

ASSESSMENT OF THE PROCESS OF MOVEMENT OF *XYLELLA FASTIDIOSA* WITHIN SUSCEPTIBLE AND RESISTANT GRAPE VARIETIES

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ABSTRACT

In an effort to better understand the colonization of grapevines by the pathogen *Xylella fastidiosa* (*Xf*), and to develop a method of screening for resistant plant genotypes, we are investigating the spatial distribution of cells of *Xf* 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 *Xf* strain or an equal mixture of two different isogenic *Xf* 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. We followed simultaneously the movement and population size of a gfp-marked strain of *Xf* (KLN59.3) in Cabernet Sauvignon, Roucaneuf and Tampa grape varieties. Very low population sizes of *Xf* 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 *Xf* is unrestricted in movement within the petiole. These results suggest 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 these two 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 *Xf* can spread and thus grow. We have produced a cyan-marked strain of *Xf* that is being used in mixed inoculation studies with the gfp-marked strain to better define the process of movement through the plant.

INTRODUCTION

Nearly all studies of *Xylella fastidiosa* (*Xf*) colonization of grapes have focused on the petioles, with little examination of *Xf* movement and distribution of in the stems. Importantly, the work from the Walker lab has noted that the mechanism of resistance to *Xf* 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.

Before initiating studies of the segregation of differentially marked strains of *Xf* 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 strain mixtures. We set out to determine how quickly *Xf* 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 *Xf* in xylem vessels as compared to tracheal elements. We specifically considered the longitudinal movement of *Xf* in the xylem vessels in the internodal stem locations and the rate at which segregation of the two strains occurs. A more detailed examination of the movement of *Xf*, using epifluorescence microscopy, was performed on stem sections at various locations and times after inoculation with *Xf* strains harboring gfp marker genes.

We followed simultaneously the movement and population size of a gfp-marked strain of *Xf* (KLN59.3) in Cabernet Sauvignon, Roucaneuf and Tampa grape varieties. Plants were inoculated with the gfp-marked strain to directly compare the movement and growth of *Xf* in resistant and susceptible grape genotypes. We then correlated the percentage of infected vessels in a stem cross section (as determined by microscopy) and bacterial populations. A strong correlation would indicate that the low bacteria population detected in the resistant genotypes is due to a low number of infected vessels.

OBJECTIVES

1. Study the process of movement of *Xf* 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 *Xf* 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 *Xf*.

RESULTS

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 *Xf* 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 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 *Xf* 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.

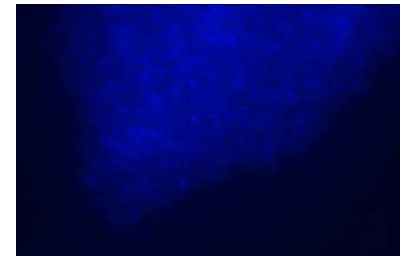
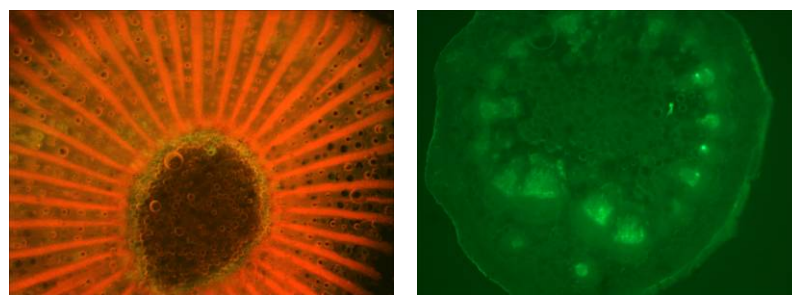


Figure 1. cyan-marked cells of *Xf*

Susceptible Cabernet Sauvignon and resistant varieties including Tampa and Roucaneuf were inoculated with the gfp-marked *Xf* strain and examined by sequential culturing and epifluorescence microscopy. Roucaneuf is a complex hybrid that includes *V. cinerea* 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 2 to 7). 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 six 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 six 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 nine sections was prepared for each stem location and photos were taken from each sample.



Figures 2-3. Roucaneuf stem section (left) and petiole section (right) inoculated with *Xf*Gfp. Week 11 post inoculation at 30 cm from point of inoculation.

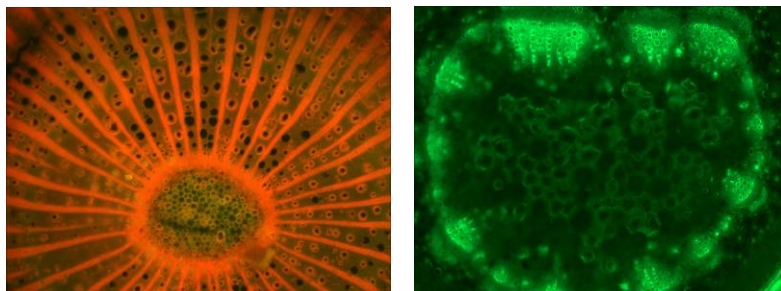
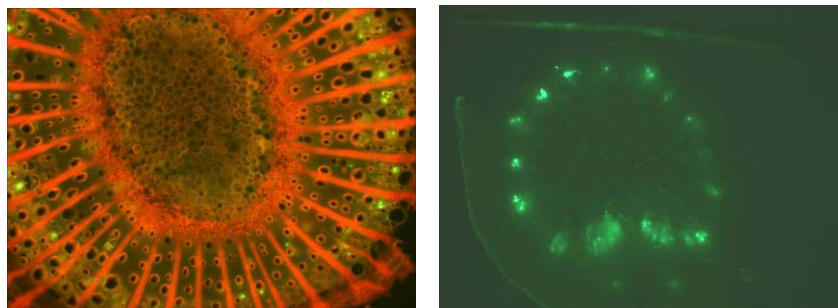


Figure 4-5. Tampa stem section (left) and petiole section (right) inoculated with *Xf* Gfp. Week 11 post inoculation at 30 cm from point of inoculation.



Figures 6-7. 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 four 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 2-7). 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 *Xf* populations in each genotype to determine cell viability as a function of time and distance from the point of inoculation (Figure 8). The direct relationship between population size of *Xf* and the proportion of vessels colonized indicates 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 many vessels.

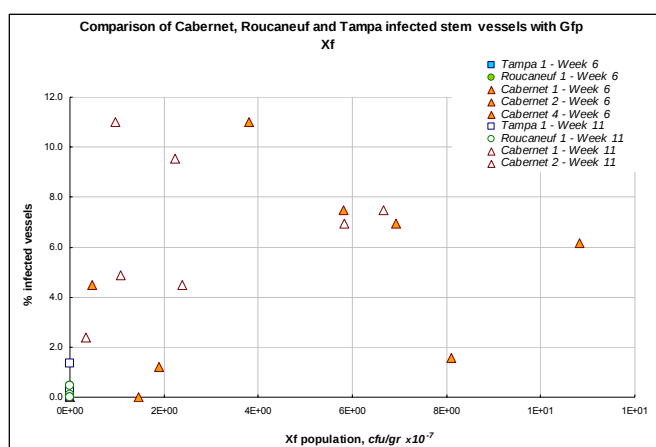


Figure 8. Direct relationship between increasing incidence of xylem colonization and population size in several grape varieties.

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 2 to 7). 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 2 to 7). 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. *Xf* cells in petioles of different grape varieties (Log cells/g)

Week	Roucanneuf	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 *Xf* 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 *Xf* 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 Roucanneuf are low and apparently spatial variable. Thus, at a given sampling time, not all one 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 (Table 1). 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 Roucanneuf and 80 cm in Tampa in the same amount of time.

To compare the spatial segregation between vessels within the grapevine stems ninety individual Roucanneuf grapevines were stem inoculated as described above, with an equal mixture of *Xf* strains Temecula and *rpfF*- mutant KLN61. In Roucanneuf 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 9). While *Xf* 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 dying after they colonize a vessel but can not move to new ones. This is being investigated using propidium iodide staining to visualize dead cells.

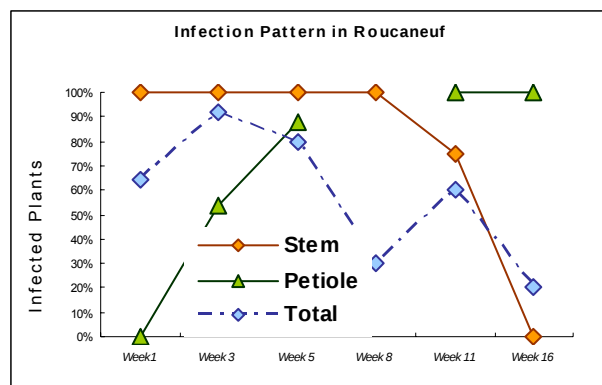


Figure 9. Incidence of isolation of *Xf* from various parts of Roucanneuf grape at various times after inoculation showing a reduced recovery from both stems and petioles after 8 weeks. This is in contrast with continued high recovery in Cabernet (data not shown).

CONCLUSIONS

These results all suggest that structural differences in the stems of grape account for resistance to Pierce's disease. These results suggest 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 these two 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 *Xf* can spread and thus in which they can grow.

FUNDING AGENCIES

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