Progress report for CDFA contract number 06-0226

Project Title: Assessment of the process of movement of *Xylella fastidiosa* within susceptible and resistant grape varieties

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Abstract:

We have followed the movement and population size of a gfp-marked strain of *X. fastidiosa* simultaneously in both the stems and petioles of Cabernet Sauvignon, Chenin Blanc, Roucaneuf and Tampa grape varieties which differ in susceptibility to Pierce's disease. Very low populations of *X. fastidiosa* and less frequent occurrence in xylem vessels in the stem were observed in the resistant varieties compared to more susceptible varieties. There was no simple relationship between the population size of *Xf* in the stem and the proportion of vessels colonized when considered over the several varieties; a much higher population size of *Xf* was observed than expected, even after accounting for the higher number of infected vessels, in susceptible varieties. To better understand the distribution of the *Xf* population, particularly in the stem vascular system, we distinguished between high moderate and low levels of cell numbers in a given infested vessel. The higher populations in susceptible genotype stems are achieved because of both higher numbers of infected vessels and particularly due to the much higher extent of colonization of those vessels that become infested with *Xf*. Lower populations in resistant genotype stems are achieved because of both lower numbers of infected vessels and also because of a lower number of cells in the vessels that are colonized. This suggests that in resistant genotypes the movement and multiplication of *Xf* in the stem are both impaired and are co-dependent phenomena. In contrast, similarly high percentages of vessels in petioles of susceptible and resistant plants were colonized, and similar population sizes were attained, suggesting that *X. fastidiosa* is unrestricted in movement and growth within the petiole. These results indicate that resistance to Pierce's disease is not due to inhibitory compounds that circulate through the xylem or to host defenses since they might be expected to operate similarly in all tissues. Also, large-scale cell agglomeration in a single vessel is not required for *Xf* to move laterally in the stem to adjacent vessels as the majority of vessels were categorized as having few cells in the vessels in all the genotypes. These results are consistent with earlier work done on Cabernet petioles. In the resistant genotype Roucaneuf we found only low numbers of cells in any vessel, although *Xf* was able to move a distance greater then the average vessel length from the Point of inoculation. We are currently also monitoring the movement of a gfp-marked strain of *Xf* in transgenic PGIP-expressing grapes provided by Dr. Dandekar at UC Davis to test our model that differences in digestibility of pit membranes are responsible for the differential movement and growth, and thus susceptibility, in grape varieties.

Work is continuing using mixtures of isogenic strains of *X. fastidiosa* to examine the apparent bottlenecks that occur when cells move from one infected vessel into other adjacent uninfected vessel. The efficiency with which cells move from one vessel to another is expected to be related directly to overall susceptibility to Pierce's disease and should be manifest as a rate of spatial segregation in the plant of the two strains that is inversely related to susceptibility to disease.

Introduction:

Nearly all studies of *X. fastidiosa* colonization of grapes have focused on the petioles, with little examination of *Xf* movement and distribution in the stems. Importantly, the work from the Walker lab has noted that resistance to *X. fastidiosa* is localized within the stem xylem and not fully functional or absent in the xylem of petioles and leaf blades. This was based on the observation that there was little difference in the colonization of the petioles and leaf blades, as opposed to the stems. They speculate that a more constitutive resistance mechanism is present in the stem xylem based on nutritional or structural differences between resistant and susceptible types. Our study was designed to examine differences in the colonization process of the stem of different grape genotypes to identify resistance mechanisms.

In an effort to better understand the process of colonization of grapevines by *X. fastidiosa*, and develop a method of screening for resistant plant genotypes, we are investigating the spatial segregation of *Xf* cells within the xylem vessel systems of different grape varieties. Single *Xf* strains or an equal mixture of two different isogenic *X. fastidiosa* strains, are being co-inoculatied in different varieties and their movement is being followed closely by culturing and epifuorescence microscopy, with time and distance from the point of inoculation to determine how rapidly spatial segregation of the cells might occur, presumably due to stochastic proceses occurring by transfer of only a few cells from one infected vessel to other uninfected vessels. Before initiating studies of the segregation of differentially marked strains of *X. fastidiosa* in various grape varieties, we explored the process of colonization of *Xf* in stems of Cabernet Sauvignon to establish control data and optimize sampling schemes for the *Xf* strain mixtures. We set out to determine how quickly *X. fastidiosa* moves within stems throughout the plant, the fraction of the xylem vessels colonized as a function of time and distance from the point of inoculation, and the relative likelihood of finding *X. fastidiosa* in xylem vessels as compared to tracheal elements. We specifically considered the longitudinal movement of *X. fastidiosa* in the xylem vessels in the internodal stem locations and the rate at which segregation of the two strains occurs.

Objectives:

1. Study the process of movement of *X. fastidiosa* cells between xylem vessels and through plant by determining the changes in proportion of genetically distinct strains of the pathogen initially inoculated into plants at an equal proportion with distance and time from point of inoculation

2. Determine if bottlenecks in movement of cells of *X. fastidiosa* from xylem vessel to xylem vessel is more extreme in resistant plants than in susceptible plants and whether this phenomenon can be exploited as a tool to screen germplasm for resistance to *X. fastidiosa*.

Results:

Objective 1:

We initiated our investigation by co-inoculating Cabernet Sauvignon stems with a mixture containing an equal number of cells of wild-type and gfp-marked (KLN59.3) *Xf* strains. This was designed specifically so that the segregation of the two strains could be tracked and correlated to resistance characteristics of the plant variety. The population size of the gfp-marked strain of *X. fastdiosa* was somewhat smaller at a given location and time after inoculation than the wild-type strain. It was known that this strain caused disease symptoms slightly slower than the wild-type strain, and this difference thus appears to be due to a slower growth in the plant. Given that future experiments will emphasize the spatial segregation of this gfp-marked strain and a similar cfp-marked strain which is expected to have a similar growth rate as the gfp-marked strain we do not expect that this lower growth compared to the wild-type strain will complicate our measurements of ratios of these two strains in up-coming experiments. To best test our model of stochastic processes influencing spatial segregation it is important that two isogenic bacterial strains used in such studies have nearly identical behavior in the plant. We thus have tested other such strain pairs for suitability for this study. It was found that an *rpfB* mutant of *Xf* was more virulent to grape and moved and multiplied somewhat better in cabernet than the wild type *Xf*. This was unexpected given that when inoculated singly they each had yielded similar disease severity and progression in the plant. Studies are underway with other isogenic strain pairs of *Xf*. These strain pairs include *Xf* harboring different marker genes introduced into the same intergenic region in *Xf* by the Igo lab, as well as random Tn5 mutants of *Xf* generated by the Kirkpatrick lab that exhibited similar virulence as the wild type strains.

Objective 2:

Colonization of susceptible Cabernet Sauvignon and resistant genotypes like Tampa and Roucaneuf by *Xf* was examined by sequential culturing and epifuorescence microscopy. Roucaneuf is a complex hybrid that includes V. c*inerea* and V. *berlandieri* and has been described as "fully-resistant" in field conditions to PD. Tampa also is a PD resistant genotype. Microscopy did not reveal any obvious differences in anatomy of the stem and petiole tissues of the resistant and susceptible varieties**.** Cabernet Savignon, Roucaneuf and Tampa plants were inoculated with a GFP-marked X. *fastidiosa* strain. We followed population growth by culturing and also visually by microscopy. Culture sampling was done at weeks 2, 3, 4, 6, and 11 following inoculation. A total of 6 plants at each time point, two from each resistant genotype and two from the susceptible genotype were evaluated. Each plant was sampled at the petiole near the point of inoculation and at 6 internodal locations 10, 20, 30, 60, 80, and 120 cm away. The sample sites were examined the same day by epifruorescence microscopy. Petioles and portions of the stems were sectioned and prepared for microscopy. An average of 9 sections was prepared for each stem location and photos were taken from each sample.

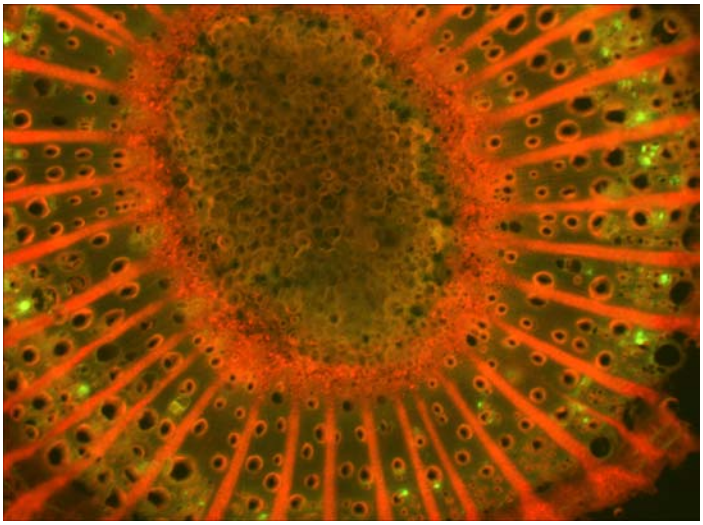


Figure 1: Visualization of colonization of Cabernet Sauvignon stems wth a gfp-marked strain of *X. fastidiosa*. The plant was sectioned 11 weeks after inoculation and this section was taken from the stem at 30 cm from the point of inoculation. This image is typical of stem tissue from susceptible grape varieties in that a relatively high proportion of vessels harbor at least some cells of *Xf* while most vessels harbor relatively few cells of the pathogen.

It was clear from our observations that a very low proportion of the stem vessels at sites away from the point of inoculation of Roucaneuf and Tampa were colonized by any cells of *Xf* compared to that of Cabernet. There was also a higher viable population

sizes of *Xf* in Cabernet in the stem tissue compared to that of Roucaneuf and Tampa. However, there was no simple relationship between the population size of *Xf* in the stem and the proportion of vessels colonized when considered over the several varieties; a much higher population size of *Xf* was observed than expected, even after accounting for the higher number of infected vessels, in susceptible varieties (Fig. 2) This raised the question as to whether cells in the resistant varieties may die as they age, or whether there was a large difference in the extent of colonization of those vessels that become infested with *Xf*.

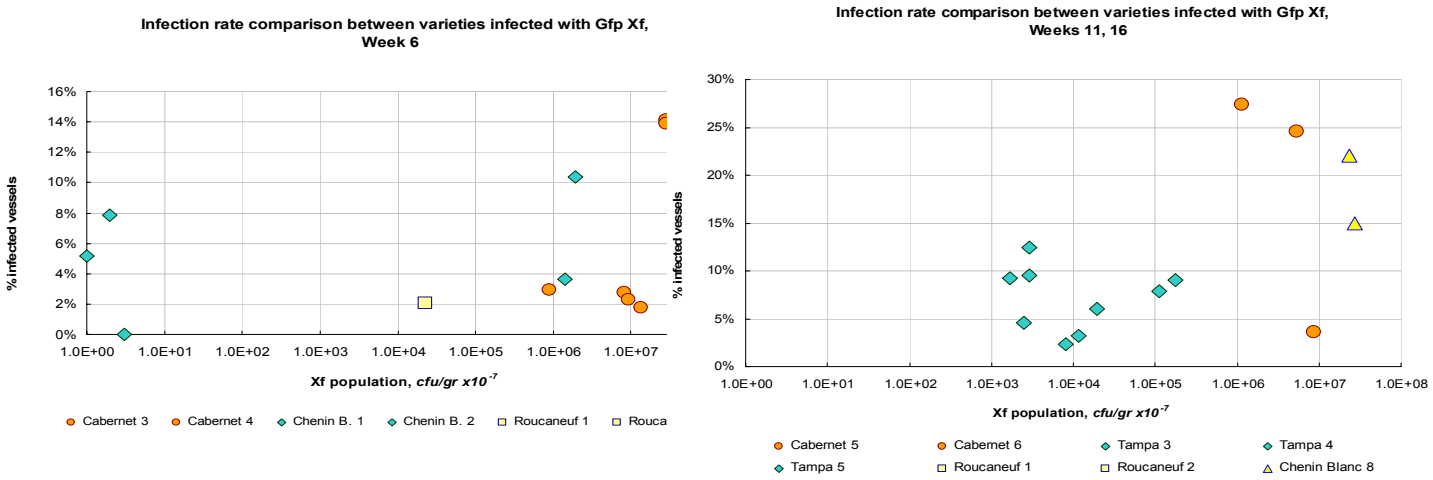


Figure 2: Relationship between incidence of colonization of stem vessels of different grape varieties by *Xf* as determined by a microscopic detection of gfp-tagged *Xf* strain (Y-axis) and the population size of *Xf* determined by culturing of small samples of tissue near the site of examination (X-axis)

In contrast to the stem tissue, visualization of cells of *Xf* in petioles of Cabernet, Roucaneuf and Tampa reveal that petioles of these plants were both equally well colonized by the gfp-tagged cells of *Xf* **.** This is in contrast with the stems of these two varieties where very few vessels of Roucaneuf were colonized but a large percentage of vessels of Cabernet were colonized. It was evident that there was no significant difference in bacterial population sizes between the resistant and susceptible genotypes in the petioles (Table 1)**.** In addition, the proportion of the total stem xylem vessels that are colonized by *X. fastidiosa* appears to be much less than that of the xylem vessels in the petiole for a given variety. Thus the petiole seems to offer little resistant to movement and or multiplication of *Xf* compared to stem tissue.

Xf Concentration, log[(cfu/g+1)]

week	Petiole		
	Roucaneuf	*Tampa*	*Cabernet Sauvignon*
3	7.77	4.86	7.60
4	7.71	5.55	7.43
6	7.18	5.22	6.26
11		6.12	8.46

Table 1: *Xf* populations in petioles of different grape varieties determined by dilution plating a a given time after inoculation.

To investigate the model that not only does *Xf* move into more vessels of susceptible varieties than resistant varieties, but it also multiplies more extensively in those vessels into it moves we performed a more robust examination of colonization of the varieties Tampa, Roucaneuf, Cabernet, as well as Chenin Blanc, a susceptible variety with a slightly more resistance to PD than Cabernet. In addition to counting number of stem vessels that were colonized by any number of *Xf* cells, we distinguished between those having high levels of colonization (which we estimated to be about 100,000 cells/ vessel (labeled "full" in the figures, those having moderate levels of colonization (about 1000 cells/vessel) (labeled "medium" in the figures) or those having minor colonization (less than 10 cells/vessel) (labeled "few" in the figures). The colonization was assessed in the stem for each variety at several different times and distances from the point of inoculation. At each sampling location and time, 12 stem sections were examined under the fluorescence microscope to obtain robust estimates of both incidence and intensity of colonization of vessels. More then 10,000 xylem vessels were observed at each sampling. It was clear that the incidence of infestation of stem xylem vessels by *Xf* was related directly to the resistance of these varieties to Pierce's disease; The highest incidence of colonization of vessels was observed in the highly susceptible Cabernet sauvignon with the lowest in the most resistant variety, Roucaneuf (Figure 3). The varieties with intermediate susceptibilities exhibited intermediate levels of colonization incidence. It is evident that near the point of inoculation the proportion of vessels that harbor any number of cells of Xf are higher than at more distal sites. With increasing time the number of vessels colonized also increase. The reduced number of colonized vessels, particularly at distal sites suggests that in resistant genotypes the

lateral movement to adjacent vessels is what it is impaired. More importantly, a large difference in the extent of colonization of vessels was observed between varieties. In all varieties the large majority of vessels harbored relatively few cells of *Xf* (Figures 4 and 5). Vessels that harbored very large numbers of *Xf* were only observed in the most susceptible variety Cabernet sauvignon (Figures 4 and 5). Likewise, the more susceptible varieties Cabernet sauvignon and Chenin Blanc both had higher numbers of vessels that harbored intermediate extents of colonization by *Xf* (Figures 4 and 5). These differences in levels of colonization were highly statistically different between varieties in most cases (Table 4). At increasing distances from the point of inoculation, the resistant genotypes respond more like each other and become more statistically divergent from Cabernet and Chenin Blanc varieties, having lower colonized vessels.

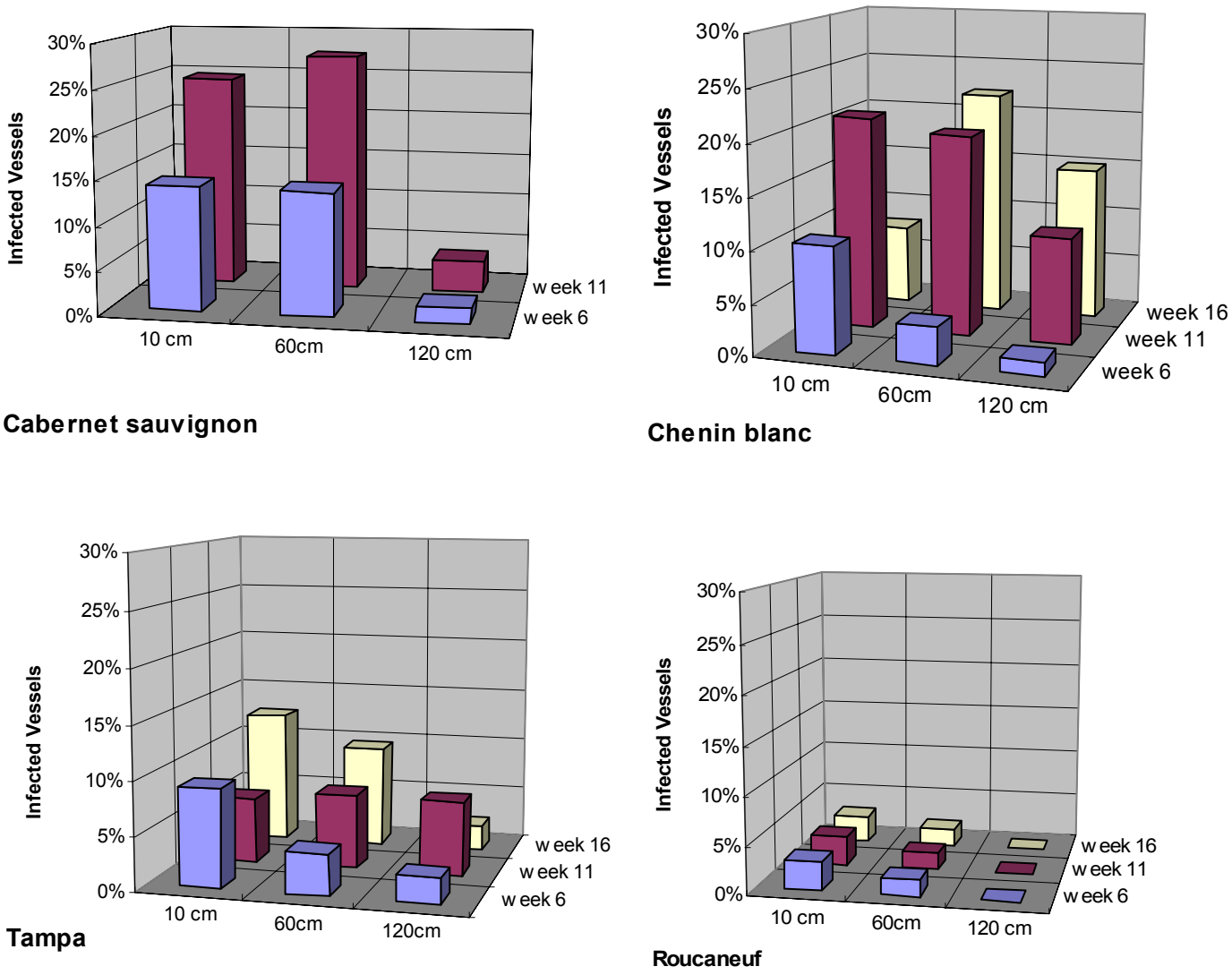


Figure 3. Percentages of infected vessels determined by microscopy (12 stem cross sections each of 28 µm thickness examined per location) sampled at different times and distances from the point of inoculation for four grape varieties.

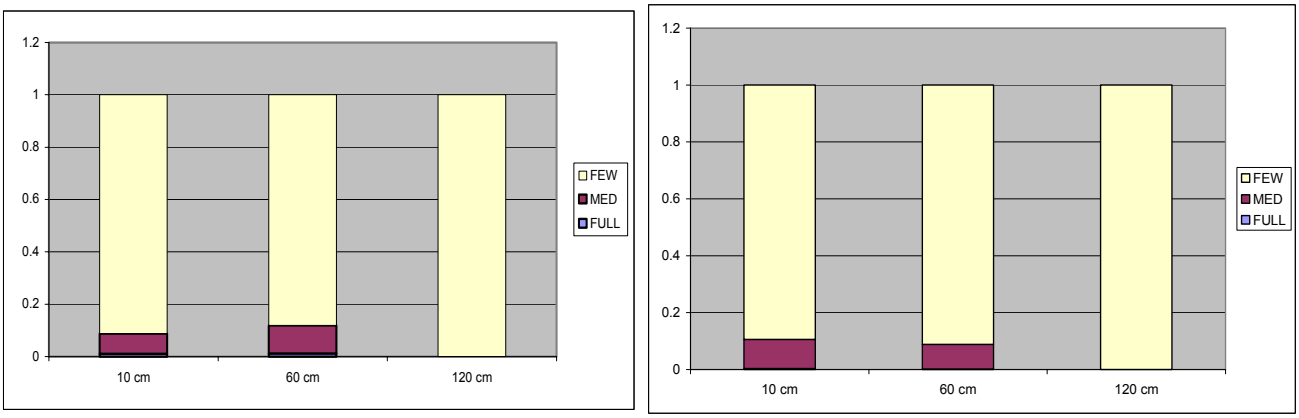
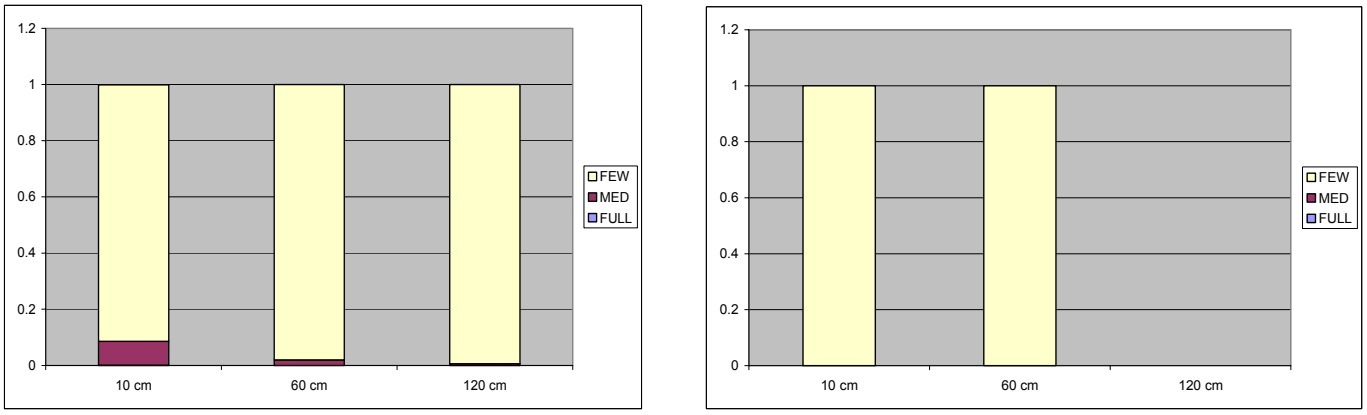


Figure 4. Proportion of colonized vessels having different extents of colonization by *Xf* in Cabernet (left) and Chenin Blanc (right)

Table 3 :*LSD test for mean number of colonized vessels*			
Few cells colonization			
	10 cm	***60 cm***	***120 cm***
Cabernet sauvignon	68.2a	75.7b	8.1a
Tampa	25.5b	13.3a	38c
Chenin Blanc	60.9c	46.9c	4.1ab
Roucaneuf	8d	4.7a	0b
Medium vessels colonization			
	10 cm	***60 cm***	***120 cm***
Cabernet sauvignon	5.7b	8.3b	0a
Tampa	3.4a	0.41a	0a
Chenin Blanc	3.2a	1.41a	0a
Roucaneuf	0c	0a	0a
Full vessel colonization			
	10 cm	***60 cm***	***120 cm***
Cabernet sauvignon	0.9b	0a	0a
Tampa	0a	0a	0a
Chenin Blanc	0a	0a	0a
Roucaneuf	0a	0a	0a

Table 3: Differences in extent of colonization of stem xylem vessels in different grape varieties determined by microscopic detection of a gfp-marked strains of *Xf* at different distances from the point of inoculation. The results of an LSD test performed on the mean number of colonized vessels 11 weeks post-infection are shown. Means followed by the same letter within a column do not differ (P<0.05). Vessels having large numbers 100,000 cells/vessel (full), moderate numbers (1000) of cells/vessel (medium) or few (<10) cells/vessel were differentiated.



F**igure 5.** Proportion of colonized vessels having different extents of colonization in Tampa (left) and Roucaneuf (right)

Table 4:LSD test for proportion mean of vessels colonizatio			
Few cells colonization			
	10 cm	*60 cm*	*120 cm*
Cabernet sauvignon	0.91a	0.88b	1a
Tampa	0.93a	0.97a	0.99a
Chenin	0.9a	0.92c	1a
Roucaneuf	0.96a	0.98a	0b
Medium vessels colonization			
	10 cm	*60 cm*	*120 cm*
Cabernet sauvignon	0.07a	0.07a	0a
Tampa	0.06a	0.16b	0a
Chenin	0.09a	0.07a	0a
Roucaneuf	0b	0c	0a
Full vessel colonization			
	10 cm	*60 cm*	*120 cm*
Cabernet sauvignon	*0.01b*	*0.01b*	*0a*
Tampa	*0a*	*0.01a*	0a
Chenin	*0.03c*	*0.07c*	0a
Roucaneuf	*0a*	*0a*	0a

Table 4: Differences in the proportion of vessels from different grape varieties that had been colonized by any cells of *Xf* that exhibited varying extents of colonization. Microscopic detection of a gfp-marked strains of *Xf* at different distances from the point of inoculation was determined. The results of an LSD test performed on the mean number of colonized vessels 11 weeks post-infection are shown. Means followed by the same letter within a column do not differ (P<0.05). Vessels having large numbers 100,000 cells/vessel (full), moderate numbers (1000) of cells/vessel (medium) or few (<10) cells/vessel were differentiated. (Data of Table 3 expressed as a proportion of the total colonized vessels)

Since we had made independent measures of both the incidence and extent of colonization of stem xylem vessels by *Xf* by microscopy as well as direct measures of viable population sizes of *Xf* by culturing of the adjacent tissue, we tested the model that cells of *Xf* had similar frequencies of viability in different grape varieties. We estimated population sizes from microscopy measurements by multiplying the number of infected vessels by the number of cells enclosed in a given vessel and with knowledge of the amount of plant material that had been examined (28 um/section examined). In locations more proximal to the point of inoculation, the total population sizes estimated by microscopy were very similar to that of the culturable population, suggesting that most of the cells were viable, irrespective of grape variety (Figure 6). Given that the numbers of *Xf* in stems of resistant varieties such as Roucaneuf are low and apparently spatially variable at a given sampling time, not all visualized stem segments (28 um/section x 12sections/sample) include detectable cells of *Xf*.

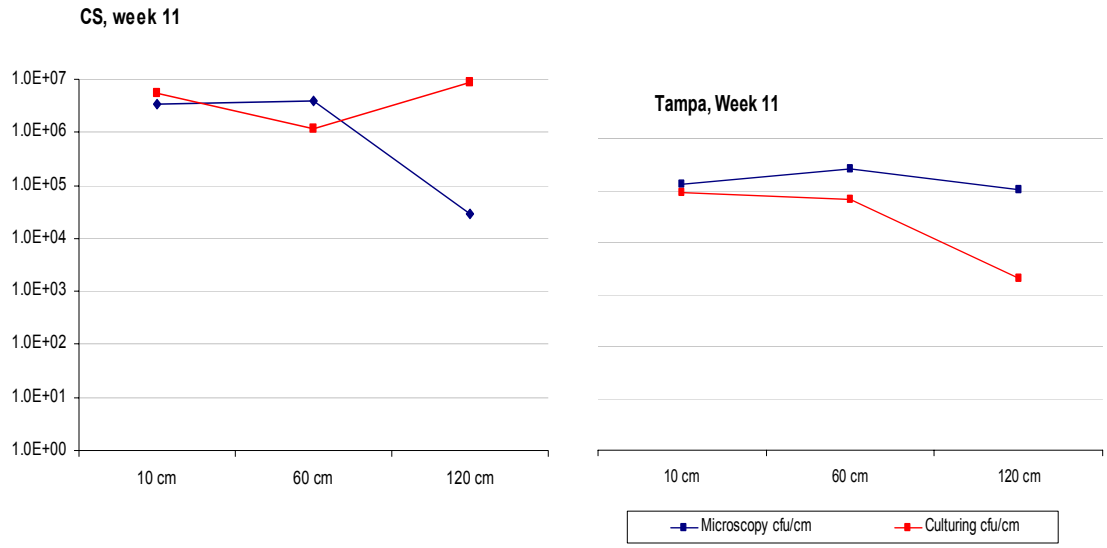


Figure 6. Comparison of population sizes of *Xf* in in Tampa and Cabernet sauvignon stem segments at different distances from the point of inoculation estimated by culturing (red) and by microscopy (blue) at week 11 post infection.

Since *Xf* populations seemed to be viable in resistant and susceptible grape varieties 11 weeks post-inoculation, the question remains as to why in resistant variety like Roucaneuf Xf does not move very far from the POI in the stem, or if it moves like in Tampa, it is incapable of multiplying to higher population densities (Figure 6). Our observations suggest that pit membranes in the stem vessels may play a major role in movement and population size dissimilarity among the varieties examined in this study. To provide evidence in support of this theory, we have initiated a new collaborative study with Dr. Dandekar at UC Davis, whose group has constructed transgenic Thompson grapes expressing PGIP (Poligolagturonase- Inhibiting protein) which would inhibit PG enzymes produced by *Xylella fastidiosa*. These transgenic plants would confer tolerance to susceptible grapes since the PGIP would inhibit the PG enzyme produced by *Xf*, which is responsible for pit membrane degradation. In this project we are following the movement of a gfp marked strain of *Xf* in stems of wild type Thompson and transgenic Thompson grapes across time and distance from the POI. The movement will be tracked using microscopy and culturing methods, similar to the methods used in our prior experiments discussed. We expect that the transgenic PGIP-expressing Thompson seedless grapes will reveal a similar pattern in the movement of *Xf* as in the resistant grape varieties Roucaneuf and Tampa. We anticipate that the movement of *Xf* in the transgenic Thompson grapes would resemble *Xf* movement behavior in the resistant grape Roucaneuf, with a much smaller *Xf* population density and fewer colonized vessels. We are going to sample stems for population by culturing and by epifluorescence microscopy of stem cross sections to calculate the incidence and intensity of infections. Plants will be sampled at weeks 6 and 10 after bacterial infection. This work is in progress.

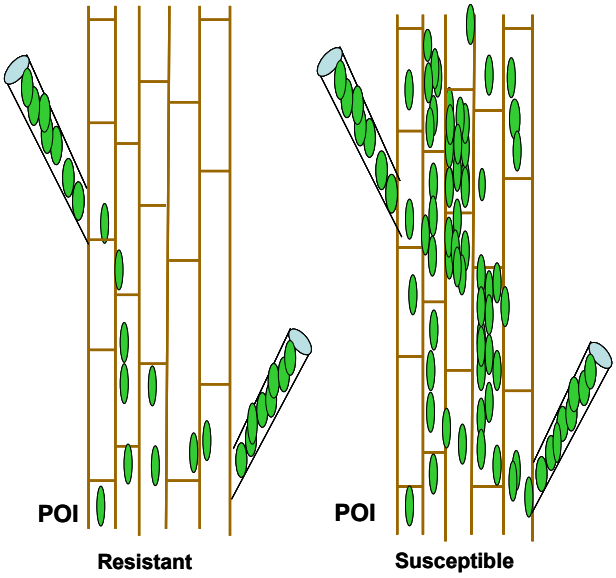


***Figure 7. P*roposed movement of *Xylella fastidiosa* in resistant and susceptible grapes.**

In this model there is limited *Xylella fastidiosa* movement and growth within stem tissues but unlimited movement and growth within petioles of susceptible and resistant grape varieties. Low *Xf* populations in the stem of resistant varieties are due to both a lower incidence of vessel colonization and a lower extent of colonization of those vessels since *Xf* is unable to degrade the pit membranes between vessels which may be main source of nutrition for the bacteria, and thus not able to attain high populations. The high *Xf* stem populations in susceptible varieties is due to higher vessels incidence and intensity of colonization since *Xf* is able to degrade pit membrane consequently multiplying more as well as subsequently invading more vessels.

Intellectual Property issues:
There are no intellectual property issues associated with this project.

Publications:
Baccari, C., and S.E. Lindow Assessment of the process of movement of *Xylella fastidiosa* within susceptible and resistant grape varieties. Phytopathology (in preparation).

Contributions to solutions for Pierce's disease in California.
Not only do these results provide considerable insight into the process of movement which, while central to the disease process, remains very poorly understood, but it should also provide new tools for screening grape germplasm for resistance to *X. fastidiosa*. Our demonstration that resistance is associated with stem tissue colonization will be useful in screening o germplasm for resistance. Furthermore, the on-going studies of spatial segregation of isogenic strains of *X. fastidiosa* should lead to a useful and quick screen for resistance if we can verify that the rate of segregation is proportional to resistance in a given grape variety.