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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 *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 P. *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).

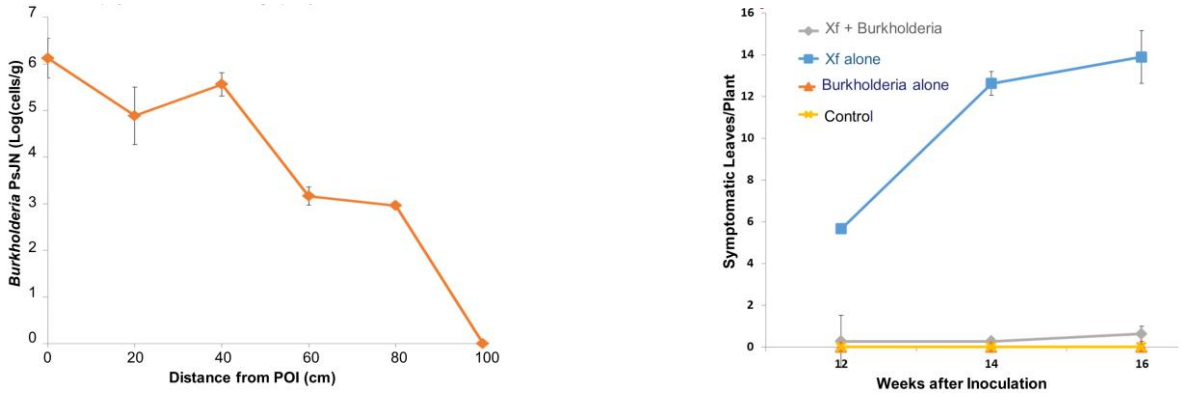

Figure 1. (Left). Population size of *P. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after 6 weeks incubation. (Right). Severity of Pierce's disease of Cabernet Sauvignon at various times after inoculation with *X. fastidiosa* alone (blue) or when co-inoculated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

While the droplet puncture method used in Figure 1 to introduce *P. phytofirmans* is an effective way to introduce bacteria into the xylem we have investigated the potential to introduce *P. phytofirmans* into the vascular tissue by topical application to leaves using 0.2% Breakthru, an organo-silicon surfactant with sufficiently low surface tension that spontaneous invasion of plant tissues can be achieved. The population size of *P. phytofirmans* in the petioles of leaves distal from the leaf on which cell suspensions in Breakthru (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 *P. 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.

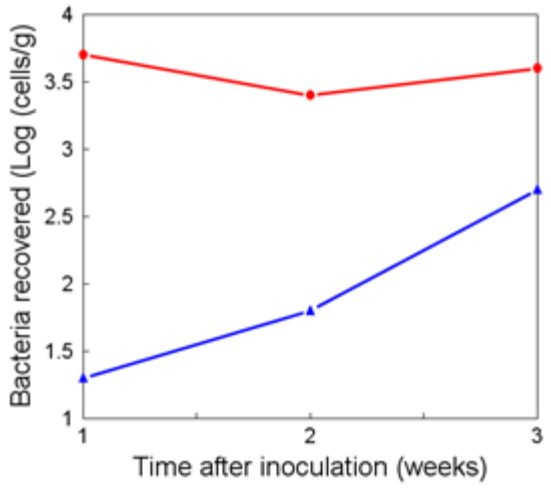

Figure 2. Population size of *Paraburkholderia phytofirmans* in petioles of Cabernet Sauvignon of plants sprayed with this strain alone (blue line) or this strain applied with 0.2% Breakthru (gray line) or of *Erwinia herbicola* strain 299R applied with 0.2% Breakthru (orange line). Vertical bars represent 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 *P. phytofirmans* we performed additional experiments in which *X. fastidiosa* was co-inoculated with *P. phytofirmans* as well as when *P. 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 *P. 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 *P. 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 *P. phytofirmans* 30 days after inoculation with the pathogen (Fig. 3), These results are quite exciting and confirmed that *P. 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 *P. 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 *P. phytofirmans* and *X. fastidiosa* are present together in the plant. Inoculation of plants with *P. 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.

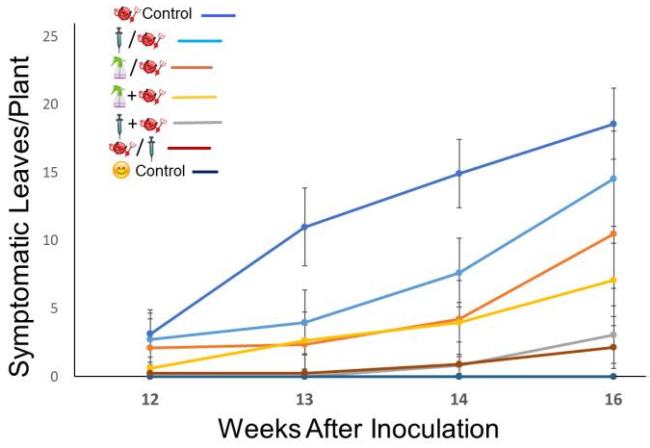

Figure 3. Severity of Pierces disease symptoms (number of symptomatic leaves/vine) on Cabernet Sauvignon plants needle inoculated only with *P. phytofirmans* (dark blue line), only with *X. fastidiosa* (Medium Blue line), or co-inoculated with *X. fastidiosa* and *P. phytofirmans* (yellow line). Also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

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 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, *P. phytofirmans* conferred a very high level of disease resistance (Figure 4). 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.

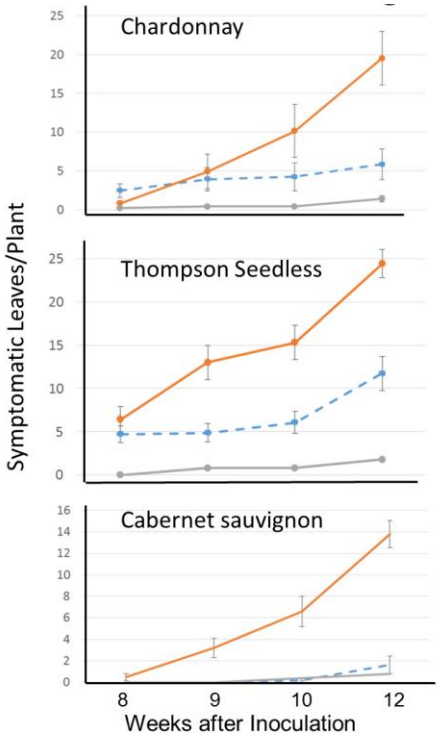

Figure 4. Severity of Pierce's disease observed in different grape varieties needle inoculated at the same time but at different locations with *X. fastidiosa* and *P. phytofirmans* (blue line) compared to that inoculated only with *X. fastidiosa* (orange line), or with *P. phytofirmans* alone (gray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

While the mechanism by which *P. 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 *P. 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 *P. phytofirmans* (Figure 5).

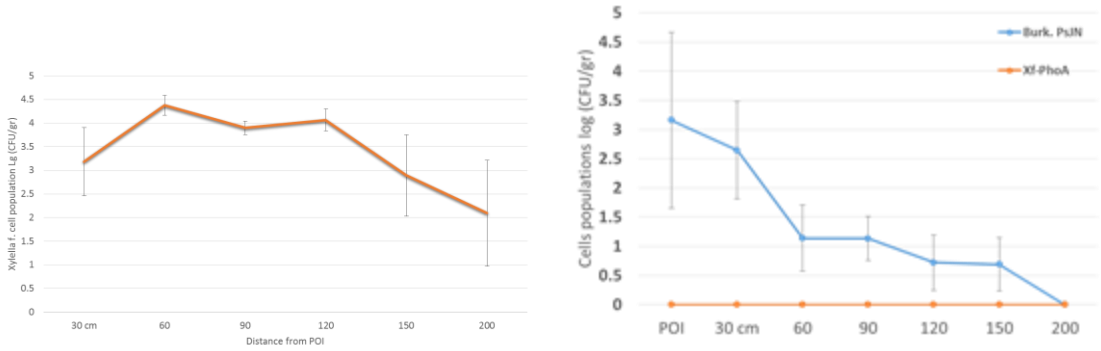

Figure 5. (left). Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation of the pathogen alone when measured 12 weeks after inoculation. (right). Population size of *X. fastidiosa* in the stems of grapes at various distances from the point of inoculation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

Surprisingly, we have frequently observed that while *P. 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 *P. phytofirmans* with either the plant or *X. fastidiosa* occur early in the infection process. The fact that the effect of inoculation of plants with *P. 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 *P. 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 *P. phytofirmans* (Figure 6). This suggests that in addition to any effect that *P. 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.

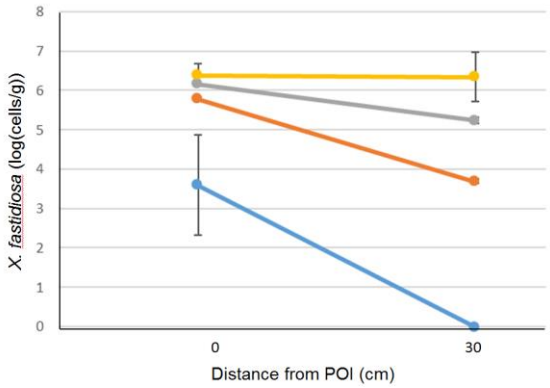

Figure 6. Population size of *X. fastidiosa* three weeks after inoculation of plants with the pathogen alone (yellow line), plants sprayed with *P. phytofirmans* on the same day that it was needle inoculated with the pathogen (gray line), plants needle inoculated with *P. phytofirmans* on the same day that it was needle inoculated with the pathogen at a nearby site (orange line), and plants needle inoculated with *P. 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 *Paraburkholderia* 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 *Paraburkholderia* had to be coincident for biological control to occur

or whether the presence of *Paraburkholderia* was mediating a distal effect in the plant. That is, could the presence of *Paraburkholderia* 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 *Paraburkholderia* 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 *Paraburkholderia* inoculated many centimeters away from the pathogen. The two bacteria, *X. fastidiosa* and *Paraburkholderia* 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 *Paraburkholderia* 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 *Paraburkholderia* was inoculated 30 cm towards the distal portion of the stem at the same time. In the converse treatment, *Paraburkholderia* 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 *Paraburkholderia 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. 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 *Paraburkholderia* (compare Figures 7 and 8); while *X. fastidiosa* reached population sizes of over 10^4 cells/g in the stem even at distances of 130 cm from the point of inoculation when inoculated alone in plants (Figure 7), it's populations were undetectably low at all stem locations when co-inoculated with *Paraburkholderia* (Figure 8). It is noteworthy that *Paraburkholderia* populations were low at most locations in plant when 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 *Paraburkholderia*, population sizes of the pathogen were only modestly reduced when *Paraburkholderia* 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 *Paraburkholderia,* but that such inhibition was context-dependent in that it apparently was maximal in locations distal from the point of the separate inoculations where these two strains would have been expected to have been coincident in the plant. These preliminary results suggest that inoculation of grape with *Paraburkholderia* 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 *Paraburkholderia* must a plant response may be occurring but they must be in relatively close proximity. Studies to test this hypothesis will be discussed below.

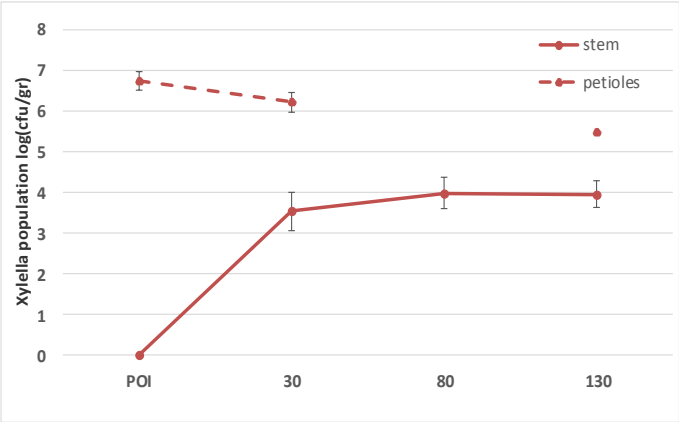

Figure 7. Population size of *Xylella fastidiosa* in grape plants inoculated only with the pathogen. The solid red line represents the bacteria populations in the stem while the dashed line represents pathogen populations in the petioles in samples taken at different centimeter locations from the point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

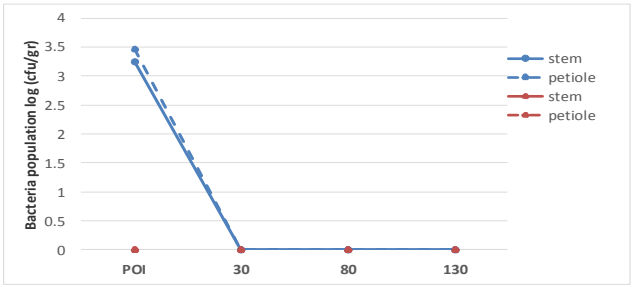

Figure 8. Population size of *Xylella fastidiosa* (red lines) and *Paraburkholderia* (blue lines) in grape plants inoculated go inoculated with the pathogen and *Paraburkholderia* at the same location. The solid lines represent bacteria populations in the stem while the dashed lines represents populations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

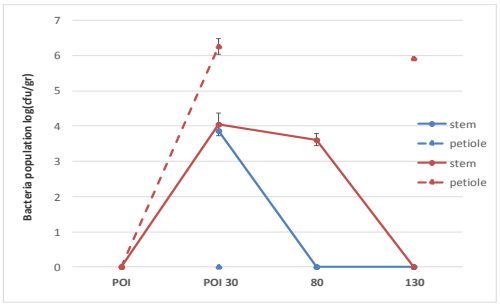

Figure 9. Population size of *Xylella fastidiosa* (red lines) and *Paraburkholderia* (blue lines) in grape plants inoculated at their base with the pathogen while *Paraburkholderia* was inoculated 30 cm distal to the point of inoculation at the same time. The lines represent bacteria populations in the 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.

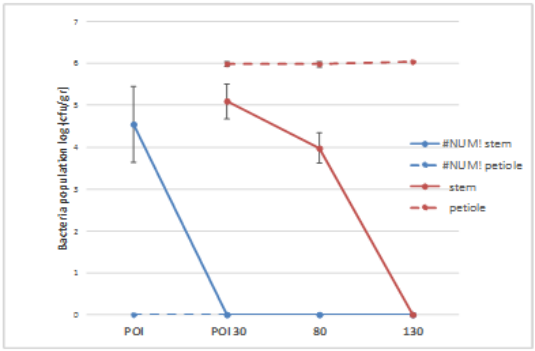

Figure 10. Population size of *Xylella fastidiosa* (red lines) and *Paraburkholderia* (blue lines) in grape plants inoculated at their base with *Paraburkholderia* while *X. fastidiosa* was inoculated 30 cm distal to the point of inoculation at the same time. The lines represent bacteria populations in the stem while the dashed lines represent 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 *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. For example, when measured 4 to 6 weeks after inoculation, very large *Paraburkholderia* 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, *Paraburkholderia* 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 *Paraburkholderia* populations in grape. We will be testing 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 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 either before or after that of the pathogen (such as was seen in experiments described in Figure 3) 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 be of great importance to the efficacy of biological control (Figure 11). Our ongoing studies will 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. This information will be central to our understanding of how to optimize biological control of Pierce's disease.

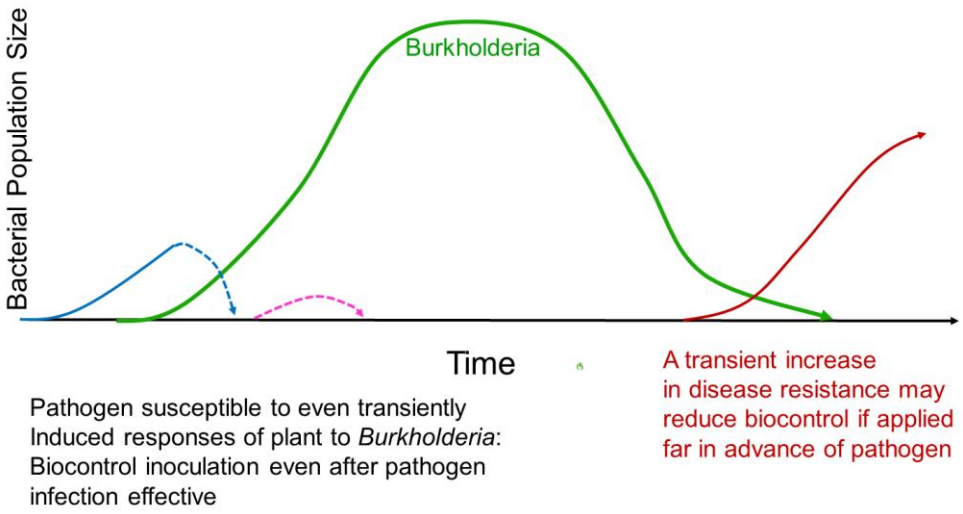

Figure 11. A model describing the expected temporal growth and persistence of *Paraburkholderia* in grape plants after inoculation (green line) and the expected effects on population sizes of *X. fastidiosa* inoculated at various times relative to that of *Paraburkholderia* (blue, pink, and red lines)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

Further support for the model developed above wherein *Paraburkholderia* is somewhat self-limiting in the plant after inoculation, rapidly rising to relatively high population sizes, but then rapidly decreasing in population sizes to a point where it is not capable of antagonizing *X. fastidiosa*, was obtained by further studies in which it was inoculated in different ways both before, at the same time as, and after that of *X. fastidiosa*. As we have consistently seen before, inoculation of plants with *Paraburkholderia* at the same time as the pathogen confers a very large reduction in the extent of disease severity compared to that of plants inoculated with the pathogen alone (Fig. 12). It is noteworthy that the extent of disease protection conferred by inoculation of *Paraburkholderia* into plants at the same time as the pathogen but at a different nearby location than the pathogen by needle inoculation (Figure 12 treatment 3) conferred the same high levels of disease protection than did co-inoculation of *Paraburkholderia* and the pathogen together with needle inoculation as a mixture in the same inoculation site (Figure 12 treatment 5). It is also noteworthy that application of *Paraburkholderia* as a topical spray at the same time as the pathogen conferred nearly the same level of disease control as needle inoculations with the pathogen (compare treatment 4 with treatments 3 and 5 in Figure 12). Also as observed before, the disease control conferred by application of *Paraburkholderia* either by spray or needle inoculation three weeks prior to that of inoculation with the pathogen was much less than that conferred by simultaneous inoculation with the pathogen (compare treatments 1 and 2 with treatments 3, 4, and 5 in Figure 12). Interestingly, and as observed before, inoculation of plants with *Paraburkholderia* either by spray or needle inoculation methods three weeks after inoculation with the pathogen reduced disease severity as much as or

more so than that from simultaneous inoculations (compare treatments 9 and 10 with treatments 3 and 4 in Figure 12). These results are quite exciting in that they suggest that inoculation of plants with *Paraburkholderia* need not anticipate the infection of the plants with *X. fastidiosa*, but instead this biological control agent might be applied even well after plants become infected with the pathogen but before symptoms develop. Further studies will be performed to determine the length of time following infection of plants by *X. fastidiosa* that inoculation with *Paraburkholderia* can block symptom development. The observations that spray inoculation of *Paraburkholderia* are equally effective as needle inoculation also indicate that field inoculation of plants could be a very simple and inexpensive way by which plants could be treated with this biological control agent. The rather unexpected observation that symptom development can be blocked even well after inoculation with the pathogen is supportive of a model in which *Paraburkholderia* induces a form of host defense against the pathogen, as discussed below.

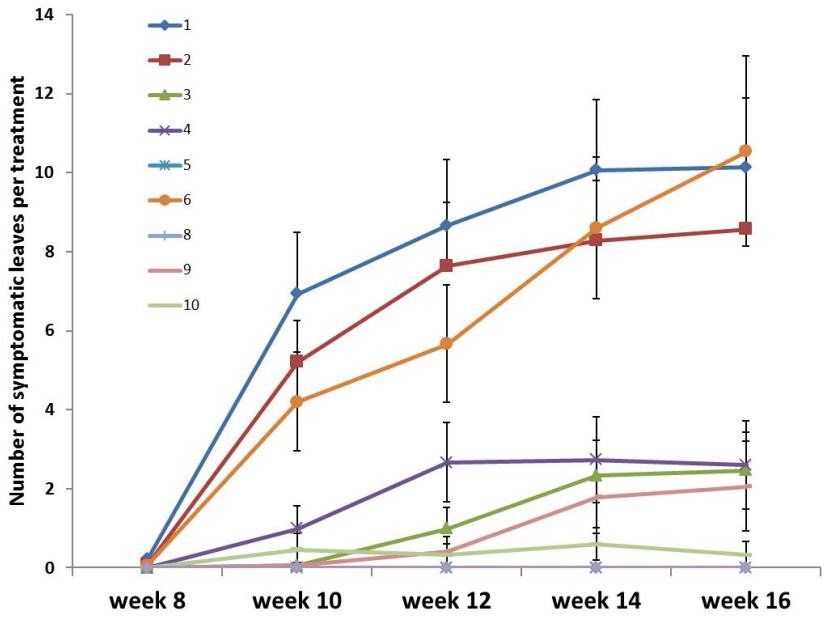

Figure 12. Severity of Pierces disease symptoms (number of symptomatic leaves/vine) on Cabernet Sauvignon plants needle inoculated only with *P. phytofirmans* (treatment 8), only with *X. fastidiosa* (treatment 6), Inoculated only with buffer (treatment 7), co-inoculated at the same time but in different locations within the same stem internode with *X. fastidiosa* and *P. phytofirmans* (treatment 3), co-inoculated at the same time and as a mixture of *X. fastidiosa* and *P. phytofirmans* inoculated at the same location within the stem (treatment 5), and sprayed with *P. phytofirmans* immediately before needle inoculation with *X. fastidiosa* (treatment 4). Also shown is disease severity on plants needle inoculated with *P. phytofirmans* 21 days before inoculation with *X. fastidiosa* (treatment 1) or sprayed with *P. phytofirmans* in a solution of 0.2% Breakthru 21 days before inoculation with *X. fastidiosa* (treatment 2) as well as on plants needle inoculated with *X. fastidiosa* 21 days after inoculation with *P. phytofirmans* (treatment 9) or sprayed with *P. phytofirmans* 21 days after inoculation with *X. fastidiosa* (treatment 10). The vertical bars represent the standard error of the determination mean disease severity.

Objective 2: Mechanisms of biological control

As discussed in Objective 2 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 phytofirmans 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 *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 several defense related genes in three groups of plants: 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*. A comparison of gene expression patterns in grape from these three treatments should enable us to determine whether *Paraburkholderia* alone can alter gene expression patterns in grape or instead, may "prime" the plant to respond to *X. fastidiosa*. Tissue samples were collected every week for 5 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.

As we had seen in previous experiments, the population size of *Paraburkholderia* increased rapidly with time at the site of inoculation and quickly could be detected as much as 40 cm away from the point of inoculation, although at somewhat lower population sizes that also tended to increase with time (Figures 13 & 14). As we have consistently seen, *X. fastidiosa* could not be detected in plants that were co-inoculated with *Paraburkholderia* at any time (Figures 13 & 14). In contrast, the population size of *X. fastidiosa* increased rapidly with time and by three and five weeks could be detected 40 cm away from the point of inoculation (Figures 13 & 14). Because of the design of this experiment, it was possible to systematically examine the population dynamics of *Paraburkholderia* as a function of time after it was inoculated into plants. An examination of Figures 13 and 14 reveal that is population size at a given site in the plant typically increased for 2 to 3 weeks before dropping by week 5 (Figure 15). This pattern is most apparent when one considers its population size at the point of inoculation as a function of time (Figure 16). It thus appears that *Paraburkholderia* increases rapidly within the plant but its population sizes then drop thereafter, suggesting that it may be somewhat self-limiting in its colonization capacity of grape. Its population and dynamics are quite different from that of *X. fastidiosa* - which increased continually with time at a given site within the plant (Figures 15 & 16). The study is being repeated so as to allow us to monitor population sizes of the pathogen and *Paraburkholderia* in plants for a longer period of time after inoculation.

Not only were populations of *Paraburkholderia* and *X. fastidiosa* measured in each of the samples, but total RNA was extracted and semi-quantitative RT-PCR performed to measure the expression of several key genes in the defense-signaling network of grape. Among them are PR1 (salicylic acid - related), Jaz1 (Jasmonic acid related), ETR1 (ethylene - related) genes. EF1α was used as an internal control as it is typically constitutively expressed in plants. While the expression of these various genes involved in plant defense were typically very low and not influenced by inoculation by *Paraburkholderia* alone, *X. fastidiosa* alone, or co-inoculation with *Paraburkholderia* and *X. fastidiosa* (data not shown), we did find evidence of induced expression of PR1 and ETR1 with and 1 to 3 weeks after inoculation, and plants go inoculated with *Paraburkholderia* and *X. fastidiosa*, but not in plants inoculated with either of these strains alone, especially those petioles near the point of inoculation (Figure 17). We interpret these results to suggest that the presence of *Paraburkholderia* somehow primed a host defense reaction toward *X. fastidiosa*, but that the pathogen alone was not capable of inducing such defenses. The induction of defense in such a successful pathogen would not have been expected. Because of the different anatomical structure of stem tissue compared petiole tissue, it may be that there was less living tissue in contact with either of these bacteria than in petioles, thus limiting our ability to measure such a defense reaction even if it had happened in the stem tissue. Given that we did not see evidence of induction of PR1 and ETR1 at distances distal from the point of inoculation, it suggests that host defenses are induced primarily locally in the presence of both *Paraburkholderia* and *X. fastidiosa*. We will be repeating these results to confirm that at least one of the effects of inoculation with *Paraburkholderia* is to induce host defenses. It is possible that such an induction of host defenses also is operative leading to its own demise in the plant with time.

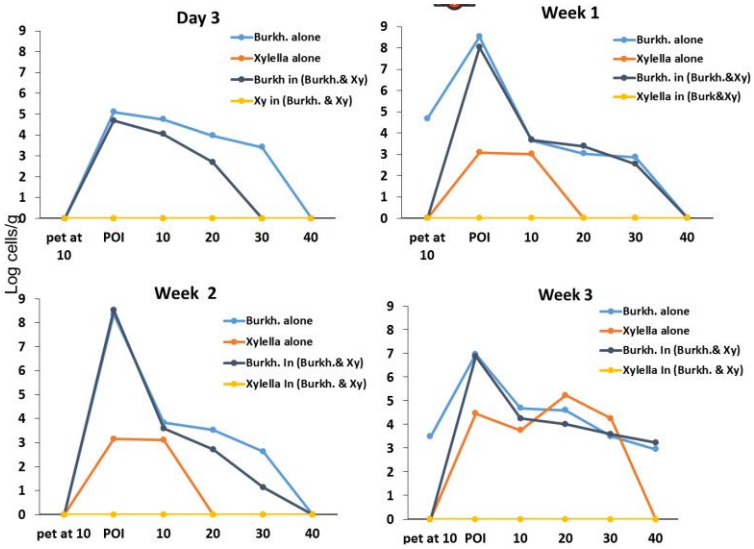

Figure 13. Population size (log cells/gram) of *Paraburkholderia* in plants inoculated only with this strain (light blue lines), *Paraburkholderia* in plants co-inoculated with *X. fastidiosa* (dark blue lines), *X. fastidiosa* one inoculated only with this strain (orange lines), and *X. fastidiosa* in plants co-inoculated with *Paraburkholderia* (yellow lines). Samples were collected at the different times shown on each graph stem segments at the point of inoculation (POI) as well at different distances (in cm) distal to the point of inoculation shown on the abscissa. Samples were also collected from petioles located 10 cm distal from the point of inoculation (pet at 10).

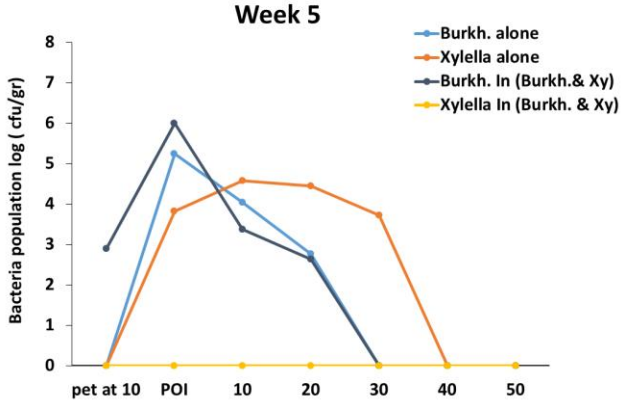

Figure 14. Population size (log cells/gram) of *Paraburkholderia* in plants inoculated only with this strain (light blue line), *Paraburkholderia* in plants co-inoculated with *X. fastidiosa* (dark blue line), *X. fastidiosa* one inoculated only with this strain (orange line), and *X. fastidiosa* in plants co-inoculated with *Paraburkholderia* (yellow line). Samples were collected five weeks after inoculation in stem segments at the point of inoculation (POI) as well at different distances (in cm) distal to the point of inoculation shown on the abscissa. Samples were also collected from petioles located 10 cm distal from the point of inoculation (pet at 10).

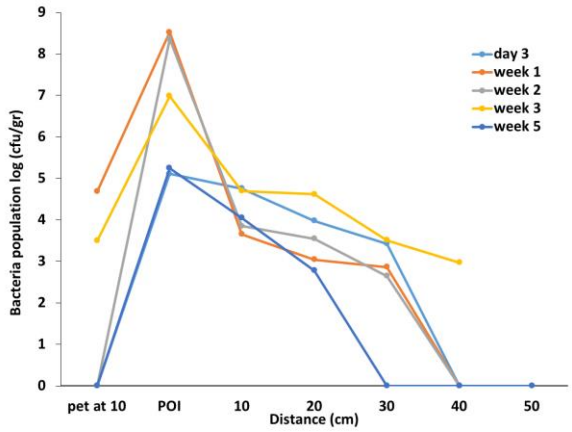

Figure 15. Population size (log cells/gram) of *Paraburkholderia* in plants inoculated only with this strain when sampled 3 days (light blue line), 1 week (orange line), 2 weeks (grey line), 3 weeks (yellow line), and 5 weeks (dark blue line) from stem segments collected at the point of inoculation (POI) as well at different distances (in cm) distal to the point of inoculation shown on the abscissa. Samples were also collected from petioles located 10 cm distal from the point of inoculation (pet at 10).

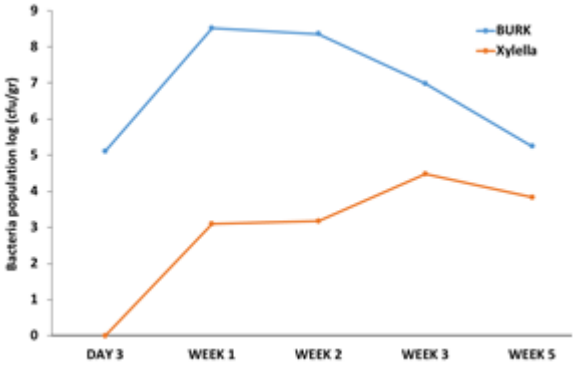

Figure 16. Population size (log cells/gram) of *Paraburkholderia* in plants inoculated only with this strain (light blue line) or with *X. fastidiosa* alone (orange line) in stem segments collected at the point of inoculation at the various times shown on the abscissa.

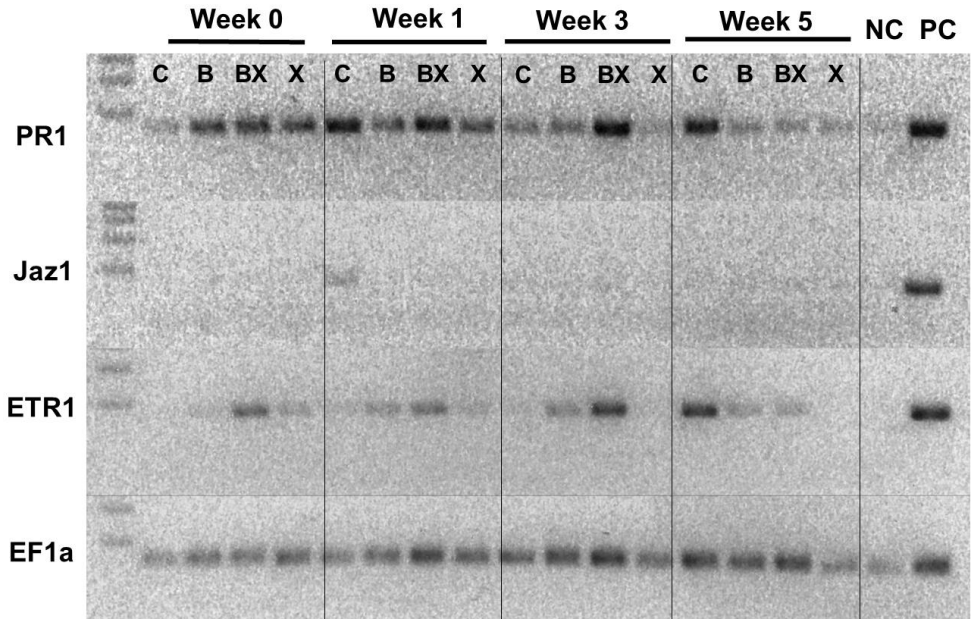

Figure 17. PCR Amplification products obtained after PCR amplification of cDNA obtained from RNA that had been subjected to reverse transcriptase that was isolated from grape plants that were inoculated (C) inoculated with *Paraburkholderia* alone (B), inoculated with both *Paraburkholderia* and *X. fastidiosa* (BX), 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 panel.

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, 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 *Paraburkholderia* 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 *Paraburkholderia*, 2) challenge plant with *Xf* several weeks after inoculation 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 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 shown in Figure 18:

	2018				2019
Treatment	April	May	June	July	
1		Xf PsJN needle			
2		PsJN&Xf mix needle			
3		Xf PsJN spray			
4	PsJN needle	Xylella			
5	PsJN spray	Xylella			
6		only Xylella (control)			
7	PsJN needle				
8	PsJN spray				
9		Uninoculate (control)			
10		Xylella	PsJN needle		
11		Xylella	PsJN spray		
12		Xylella BREAK 0.2%			
13		Xf PsJN needle	PsJN needle	PsJN needle	
14		Xf PsJN spray	PsJN spray	PsJN spray	
15		Xf PsJN needle	Xy PsJN needle	PsJN needle	
16		Xf PsJN spray	Xy PsJN spray	PsJN spray	
17		only Xf (control)	only Xf (control)		
18		Xf PsJNvine trunk			
19		Xf soap 2%& Break	soap 2%	soap 2%	
20			prime with PsJN needle		year 2
21			prime with PsJN spray		year 2

Figure 18: Treatments being evaluated in a large replicated field trial at UC Davis. Each of these treatments is being performed during 2018 on Cabernet Sauvignon and Pinot Noir grapes. Treatment codes consist of: (PsJN needle) = Cell suspensions (ca. 10^8 cells/ml) of *Paraburkholderia phytofirmans* applied to grape stems using a droplet (10 µl) puncture method. (Xf) = Cell suspensions (ca. 10^8 cells/ml) of *Xylella fastidiosa* applied to grape

stems using a droplet puncture method. (PsJN spray) = Cell suspensions (ca. 10^8 cells/ml) of *Paraburkholderia phytofirmans* containing 0.2% Breakthru® were sprayed onto leaves until wetness. (PsJN&Xf needle mix)= cell suspensions of *Paraburkholderia phytofirmans* and *Xylella fastidiosa* (ca. 10^8 cells/ml each) were mixed and applied to grape stems by the droplet puncture method. (Soap)= leaves were sprayed to wetness with a solution of 2.0% soap prepared by saponification of macadamia nut oil. (*Xylella* Break 0.2%)= plants were sprayed to wetness with a solution of 0.2% breakthrough immediately following inoculation of plants with Xylella fastidiosa by the droplet puncture method.

To obtain an APHIS to allow the field use of *P. phytofirmans* we had to demonstrate the presence of microorganisms closely related to *P. phytofirmans* in California or nearby states. We thus collected 82 plant and soil samples from various sites in Solano, Yolo, Nevada, and Sutter counties which were interrogated for the presence of full-length bacterial 16S ribosomal RNA genes identical that were very closely related (>99.5%) to that of *P. phytofirmans*. Samples of rhizosphere material as well as macerated roots, and separately soil, were plated onto media differentially selective for various *Burkholderia* and *Paraburkholderia* species. The identity of the many colonies recovered on the selective medium was investigated by bulk harvesting of all of the bacteria recovered from selective medium plates followed by bulk isolation of DNA, and amplification of full-length 16S rRNA genes using universal primers. The mixture of full-length 16S rRNA amplicons were assessed by PacBio sequencing at the University of California, Davis genome Center, DNA technologies Core. Of a total of about 7000 full-length 16S RNA gene sequences, obtained 12 were found to be nearly identical in sequence to that of *Paraburkholderia phytofirmans* PsJN. A permit was therefore granted for its field use.

So-called "Uber" plants for the study were generously provided by Duarte Nurseries and were planted in late April, 2017 (due to the presence of wet soils) at the UC Davis field site. These large "Uber" plant group quite rapidly during the 2017 growing season and were sufficiently large to enable inoculation with the treatments early in the 2018 growing season. Figure 19.

Figure 19. Appearance of Pinot Noir grapes on April 30, 2018 when initial injection and spray applications of *Paraburkholderia phytofirmans* were applied.

The first applications of *Paraburkholderia phytofirmans* were applied to Pinot Noir grape on April 30, 2018, with subsequent applications of this strain as well as the pathogen made on May 21, 2018. Additional applications of bacteria were made to Pinot Noir on June 11 and July 9, 2018. Initial applications of *Paraburkholderia phytofirmans* were made to Cabernet Sauvignon grapes on May 7, 2018, with subsequent applications of this strain as well as the pathogen made on May 30, 2018. Additional bacterial inoculations were made on Cabernet Sauvignon on June 18, 2018 and July 9, 2018. Grape stems were approximately 20 to 30 cm

in length at the time that *X. fastidiosa* was inoculated by droplet puncture on each variety when they were inoculated on either May 21 or May 30, 2018 as appropriate (Figure 20).

Figure 20. Appearance of Pinot Noir plants on May 21, 2018 at the time of inoculation with *Xylella fastidiosa* or *Paraburkholderia phytofirmans.*

Spray inoculation of *Paraburkholderia phytofirmans* in a solution containing 0.2% Breakthru was a very effective method for introducing this bacterium into grape leaves. When bacterial suspensions containing 10^8 cells per milliliter in the surfactant were sprayed onto leaves until the leaf surface was wet using a hand-held sprayer, the leaves became water -soaked within about 20 seconds, indicating the spontaneous infiltration of the bacterial suspension into the apoplast of the leaves (Figure 21). The water-soaked appearance of the leaves was most apparent on the underside of the leaf, and it was noted that the watersoaking process was much more efficient when bacterial suspensions were applied to the underside of the leaf. Surprisingly, the process of introducing bacteria into the leaf using the penetrating surfactant seemed to be more effective under field conditions than it had been in greenhouse studies. Plants typically remained water-soaked for about five minutes after spraying, depending on ambient temperature and wind conditions, thereby depositing the inoculated bacteria within the leaf.

Figure 21. Appearance of Pinot Noir grape leaves approximately two minutes after spray application of *Paraburkholderia phytofirmans* in a solution of 0.2% Breakthru. Note the water-soaked areas on the leaf on the right indicating the spontaneous infiltration of the bacterial suspension into the leaf.

Relatively large population sizes of *Paraburkholderia phytofirmans* were introduced into leaves during the process of spraying of bacterial suspensions in the presence of the organosilicon surfactant Breakthru. Internal populations of the bacteria within the leaf were determined by maceration of the leaf lamina or petioles separately after surface sterilization of the leaf with sequential topical applications of 70% ethanol, and 0.5% sodium hypochlorite. Population sizes of *P. phytofirmans* were typically about 100-fold higher in the lamina compared to that of the petiole (Figure 22). Population sizes of greater than 10^5 cells/g were achieved in the leaf lamina immediately after spray application during the process of watersoaking of the interior of the leaf. The population sizes were maintained at a similar high population size for over 30 days after inoculation (Figure 22). In greenhouse studies in which *P. phytofirmans* was applied in buffer alone (without surfactant), fewer than about 10 bacteria per gram were ever detected after inoculation (data not shown). Thus, not only can we apparently introduce *P. phytofirmans* into the vascular tissue within the petiole, but much larger populations can be introduced into the apoplast of the leaf. If the mechanism by which *P. phytofirmans* confers resistance to *X. fastidiosa* involves priming of disease-resistant mechanisms, the large apoplastic population might be expected to be particularly effective in triggering such a response.

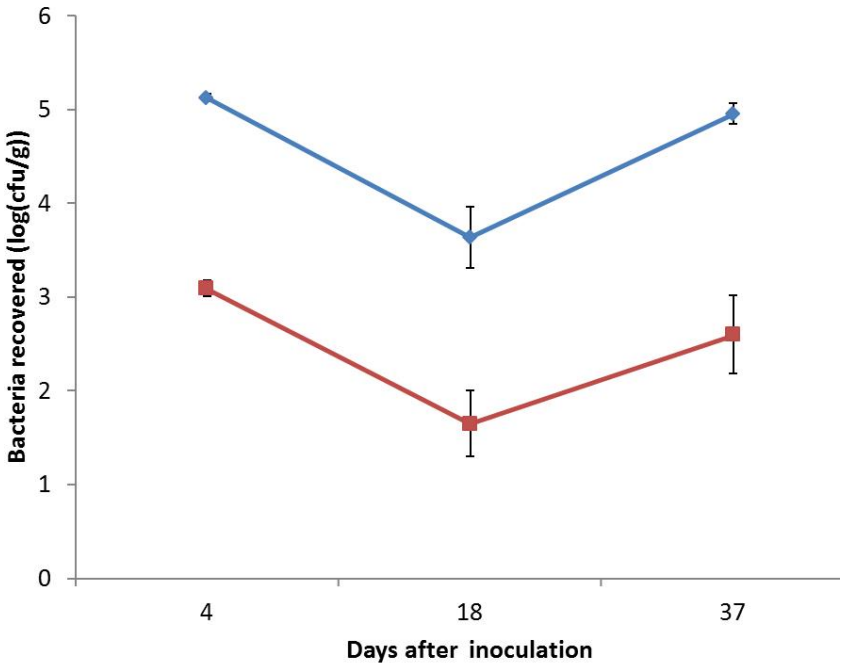

Figure 22. Population sizes of *Paraburkholderia phytofirmans* within surface sterilized leaf lamina of Pinot Noir grape (blue line), or within surface sterilized petioles (red line) at various times after leaves were treated in a field plot with suspensions of this bacteria in a solution of 0.2% Breakthru. The vertical bars represent the standard error of the determination of log- transformed bacterial population sizes per gram fresh weight of leaf tissue.

As of mid-August, 2018 symptoms of Pierce's disease were beginning to become apparent on both Pinot Noir and Cabernet grape plants. The severity of Pierce's disease on each of the 4 vines for each plant (10 plants per treatment) that were inoculated with the pathogen will be assessed. The total number of leaves on a given inoculated vine as well as the number of symptomatic leaves will be counted to enable the calculation of the fraction of leaves of a given vine that are symptomatic. In addition, any evidence of Pierce's disease on other vines emerging from cordons of each plant will also be noted to determine if treatments reduced to spread of *X. fastidiosa* between vines on a given plant.

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.

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:

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 does 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.