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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 a *Burkholderia phytofirmans* strain was capable of extensive growth and movement within grape. 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 *Burkholderia* 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 *Burkholderia* and *X. fastidiosa* in grape inoculated in different ways with this biological control agent lead to disease control.
2) Identify the mechanisms by which *Burkholderia* 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 *Burkholderia 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 *Burkholderia 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 *Burkholderia* species and the genome sequence of *B. phytofirmans* revealed that it has a homologue of *Xf rpfF*. While we have no evidence for its production of a DSF species to which *X. fastdiosa* could respond, the promiscuous nature of RpfF in *X. fastdiosa* and other species suggested that it might make DSF species to which *X. fastidiosa* would respond under some circumstances, such as when growing within plants. Preliminary results suggest that co-inoculation of *X. fastidiosa* and *B. phytofirmans* resulted in greatly reduced disease symptoms compared to plants inoculated with *X. fastdiosa* alone; whereas the number of infected leaves of plants inoculated with *X. fastidiosa* alone increased rapidly after week 12, very little disease was observed in plants inoculated with *X. fastidiosa* and *B. phytofirmans* (Figure 1).

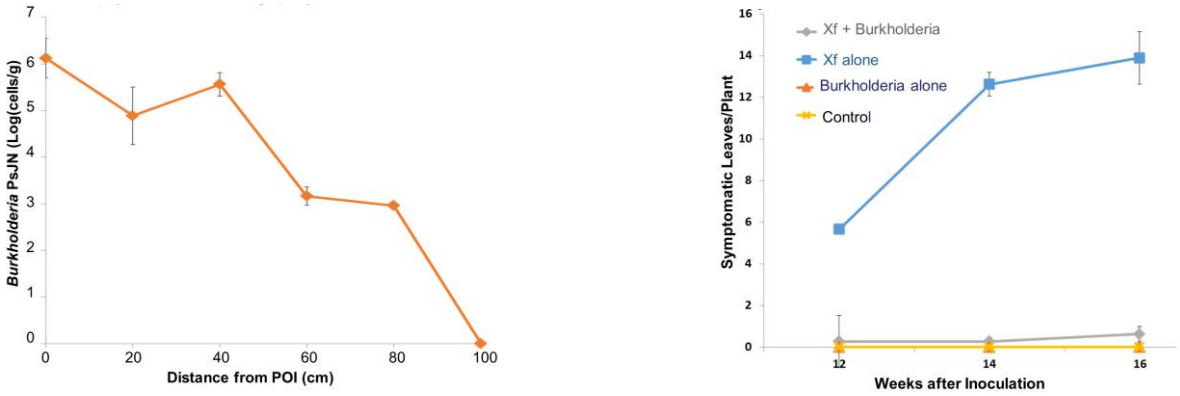


Figure 1. (Left). Population size of *B. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after 6 weeks incubation. (Right). Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *X. fastidiosa* alone (blue) or when co-inoculated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

While the droplet puncture method used in Figure 1 to introduce *B. phytofirmans* is an effective way to introduce bacteria into the xylem we have investigated the potential to introduce *B. phytofirmans* into the vascular tissue by topical application to leaves using 0.2% Brekthru, an organo-silicon surfactant with sufficiently low surface tension that spontaneous invasion of plant tissues can be achieved. The population size of *B. phytofirmans* in the petioles of leaves distal from the leaf on which cell suspensions in Brekthru (10^8 cells/) have been applied were used as a measure of growth and movement potential from such an inoculation site. Substantial numbers of cells of *B. phytofirmans* could be recovered from petioles within one or two weeks after topical application to leaves in the presence of Silwet L77 or Breakthru (Figure 2). Very few cells were present within petioles when the bacterium was applied without a penetrating surfactant. Topical application of such an endophyte thus appears to be a very practical means of inoculating plants in the field.

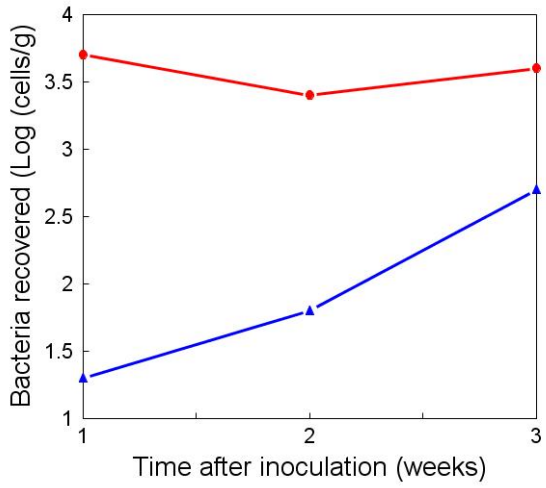


Figure 2. Population size of *Burkholderia phytofirmans* in petioles of Cabernet Sauvignon of plants sprayed with this strain alone (blue line) or this strain applied with 0.2% Breakthru (gray line) or of *Erwinia herbicola* strain 299R applied with 0.2% Breakthru (orange line). Vertical bars represent the mean of log population size at a given sampling time.

Given the promising results of the reduction of severity of Pierces disease in grape treated with *B. phytofirmans* we performed additional experiments in which *X. fastidiosa* was co-inoculated with *B. phytofirmans* as well as when *B. phytofirmans* both preceded or followed inoculation of plants with *X. fastidiosa* by 30 days. As observed before, the severity of Pierces disease of plants co-inoculated with *B. phytofirmans* and *X. fastidiosa* was greatly reduced at all times after inoculation compared to that on plants inoculated with the pathogen alone (Fig. 3). Importantly, the severity of Pierces disease was also substantially less on plants in which inoculation with *B. phytofirmans* followed inoculation with the pathogen by 30 days then on control plants inoculated only with the pathogen (Fig. 3). Almost no disease was observed on plants inoculated with *B. phytofirmans* 30 days after inoculation with the pathogen (Fig. 3), These results are quite exciting and confirmed that *B. phytofirmans* can confer high levels of disease resistance in grape - both when co-inoculated with the pathogen and also when inoculated into plants already infected with *X. fastidiosa*. It might have been anticipated that pre-inoculation of plants with *B. phytofirmans* would have yielded the largest degree of disease resistance. However, this and other studies have shown that disease incidence and severity is reduced whenever *B. phytofirmans* and *X. fastidiosa* are present together in the plant. Inoculation of plants with *B. phytofirmans* after that of the pathogen would, by definition, place them both in the plant together while pre-inoculation could result in a situation where the biological control agent may not be present in a plan, particularly if it did not continuously colonize the plant.

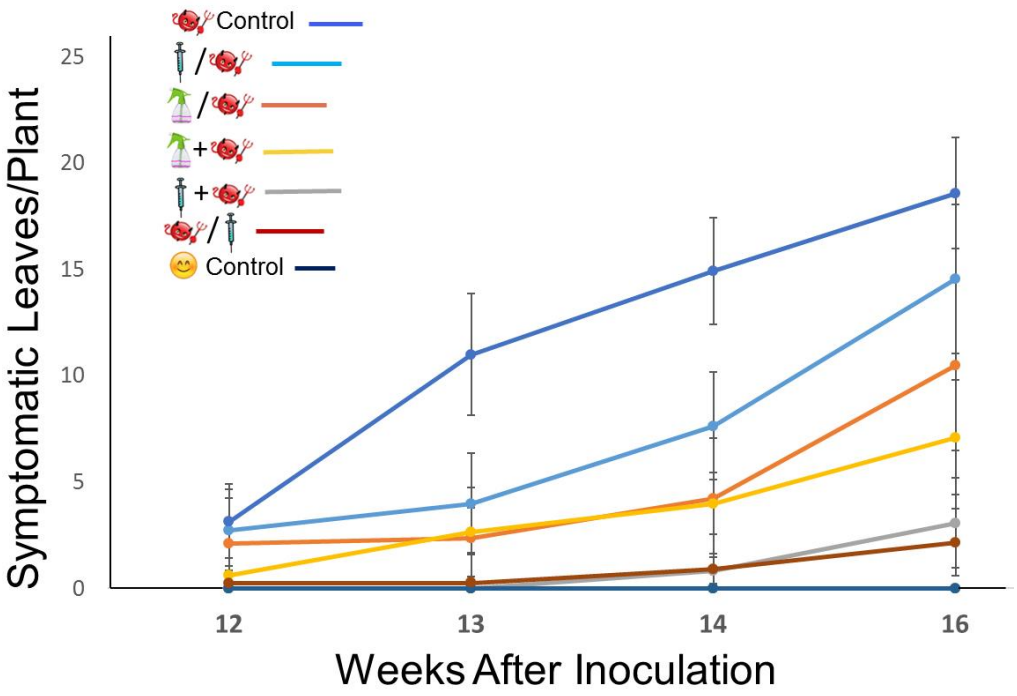


Figure 3. Severity of Pierces disease symptoms (number of symptomatic leaves/vine) on Cabernet Sauvignon plants needle inoculated only with *B. phytofirmans* (dark blue line), only with *X. fastidiosa* (Medium Blue line), or co-inoculated with *X. fastidiosa* and *B. phytofirmans* (yellow line). Also shown is disease severity on plants needle inoculated with *B. phytofirmans* 30 days before inoculation with *X. fastidiosa* (light blue line) or sprayed with *B. phytofirmans* in a solution of 0.2% Breakthru 30 days before inoculation with *X. fastidiosa* (orange line) as well as on plants needle inoculated with *X. fastidiosa* 30 days after inoculation with *B. phytofirmans* (maroon line). The vertical bars represent the standard error of the determination mean disease severity.

B. 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 10 cm of the side 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 4). 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, *B. phytofirmans* conferred a very high level of disease resistance (Figure 4). It thus appears that the beneficial effect of *B. phytofirmans* is not variety specific, and that it should confer high levels of resistant in all grape varieties.

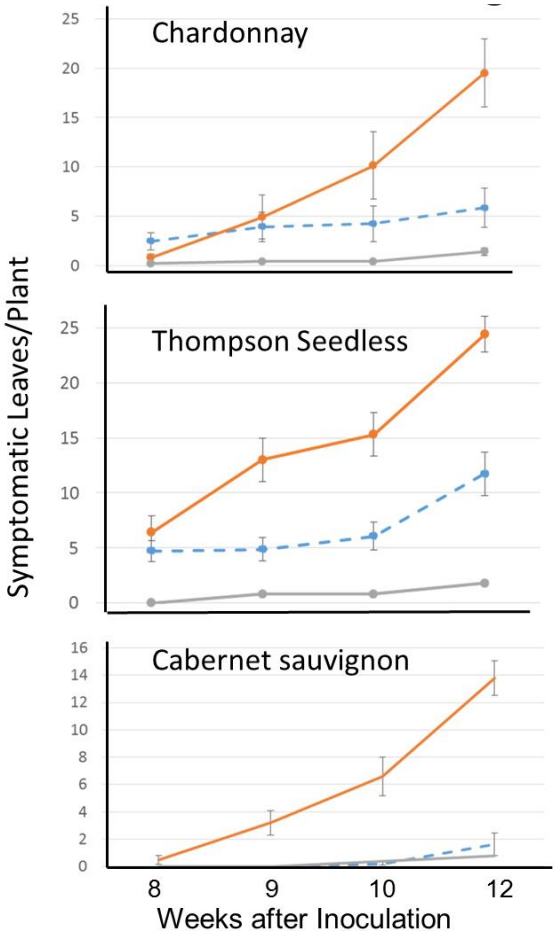
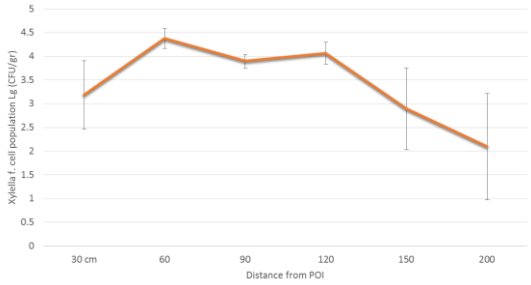


Figure 4. Severity of Pierce's disease observed in different grape varieties needle inoculated at the same time but at different locations with *X. fastidiosa* and *B. phytofirmans* (blue line) compared to that inoculated only with *X. fastidiosa* (orange line), or with *B. phytofirmans* alone (gray line). The vertical bars represent the standard error of the determination mean disease severity.

While the mechanism by which *B. phytofirmans* reduces the severity of Pierces disease remains somewhat unclear, the biological control activity conferred by this bacterium is associated with its ability to reduce the population size of *X. fastidiosa* in inoculated plants. Relatively high population sizes of *X. fastidiosa* were recovered from stem segments collected from 30 to 300 cm away from the point of inoculation in plants inoculated only with the pathogen (Fig. 5). As expected, the highest population sizes were seen within the first 120 cm, but population sizes greater than 100 cells per gram were observed as much as 200 cm away from the point of inoculation. In contrast, the population size of *X. fastidiosa* was much lower at a given distance away from the point of inoculation in plants co-inoculated with *X. fastidiosa* and *B. phytofirmans* (Fig. 5). Whereas population sizes of the pathogen were usually in excess of 10^4 cells per gram in stem segments within 120 cm of the point of inoculation in plants inoculated with the pathogen alone, the pathogen population sizes were much lower, decreasing from a high of $10^{2.5}$ to less than 10 cells per gram in plants co-inoculated with *B. phytofirmans* (Figure 5).



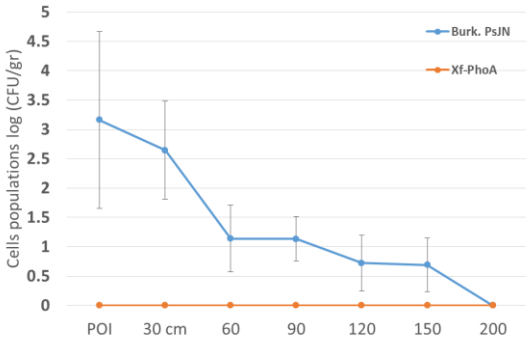


Figure 5. (left). Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation of the pathogen alone when measured 12 weeks after inoculation. (right). Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation of the pathogen when co-inoculated with *B. phytofirmans* (blue) or populations of *B. phytofirmans* (orange).The vertical bars represent the standard error of the mean population size/g.

Surprisingly, we have frequently observed that while *B. phytofirmans* rapidly achieves high population sizes and spreads extensively with plants after inoculation, when assessed several weeks after inoculation, its population sizes in inoculated plants, irrespective of whether *X. fastidiosa* was also inoculated into the grape plants is often quite low. These results suggest that the interactions of *B. phytofirmans* with either the plant or *X. fastidiosa* occur early in the infection process. The fact that the effect of inoculation of plants with *B. phytofirmans* reduce population sizes of *X. fastidiosa* most at sites distal to the point of inoculation suggest that it had reduced the motility of the pathogen. Such an effect would be expected if it stimulated DSF-mediated quorum sensing. That is, the behavior of *X. fastidiosa* in plants treated with *B. phytofirmans* was similar to that seen in transgenic plants harboring *X. fastidiosa rpfF* that produce DSF. It is curious however that the population size of *X. fastidiosa* is often lower even near the point of inoculation in plants also treated with *B. phytofirmans* (Figure 6). This suggests that in addition to any effect that *B. phytofirmans* has on changing the signaling, behavior of *X. fastidiosa*, possibly by altering DSF signaling, that it might also be either directly antagonistic to the pathogen in the plant or, more likely, triggering a host defensive reaction that inhibits the growth or survival of the pathogen. Experiments are underway to distinguish these different possibilities.

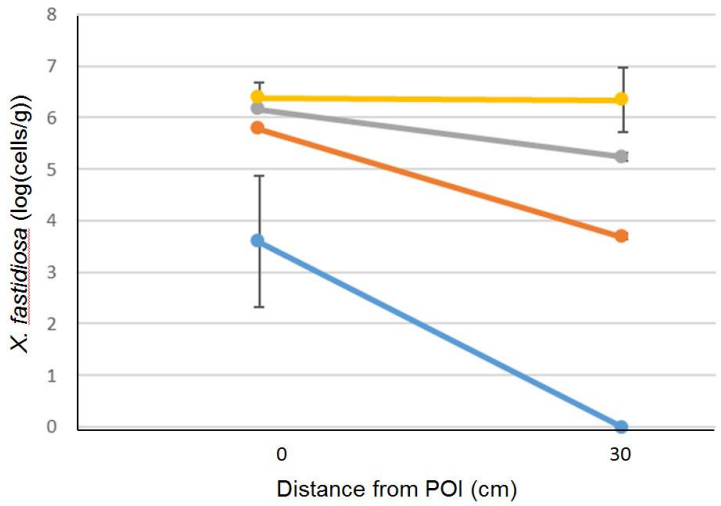


Figure 6. Population size of *X. fastidiosa* three weeks after inoculation of plants with the pathogen alone (yellow line), plants sprayed with *B. phytofirmans* on the same day that it was needle inoculated with the pathogen (gray line), plants needle inoculated with *B. phytofirmans* on the same day that it was needle inoculated with the pathogen at a nearby site (orange line), and plants needle inoculated with *B. phytofirmans* three weeks prior to being needle inoculated with the pathogen at a nearby site (blue line). The vertical bars represent the standard error of the determination of log- transformed population sizes.

The dramatic reductions in both the population size of *X. fastidiosa* as well as Pierce's disease symptoms both in plants in which the pathogen and *Burkholderia* were simultaneously inoculated (either together as a mixture or in close proximity) as well as when inoculated at different times relative to one another in grape raise the question as to whether the pathogen and *Burkholderia* had to be coincident for biological control to occur or whether the presence of *Burkholderia* was mediating a distal effect in the plant. That is, could the presence of *Burkholderia* in the plant. Having an effect on *X. fastidiosa* even at a distance, perhaps by initiating a host-mediated defense against the pathogen, perhaps on a systemic level. Experiments were therefore conducted to provide evidence to distinguish between these possibilities. In this experimental design, the pathogen and *Burkholderia* were inoculated simultaneously but at spatially distant locations in the plant to ascertain whether a systemic resistance to the growth and movement of *X. fastidiosa* or disease symptoms could be conferred by *Burkholderia* inoculated many centimeters away from the pathogen. The two bacteria, *X. fastidiosa* and

Burkholderia were either co-inoculated or inoculated in the same grape plant at the same time but 30 centimeters from each other. The experiment used rooted cuttings of Cabernet Sauvignon inoculated when the plants were approximately 50-70 cm tall. Grapes were either needle droplet puncture inoculated with *Burkholderia* alone, with *X. fastidiosa* alone, or with an equal mixture of the two bacteria as in earlier studies. However in addition, in one treatment plants were inoculated at their base with *X. fastidiosa* while *Burkholderia* was inoculated 30 cm towards the distal portion of the stem at the same time. In the converse treatment, *Burkholderia* was inoculated at the base of the plant while *X. fastidiosa* was inoculated at the same time 30 cm distal along the stem. The population size of both *Burkholderia p.* and *X. fastidiosa* was determined at 8 weeks post inoculation in petioles collected various points on the plant as well as at various locations in the stem. The two different bacterial strains could be distinguished on different culture media. Population size of X. *fastidiosa* was determined by dilution plating on PWG medium containing natamycin and 50 ug/ml and gentamycin of *X. fastidiosa* and that of Burkholderia was determined on Kings medium B containing 100 ul/ml natamycin (fungicide) and rifampicin. As has been seen in all experiments, the population size of the pathogen was greatly reduced at all locations in the plant when co-inoculated with *Burkholderia* (compare Figures 7 and 8); while *X. fastidiosa* reached population sizes of over 10^4 cells/g in the stem even a distances of 130 cm from the point of inoculation when inoculated alone in plants (Figure 7), it's populations undetectably low at all stem locations when co-inoculated with *Burkholderia* (Figure 8). It is noteworthy that Burkholderia populations were low at most locations in plant and measured eight weeks after inoculation (Figure 8), although much higher populations were detected earlier in the experiment (data not shown). In contrast to the great reduction in populations of *X. fastidiosa* seen when co-inoculated with *Burkholderia*, population sizes of the pathogen were only modestly reduced when *Burkholderia* was inoculated either 30 cm towards the base or 30 cm towards the apex of the grape plant relative to that of the pathogen (Figures 9 and 10). In both cases however, the population sizes of *X. fastidiosa* were reduced greatly at locations furthest from the point of inoculation of the pathogen (Figs. 9 and 10) indicating that the growth and movement of the pathogen was strongly influenced by Burkholderia, but that such inhibition was context-dependent in that it apparently was maximal in locations distal from the point of the separate inoculations were these two strains would have been expected to have been coincident in the plant.. These preliminary results suggest that inoculation of grape with *Burkholderia* does not lead to a strong, systemic resistance to the colonization of the plants by *X. fastidiosa*, and thus to symptom development. Instead, it suggests that *X. fastidiosa* and *Burkholderia* must be in relatively close proximity for an inhibition of pathogen growth and movement to occur. Further studies to investigate this phenomenon are underway. This model does not however rule out the possibility that *Burkholderia* is mediating a local resistance to *X. fastidiosa* as it grows and moves in the plant. Studies to test this hypothesis will be discussed below.

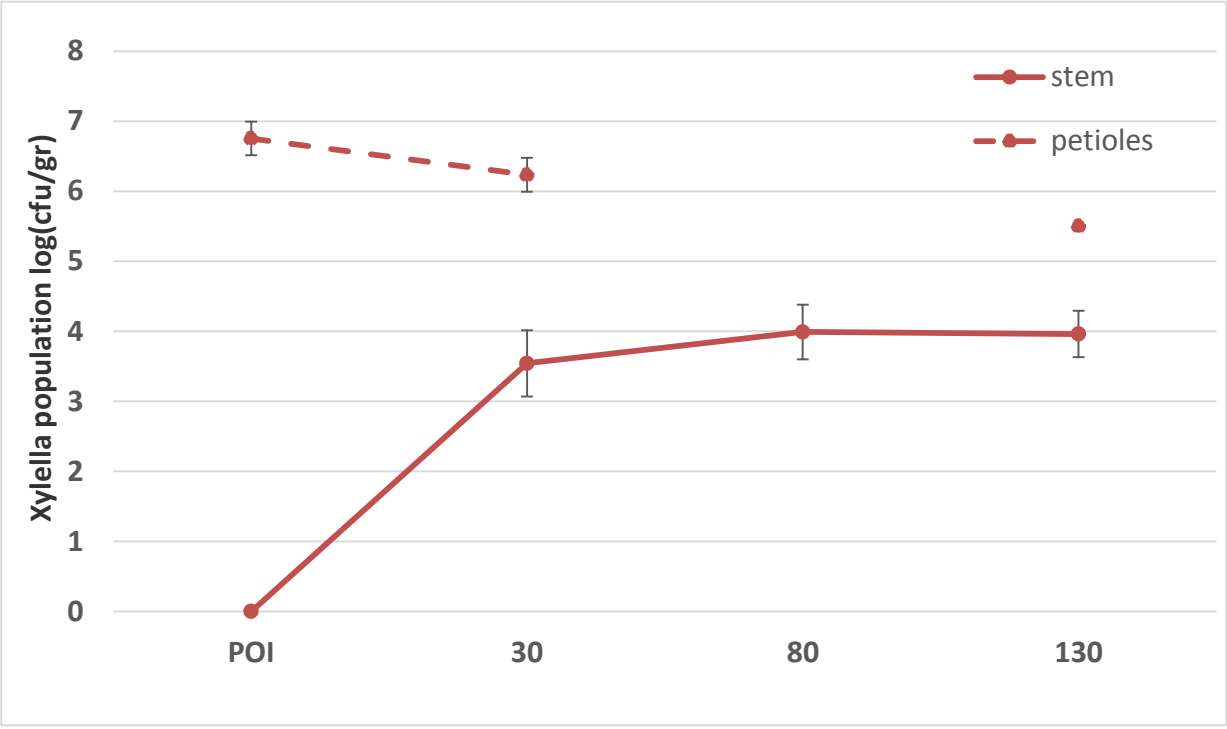


Figure 7. Population size of *Xylella fastidiosa* in grape plants inoculated only with the pathogen. The solid red line represents the bacteria populations in the stem while the dashed line represents pathogen populations in the petioles in samples taken at different centimeter locations from the point of inoculation shown on the abscissa. The vertical bars represent the standard error of log transformed population size per gram.

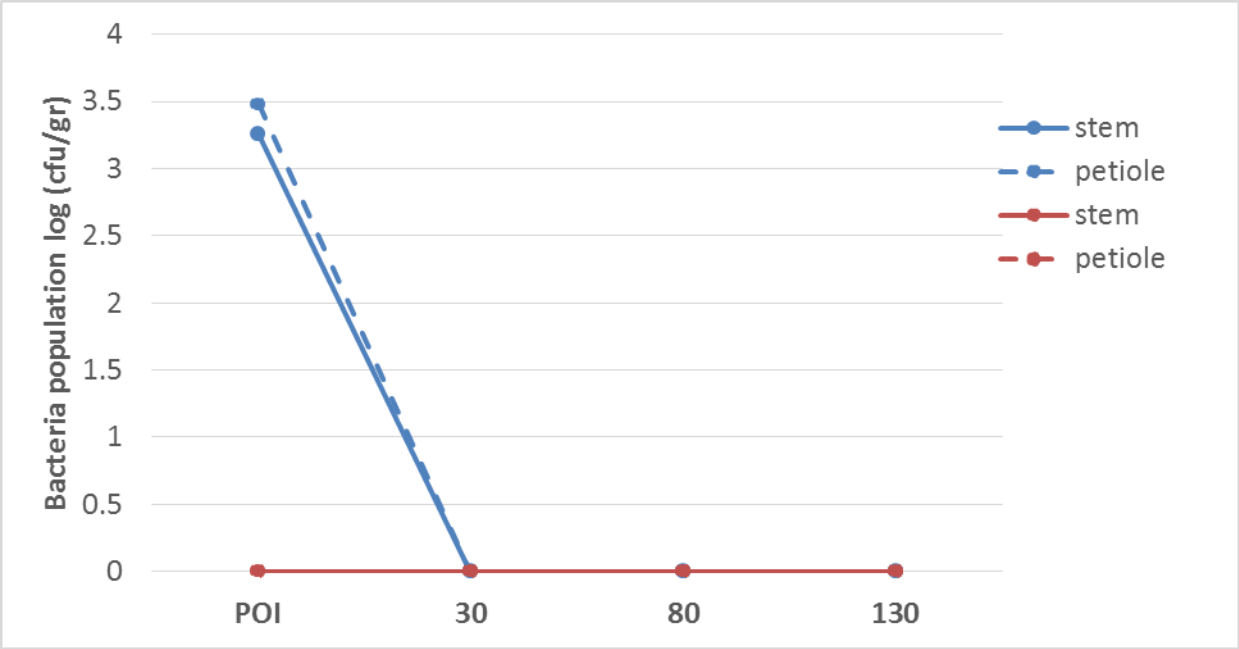


Fig.

Figure 8. Population size of *Xylella fastidiosa* (red lines) and *Burkholderia* (blue lines) in grape plants inoculated go inoculated with the pathogen and *Burkholderia* at the same location. The solid lines represent bacteria populations in the stem while the dashed lines represents populations in the petioles in samples taken at different centimeter locations from the point of inoculation shown on the abscissa.

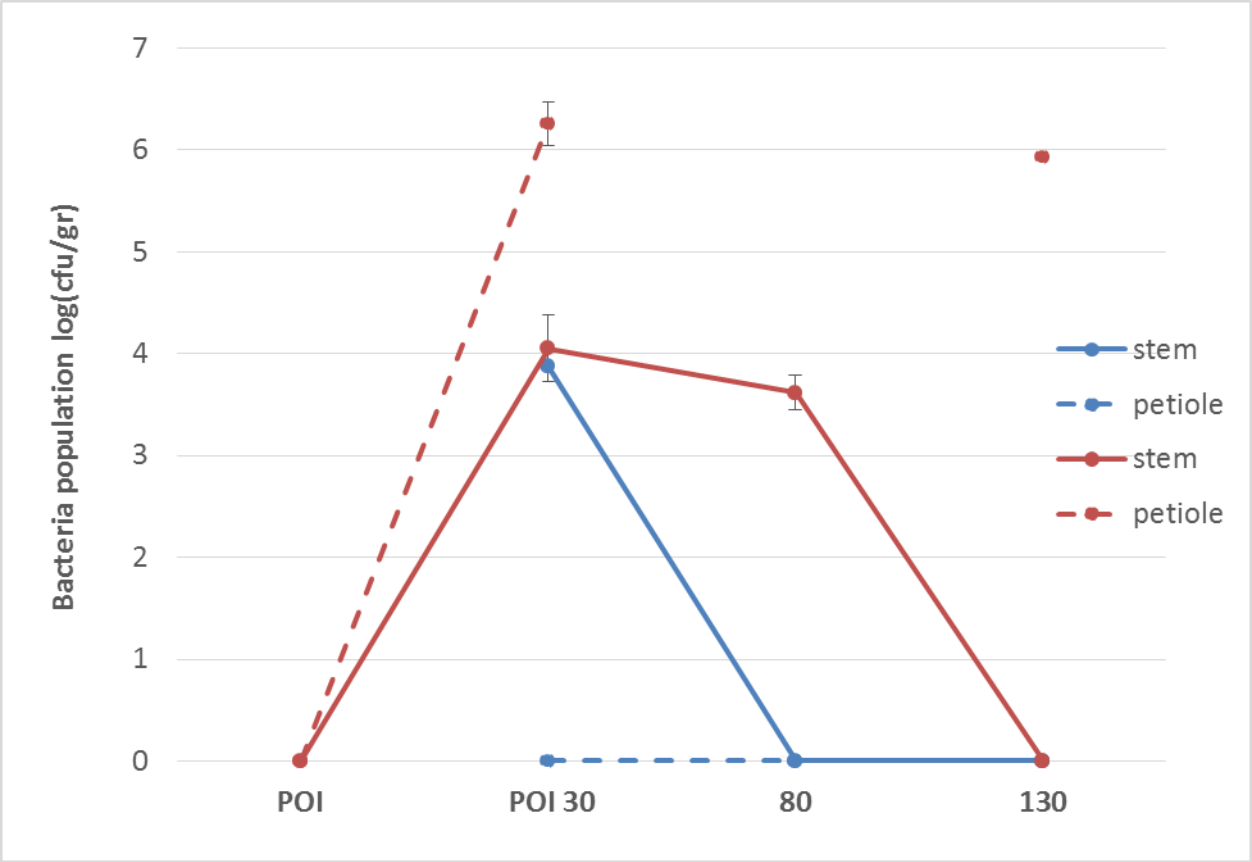


Figure 9. Population size of *Xylella fastidiosa* (red lines) and *Burkholderia* (blue lines) in grape plants inoculated at their base with the pathogen while *Burkholderia* was inoculated 30 cm distal to the point of inoculation at the same time. The lines represent bacteria populations in the stem while the dashed lines represents populations in the petioles in samples taken at different centimeter locations from the point of inoculation shown on the abscissa. The vertical bars represent the standard error of the determination of log-transformed population sizes per gram.

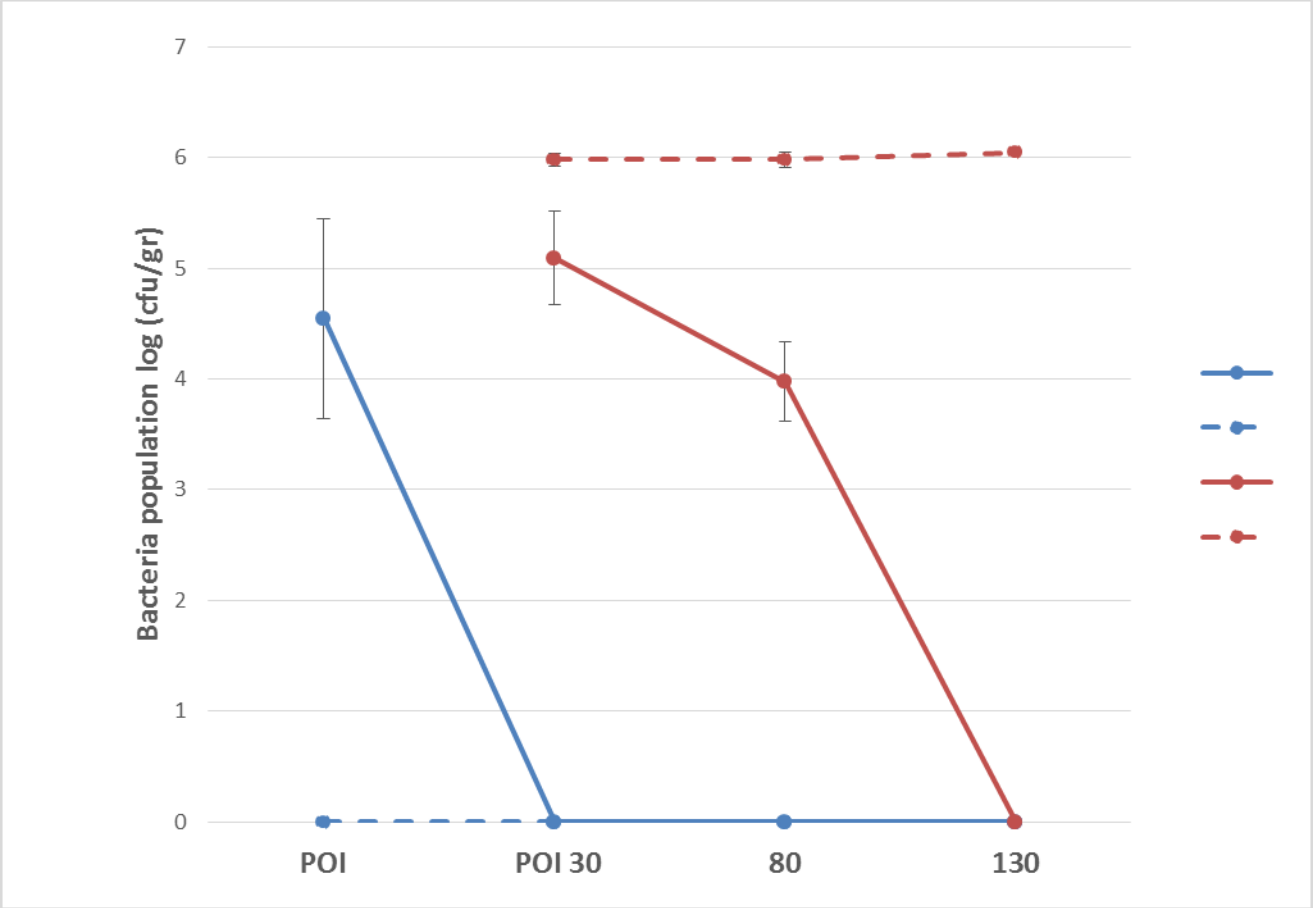


Figure 10. Population size of *Xylella fastidiosa* (red lines) and *Burkholderia* (blue lines) in grape plants inoculated at their base with Burkholderia while *X. fastidiosa* was inoculated 30 cm distal to the point of inoculation at the same time. The lines represent bacteria populations in the stem while the dashed lines represents populations in the petioles in samples taken at different centimeter locations from the point of inoculation shown on the abscissa. The vertical bars represent the standard error of the determination of log-transformed population sizes per gram.

We have observed in the many experiments in which grape has been inoculated with *Burkholderia* 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. For example, when measured 4 to 6 weeks after inoculation, very large *Burkholderia* populations are often observed a meter or more away from the point of inoculation (Figure 1). However, we have often observed that when measured many weeks after inoculation, such as in the experiments described in Figures 7-10, *Burkholderia* population sizes throughout the plant are much lower than they had been earlier. Intensive experiments are underway to systematically examine the temporal and spatial dynamics of *Burkholderia* populations in grape. We will be testing the hypothesis that *Burkholderia 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 *Burkholderia* in a way that leads to a reduction in its population size. In fact, it may be this response of the plant to *Burkholderia* that is also responsible for the dramatic reductions in *X. fastidiosa* populations in plants inoculated with *Burkholderia*. If, as we hypothesize, such a host response is relatively local to the plant region colonized by *Burkholderia*, the patterns of biological control that we have observed could be explained. Specifically, biological control of Pierce's disease would be expected if *Burkholderia* was applied either before or after that of the pathogen (such as was seen in experiments described in Figure 3) if the rapid movement of *Burkholderia* 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 *Burkholderia* in the plant would be of great importance to the efficacy of biological control (Figure 11). Our ongoing studies to investigate the spatial movement and temporal persistence of *Burkholderia* in plants after inoculation been 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.

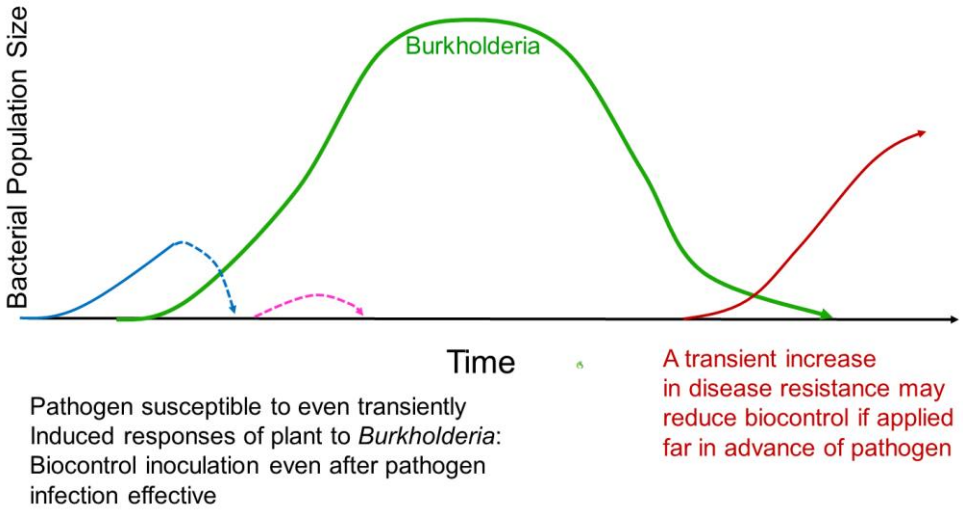


Figure 11. A model describing the expected temporal growth and persistence of *Burkholderia* in grape plants after inoculation (green line) and the expected effects on population sizes of *X. fastidiosa* inoculated at various times relative to that of *Burkholderia* (blue, pink, and red lines) based on the hypothesis that *Burkholderia* mediates a local inhibitory effect on pathogen populations.

Objective 2: Mechanisms of biological control

Considerable effort has been made during this reporting period to better understand the mechanisms by which *B. phytofirmans* alters the behavior of *X. fastidiosa* in plants. Some studies of the mechanism of biological control (possibilities of induce plant resistance) have already been discussed above as part of Objective 1. In addition, Burkholderia also appears to produce compounds that might directly affect pathogen behavior. DSF production has been described in other *Burkholderia* species including *Burkholderia ceonocepacia*. Furthermore, the genome sequence of *B. phytofirmans* PSJN has been determined, allowing us to putatively identify a gene with some homology to *X. fastidiosa* and *Xanthomonas campestris rpfF*, that thus might be expected to lead to the production of fatty acids capable of conferring signaling activity like that of DSF species. We therefore made a site-directed deletion mutant of the putative *rpfF* gene in *B. phytofirmans*. We subsequently investigated whether ethyl acetate extracts of wild type *B. phytofirmans* culture supernatants or *rpfF* mutants of *B. phytofirmans* could alter the expression of genes in either *Xanthomonas campestris* or *X. fastidiosa* that were known to be regulated by the presence of various DSF species. Interestingly, relatively strong induction of the *eng:gfp* reporter gene fusion in *Xanthomonas campestris* was observed when the biosensor was exposed to extracts of both the wild type and *rpfF* mutant of *B. phytofirmans* (Figure 12). These results suggest that indeed *B. phytofirmans* was capable of producing a DSF-like molecule that *Xanthomonas campestris* could respond to. It also suggested however that the putative *rpfF* gene that we had removed was not responsible for producing the putative signal molecule. In contrast to the results that revealed that *Xanthomonas campestris* could respond to way putative signal molecule from *B. phytofirmans*, little or no change in expression of the *phoA* reporter gene was observed when the *X. fastidiosa Xf:phoA* biosensor was exposed to ethyl acetate extracts of either the wild type or *rpfF* mutant of *B. phytofirmans* (data not shown). Given that *X. fastidiosa* and *Xanthomonas campestris* respond to different DSF species, it was not unexpected that they might differentially respond to the signal molecule apparently made by *B. phytofirmans.*

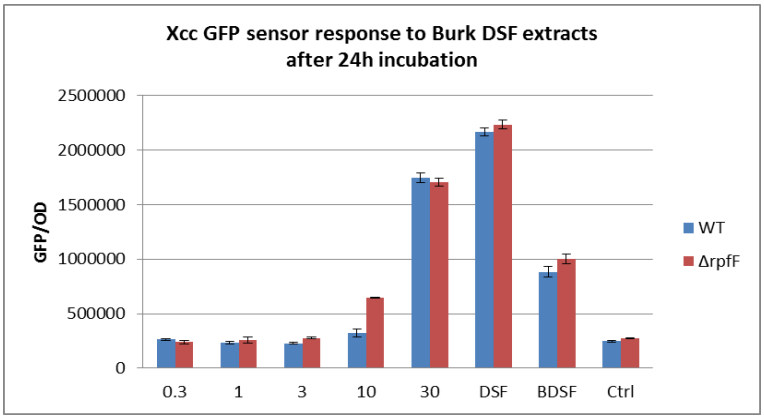


Figure 12. Normalized GFP fluorescence exhibited by the *Xanthomonas campestris* pv. campestris DSF biosensor strain harboring an *eng:gfp* reporter gene when exposed to different concentrations of ethyl acetate extracts (100 ml of supernatant extracted into 1 ml of solvent) from a wild type *B. phytofirmans* (blue bars) or an *rpfF* mutant (red bars). Shown on the abscissa are different ul aliquots of the extract added to a 1 mL culture of the biosensor as well as a culture of the biosensor exposed to 1 uM DSF, 1 uM BDSF,or to no added material (ctrl).

While we did not detect a change in apparent expression of the *hxfA* promoter linked to the *phoA* reporter gene in the *X. fastidiosa Xf:phoA* biosensor when it was exposed to either ethyl acetate extracts of culture supernatants of *B. phytofirmans* or small amounts of culture supernatant themselves, we observed that the biofilm formation (apparent adhesiveness) of *X. fastidiosa* was dramatically higher when either ethyl acetate extracts of culture supernatant or culture supernatant itself from *B. phytofirmans* was added to cultures of either wild type or rpfF* mutants of *X. fastidiosa* (Figure 13). Not only was the amount of bacterial biomass that accumulated in the "ring" which formed at the media/air interface and shake cultures greater, but more importantly, substantial numbers of cells of *X. fastidiosa* adhered to the walls of class culture flasks below the ring - in the area exposed to turbulent mixing of the culture during shaking (Figure 13). These results suggested that the adhesiveness of *X. fastidiosa* was dramatically higher in the presence of some component of the culture supernatant of *B. phytofirmans.* Furthermore, the fact that biofilm formation was by extracts of both the wild type and putative *rpfF* mutant of *B. phytofirmans*, suggested that the putative *rpfF* gene of *B. phytofirmans* was not involved in production of the signal molecule that induced biofilm formation.

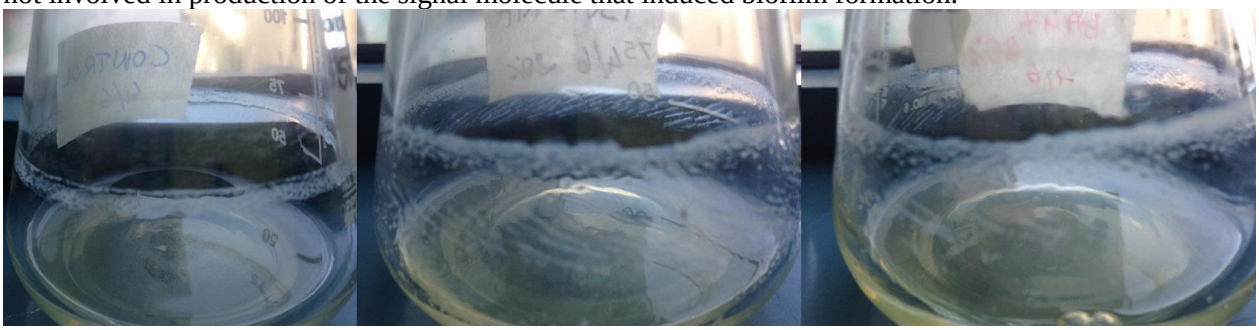


Figure 13. Biofilm formation of wild type *X. fastidiosa* grown in PD three media alone (left), or in media containing 20%v/v of culture supernatant of wild type *B. phytofirmans* (center) or a putative *rpfF* mutant of *B. phytofirmans* (right).

Interestingly, a large increase in biofilm formation could be conferred by relatively small amounts of extracts of either wild type or the *rpfF* mutant of *B. phytofirmans*, while higher concentrations appeared to lead to some inhibition of *X. fastidiosa* growth, and hence biofilm formation. These results are quite interesting in that it suggests strongly that *B. phytofirmans* produces a signal molecule to which *X. fastidiosa* responds, leading to its increased adhesiveness. It is unclear whether the signal molecule is a fatty acid related to DSF. It is quite possible that *X. fastidiosa* can perceive the putative signal molecule of *B. phytofirmans* using receptors different from those used to detect DSF itself, and that detection of the putative signal molecule of *B. phytofirmans* might lead to expression of somewhat different genes that of DSF itself. Work to determine the identity of the signal molecule is underway. Dramatic ability of the factor produced by Burkholderia to increase the biofilm

formation of *X. fastidiosa* has facilitated our preliminary purification of the molecules involved. Compounds found in cell free culture extracts of Burkholderia were subjected to partitioning into different concentrations of methanol. These preliminary results suggest wrongly that the factor that mediates biofilm formation is quite hydrophobic, being released from hydrophobic fractionation columns only at relatively high concentrations of methanol (Figure 14). Further work on its chemical purification is underway. The ability of this putative signal molecule to increase the apparent adhesiveness of *X.fastidiosa* is likely contributing to the biological control of disease conferred by co-inoculation or pre- or post-inoculation plants with *B. phytofirmans*. As with DSF itself, increasing the adhesiveness of *X. fastidiosa* would restrict its ability to move within the plant. Given that the putative signal molecule made by *B. phytofirmans* is both a small molecule and active at quite low concentrations, it suggests that it might be readily diffusible throughout the plant, again explaining why biological control conferred by *B. phytofirmans* appears to be so robust. Experiments are underway to determine the relative importance of such putative signal molecules and possible host-mediated defenses elicited by *B. phytofirmans* in biological control.

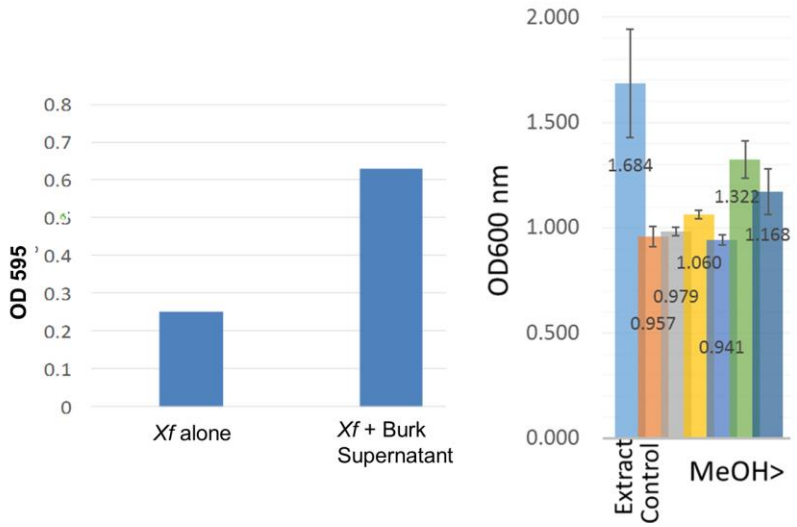


Figure 14. (Left) Quantification of biofilm formation by broth cultures of *X. fastidiosa* to which 1% by volume of a culture supernatant of a *Burkholderia* culture had been added. Biofilm formation was quantified by crystal violet staining and measured at 595 nm. (Right) Characteristics of a compound found in culture supernatants of a *Burkholderia* culture that induced biofilm formation in *X. fastidiosa*. A methanol extract of a *Burkholderia* culture supernatant was absorbed onto a hydrophobic carbon which was then subject to increasing concentrations of methanol and water. The eluates were then evaluated for their ability to induce biofilm formation in *X. fastidiosa*. Increasing ability of eluates to induce biofilm formation was observed with increasing concentrations of methanol used to the compound from the hydrophobic column.

As discussed in Objective 2 it seemed possible that *Burkholderia* 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 *Burkholderia*. Certain beneficial microorganisms such as *Burkholderia 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 are thus exploring whether the reduced disease symptoms and lower pathogen population seen in plants inoculated with *Burkholderia* either before or after that of *X. fastidiosa* is mediated by the activation of plant innate immunity. To test this hypothesis we are measuring the expression of several defense related genes in three groups of plants: 1) control plants with no treatment, 2) plants injected with the *Burkholderia* strain alone, 3) plants injected with both *Burkholderia* and *X. fastidiosa* strains simultaneously, and 4) plants inoculated only with *X. fastidiosa*. A comparison of gene expression patterns in grape from these three treatments will enable us to determine whether *Burkholderia* alone can alter gene expression patterns in grape or instead, may "prime" the plant to respond to *X. fastidiosa*. Tissue samples are being collected every week for 4 weeks and included stem segments, petioles, and a leaf blade tissue starting from the point of inoculation and continuing every 10 cm up

to 50 cm from the point of inoculation. Total RNA is being extracted and semi-quantitative RT-PCR is being performed to measure the expression of several key genes in the defense-signaling network of grape. Among them are PR1 and NPR1 (salicylic acid - related), Jaz1 (jasmonic acid related), ETR1 (ethylene - related) genes. EF1α and Actin are being used as internal controls. These studies are well underway and results should soon reveal whether either local or systemic changes in gene expression is being modulated by *Burkholderia* in grape, and the extent to which these could explain the biological control that it confers against Pierce's disease.

Objective 3: Field efficacy of biological control of PD.

While we have already obtained strong evidence of effective biological control of PD in the greenhouse, and further details of how this process can be exploited will be addressed in Objective 1, we feel it will be important to demonstrate that the process of biological control is robust under field conditions since greenhouse plants and field plants could differ. Therefore we are evaluating the extent to which the factors which control the efficacy of biological control and the greenhouse are directly applicable to a field setting. The study would also allow us to evaluate the effectiveness of spray application of *Burkholderia* relative to that of direct needle inoculation. An extensive field study has been initiated in which we will: 1) challenge plants of three different grape varieties (Chardonnay, Cabernet Sauvignon and Pinot Noir) with *Xf* relatively soon after needle inoculation or topical treatment with *Burkholderia*, 2) challenge plant with *Xf* several weeks after inoculation with *Burkholderia* in different ways, 3) inoculate *Burkholderia* 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 *Burkholderia* 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 indicated that topical applications of a DSF-like molecule, palmitoleic acid, with a penetrating surfactant can also confer disease resistance. This treatment will therefore be compared with biological control treatments. Studies are being done in a replicated field site managed by the Department of Plant Pathology at the University of California, Davis. Each treatment consists of 10 plants for a given grape variety. The experimental design is as follows:

May 2018	June 2018	July 2018	May 2019
Needle Burkholderia	Xf		
Spray Burkholderia	Xf		
	Xf control		
Needle Burkholderia			
Spray Burkholderia			
Needle Burkholderia		Xf	
Spray Burkholderia		Xf	
		Xf control	
Needle Burkholderia	Xf	Xf	Xf
Spray Burkholderia	Xf	Xf	Xf
	Xf	Xf	Xf
	Xf	Needle Burk	
	Xf	Spray Burk	
Needle Burkholderia			Xf
Spray Burkholderia			Xf
			Xf control
Burkholderia Rootstock	Xf		
Rootstock control	Xf		
10 mM Palmitoleic acid + 0.2% Breakthru	Xf		
0.2% Breakthru control			
Uninoculated control			

So-called "Uber" plants for the study have been provided by Duarte Nurseries. These plants will be acquired on April 3, 2017, and will be planted the next day at the UC Davis field site. These large "Uber" plant should allow

for rapid establishment of plants in the field trial, enabling experimentation to proceed as planned starting in the spring of 2018.

PUBLICATIONS AND PRESENTATIONS:

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.

RESEARCH RELEVANT STATEMENT:

The studies underway directly address practical strategies of control of Pierce's disease. Our results reveal that *Burkholderia 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 the biological control agent is a naturally occurring strain, the regulatory requirements for its commercial adoption should be relatively modest.

LAYPERSON SUMMARY:

A naturally occurring *Burkholderia* 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 *Burkholderia* strain was inoculated either simultaneously with, prior to, or even after that of *X. fastidiosa*. The biological control agent can be applied either by direct introduction into the xylem by droplet puncture or by spray application to foliage using a penetrating surfactant. These results are quite exciting in that they reveal that biological control of Pierce's disease using *B. phytofirmans* is both robust and may be relatively easy to employ by various ways of inoculation.

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 US patent 8,247,648 B2 entitled “Biological control of pathogenicity of microbes that use alpha, beta unsaturated fatty acid signal molecules” was approved in June, 2012 and was issued on August 21, 2012. While this patent is not specifically address biological control, depending on the outcome of our studies investigating the mechanisms of biological control, it is possible that some of the practices leading to control of Pierce’s disease to be demonstrated here could be covered by this patent if signaling molecules produced by *Burkholderia* are involved in the biological control effect.