BIOLOGICAL CONTROL OF PIERCE'S DISEASE OF GRAPE WITH AN ENDOPHYTIC BACTERIUM

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INTRODUCTION

Much of our previous work on *X. fastidiosa* and the control of Pierce's disease has dealt with a cell density-dependent gene expression system mediated by a family of small signal molecules called diffusible signal factor (DSF) which includes 2-Z-tetradecenoic acid (C14-cis), and 2-Z-hexadecenoic acid (C16-cis). This work revealed that cell density signaling modulated the adhesiveness of cells in the plant, and that movement of the pathogen is essential for its virulence and that artificially increasing DSF levels in transgenic plants greatly increased the resistance of these plants in both greenhouse and field studies to Pierce's disease by limiting the spread of the pathogen after infection. While endophytic bacteria might be exploited to produce DSF in plants, until recently, no strains capable of growth or movement in grape had been found. We found however that *Burkholderia phytofirmans* strain PsJN was capable of extensive growth and movement within grape. *Burkholderia phytofirmans* stain PsJN has 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 known not to be plant pathogens. Our intention therefore was to use such a strain as a surrogate host for the *rpfF* gene from *X. fastidiosa* that encodes DSF synthase. We found however that this *Paraburkholderia* strain itself was capable of mediating very high levels of control of Pierce's disease. Our continuing results from greenhouse studies show remarkable ability of this biological control agent to move within plants and to inhibit the movement of *X. fastidiosa*, thus achieving very high levels of disease control. The current work is providing a better understanding of the ways in which this biological control agent can be used for disease control, and extensive field evaluations to exploit the information learn from greenhouse studies are underway. Preliminary results suggest that the biological control agent will be highly efficacious, and that it could be used in conjunction with other disease control strategies such as DSF-mediated pathogen confusion in transgenic plants or by topical application of signaling molecules, as well as with other resistant plants that are being developed in other laboratories.

OBJECTIVES:

1) Determine how the temporal and spatial interactions of *Paraburkholderia* and *X. fastidiosa* in grape inoculated in different ways with this biological control agent lead to disease control.
2) Identify the mechanisms by which *Paraburkholderia* confers biological control of Pierce's disease.
3) Evaluation of biological control of Pierce's disease in field trials in comparison with other strategies of pathogen confusion.

RESULTS AND DISCUSSION:

Objective 1: Biological control with *Paraburkholderia phytofirmans* PsJN.
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 *Paraburkholderia phytofirmans* stain PsJN, which had been suggested to be an endophyte of grape seedlings, multiplied and moved extensively in mature grape plants (Figure 1). Its population size and spatial distribution in grape within six weeks of inoculation was similar to that of *X. fastidiosa* itself, suggesting that it is an excellent grape colonist. Furthermore, DSF production has been demonstrated in certain other *Paraburkholderia* 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. Our studies have shown that co-inoculation of *X. fastidiosa* and *B. phytofirmans* resulted in greatly reduced disease symptoms compared to plants inoculated with *X. fastdiosa* alone; whereas the number of infected leaves of plants inoculated with *X. fastidiosa* alone increased rapidly after week 12, very little disease was observed in plants inoculated with *X. fastidiosa* and *B. phytofirmans* (Figure 1).

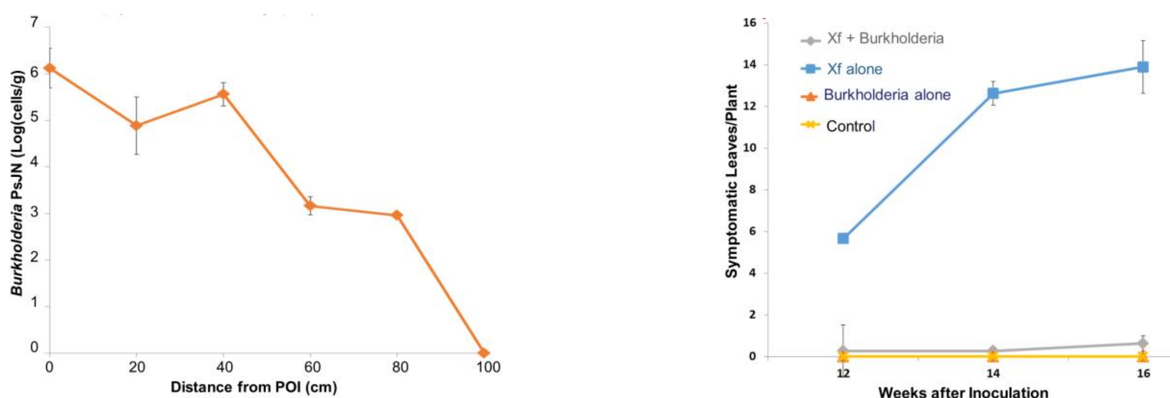

Figure 1. (Left). Population size of *B. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after 6 weeks incubation. (Right). Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *X. fastidiosa* alone (blue) or when co-inoculated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

P. phytofirmans was able to inhibit Pierce's disease development in all grape varieties in which it was evaluated. When inoculated simultaneously into different grape varieties (although not at the same location, but within about 1 cm of the site of inoculation with the pathogen) the progression of Pierce's disease was greatly suppressed compared to that of plants inoculated with *X. fastidiosa* alone (Figure 2). While the greatest reduction in disease severity was conferred in Cabernet Sauvignon, a variety somewhat more resistant to Pierce's disease than either Thompson seedless or Cabernet, *P. phytofirmans* conferred a very high level of disease resistance (Figure 2). It thus appears that the beneficial effect of *P. phytofirmans* is not variety specific, and that it should confer high levels of resistant in all grape varieties.

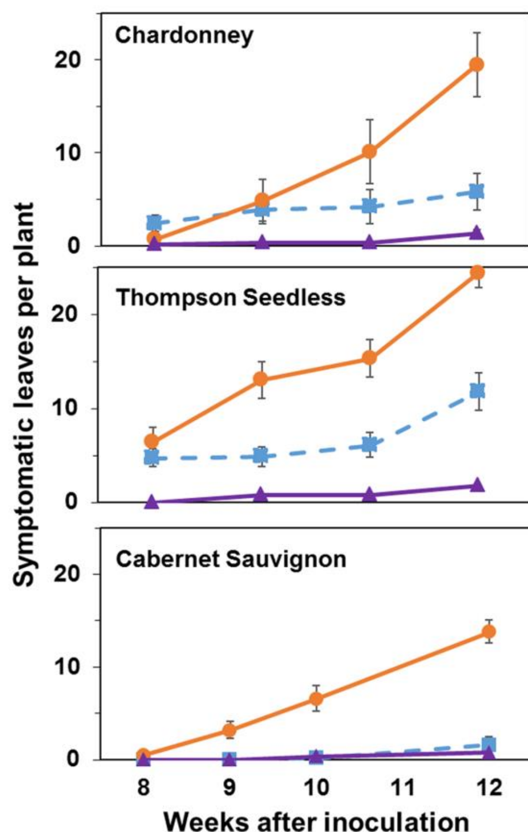

Figure 2. Severity of Pierce's disease observed in different grape varieties needle inoculated at the same time but at different locations with *X. fastidiosa* and *P. phytofirmans* (blue line) compared to that inoculated only with *X. fastidiosa* (orange line), or with *P. phytofirmans* alone (purple line). The vertical bars represent the standard error of the determination mean disease severity.

The large reductions in the severity of disease when *X. fastidiosa* was co-inoculated with *P. phytofirmans* PsJN was associated with the apparent elimination of viable cells of the pathogen both at the point of inoculation as well as at various distances distal to the point of inoculation either 4 or 8 weeks after inoculation (Fig. 3). In contrast, the population size of *X. fastidiosa* increased progressively after its inoculation into grape in the absence of *P. phytofirmans*, reaching population sizes of approximately 10^6 cells at all sites within about 60 cm from the point of inoculation, and moved to at least 120 cm from the point of inoculation within 8 weeks after inoculation (Fig. 3). Such large populations throughout the plant were associated with a high severity of disease, which increased between 11 and 14 weeks after inoculation (Fig. 4). In contrast, no viable cells of *X. fastidiosa* were detected at any location in these plants either 4 or 8 weeks after inoculation together with *P. phytofirmans* (Fig. 4) and no evidence of Pierce's disease was observed even by 14 weeks after inoculation (Fig. 4). By 4 weeks after inoculation, population sizes of *P. phytofirmans* of about 10^4 cells/g were observed at all points up to 60 cm distal to the point of inoculation (Fig. 3). Curiously, while readily detected up to 90 cm or more from the point of inoculation when assessed 8 weeks after inoculation, *P. phytofirmans* population sizes were consistently about 10-fold lower at a given distance from the point of inoculation than at 4 weeks (Fig. 3). *P. phytofirmans* population sizes were often slightly lower at a given sampling time and location when co-inoculated into plants with the pathogen compared to when it was inoculated alone (Fig. 3). Large reductions in population sizes of *X. fastidiosa*, often to undetectably low numbers, in plants inoculated with *P. phytofirmans* at various times, and in various ways, was always observed in the many experiments undertaken.

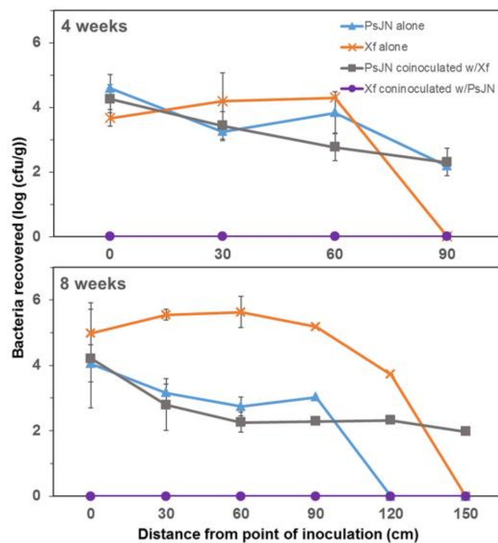

Figure 3. Population size of P. *phytofirmans* PsJN in Cabernet Sauvignon grape stems when needle inoculated alone (Triangles, blue line) or together with *X. fastidiosa* (Squares, black line), and *X. fastidiosa* when inoculated alone (X's, orange line), or together with PsJN (Circles, purple line) at various distances from the point of inoculation after 4 weeks incubation (top panel) or after 8 weeks incubation (bottom panel). The vertical bars represent the standard error of mean log-transformed bacterial population sizes.

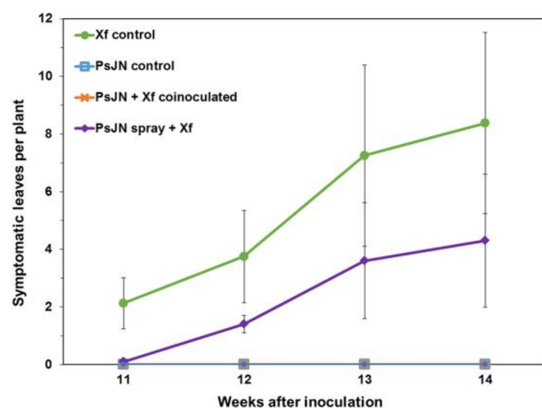

Figure 4. Severity of Pierces disease symptoms on Cabernet Sauvignon grape in the experiment also described in Figure 5 inoculated only with *X. fastidiosa* (Circles, green line), needle inoculated with a mixture of *X. fastidiosa* and *P. phytofirmans PsJN* (X's orange line), inoculated with *X. fastidiosa* immediately after spray inoculation of PsJN in 0.2% Breakthru (Diamonds, purple line), or needle inoculated only with PsJN (squares, blue line). The vertical bars represent the standard error of the mean number of symptomatic leaves at a given assessment time.

To determine whether the inhibitory effects of *P. phytofirmans* on the process of Pierce's disease was dependent on any direct interactions between it and *X. fastidiosa* that might have occurred because of their mixture together at the point of inoculation, we compared the dynamics of disease process in plants in which the pathogen and strain PsJN were applied as mixed inoculation in the same site with that in plants in which they were inoculated separately up to 10 cm apart but at the same time. As previously observed, the severity of Pierce's disease in plants in which the pathogen and strain PsJN were applied as mixed inoculum in the same site in the plant was greatly reduced at a given time after inoculation compared to plants inoculated only with the pathogen (Fig. 5). Importantly, disease severity for plants inoculated at the same time but at different locations with these two strains was usually only nearly as low as that in plants receiving a mixed inoculum. Both treatment schemes resulted in very large reductions in disease severity compared to that of control plants inoculated only with the pathogen (Fig. 5).

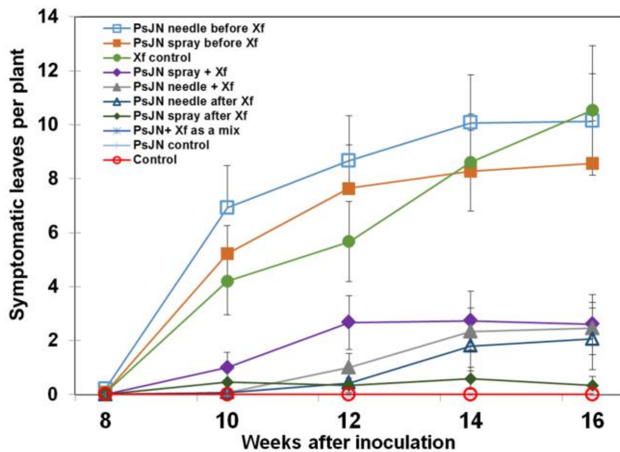

Figure 5. Severity of Pierces disease symptoms on Cabernet Sauvignon grape inoculated only with *X. fastidiosa* (filled circles, green line), needle inoculated with a mixture of *X. fastidiosa* and P. *phytofirmans PsJN* at the same site (X's light blue line), needle inoculated at the same time with *X. fastidiosa* and P. *phytofirmans PsJN* but at different sites on the base of the plant (filled triangles, grey line), inoculated with *X. fastidiosa* immediately after spray inoculation of PsJN in 0.2% Breakthru (filled diamonds, purple line), needle inoculated with PsJN 30 days before inoculation with *X. fastidiosa* (open squares, light blue line), Sprayed with PsJN in 0.2% Breakthru 30 days before inoculation with *X. fastidiosa* (filled squares, brown line); sprayed with PsJN in 0.2% Breakthru 30 days after inoculation with *X. fastidiosa* (filled diamonds, green line), needle inoculated with PsJN 30 days after inoculation with *X. fastidiosa* (open triangles, blue line), needle inoculated only with PsJN (vertical slash, light blue line), or on un-inoculated plants (open circles, red line). The vertical bars represent the standard error of the mean number of symptomatic leaves assessed on each of 15 replicate plants for each treatment at a given assessment time.

Given that close physical proximity of *X. fastidiosa* and *P. phytofirmans* at the time of inoculation of the pathogen is apparently not required to achieve large reductions in disease, we explored methods of inoculation of plants with strain PsJN that might ultimately prove more practical for implementation under field conditions than injection into stems. Spray application of bacterial inoculum might readily be adoptable by growers because of similarities in methodology and equipment used in other pest management procedures. Topical application of suspensions of *P. phytofirmans* of 10^8 cells/ml in buffer alone resulted in only very low internalized population sizes of this strain within either petioles or leaf lamina when assessed at different times after spray application (Fig. 6). In contrast, the population size of strain PsJN applied in buffer containing 0.2% BreakThru®, an organo-silicon surfactant conferring extremely low surface tension to aqueous solutions, (similar to that of Silwet L77), were about 1000-fold higher than that within leaves sprayed with bacterial suspensions in buffer alone (Fig. 6). Furthermore, the population size of strain PsJN was about 100-fold higher within the lamina of the leaf compared to that within the petioles. Not only were large internalized populations of *P. phytofirmans* achieved immediately after inoculation (> 10^3 to 10^5 cells/g), but these endophytic bacterial population sizes increased with time for at least 9 days after spray inoculation (Fig. 6). In many other experiments in which strain PsJN was topically applied with 0.2% Breakthru® the population size of strain PsJN within leaves immediately after inoculation was as high as 10^5 cells/g (data not shown). The leaves sprayed with bacterial suspensions containing this surfactant immediately acquired a water-soaked appearance, indicative of water infiltration into the leaf (Figure 7). The number of bacteria introduced into the plant by such sprays appeared to be influenced by the water status of the plant and whether stomata were fully open, both of which influenced the degree of water infiltration. It thus appears that *P. phytofirmans* can be readily inoculated into grape by simple spray application when appropriate surfactants are used.

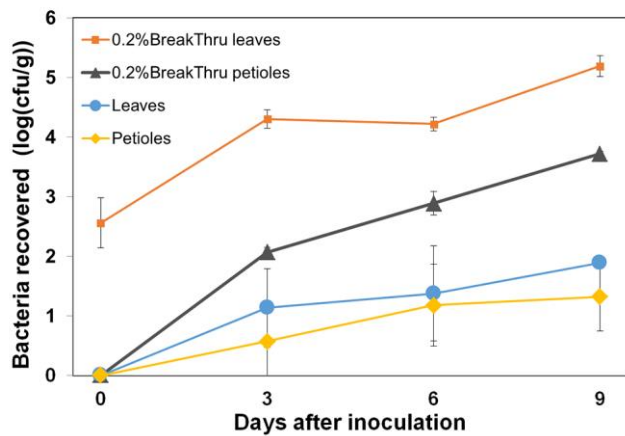

Figure 6. Population size of *Paraburkholderia phytofirmans* PsJN within leaves (Squares, orange line) or petioles (Triangles, black line) when sprayed onto Cabernet sauvignon grape with 0.2% BreakThru® or within leaves (Circles, blue line) or petioles (Diamonds, yellow line) when sprayed onto plants in buffer alone, when sampled at various times after inoculation shown on the abscissa. The vertical bars represent the standard error of the mean of long-transformed population sizes.

Figure 7. Water-soaked spots in leaves of Cabernet Sauvignon grape 10 minutes after topical applications of a suspension of *Paraburkholderia phytofirmans* PsJN suspended in 0.2% Breakthru.

The severity of Pierce's disease on plants sprayed with *P. phytofirmans* immediately before inoculation with *X. fastidiosa* was significantly lower than on control plants inoculated with the pathogen alone (Figs. 4, 5 and 8). While disease severity of plants sprayed with *P. phytofirmans* at the same time as inoculation with the pathogen was often slightly higher than that on plants that were puncture-inoculated with this strain as the pathogen when assessed at a given time, the severity of disease severity in both treatments were always much less than that of control plants inoculated only with the pathogen, and often did not differ significantly. It appears that topical application of *P. phytofirmans* with a surfactant that allows spontaneous stomatal infiltration is nearly as effective in mediating control of Pierce's disease as direct inoculation of this biological control agent into xylem tissue.

While *X. fastidiosa* and *P. phytofirmans* apparently do not need to be entirely spatially coincident at the time of inoculation of the pathogen in order to achieve suppression of Pierce's disease symptoms, and substantial disease control was inevitably obtained when the two strains were inoculated at the same time into plants by various ways, insights as to the possible mechanisms contributing to disease control were obtained by inoculating strain PsJN into plants at various times relative to that of the pathogen. Surprisingly, the extent to which the severity of Pierce's disease was reduced when *P. phytofirmans* was inoculated into plants either by injection or spray application 4 weeks prior to inoculation with *X. fastidiosa* was invariably less than when the two strains were applied at the same time when made by the same method of PsJN application. For example, in some experiments, Pierce's disease severity in plants treated with *P. phytofirmans* either by needle inoculation or spraying 4 weeks before that of the pathogen did not differ from that of plants inoculated with the pathogen

alone, while simultaneous inoculation with strain PsJN by either method conferred very large reductions in disease severity compared to control plants (Fig. 5). In other experiments, pre-treatments of plants with *P. phytofirmans* either by needle inoculation or spraying conferred significant reductions in disease severity compared to that of control plants, yet the extent of disease control was substantially less than that conferred by corresponding needle or spray inoculation at the same time as the pathogen (Figure 8). Disease severity in plants sprayed with *P. phytofirmans* was however consistently less than that in plants to which strain PsJN had been inoculated by puncturing before the pathogen (Figs. 5 and 8). Even more surprising however was the observation that disease control achieved by puncture or spray inoculation of *P. phytofirmans* into plants 3 to 4 weeks after inoculation of the pathogen was as great as, and often greater than, that achieved by simultaneous inoculation by a given method (Figs. 5 and 8). Given that population sizes of *X. fastidiosa* typically increase and spread extensively in inoculated plants within four weeks (Fig. 3), it was remarkable to find, as in other experiments, very low or undetectable population sizes of *X. fastidiosa* subsequent to such treatments with *P. phytofirmans*, even though it was applied 4 weeks after that of the pathogen (data not shown).

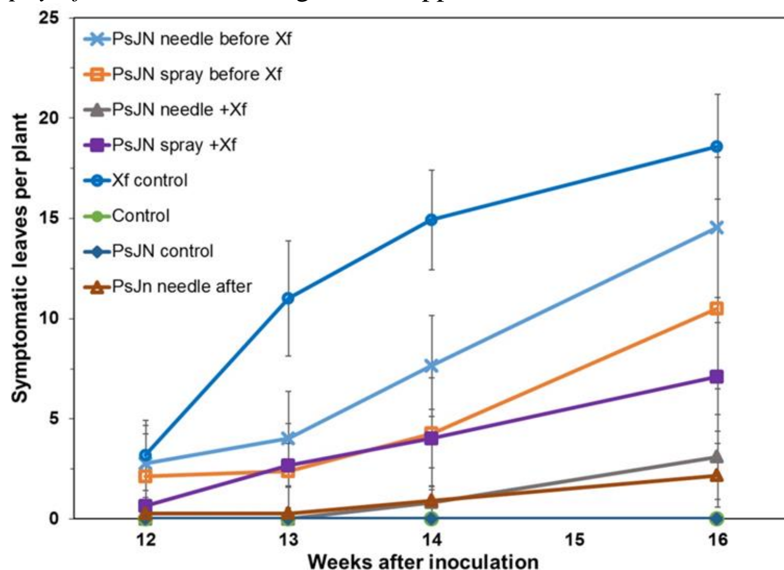

Figure 8. Severity of Pierces disease symptoms on Cabernet Sauvignon grape inoculated only with *X. fastidiosa* (Circles, dark blue line), needle inoculated with a mixture of *X. fastidiosa* and P. *phytofirmans PsJN* (Filled triangles, grey line), inoculated with *X. fastidiosa* immediately after spray inoculation of PsJN in 0.2% Breakthru (Filled squares, purple line), needle inoculated with PsJN 30 days before inoculation with *X. fastidiosa* (X's, light blue line), sprayed with PsJN in 0.2% Breakthru 30 days before inoculation with *X. fastidiosa* (Open squares, orange line), inoculated with *X. fastidiosa* 30 days after needle inoculation with PsJN (Open triangles, red line), or needle inoculated only with PsJN (Diamonds, dark blue line), or on un-inoculated plants (Filled circles, green line). The vertical bars represent the standard error of the mean number of symptomatic leaves at a given assessment time.

Insight as to the surprising observation that pre-treatment of plants with *P. phytofirmans* inevitably reduced its efficacy in biological control of Pierce's disease compared to simultaneous or post inoculation treatments was provided by analysis of the temporal patterns of colonization of plants by strain PsJN. We frequently observed that while relatively large population sizes of *P. phytofirmans* could be detected throughout grape within a few weeks after inoculation, this population size often subsequently decreased, often dramatically so (Fig. 3; data not shown). A more systematic examination of *P. phytofirmans* populations when co-inoculated with *X. fastidiosa* in grape as a function of time revealed that its population size and distribution distal to the point of inoculation both increased for at least 3 weeks after inoculation, but then started to decrease by 5 weeks (Fig. 9). As in most other experiments, viable cells of *P. phytofirmans* often became undetectably low within 10 weeks after inoculation (data not shown). As in all experiments, when inoculated in the absence of *P. phytofirmans* both the population size and extent of distribution of *X. fastidiosa* distal to the point of inoculation tended to increase with time (Fig. 9) while viable cells of the pathogen were not detected at any time or distance from the point of inoculation when co-inoculated with strain PsJN (Fig. 9).

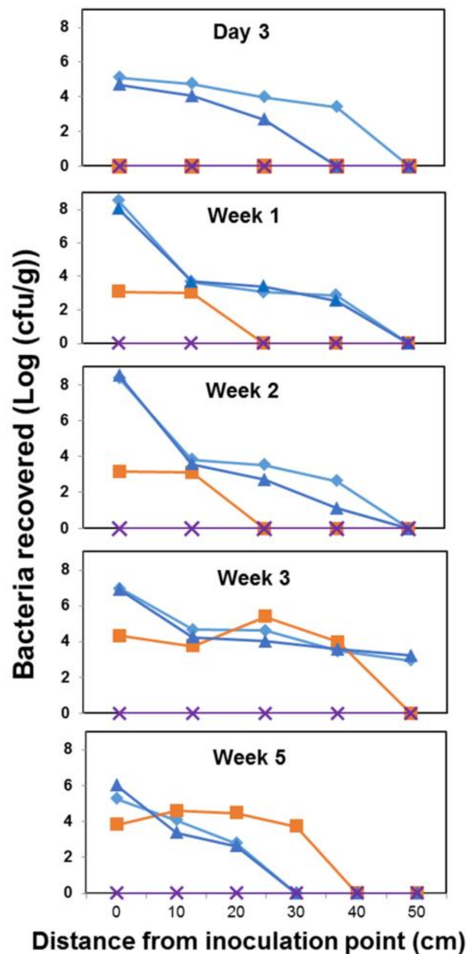

Figure 9. Population size of *P. phytofirmans* PsJN in Cabernet Sauvignon grape stems when needle inoculated alone (Diamonds) or together with *X. fastidiosa* (Triangles), and *X. fastidiosa* when inoculated alone (Squares, orange line), or together with PsJN (X's, purple line) at various distances from the point of inoculation. Each panel shows population sizes at a given time after inoculation.

Objective 2: Mechanisms of biological control

As discussed in Objective 1 it seemed possible that *Paraburkholderia* may alter the behavior and survival of *X. fastidiosa* by inducing changes in grape plants themselves, such as by stimulating innate plant community. Plant innate immunity serves as an important mechanism by providing the first line of defense to fight against pathogen attack. While grape apparently does not successfully recognize and therefore defend against infection by *X. fastidiosa*, it might be possible that plants could be "primed" to mount a defense against *X. fastidiosa* by another organism such as *Paraburkholderia*. Certain beneficial microorganisms such as *Paraburkholderia phytopirmans PsJN* have been shown to prime innate defenses against various pathogens in model plant system such as Arabidopsis, and a recent study suggest that it could also do so in grapevine. Further, the bacterium induces plant resistance against abiotic stresses, apparently by changing patterns of gene expression in host plants. We thus explored whether the reduced disease symptoms and lower pathogen population seen in plants inoculated with *Paraburkholderia* either before or after that of *X. fastidiosa* is mediated by the activation of plant innate immunity. To test this hypothesis we measured the expression of various genes in grape that are responsible for, or reflective of, responses to pathogens, mechanical, and abiotic stresses in 1) Control plants with no treatment, 2) Plants injected with the *Paraburkholderia* strain alone, 3) Plants injected with both *Paraburkholderia* and *X. fastidiosa* strains simultaneously, and 4) Plants inoculated only with *X. fastidiosa*.

The abundance of PR1 indicative of induction of salicylic acid-mediated host defenses, JAZ1 indicative of jasmonic acid-mediated host defenses, and ETR1 reflecting ethylene-dependent responses were determined in RNA isolated from petioles collected from near the point of inoculation of plants by semi-quantitative RT-PCR. The abundance of EF1a, expected to be constitutively expressed, was used as an internal control to account for

the efficiency of RNA isolation. The abundance of these indicator transcripts was compared in plants inoculated only with *P. phytofirmans*, *X. fastidiosa*, or co-inoculated with the pathogen and strain PsJN weekly after inoculation as well as in mock-inoculated plants. Little expression of JAZ1 was detected in any of the plants, irrespective of the sampling time after inoculation (Fig. 10). In contrast, some PR1 transcript was seen soon after inoculation of plants only with *P. phytofirmans*, with lesser amounts subsequently detected. Low levels of PR1 transcript were also observed within 1 week of inoculation of plants only with *X. fastidiosa*, with reductions thereafter. Most notably, the highest levels of PR1 transcript were observed in plants co-inoculated with *P. phytofirmans* and *X. fastidiosa*, with the apparent abundance of this transcript increasing with time up to 3 weeks (Fig. 10). The abundance of PR1 transcript in these plants decreased rapidly thereafter (data not shown). Very low levels of ETR1 transcript were observed in all plants except those co-inoculated with *P. phytofirmans* and *X. fastidiosa* (Fig. 10). This suggests that an interaction between *P. phytofirmans* and *X. fastidiosa* induces both the SA- and ethylene-dependent signal transduction pathways in grape to levels higher than that mediated by either strain alone.

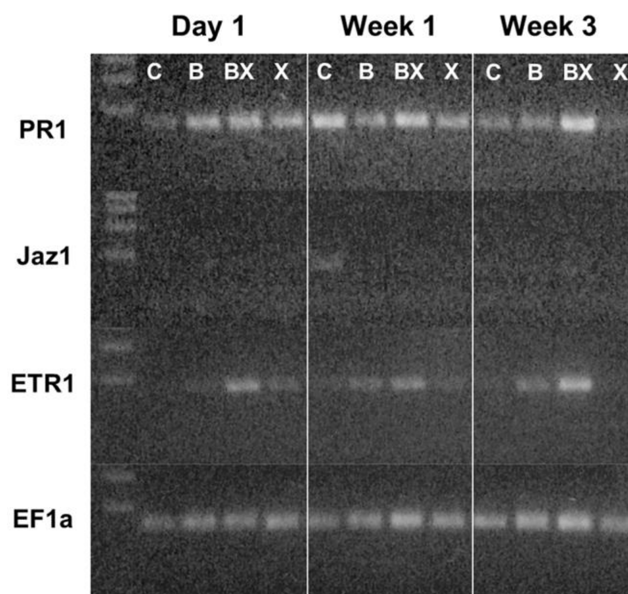

Figure 10. Products obtained after PCR amplification of cDNA obtained from RNA that had been subjected to reverse transcriptase that was isolated from petioles of Cabernet Sauvignon grape near the point of inoculation of plants that were inoculated only with buffer (C), inoculated with P. phytofirmans PsJN alone (B), inoculated with both PsJN and *X. fastidiosa* (BX), or were inoculated with *X. fastidiosa* alone (X). Shown are bands corresponding to amplification products of PR1, Jaz1, ETR1, and EF1a from RNA sampled from plants harvested at the various times shown above each lane.

Ethylene and salicylic acid mediated host defenses often involve production of reactive oxygen species such as hydrogen peroxide. It is a production of such compounds that are associated with the inhibition of pathogen growth. To move beyond the simple measurement of gene expression changes in grape associated with the presence of both *Paraburkholderia* and *X. fastidiosa* in the plant, we measured hydrogen peroxide levels in plants that were treated with *Paraburkholderia* immediately before *X. fastidiosa*. Thompson seedless grapes were needle inoculated on the stem with 10 ul of *X. fastidiosa* cell suspension (10^8 cells/ml) or with 10ul of a suspension of *Paraburkholderia* PsJN at the same concentration, or with 10 ul of a mixture of the two bacteria (each at 10^8 cells/ml) by the droplet puncture method. Plants were also inoculated with 10 µL of buffer as a negative control. After 15 minutes, the petioles closest to the point of inoculation on each plant was removed and freehand sections were made using a razor blade. The cut end of each petiole was then pressed onto a nitrocellulose membrane previously soaked in 5ml/ml BAD-HCL solution (PH 3.8) and then dried at room temperature in the dark before the use. After 10 seconds, the petiole was removed from the nitrocellulose membrane and immediately washed with 100% EtOH to remove any plant material and photographed under a stereoscope after 5 m at room temperature. The presence of hydrogen peroxide in the plant tissue reacts with the BAD reagent to produce a brown insoluble product that is readily visible on the surface of the white nitrocellulose membranes. Digital images one of the impregnation spot were obtained with a stereo microscope and the digital images were then were analyzed using Image J to determine the intensity of the brown product,

which in turn, is proportional to the concentration of hydrogen peroxide in the plant tissue. These stained images were converted to grayscale with a range of 0-255 (255 being white and 0 being black) and pixel intensity determined. Very low hydrogen peroxide concentrations, similar to those observed in plants inoculated with buffer alone, were observed in plants inoculated with *Paraburkholderia phytofirmans* alone, as well as *X. fastidiosa* alone (Figure 11). Importantly, a much higher concentration of hydrogen peroxide was observed in petioles of plants that had been inoculated with both *Paraburkholderia* and *X. fastidiosa* (Fig. 11) (note that the smaller value represents a darker image and hence higher concentration of hydrogen peroxide). These exciting results help to confirm that the presence of *Paraburkholderia* is priming grape for a strong defensive reaction to the presence of the pathogen, whereas the plant appears to have no response to the presence of the pathogen alone. The induction of reactive oxygen species in such primed plants provide strong support for the observations noted above that viable cells of *X. fastidiosa* are often not detected in plants co-inoculated with *Paraburkholderia*, and also could explain why the population size of *Paraburkholderia* itself tends to decrease with time, especially when inoculated into plants with the pathogen. This method for detection of reactive oxygen species appears to be one that can be readily scaled up and we look forward to investigating both the temporal and spatial patterns of reactive oxygen species in plants under field conditions in which *Paraburkholderia* is inoculated into plants at various times relative to that of inoculation with the pathogen.

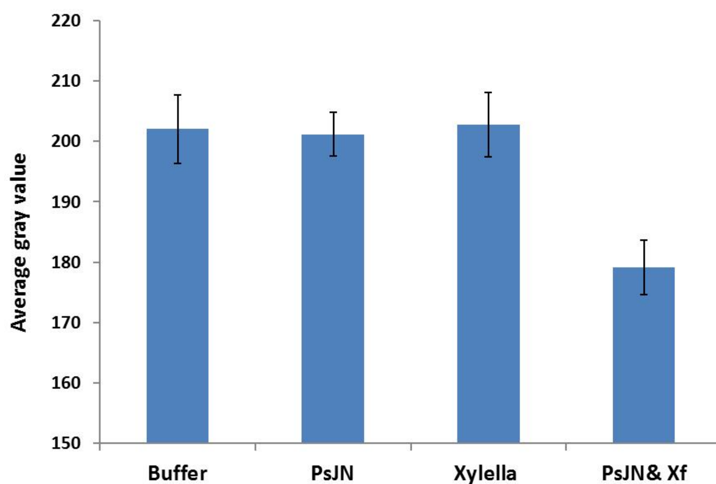

Figure 11. Estimations of reactive oxygen species in plants inoculated with buffer alone, *Paraburkholderia* alone, *X. fastidiosa* alone, and with both *Paraburkholderia* and *X. fastidiosa* determined by average of gray scale values for DAB mediated tissue printing. The vertical bars represent the standard error of oxidized DAB determined from the general analysis of images. Note that the smaller value represents a darker image and hence higher concentration of hydrogen peroxide.

We have observed in the many experiments in which grape has been inoculated with *Paraburkholderia* that population sizes of this biological control agent are maximal in plants within a few weeks after inoculation, but that populations in the plant seem to decrease thereafter. We are continuing work to test the hypothesis that *Paraburkholderia i*s a very efficient colonizer of grape, but one that may be self-limiting. Specifically, we hypothesize that the plant may locally recognize and respond to the colonization of *Paraburkholderia* in a way that leads to a reduction in its own population size. In fact, it may be this response of the plant to *Paraburkholderia* that is also responsible for the dramatic reductions in *X. fastidiosa* populations in plants inoculated with *Paraburkholderia*. If, as we hypothesize, such a host response is relatively local to the plant region colonized by *Paraburkholderia*, the patterns of biological control that we have observed could be explained. Specifically, biological control of Pierce's disease would be expected if *Paraburkholderia* was applied at the same time as or even after that of the pathogen if the rapid movement of *Paraburkholderia* throughout the plant mediated a defensive reaction either before the plant had been colonized by *X. fastidiosa* or before the pathogen had achieved population sizes sufficient to incite disease symptoms. In this model, the spatial movement and persistence of *Paraburkholderia* in the plant would determine the efficacy of biological control (Fig. 12). Our ongoing studies to investigate the spatial movement and temporal persistence of *Paraburkholderia* in plants after inoculation relative to that of the pathogen when inoculated at different times and locations are central to our understanding of how to optimize biological control of Pierce's disease.

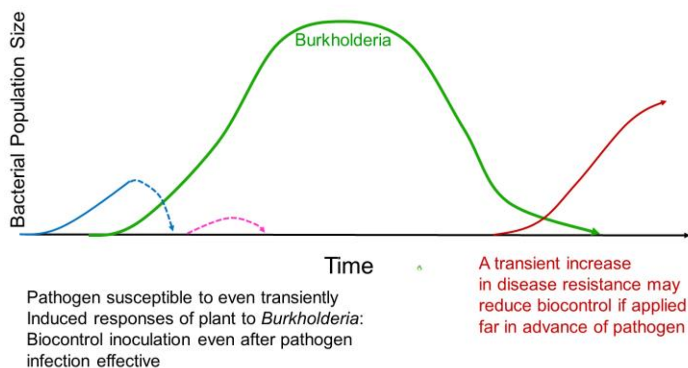

Figure 12. A model describing the expected temporal growth and persistence of *Paraburkholderia* in grape plants after inoculation (green line) and the expected effects on population sizes of *X. fastidiosa* inoculated at various times relative to that of *Paraburkholderia* (blue, pink, and red lines) based on the hypothesis that *Paraburkholderia* mediates a local inhibitory effect on pathogen populations.

Objective 3: Field efficacy of biological control of PD.

Large-scale field studies in a replicated field site managed by the Department of Plant Pathology at the University of California, Davis were initiated in 2018 that evaluated the extent to which the factors which we found to control the efficacy of biological control under greenhouse conditions were directly applicable to the control of Pierce's disease in a field setting. The study was also designed to enable us to evaluate the effectiveness of spray application of *Paraburkholderia* relative to that of direct needle inoculation. A large planting of Chardonnay, Cabernet Sauvignon, and Pinot Noir were established from so-called "Uber" plants generously provided by Duarte Nurseries and were planted in late April, 2017 and were sufficiently large by the spring of 2018 to inoculate with the pathogen and *Paraburkholderia*. In 2018 both Cabernet Sauvignon and Pinot Noir were inoculated, while Chardonnay will be inoculated in 2019. The overall experimental design involve the following treatments 1) challenge plants with *Xf* relatively soon after needle inoculation or topical treatment with *Paraburkholderia,* 2) challenge plants with *Xf* several weeks after inoculation of plants with *Paraburkholderia* in different ways, 3) inoculate *Paraburkholderia* into plants in different ways only after challenge inoculation with *Xf* to assess the potential for "curative effects" after infection has occurred, and 4) challenge inoculate plants treated with *Paraburkholderia* with *Xf* on multiple occasions, spanning more than one growing season to reveal the persistence of the biological control phenomenon. Greenhouse studies in our current project have also indicated that topical applications of a DSF-like molecule, palmitoleic acid, with a penetrating surfactant can also confer disease resistance. This treatment was therefore compared with the various biological control treatments. Each treatment consisted of 10 plants for a given grape variety. For individual vines (one on each of the four cordon arms for a given plant) were inoculated. The details of the experimental design are shown in figure 13.

	April	May	June	July	August rating	Sept rating	2019
	Xy STL						
1		Xy B needle					
2		B&Xy mix					
3		Xy B spray					
4	B needle	Xylella					
5	B spray	Xylella					
6		only Xylella (control)					
7	B needle						
8	B spray						
9		UNINOCULATE (control)					
10		Xylella	B needle				
11		Xylella	B Spray				
12		Xylella BREAK					
13		Xy B needle	B needle	B needle			
14		Xy B spray	B Spray	B Spray			
15		Xy B needle	Xy B needle	B needle			
16		Xy B spray	Xy B spray	B Spray			
17		only Xy (control)	only Xy				
18		Xy B TRUNK					
19		Xy & soap	soap	soap			
20		prime with B needle					year 2
21		prime with B spray					year 2

Figure 13. Experimental design and treatment listed for field trials conducted in 2018. Columns represent treatments made at a given time indicated in the headings. Note that on some occasions more than one treatment

was applied at a given inoculation time. Unless otherwise noted, all inoculations made at the base of vines. Xy or *Xylella* = inoculation made with *X. fastidiosa* strain STL via droplet puncture. B needle = inoculation made with *P. phytofirmans* PsJN via droplet puncture. B and Xy mix = inoculum of both *P. phytofirmans* and *X. fastidiosa* were mixed in inoculated as a single droplet puncture. Bspray = inoculation made by spraying *P. phytofirmans* PsJN in 0.2% Breakthru. B trunk = inoculation of the trunk of vines (ca. 30 cm from soil level) made with *P. phytofirmans* PsJN via droplet puncture. Soap = spray application of 2% Palmitoleic acid. Year 2 = challenge inoculation with *Xylella fastidiosa* to be made in spring, 2019 in plants that were inoculated in spring, 2018 with *P. phytofirmans* in different ways.

As was observed under greenhouse conditions, topical applications of *P. phytofirmans* with 0.2% Breakthru to leaves was found to be an efficient way to introduce this bacterium into grape tissues under field conditions. Watersoaking was quite apparent within one minute after application to leaves (Figure 14). Despite the fact that the water suspensions dried relatively rapidly on the leaves under the relatively warm and often windy conditions in which they were applied, watersoaking was quite extensive and persisted for approximately 15 minutes after inoculation. Large population sizes of strain PsJN to be immediately introduced into leaves in this process, and these populations remained high for many days after inoculation (Figure 14).

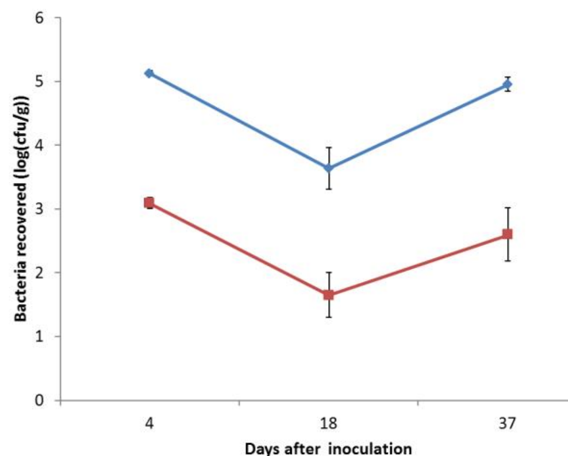

Figure 14. Left) watersoaking appearance of Cabernet Sauvignon leaves approximately two minutes after topical application of a suspension of *P. phytofirmans* PsJN in 0.2% Breakthru in a field trial. Right) population size of *P. phytofirmans* PsJN recovered from surface sterilized lamina of spray-inoculated leaves (blue line) or surface sterilized petioles (red line) at various times after spray inoculation. The vertical bars represent the standard error of log-transformed Bible bacteria recovered per gram of plant tissue.

Substantial levels of disease control were conferred by application of *P. phytofirmans* PsJN in various ways to both Cabernet Sauvignon and Pinot Noir grape either before or after challenge inoculations with *X. fastidiosa* (Figure 15). At the time of this report, statistical analysis of disease assessments of Pinot Noir were still underway, and so disease control obtained in Cabernet Sauvignon will be discussed. Disease severity was measured as the proportion of the total leaves on a given inoculated shoot that exhibited symptoms of leaf scorching. Disease severity was measured approximately every three weeks beginning in mid-August; three separate assessments of disease severity were made. Differences in disease severity were determined after calculating the area under the disease progress curve (AUDPC) for disease measured over time. Very high levels of disease were observed in control vines in which *X. fastidiosa* was inoculated a single time (treatment #6) or on two occasions (treatment #17) or was treated only with the surfactant Breakthru after inoculation (treatment #12). As expected, no symptoms of Pierce's disease were observed on control plants that were not inoculated with *X. fastidiosa* (treatments #7, 8, and 9). Very high levels of disease control were observed in plants treated with *P. phytofirmans* applied in different ways. While the greatest degree of disease control was achieved when both *P. phytofirmans* and *X. fastidiosa* were co-inoculated together at a single site into vines (treatment #2), a very high degree of disease control was also observed when *P. phytofirmans* was either injected or sprayed onto plants several weeks after inoculation with *X. fastidiosa* (treatments #10 and #11, respectively) or inoculated at the same time as, but at different locations within a vine (treatments #1 and #3). Surprisingly, disease control

conferred by a single inoculation of *P. phytofirmans* made after that of the pathogen, provided higher levels of disease control then multiple such applications (compare treatments # 10 and #11 with treatments #13 and #14). In contrast to what had been observed in greenhouse studies, injection of *P. phytofirmans* into plants three weeks before they were inoculated with *X. fastidiosa* also led to high levels of disease control (treatment #4). Given that field-grown plants have a large trunk on which cordons on which the vines are born, unlike the single stems resulting from rooted cuttings in greenhouse studies, we evaluated the direct injection of *P. phytofirmans* into the base of the trunk to determine a very somewhat systemic and distal effect on disease control could be conferred. Disease reductions from trunk injection were similarly large as those made directly into the vines in which *X. fastidiosa* was inoculated (compare treatment #18 with treatments #1 and #10). Repeated topical application of palmitoleic acid also appeared efficacious for disease control (treatment #19).

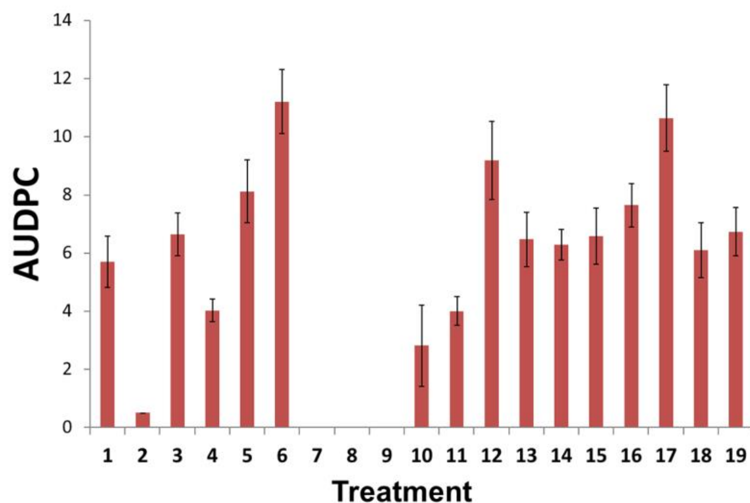

Figure 15. Disease severity of Cabernet Sauvignon grape shown as the area under the disease progress curve for disease assessments made on three occasions in the summer of 2018. The treatment numbers refer to the treatments described in Figure 13.

In addition to measuring the severity of disease (as the proportion of symptomatic leaves on a given inoculated shoot) as shown in Figure 15, we also assessed the extent to which the pathogen moved from each of the four inoculated shoots on a given plant to infect and cause symptoms on adjacent shoots. We thus counted the number of additional shoots on a given plant that exhibited symptoms of Pierce's disease (Figure 16). Even within the short time since plants were inoculated with the pathogen alone (Treatments #6, 12, and 17) symptoms could be observed on a large number of adjacent vines on a given plant (Figure 16). In contrast, many fewer adjacent vines exhibited any symptoms of Pierce's disease on plants treated with *P. phytofirmans* in various ways. Generally, those treatments such as treatment #2 that conferred the greatest reduction in disease severity on inoculated vines also conferred the greatest reduction in spread of disease symptoms to adjacent vines on a given plant (Figure 16). It was noteworthy that the direct inoculation of *P. phytofirmans* into the trunk of these mature plants also greatly reduced any spread of disease symptoms away from the inoculated vines (treatment #18), suggesting that it's basal inoculation site may have maximized any potential systemic induction of disease resistance that is postulated as a mechanism of action of *P. phytofirmans*. The high levels of disease control seen after inoculation with *P. phytofirmans* are exciting and suggest that even higher levels of disease control could be conferred after further exploration of practical questions of optimum timing and application methods.

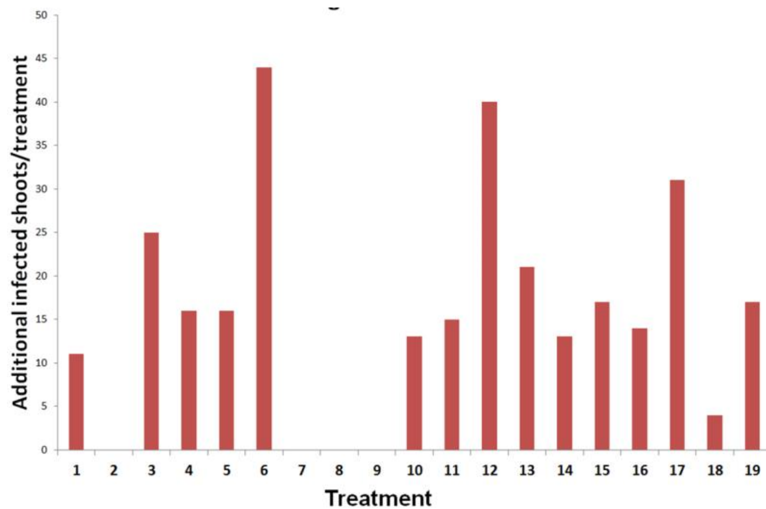

Figure 16. The number of additional shoots on a given plant that were not directly inoculated with *X. fastidiosa* that exhibited symptoms of Pierce's disease by late September, 2018. Shown is the total number of shoots on inoculated with *X. fastidiosa* on the 10 plants receiving a given treatment (described in Figure 13) that exhibited symptoms of Pierce's disease.

Since 40 individual shoots on the 10 replicate plants in the field study received a given treatment of both *Paraburkholderia* and *X. fastidiosa*, it was productive to investigate the patterns of disease that resulted among this large collection of individual shoots. Most commonly, all of the leaves on a shoot that was inoculated only with *X. fastidiosa* became symptomatic by 14 weeks after inoculation, although a few shoots (<10%) were unsuccessfully inoculated with the pathogen and a few exhibited high but not 100% disease severity (Fig. 17). In contrast, a very high proportion of the shoots that were inoculated with both *Paraburkholderia* and *X. fastidiosa* in various ways exhibited no evidence of disease, with a small proportion of vines exhibiting some disease (Fig. 17). That is, inoculation of grape with *Paraburkholderia* greatly decreased the probability that inoculation with the pathogen would be successful, presumably by eradicating the pathogen before systemic infection could occur, or eradicated infections after they had occurred within a given vine and before disease symptoms could result - rather than reducing the severity of symptom development in plants that would have become infected with the pathogen. In other words, inoculation with *Paraburkholderia* in various ways appears to act as an eradicant of *X. fastidiosa* after it is inoculated into plants, thus preventing successful systemic infection/movement and therefore symptom development. The likelihood that inoculation with *X. fastidiosa* leads to infection was therefore reduced 3-fold or more - an outcome very distinct from, and much more practical, than simply reducing the level of symptoms that would have occurred in plants that would have become infected. It is very noteworthy that infection can be so dramatically reduced in these plants in the field despite the fact that they were inoculated with VERY high levels of the pathogen ($>10^7$ cells/ inoculation site). We presume that viable cells of *X. fastidiosa* were eliminated in those vines in which disease symptoms could not occur since symptoms never developed, even after prolonged observation throughout the summer. Such a finding is consistent with that of greenhouse studies which revealed that viable cells of *X. fastidiosa* were typically not detectable in plants that had been inoculated in a similar manner.

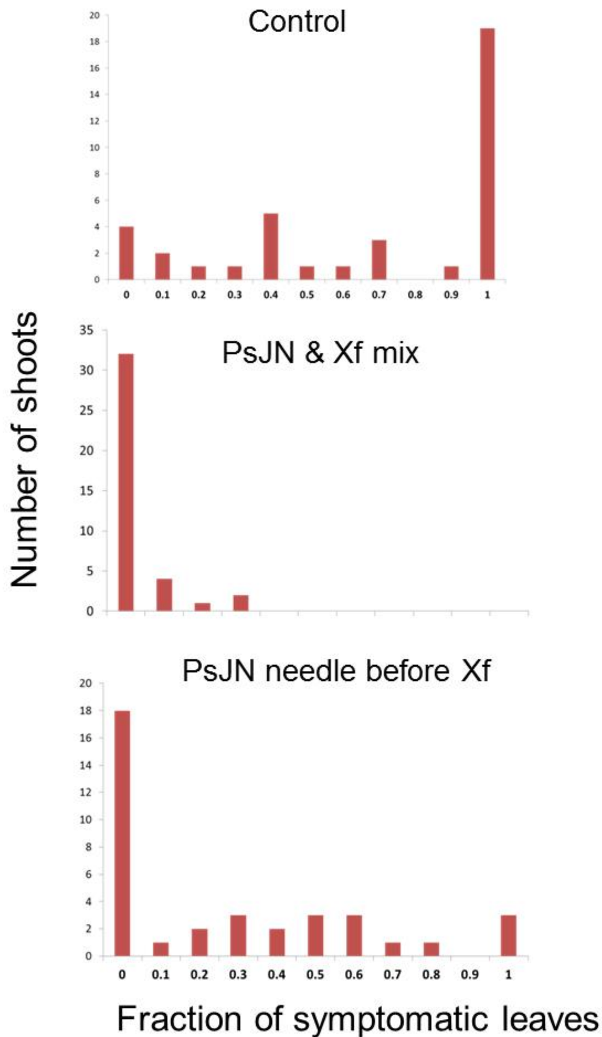

Figure 17. Frequency histogram of the distribution of disease severity observed in 40 individual shoots inoculated only with X.fastidiosa (top panel), inoculated simultaneously with a mixture of *Paraburkholderia* and *X. fastidiosa* (middle panel), or inoculated by droplet puncture three weeks before that of *X. fastidiosa* (bottom panel).

PRESENTATIONS MADE:

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

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

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

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

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

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

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

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

Presentation made at the annual meeting of the International Society for Extracellular Vesicles entitled "novel roles of quorum sensing regulated extracellular vesicles produced by *Xylella fastidiosa* and their role in virulence to plants". May, 2017.

Presentation made at the Department of Plant and Microbial Biology, the University of Zürich, November, 2017.

Presentation at the Department of Plant pathology, Auburn University entitled "The many cell density -dependent behaviors of *Xylella fastidiosa*: achieving plant disease control by pathogen confusion", March, 2018.

Presentation made at the 6th Xanthomonas genetics conference, Halle, Germany entitled "The many cell density -dependent behaviors of *Xylella fastidiosa*: achieving plant disease control by pathogen confusion", July, 2018.

Keynote presentation made at the 11th International Congress of Plant Pathology, Boston Massachusetts, entitled "The many cell density -dependent behaviors of *Xylella fastidiosa*: achieving plant disease control by pathogen confusion", July, 2018.

Presentation made at the 2018 Pierce's disease symposium, San Diego California, December, 2018.

Presentation made at the 21st annual IPM seminar for Lake and Mendocino County. Research and Extension Ctr., November 16th, 2018 entitled "Novel approaches to controlling Pierces disease and when grapevines".

Presentation made at the AG Unlimited 23rd annual girl remaining entitled "Control of frost damage and Pierce's disease in grapes", February 28, 2019.

Presentation made at the Fifth International Meeting of the International Committee on Huanglongbing disease entitled "detailed studies of plant pathogens can leave to novel methods of disease control: the case of *Xylella fastidiosa* in grapes in citrus", Riverside California, March, 2019.

RESEARCH RELEVANCE STATEMENT:

The studies underway directly address practical strategies of control of Pierce's disease. Our results reveal that *Paraburkholderia phytofirmans* continues to provide levels of biological control under greenhouse conditions that is even greater than what we would have anticipated, and the encouraging results of practical means to introduce this strain into plants such as by spray applications as well as the fact that it seems to be active even when not co-inoculated with the pathogen is a very promising result that suggests that this method of disease control might also be readily implemented. Given that this well-studied biological control agent is a naturally occurring strain recognized as a beneficial organism, the regulatory requirements for its commercial adoption should be relatively modest.

LAYPERSON SUMMARY:

A naturally occurring *Paraburkholderia* strain capable of production of DSF-like molecules that is also capable of growth and movement within grape has been found that can confer increased resistance to Pierce's disease. We are exploring 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 *Paraburkholderia* 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. Spray application of the bacterium onto leaves with a surfactant that achieves low surface tension appears to be a particularly effective method of inoculation under field conditions. These results are quite exciting in that they reveal that biological control of Pierce's disease using *P. phytofirmans* is both robust and may be relatively easy to employ by various ways of inoculation.

STATUS OF FUNDS:

Work has been progressing well and we are very excited about the findings. The major component of this project involved establishing a grape field trial at UC Davis wherein we could inoculate three different varieties of grape with *Paraburkholderia phytofirmans* to evaluate its ability to confer biological control of Pierce's disease. The rest of the study objectives have been completed. The field trial was established on schedule in year 1; Cabernet Sauvignon, Chardonnay, and Pinot Noir plants were planted in spring, 2017, and grew well in their first year, enabling us to initiate inoculations in the spring of 2018 on schedule. As I reported at the Pierce's disease research conference in December, we obtained very exciting results that revealed that the control of disease by inoculations with *Paraburkholderia phytofirmans* made even after inoculation of plants with the pathogen in the field were as dramatic as we had observed in the greenhouse studies. The proposed experimental design however allowed us to evaluate continuing disease control in the 2019 growing season in plants that were inoculated in 2018. Furthermore, three of the treatments evaluated a "memory effect" wherein plants that were inoculated in 2018 with *Paraburkholderia phytofirmans* would be challenge inoculated for the first time with *Xylella fastidiosa* for the first time in 2019 to determine whether a prior treatment of plants with the biological control agent could still confer disease control, as it had when it was inoculated at times closer to that of the time of inoculation with the pathogen. These additional inoculations and all disease assessments will need to be made after the current June 30, 2019 end of the project. More importantly, our field experiments in 2018 proved to be much more laborious and time consuming than we had anticipated, and for that reason, we chose to inoculate only the Cabernet Sauvignon and Pinot Noir plants in 2018. It will be these two varieties that we will continue to evaluate during the 2019 season as discussed above. The decision was made to not inoculate the Chardonnay plants in 2018 for the logistical reasons noted above, but also, and more importantly, so that we could make more informed decisions about treatments to evaluate when they would be inoculated in the 2019 growing season. That is, we could better learn from our results of 2018 to design a better experiment 2019. Indeed, we learned a lot in our 2018 inoculations of Cabernet Sauvignon and Pinot Noir about the relative efficacy of the timing of application of *Paraburkholderia phytofirmans* both before and after that of the pathogen to identify treatment times that we wish to test in 2019 to better refine the "optimum window" for application of the biological control agent. We also learned a lot about the optimum inoculation methods. Thus in addition to continued treatment and evaluation of the Cabernet Sauvignon and Pinot Noir plants as discussed above, we have planned a very ambitious set of treatments in the field plot on the Chardonnay plants that will complete the originally proposed work that will require us to conduct work throughout 2019 to complete the study. Thus I need to request a no-cost extension in the project period until June 30, 2020 to enable me to complete the work proposed. At this time we estimate that there will be about $147,000 of unexpended funds from this project as of June 30, 2019, and I am requesting permission to carry these funds forward to spend before June 30, 2020 to enable us to complete the work. These funds should be sufficient to enable me to cover the salaries of Dr. Clelia Bacarri and Ms. Renee Koutsoukis as well as to pay for the supplies and travel needed to complete the study.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY:

A patent disclosure for the invention "Biological control of Pierce's disease of grape with a beneficial bacterium" was made to the University of California on December 12, 2018 and was assigned UC case number BK-2019-069. If granted, this patent should facilitate the further commercial development and registration of this disease control method, and thus make it available to growers. Information regarding UC-Berkeley IP policies can be found at: https://ipira.berkeley.edu/.