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

B. CDFA contract number: 06-0226

C. Time period covered by the progress report: The results reported here are based upon work conducted between July 2007 and February 2008.

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E. Objectives:

1. Study the process of movement of *X. fastidiosa* cells between xylem vessels and through plants 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*.

F. Research accomplishments and results for each objective:

Objective 1: Temporal and spatial segregation of cells of *X. fastidiosa* during movement through grape stems. We are in the process of characterizing the spatial segregation of cells of *X. fastidiosa* within the grape xylem vessel system after co-inoculation of different varieties with equal mixtures of two different isogenic *X. fastidiosa* strains with time (and hence by distance from the point of inoculation) by two different strategies.

Experiments were done in Cabernet sauvignon and Roucaneuf grape genotypes using *Xf* wild type strain and mutants. We initiated our investigation by co-inoculating Cabernet Sauvignon stems with a mixture containing an equal amount of the wild-type and a gfp-marked (KLN59.3) *Xf* strain. This was designed specifically so that the temporal and spatial patterns of 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. fastidiosa* 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. In successive experiments we used rpfF-mutant (KLN61) and rpfB-mutant (KLN57). KLN57 has been shown behave much like the wild type. In both cases plants were mostly colonized by the mutants and in less extent by the wild type suggesting that the mutant was moving faster. 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 ongoing experiments. The results of these initial experiments has provided us the needed information on the speed with which these bacterial move through the plant and the rapidity with which segregation is occurring; such results have enabled us to develop more detailed experiments where intensive sampling will provide the needed information on the rapidity with which bottlenecks occur in *X. fastidiosa* populations during the movement process. We also recently co-inoculated the gfp-marked strain with a strain (SC1) that we have recently constructed that harbors a constitutively-expressed cyan fluorescent protein (CFP) driven by a kan promoter and located in the same insertion site in the chromosome as the GFP reporter gene is located in KLN59.3. (Figure 1). These two strains thus should exhibit identical behavior in plants but be differentiated by their different colors of fluorescence emission. On-going studies involve inoculation of this combination of two marked strain into both resistant and tolerance genotypes.

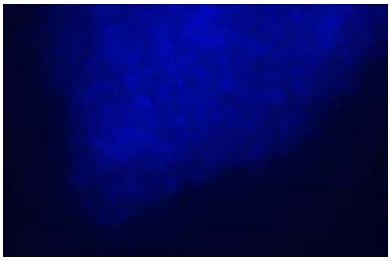


Figure 1. Cyan-marked cells of *X.fastidiosa*

We are also testing in plants mixtures of NS1::Gm and NSI::Cm transposome generated mutants from Dr. Michele Igo's laboratory which have been described as very comparable to wild type behavior regarding movement in planta and PD symptoms and may be very helpful to this study. Work to study the spatial and temporal segregation of the two strains in these various mixtures is in progress.

To compare the spatial segregation between vessels within a resistant grapevine stems, ninety individual Roucaneuf grapevines were stem inoculated as described above, with an equal mixture of X *fastidiosa* strains Temecula and rpfF- mutant KLN61. In Roucaneuf bacterial growth was notably lower in both distance and time compared to susceptible Cabernet. Most importantly the incidence of recovery of viable cells decreased greatly with time (Figure 2). While *X. fastidiosa* could be recovered from a high proportion of plants within the first 5 to 8 weeks after inoculation, the proportion dropped precipitously by weeks 11 and 16. This suggests that cells in such resistant plants are not growing, and are dieing after they colonize a vessel but can not move to new ones.

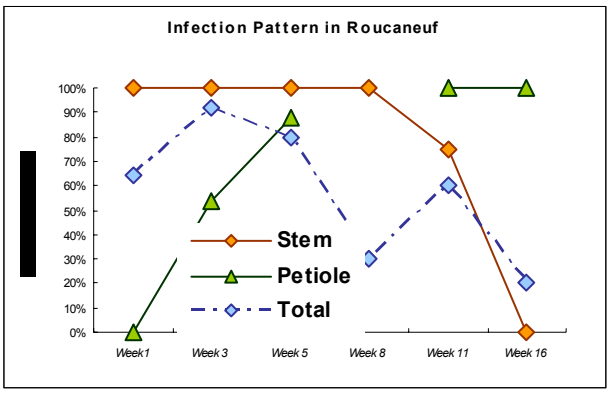


Figure2. Incidence of isolation of X. fastidiosa from various parts of Roucaneuf grape at various times after inoculation showing a reduced recovery from both stems and petioles after 8 weeks. This is in contrast with continued high recovery in Cabernet (data not shown).

To better understand the fate of cells within grapevines, in particular in PD resistant varieties (Figure 2), we investigated differentiate dead and live cells in petioles and stems using

staining techniques. Initially, samples were directly examined using microscopy after being stained for 15 minutes with propidium iodine (PI). This method had some limitations given that some plants tissues, particularly those in the xylem vessels, were highly stained by this reagent, giving high background fluorescence making it hard to quantify bacterial numbers.
We have since been using a new method that permits us to extract cells from the plants parts by sonication. After bacteria cells are extracted from the infected plant parts, we conveniently stain them with Backlight Kit, which stain all live cells green and all dead cells red. The numbers of live and dead cells are directly captured by high magnification epifluorescence microscopy (Figure 3, 4A-4B)

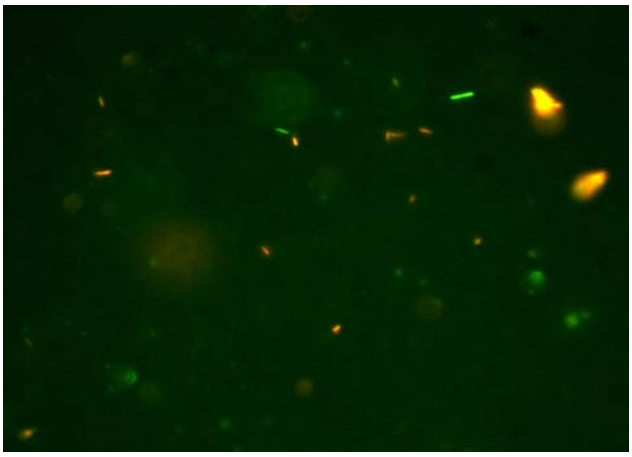


Figure 3: *Xf* Wt cells stained with backlight kiT.
Live cells are green, dead cells are red. (Magnification, 100X)

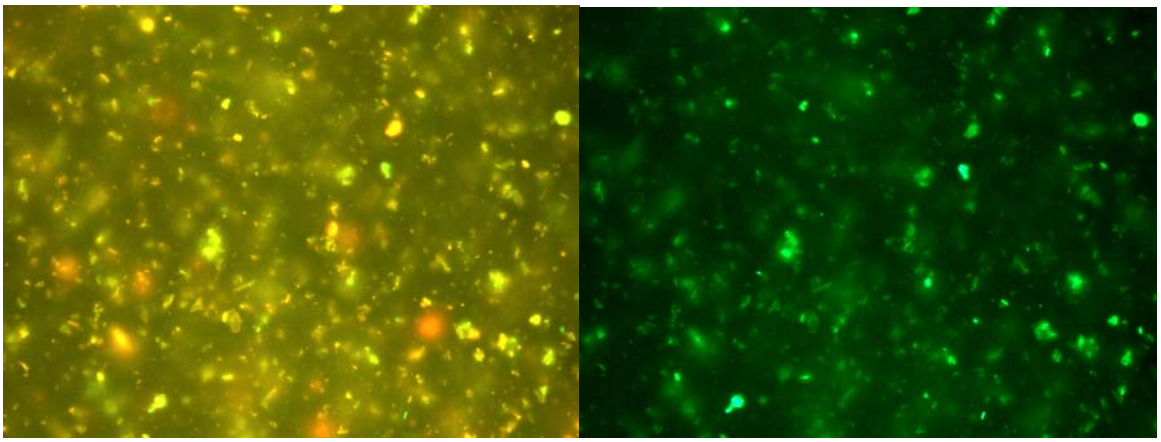
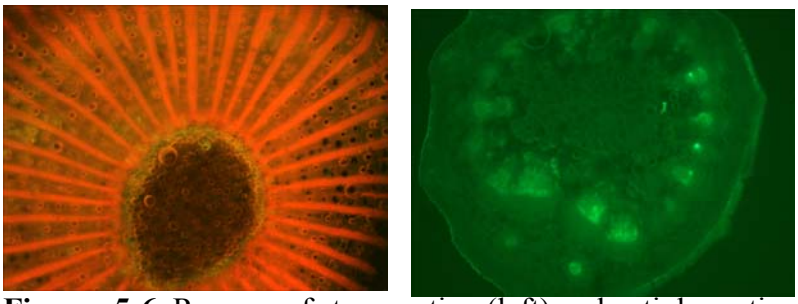


Fig 4;A: FITC filter (Magnification, 63X) **Fig 4; B**: GFP filter.(Magnification, 63X)
Xf Gfp-mutant cells extracted from sick petiole and stained with 10 µl/ml solution PI in glycerol

Objectives 2:
Susceptible Cabernet Sauvignon and resistant varieties including Tampa and Roucaneuf were inoculated with the gfp-marked *X. fastidiosa* strain and 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 (A.F.Krinvanek

at all. 2004). Tampa also is a PD resistant genotype. Microscopy did not reveal any obvious differences in the stem and petiole anatomy of resistant and susceptible varieties **(Figures 5 to 10).** We followed population growth by culturing and also visually by microscopy of numerous cross sections of both stems and petioles. 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 epifluorescence microscopy of numerous sections near the site of culturing. An average of 9 sections was prepared for each stem location and photos were taken from each sample.



Figures 5-6. Roucaneuf stem section (left) and petiole section (right) inoculated with *Xf* Gfp. Week 11 post inoculation at 30 cm from point of inoculation.

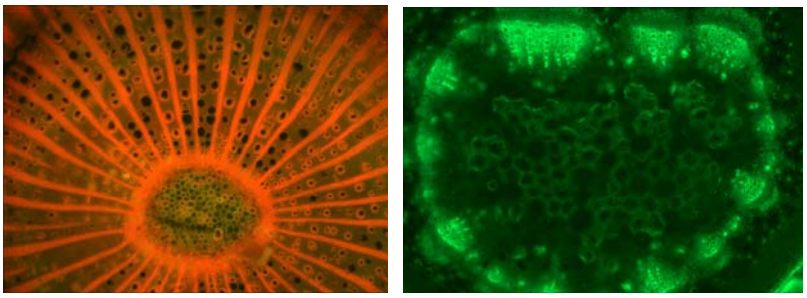
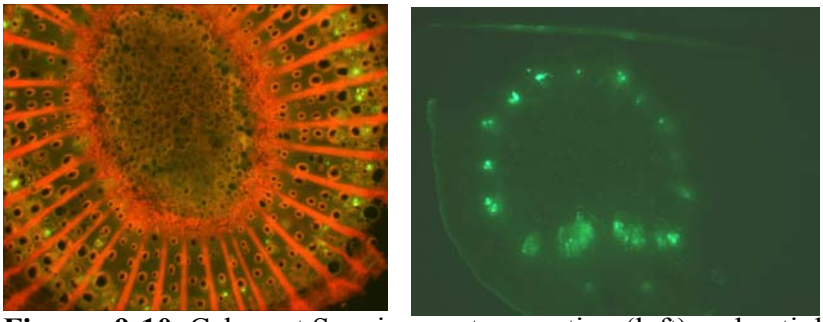


Figure 7-8. Tampa stem section (left) and petiole section (right) inoculated with *Xf* Gfp. Week 11 post inoculation at 30 cm from point of inoculation.



Figures 9-10. Cabernet Sauvignon stem section (left) and petiole section (right) inoculated with *Xf Gfp.* Week 11 post inoculation at 30 cm from point of inoculation.

The proportion of infested vessels in five microscopy stem cross sections per genotype assessed per sampling point for each plant genotype at 6 and 11 weeks post-inoculation, in different internodes locations. The percentage was calculated counting an average of five cross sections at 4 different locations from the point of inoculation. The vessels were counted positive if any presence of gfp-marked cells were noted**.** It was clear that very few 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 (**Figures 5-10**). This correlates well with the higher viable population sizes of *Xf* in Cabernet compared to that of Roucaneuf and Tampa. This procedure allowed us to compare the X. *fastidiosa* populations in each genotype to determine cell viability as a function of time and distance from the point of inoculation (Figure 11). The direct relationship between population size of X. fastidiosa and the proportion of vessels colonized indicates that that the low bacteria population detected in the resistant genotypes is due to a low number of infected vessels that each were colonized to similar levels as in susceptible varieties, rather than poor growth in a many vessels.

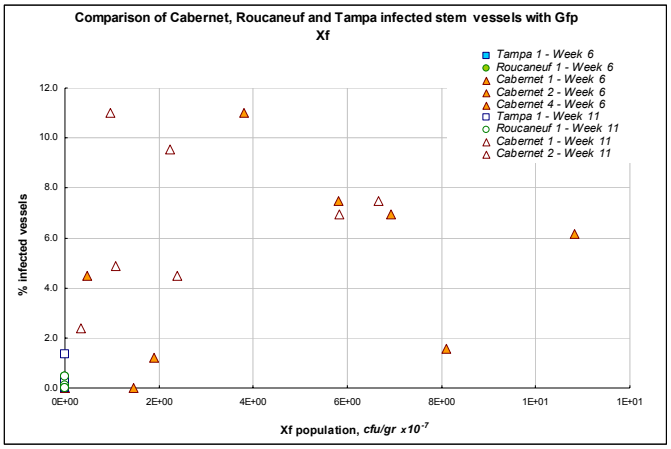


Figure 11. Direct relationship between increasing incidence of xylem colonization and population size in several grape varieties.

It was also clear from visualization of cells of *Xf* in petioles of Cabernet and Roucaneuf and Tampa that petioles of these plants were all equally well colonized by the gfp-tagged cells of *Xf* **(Figures 5-10).** 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 **(Figures 5-10)** It was evident that there was no significant difference in bacteria population between the resistant and susceptible genotypes in the petioles **(Table 1).**

Table 1. X. fastdiosa cells in petioles of different grape varieties (Log cells/g)

Week	Roucaneuf	Tampa	Cabernet Sauvignon
3	7.77	4.86	7.6
4	7.71	5.55	7.43
6	7.18	4.79	6.26
11		5.59	8.46

The proportion of total stem xylem vessels that are colonized by *X. fastidiosa* appears to be similar to that of xylem vessels in the petiole. That is, between 6-11 % of the total stem xylem vessels were colonized whereas 12-15% of the petioles of the same plant were colonized in Cabernet Sauvignon. We also observed that population sizes of *X. fastdiosa* determined by plating, reached higher values in the petioles in the same amount of time after inoculation.

It seems clear that the numbers of *Xf* in stems of resistant varieties such as Roucaneuf are low and apparently spatial variable. Thus, at a given sampling time, not all 1 cm stem segments

include detectable cells of *Xf.* Since *Xf* was frequently detected in petioles, even some distance from the point of inoculation, it appears that *Xf* follows a sinuous path up the vessels in the stem, never colonizing a large number of vessels, but when it enters the petiole it can multiply to high numbers (Table1) . In contrast, the population in the Cabernet Sauvignon remained stable and *Xf* moved much further from the POI, reaching a distance of 120 cm from the inoculation point after 11 weeks compared the 30 cm reached in Roucaneuf and 80 cm in Tampa in the same amount of time.

G. Publications and Presentations of research:

Poster presentation entitled: "Assessment of the process of movement of *Xylella fastidiosa* within susceptible and resistant grape varieties" at the Pierce's Disease Research Symposium. December 12-14, 2007, San Diego, California

H. Research relevance statement:

In an effort to better understand the colonization of grapevines by the pathogen *X. fastidiosa*, and to develop a method of screening for resistant plant genotypes, we are investigating the spatial distribution of cells of *X. fastidiosa* within susceptible and resistant grape varieties and to examine the spatial segregation of mixtures of Xf cells within the xylem vessel systems of different grape varieties. A single *X. fastdiosa* strain or an equal mixture of two different isogenic *X. fastidiosa* strains, were co-inoculated into different varieties and their movement was followed closely by culturing and epifluorescence microscopy, with time and distance from the point of inoculation. As nearly all studies of *X. fastidiosa* colonization of grapes have focused on the petioles, little examination of *X. fasitdiosa* movement and distribution of in the stems. Importantly, the work from the Walker lab has noted that the mechanism of 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. The goal is to determine whether simple measures of spatial segregation of cells of mixed inoculum or of proportions of dead cell in resistant varieties can be used as a rapid screening mechanism for identifying resistant germplasm in breeding programs.

I. Summary:

We followed simultaneously the movement and population size of a gfp-marked strain of *X. fastidiosa* (KLN59.3) in Cabernet Sauvignon, Roucaneuf and Tampa grape varieties. Very low population sizes of *X. fastidiosa* and infrequent occurrence in xylem vessels in the stem were seen in the resistant varieties. The percentage of infected vessels in a stem cross section (as determined by microscopy) and bacterial populations were very strongly and directly related indicating that the low bacteria population detected in the resistant genotypes is due to a low

number of infected vessels that each were colonized to similar levels as in susceptible varieties, rather than poor growth in a many vessels. 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 within the petiole.
These results suggest that resistance to Pierces 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. Resistance to movement thus appears to be due to structural differences in the vessels of the resistant varieties and is associated with a limitation of the number of vessels into which *X. fastidiosa* can spread and thus in which they can grow. In addition to the apparent lower mobility of *X. fastidiosa* in resistant plants, we are evaluating whether its restricted movement in resistant grape genotypes is due to reduced cell viability within the plant, as compared to the non-resistant genotypes. An increasing proportion of cells of *X. fastidiosa* are dead as symptom development increases in plants, and we will determine if cells die more rapidly in resistant than susceptible plant varieties.

J. Summary and status of intellectual property produced during this research

None produced in this period.