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, 2014

ABSTRACT:
A cell density-dependent gene expression system in *X. fastidiosa* mediated by a small signal molecule called diffusible signal factor (DSF) which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) and 2-Z-hexadecenoic acid (C16-cis) controls the behavior of *X. fastidiosa*. The accumulation of DSF attenuates the virulence of *X. fastidiosa* by stimulating the expression of cell surface adhesins such as HxfA, HxfB, XadA, and FimA (that make cells sticky and hence suppress its movement in the plant) while down-regulating the production of secreted enzymes such as polygalacturonase and endogluconase which are required for digestion of pit membranes and thus for movement through the plant. Artificially increasing DSF levels in plants in various ways increases the resistance of these plants to Pierce's disease. Disease control in the greenhouse can be conferred by production of DSF in transgenic plants expressing the gene for the DSF synthase from *X. fastidosa*; such plants exhibit high levels of disease resistance when used as scions and confer at least partial control of disease when used as rootstocks. This project is designed to test the robustness of disease control by pathogen confusion under field conditions where plants will be exposed to realistic conditions in the field and especially under conditions of natural inoculation with insect vectors. We are testing two different lineages of DSF-producing plants both as own-rooted plants as well as rootstocks for susceptible grape varieties in two field sites. Plants were established in one field site in Solano County on August 2, 2010. Plants were planted at a Riverside County site on April 26, 2011. All plants at the Solano County experimental site were needle-inoculated with a suspension of *X. fastidiosa* in May, 2012; at least 4 vines per plant were inoculated, each at a given site with a 20 ul droplet of *X. fastidiosa* containing about 10^6 cells of *X. fastidiosa*. The incidence of infection of the inoculated vines was reduced about 3-fold in assessments made in August and September. Disease was observed only near the point of inoculation in transgenic Freedom, but had spread extensively in wild-type Freedom grape. 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 incidence 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. Plants at the Riverside County plot were subject to high levels of natural infection in 2012. The incidence of infection of transgenic DSF-producing Freedom was about 3-fold less than that of wild-type Freedom grape, while the number of infected leaves per vine was about 5-fold less, 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. The incidence 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.

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, however, 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).

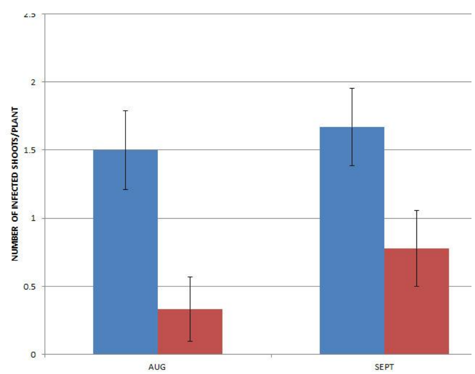


Figure 1. Incidence of vines of DSF-producing transgenic Freedom grape (red) or wild type Freedom having any symptoms of Pierce's disease when rated in August or September, 2012. A total of 3 vines per plant were assessed. The vertical bars represent the standard error of the mean.

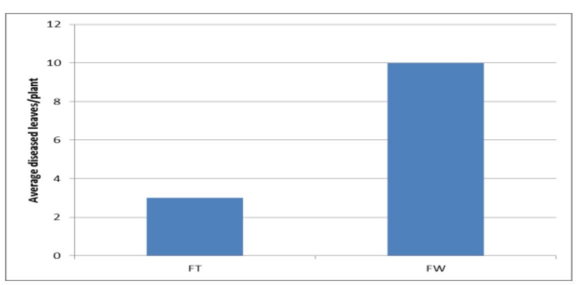


Figure 2. Severity of Pierce's disease on transgenic Freedom grape (FT) and on wild type Freedom grape assessed in August 2012 in the Solano country trial.

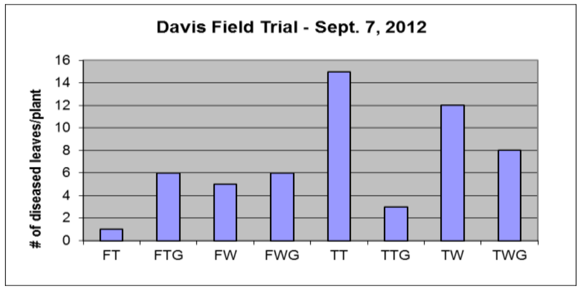


Figure 3. Severity of Pierce's disease on grape assessed in September 2012 in the Solano country trial. See treatment codes above for treatment comparisons.

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.

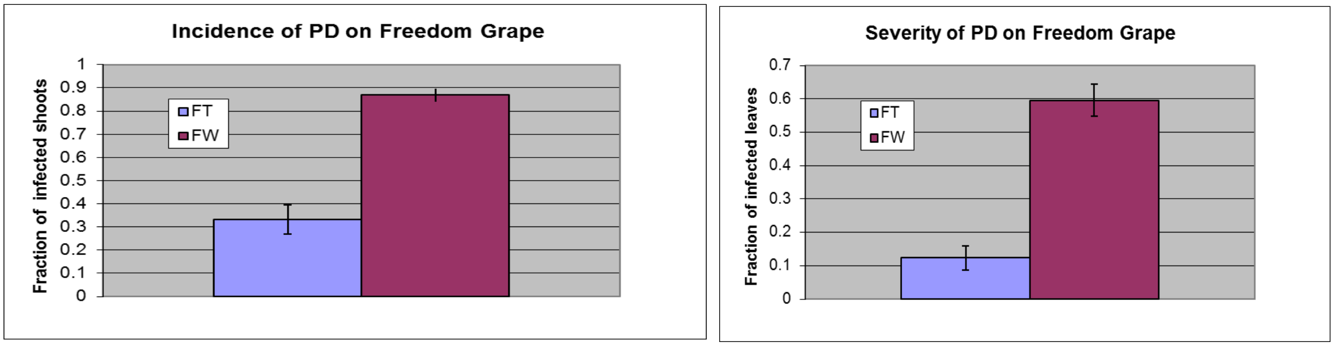


Figure 6. Incidence of Pierce's disease of transgenic DSF-producing Freedom grape (blue bars) or wild type Freedom (red Bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right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

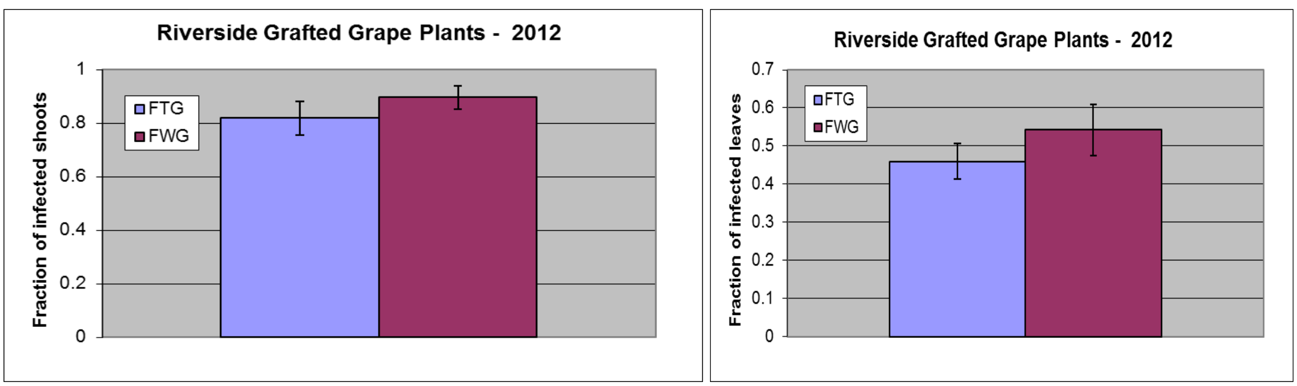


Figure 7. Incidence of Pierce's disease of normal Thompson seedless grape grafted onto transgenic DSF-producing Freedom grape rootstocks (blue bars) or wild type Freedom rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured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

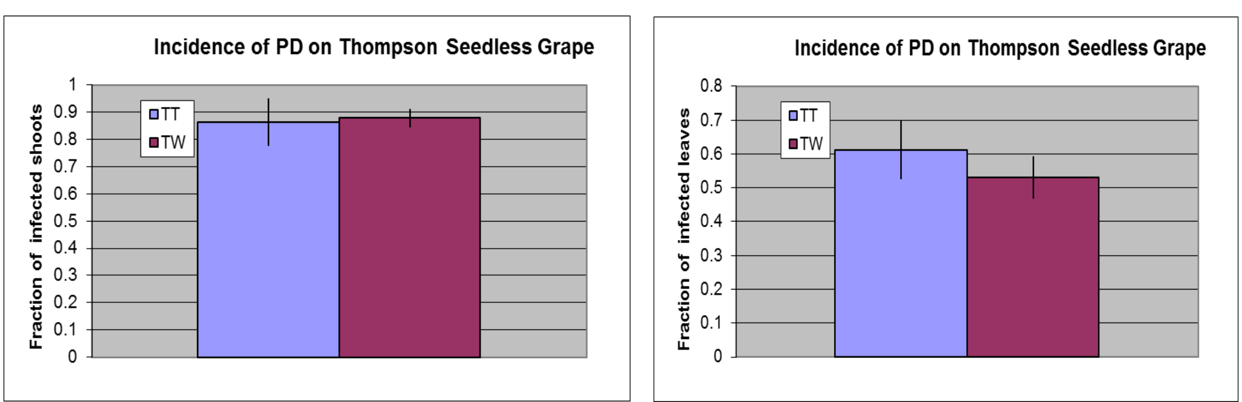
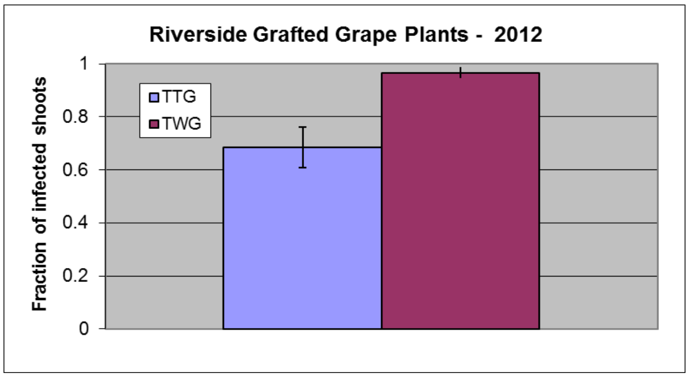


Figure 8. Incidence of Pierce's disease of transgenic DSF-producing Thomson seedless grape (blue bars) or wild type Thompson seedless (red Bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited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



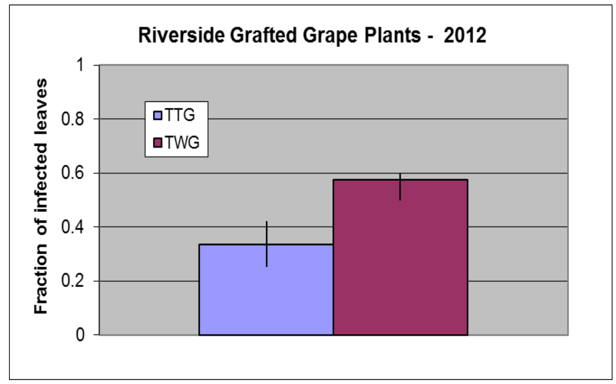


Figure 9. Incidence of Pierce's disease of normal Thompson seedless grape grafted onto transgenic DSF-producing Thompson seedless grape rootstocks (blue bars) or wild type Thompson seedless rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity 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.

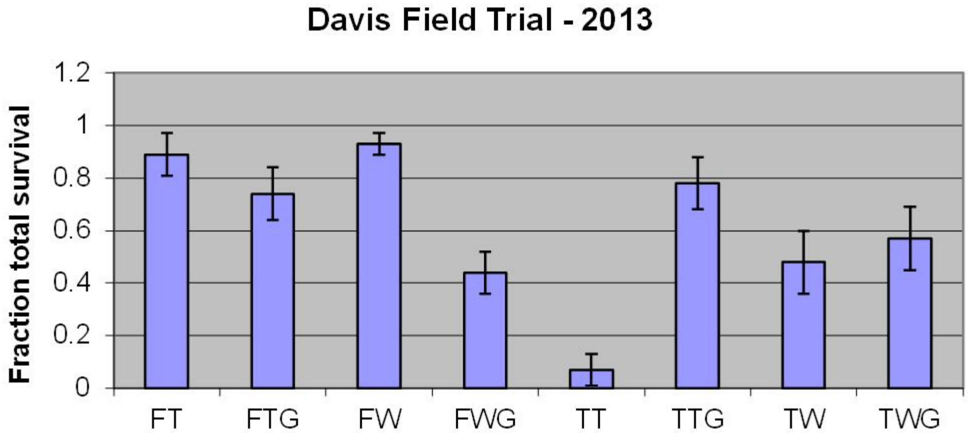


Figure 10. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xylella fastidiosa* that gave rise to at least one new shoot by May, 2013. Treatments include: transgenic DSF producing Freedom as an own-rooted plant (FT); wild type freedom as an own-rooted plant (FW); Thompson 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.

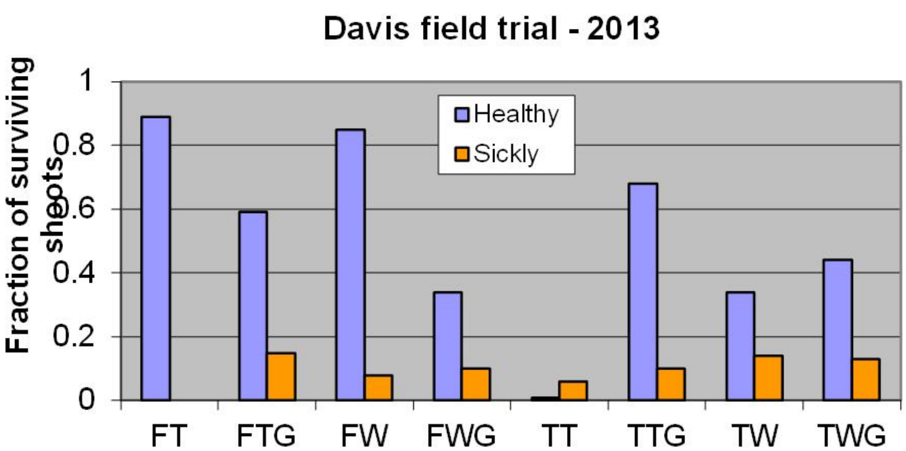


Figure 11. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xylella fastidiosa* that gave rise to at least one new shoot by May, 2013 that exhibited some abnormalities possibly indicative of early stages of Pierces disease infection (orange bars). Treatments include: transgenic 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).

All plants at the Riverside trial were also rated on October 14, 2013. Both the severity and incidence of Pierce's disease symptoms on transgenic DSF-producing Freedom grape was much lower than that on wild type Freedom plants, as had been seen in 2012. In addition, the severity of Pierce's disease symptoms on

Thompson seedless scions grafted onto transgenic DSF-producing Thompson seedless rootstocks was also lower than that of Thompson seedless scions grafted onto wild type Thompson seedless rootstocks, again as was noted in 2012.

Dormant cuttings of inoculated vines of the transgenic Freedom were collected during the winter months of 2014 from the Solano County site for use in grafting experiments at Berkeley in the Greenhouse to establish plants with exceptionally large rootstocks to assess the role of rootstock size in transfer of DSF to scions.

Publications:

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.

RESEARCH RELEVANCE STATEMENT:

Substantial disease control was conferred by transgenic DSF-producing Freedom grape in both the Solano county and Riverside county field trails. In neither trial did the transgenic Freedom rootstock confer substantial disease control, similar to the observations seen in greenhouse trials. While the transgenic Thompson seedless scion was similar in susceptibility to the wild type Thompson grape, it conferred substantial disease control when used as a rootstock. This work is a direct demonstration of the utility of disease control by a process of pathogen confusion. The work demonstrates that in two different grape varieties to behavior of the pathogen can be altered by the production of the signal molecule in plants. The results are quite promising and provide substantial evidence that this strategy will be effective in reducing the severity of Pierce disease in a wide variety of 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. No mortality of the transgenic Freedom plants has been seen, and they remain more highly resistant to Pierce disease than the untransformed plants.

Status of Funds:

Nearly all of the limited funds allocated for the continuation of these field studies have been utilized for both travel to field sites and for analysis of plant samples.

Summary and status of intellectual property associated with the project:

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.