Treatments include: transgenic DSF producing Freedom as an own-rooted plant (FT); wild type freedom as an own-rooted plant (FW); Thompson seedless scions grafted onto transgenic DSF producing Freedom rootstocks (FTG); Thompson seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF producing Thompson seedless as own-rooted plants (TT); normal Thompson seedless as own-rooted plants (TW); Thompson seedless scions grafted onto transgenic DSF producing Thompson seedless rootstocks (TTG); and Thompson seedless scions grafted onto normal Thompson seedless rootstocks (TWG). The vertical bars represent the standard error of the mean fraction of inoculated vines that gave rise to new shoots in 2013.

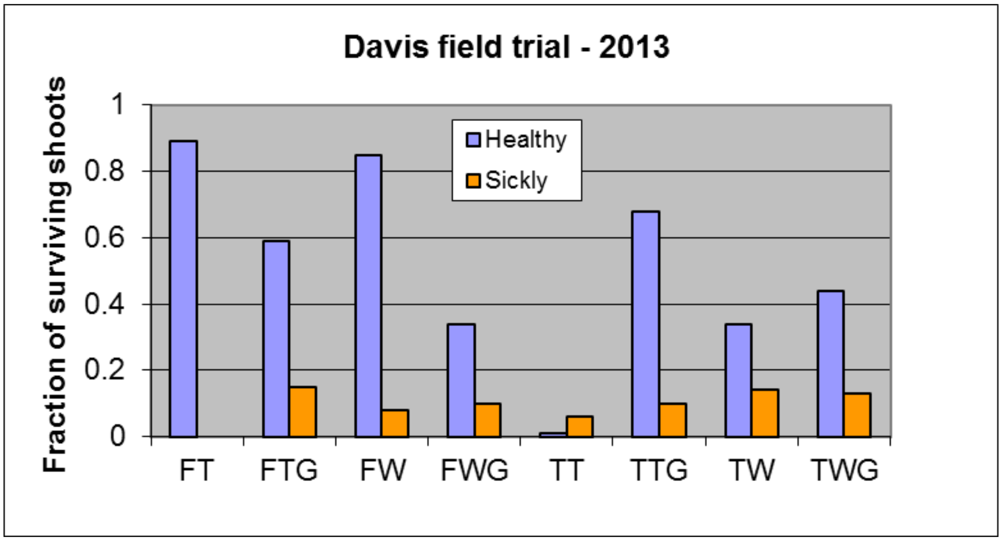

Figure 11. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xylella fastidiosa* that gave rise to at least one new shoot by May, 2013 that exhibited some abnormalities possibly indicative of early stages of Pierces disease infection (orange bars). Treatments include: transgenic DSF producing Freedom as an own-rooted plant (FT); wild type freedom as an own-rooted plant (FW); Thompson seedless scions grafted onto transgenic DSF producing Freedom rootstocks (FTG); Thompson seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF producing Thompson seedless as own-rooted plants (TT); normal Thompson seedless as own-rooted plants (TW); Thompson seedless scions grafted onto transgenic DSF producing Thompson seedless rootstocks (TTG); and Thompson seedless scions grafted onto normal Thompson seedless rootstocks (TWG).

Vines of transgenic and wild type Freedom, as well as wild type and transgenic Thompson seedless, and Thompson seedless scions grafted onto the various transgenic or wild type rootstocks that were apparently healthy and derived from cordons not showing disease in 2013 were again inoculated with *X. fastidiosa* at the Solano County trial on May 28, 2014. The goal of these continuing experiments is to verify the enhanced disease resistance exhibited by transgenic Freedom, and to further quantify the differential susceptibility of Thompson seedless scions grafted onto various transgenic rootstocks. Disease severity was assessed on August 8 and Sept. 15. In addition, disease incidence and severity that developed in 2014 from vines inoculated in previous years, was measured. A uniform rating scale for rating of all vines in both the Solano and Riverside County trials was developed by Lindow and Kirpatrick. This rating scale will allow the severity of disease on inoculated vines in the year of inoculation to be assessed as the fraction of leaves on a given inoculated vine that are symptomatic. Furthermore, on vines that have been infected for more than one year, this new 0-5 rating scale accounts for return growth and vigor of growth of vines in years subsequent to that year in which it was originally inoculated.

Disease incidence and severity on plants was rated on both August 8 and September 15, 2014. No symptoms were apparent on inoculated vines of either wild type or transgenic Freedom plants. However, symptoms were apparent on Thompson seedless vines that had been inoculated earlier in the season. A lower incidence of symptomatic leaves were found on Thompson seedless vine grafted onto transgenic Freedom rootstocks compared to those on wild type Freedom rootstocks (Figure 12). The incidence of symptomatic leaves on Thompson seedless vines grafted onto wild type Thompson seedless rootstocks did not differ from that on transgenic Thompson seedless rootstocks. Similarly, the incidence of symptomatic leaves was similar on own rooted Thompson seedless plants compared to that on transgenic Thompson seedless plants (Figure 12). The overall vigor of Thompson seedless scions grafted onto transgenic Freedom rootstocks was similar to that grafted onto wild type Thompson seedless rootstocks (Figure 13). The overall disease severity exhibited by wild type and transgenic Thompson seedless plants was also similar, and disease severity on Thompson seedless scions grafted onto either wild type or transgenic Thompson seedless rootstocks also did not differ (Figure 13). Thus, some evidence for protection of scions grafted onto RpfF-expressing freedom rootstocks was again seen in 2014 as in earlier years.

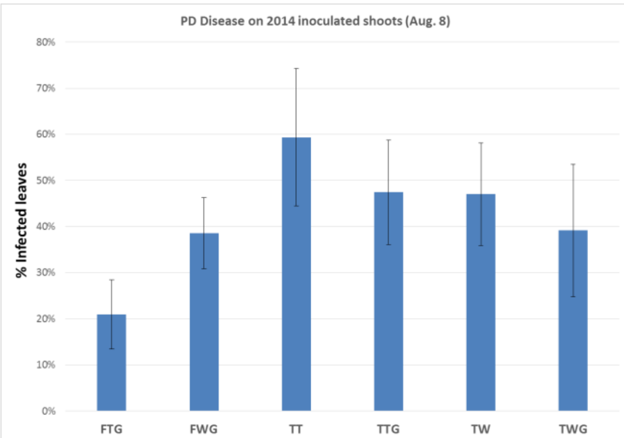

Figure 12. The percentage of leaves of vines in the Solano County field trial inoculated in 2014 with *Xylella fastidiosa* that exhibited symptoms of Pierce's disease on August 8, 2014. Treatments include: Thompson seedless scions grafted onto transgenic DSF producing Freedom rootstocks (FTG); Thompson seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF producing Thompson seedless as own-rooted plants (TT); normal Thompson seedless as own-rooted plants (TW); Thompson seedless scions grafted onto transgenic DSF producing Thompson seedless rootstocks (TTG); and Thompson seedless scions grafted onto normal Thompson seedless rootstocks (TWG). The vertical bars represent the standard error of the mean.

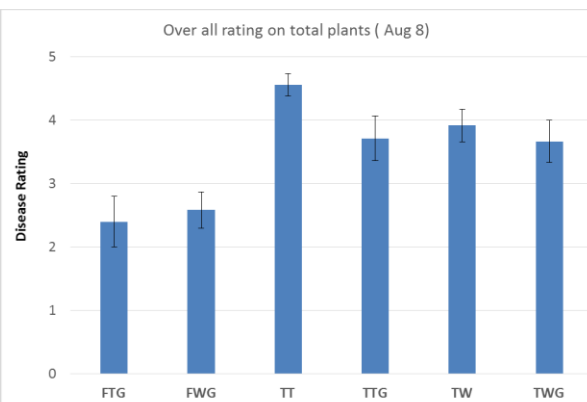

Figure 13. The overall disease rating of vines in the Solano County field trial when assessed on August 8, 2014. Treatments include: Thompson seedless scions grafted onto transgenic DSF producing Freedom rootstocks (FTG); Thompson seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF producing Thompson seedless as own-rooted plants (TT); normal Thompson seedless as own-rooted plants (TW); Thompson seedless scions grafted onto transgenic DSF producing Thompson seedless rootstocks (TTG); and Thompson seedless scions grafted onto normal Thompson seedless rootstocks (TWG). The vertical bars represent the standard error of the mean.

The incidence of symptomatic leaves had increased by September 15 from the low levels seen in August. A dramatic difference in the incidence of symptomatic leaves was observed between wild type and RpfF-expressing Freedom grape. While no symptomatic leaves were observed on the transgenic freedom plants, over

15% of the leaves on the vines of wild type Freedom plants that had been inoculated in May were showing symptoms of pierces disease (Figure 14). As observed in the August evaluation, the incidence of leaves on Thompson seedless vines grafted to a transgenic Freedom rootstock was lower than that on Thompson seedless vines grafted onto a wild type Freedom rootstock (Figure 14). An assessment was also made in September of the overall appearance of plants. The disease rating for transgenic freedom plants was significantly lower than that for wild type freedom plants (figure 15). In contrast, while numerically lower, the severity of Thompson seedless scions grafted onto transgenic Freedom rootstocks did not differ from that of Thompson seedless scions grafted onto wild type Freedom rootstocks (Figure 15). Thus, the transgenic RpfF-expressing Freedom plants continue to show relatively high resistance to Pierce's disease both in the same season that they are inoculated as well as over several years compared to the wild type Freedom plants.

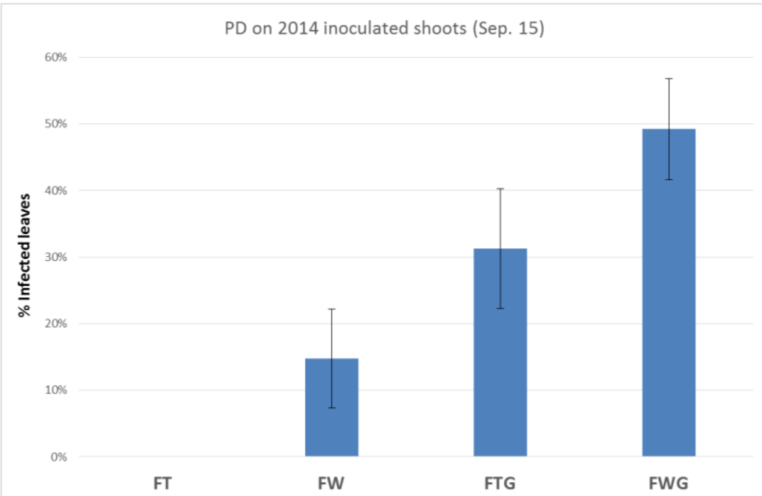

Figure 14. The percentage of leaves of vines in the Solano County field trial inoculated in 2014 with *Xylella fastidiosa* that exhibited symptoms of Pierce's disease on September 15, 2014. Treatments include: transgenic DSF producing Freedom as an own-rooted plant (FT); wild type freedom as an own-rooted plant (FW); Thompson seedless scions grafted onto transgenic DSF producing Freedom rootstocks (FTG); and Thompson seedless scions grafted onto normal Freedom rootstocks (FWG. The vertical bars represent the standard error of the mean.

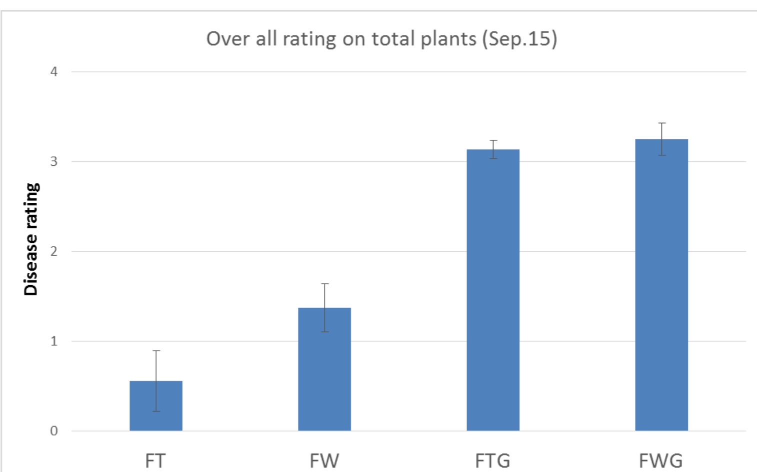

Figure 15. The overall disease rating of vines in the Solano County field trial that exhibited symptoms of Pierce's disease on September 15, 2014. Treatments include: transgenic DSF producing Freedom as an own-rooted plant (FT); wild type freedom as an own-rooted plant (FW); Thompson seedless scions grafted onto transgenic DSF producing Freedom rootstocks (FTG); and Thompson seedless scions grafted onto normal Freedom rootstocks (FWG. The vertical bars represent the standard error of the mean.

Disease was assessed in early October, 2014 at the Riverside County trial. In general, the plants had not grown well, with very little new growth, even on plants that were not infected. Overall, the plants did not look thrifty, and appeared to be suffering from other growth limitations such as nematode damage. In many cases, vines did not emerge from a given cordon. The overall disease severity of these plants was high and similar between all treatments (Figure 17). Because Freedom plants tend to have many shoots arising from a given cordon, we assessed the disease state of each should arising from a given cord on to yield an overall disease severity estimate for these plants. That is, if a given cordon had 10 shoots, two of which having symptoms of Pierces disease, disease incidence would have been assessed as 20%. While most of the shoots on some plants were healthy, on other plants, most of the shoots from a given cord on were infected. Overall, the disease incidence of plants of different treatments were similar, although the incidence of infection of shoots emerging from plants grafted onto transgenic Freedom were somewhat lower than those on plants grafted onto wild type Freedom rootstocks, as has been observed in ratings in previous years (Figure 16).

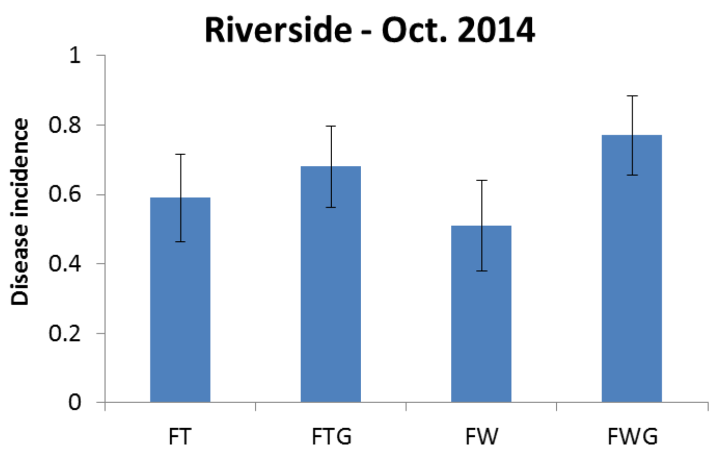

Figure 16. The percentage of vines in the Riverside County field trial that exhibited symptoms of Pierce's disease on October 6, 2014. Treatments include: transgenic DSF producing Freedom as an own-rooted plant (FT); wild type freedom as an own-rooted plant (FW); Thompson seedless scions grafted onto transgenic DSF producing Freedom rootstocks (FTG); and Thompson

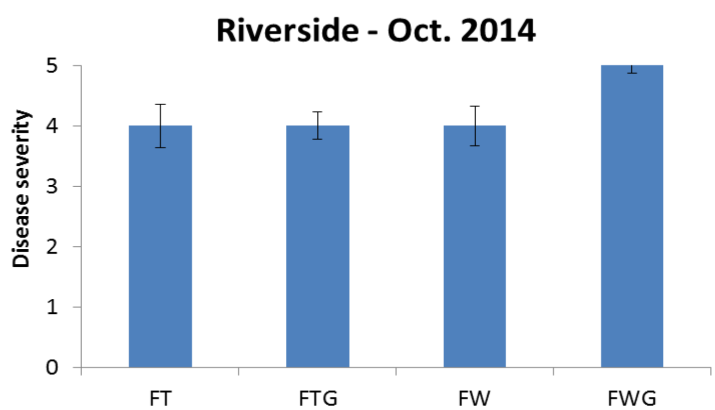

Figure 17. The overall disease rating of vines in the Riverside County field trial that exhibited symptoms of Pierce's disease on October 6, 2014. Treatments include: transgenic DSF producing Freedom as an own-rooted plant (FT); wild type freedom as an own-rooted plant (FW); Thompson seedless scions grafted onto transgenic DSF producing Freedom rootstocks (FTG); and Thompson seedless scions grafted onto normal Freedom rootstocks (FWG. The vertical bars represent the standard error of the mean.

PUBLICATIONS AND PRESENTATIONS

Retchless, A.C., Labroussaa, F., Shapiro, L., Stenger, D.C., Lindow, S.E. and Almeida, R.P.P. 2015. Genomic insights into *Xylella fastidiosa* interactions with plant and insect hosts. In: Genomics of plant-associated bacteria. Ed. Gross, D., Lichens-Park, A. and Kole, C. Springer (in press).

Roper, C and Lindow S.E. 2015. Xylella fastidiosa: Insights into the lifestyle of a xylem-limited bacterium. Pp. XX in (N. Wang, J. Jones, G. Sundin, F. Whie, S. Hogenhout, C. Roper, L. De La Fuente, and J.H. Hams, eds.) Virulence mechanisms of plant pathogenic bacteria. APS Press. St. Paul, MN. (in press)

Lindow, S.E., Newman, K., Chatterjee, S., Baccari, C., Lavarone, A.T., and Ionescu, M. 2014. Production of *Xylella fastidiosa* diffusible signal factor in transgenic grape causes pathogen confusion and reduction in severity of Pierce's disease. Molec. Plant-Microbe Interact. 27:244-254.

Presentation at the 13th International Conference on Plant Pathogenic Bacteria, Shanghai, China, entitled "The complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion".

Presentation at the International Symposium on the European Outbreak of *Xylella fastidiosa* in Olive. Gallipoli, Italy, October, 2014.

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". These field trials are direct demonstration projects to test the field efficacy of plants producing DSF to alter pathogen behavior in a way that symptom development is minimized. Results that both the Solano County and Riverside County trials provide solid evidence that pathogen confusion can confer high levels of disease control - both to plants artificially inoculated had Solano County, and especially to plants infected naturally with infested sharpshooter vectors. The work therefore has provided solid evidence that this strategy is a useful one for managing Pierces disease. These results justify the further examination of this strategy in other grape varieties.

LAYPERSON SUMMARY:

X. fastidiosa 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 grape by introducing the *rpfF* gene which encodes a DSF synthase reduces disease severity in greenhouse trials. We are testing two different lineages of DSF-producing plants both as own-rooted plants as well as rootstocks for susceptible grape varieties. Plots on both Solano and Riverside Counties reveal that DSF producing Freedom grape, which was highly resistant to Pierce's disease in greenhouse trials is also much less susceptible to disease in field trials, especially in plants naturally infected by sharpshooter vectors.

STATUS OF FUNDS

The project as proposed is proceeding on schedule. The funds remaining are sufficient to complete the project as proposed.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY

A patent application (12/422,825) entitled "biological control of pathogenicity of microbes that use alpha, beta unsaturated fatty acid signal molecules" had been submitted March 13, 2009. While many of the claims had been rejected earlier, the University of California patent office has filed on March 13, 2012 a motion requesting reconsideration of the application with clarification of, and justification for, claims related to the production of transgenic plants transformed with the *rpfF* gene from *Xylella fastidiosa*. This petition was approved in June, 2012 and patent US 8,247,648 B2 was issued on August 21, 2012.