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 green fluorescent protein (gfp)-marked strain of *Xylella fastidiosa (Xf),* 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 (PD). Very low populations of *Xf* 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 *Xf* is unrestricted in movement and growth within the petiole. These results indicate that resistance to PD 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.

Work is continuing using mixtures of isogenic strains of *Xf* 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 PD 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 *Xf* colonization of grapes have focused on the petioles, with little examination of *Xf* movement and distribution in the stems has been made. 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.

In an effort to better understand the process of colonization of grapevines by *Xf*, 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 *Xf* 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 *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 *Xf* 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.

OBJECTIVES

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

Objective 1:

We initiated our investigation by co-inoculating Cabernet Sauvignon stems with a mixture containing an equal amount 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 *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 cyan fluorescent protein (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 *Vitis*. 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 Xf strain. We followed population growth by culturing and also visually by microscopy (**Figure 1**). Culture sampling was done at weeks two, three, four, six, 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 epifruorescence microscopy. Petioles and portions of the stems were sectioned and prepared for microscopy. An average of nine sections was prepared for each stem location and photos were taken from each sample.

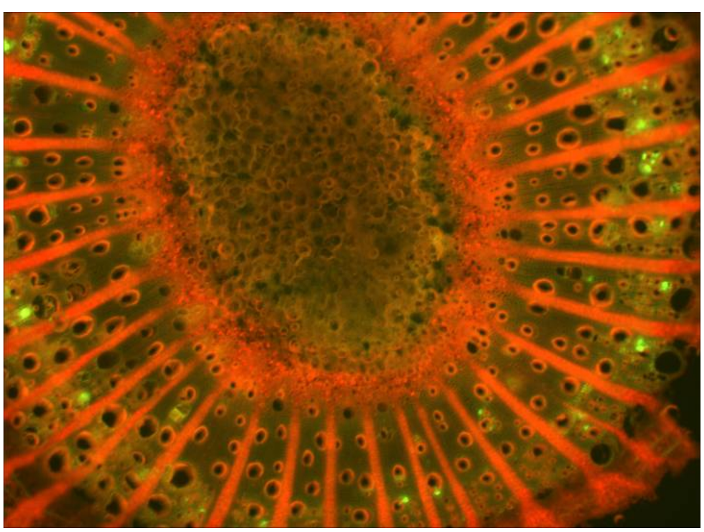


Figure 1: Visualization of colonization of Cabernet Sauvignon stems wth a gfp-marked strain of *X. fastidiosa*. The plant was sectioned 11 weeks after inoculation and this section was taken from the stem at 30 cm from the point of inoculation. This image is typical of stem tissue from susceptible grape 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 (**Figure 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*.

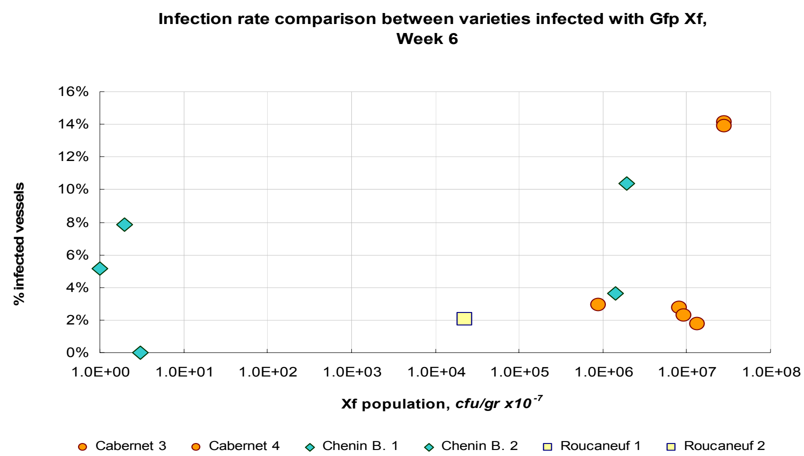
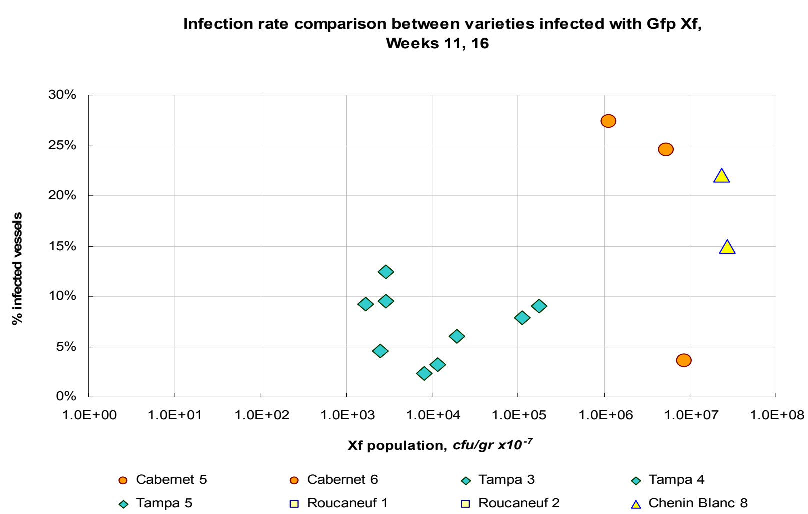


Figure 2: Relationship between incidence of colonization of stem vessels of different grape varieties by *Xf* as determined by a microscopic detection of gfp-tagged *Xf* strain (Y-axis) and the population size of *Xf* determined by culturing of small samples of tissue near the site of examination (X-axis).



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 bacteria population between the resistant and susceptible genotypes in the petioles (**Table 1**) which is consistent with the work of the Walker lab**.** In addition, The proportion of the total stem xylem vessels that are colonized by *Xf* 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.

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

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

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 which 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 plant 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 PD; 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 extent 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 statistical divergent from Cabernet and Chenin Blanc varieties, having lower colonized vessels.

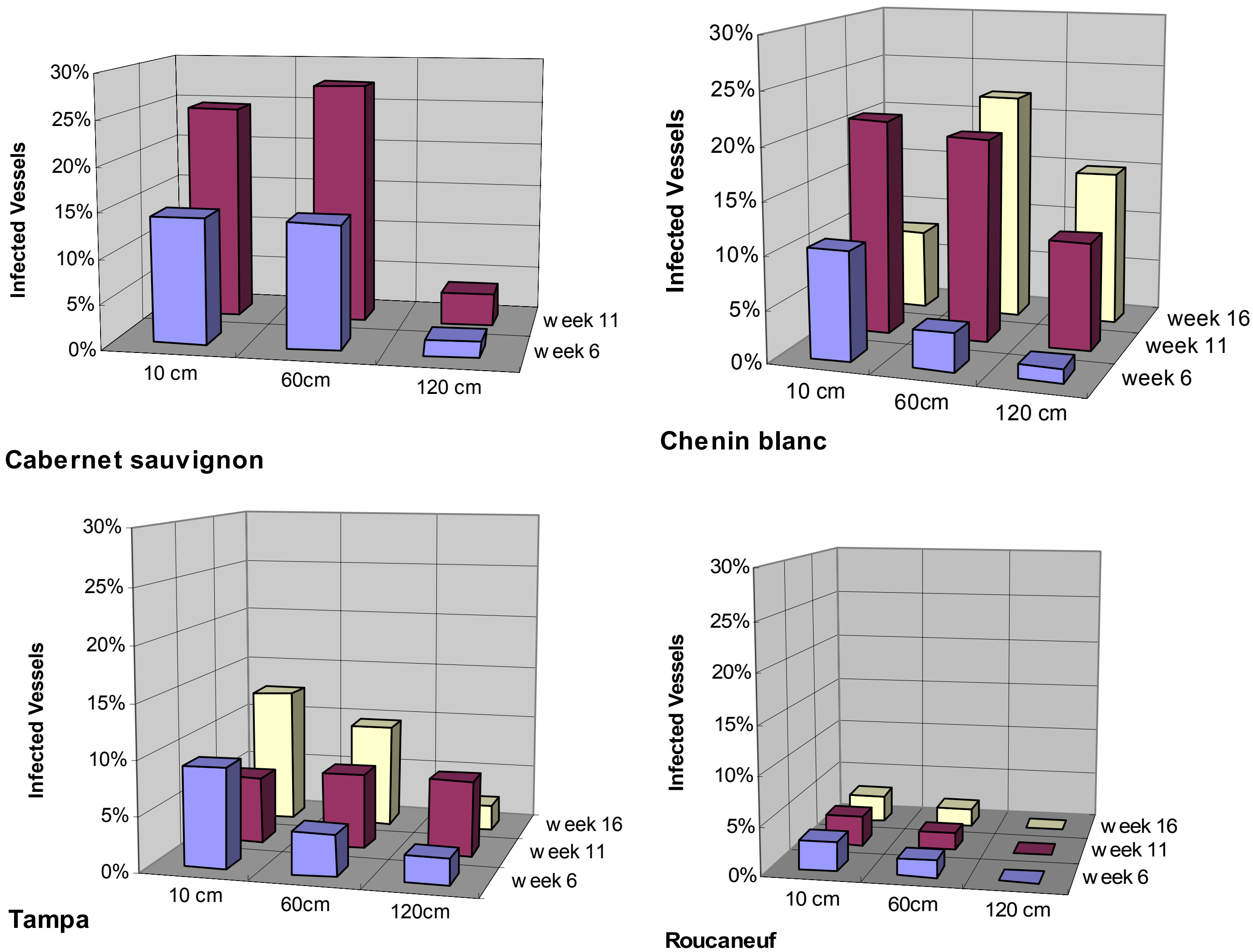


Figure 3. Percentages of infected vessels determined by microscopy (12 stem cross sections each of 28 μm thickness examined per location) sampled at different times and distances from the point of inoculation for four grape varieties.

Table 2: 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.

Table 2:*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

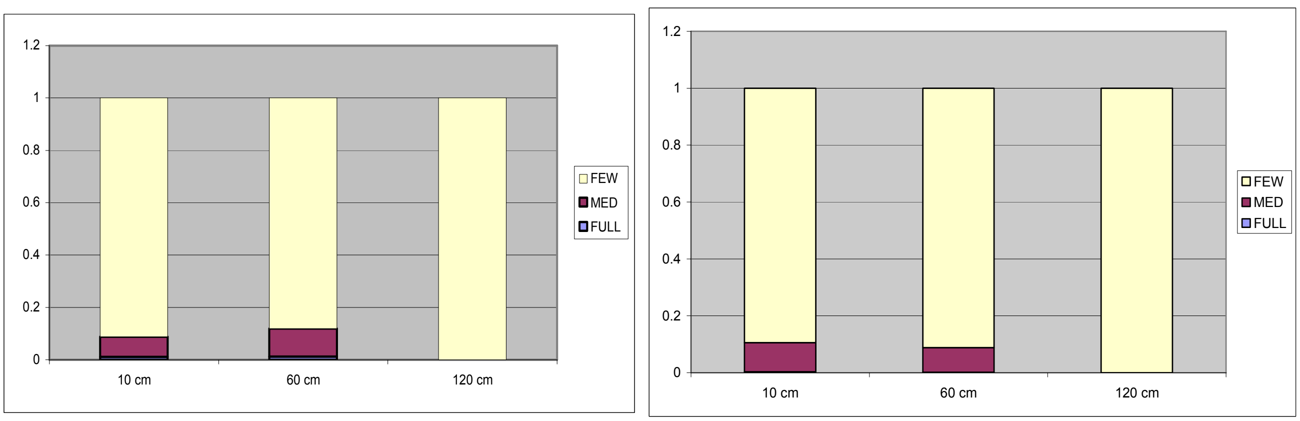


Figure 4. Proportion of colonized vessels having different extents of colonization by *Xf* in Cabernet (left) and Chenin Blanc (right).

Table 3: Differences in extent of colonization of stem xylem vessels in different grape varieties determined by microscopic detection of a gfp-marked strain 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.

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Medium vessels colonization

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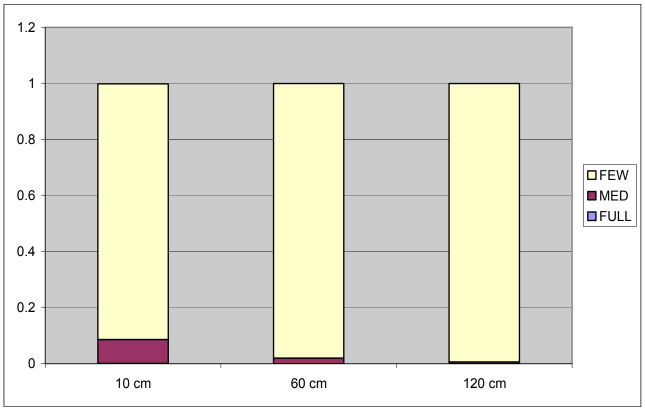
Tampa	3.4a	0.41a	0a
Chenin Blanc	3.2a	1.41a	0a
Roucaneuf	0c	0a	0a

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



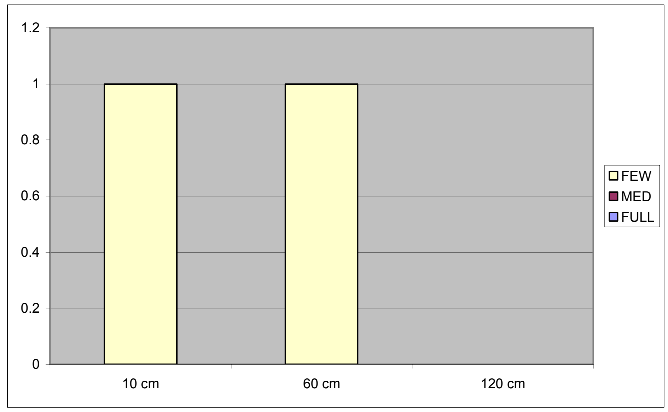


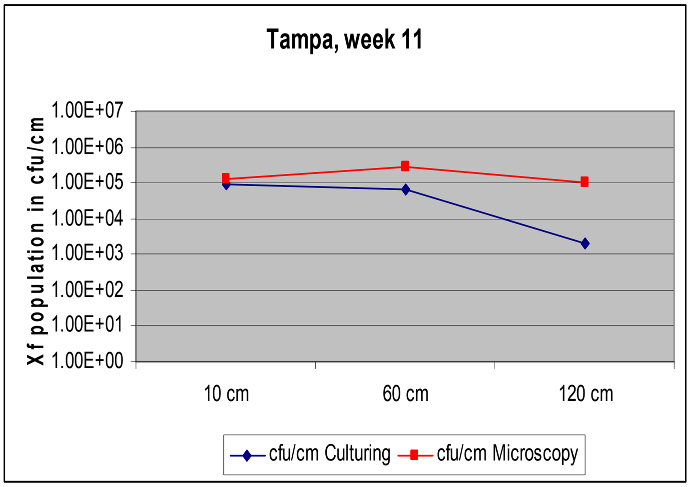
Figure 5. Proportion of colonized vessels having different extents of colonization in Tampa (left) and Roucaneuf (right).

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 strain 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).

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

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 *X*f 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 point of infection (POI), the total populations 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**). At the most distal sites from the point of inoculation, the numbers of *Xf* estimated by microscopy were somewhat lower than the culturable populations; we believe this is due to sampling issues since the relatively few vessels that were colonized by *Xf* at such distances (**Table 3**) made accurate estimates of incidence and extent of colonization difficult and subject to underestimation. Given that the numbers of *Xf* in stems of resistant varieties such as Roucaneuf are low and apparently spatial variable, at a given sampling time, not all visualized stem segments (28 um/section x 12sections/sample) include detectable cells of *Xf*.



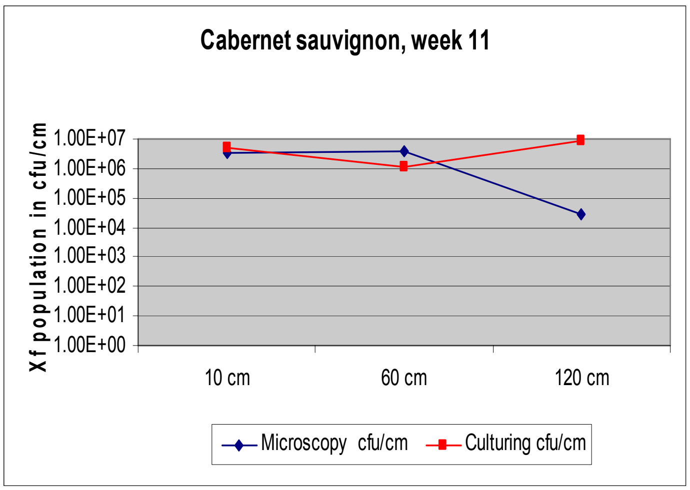


Figure 6. Comparison of population sizes of *Xf* in in Tampa and Cabernet sauvignon stem segments at different distances from the point of inoculation estimated by culturing (blue) and by microscopy (red) at week 11 post-infection.

CONCLUSIONS

Resistance to movement of *Xf* in different grape varieties appears to be restricted to the stem tissue and is 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. It is apparent that the relatively high populations in susceptible genotype stems are achieved because of higher numbers of infected vessels and also due to more extensive colonization of the vessels into which it moves. Since *Xf* was frequently detected in petioles, even in resistant varieties and at 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. The similar populations, estimated by microscopy or plating suggest that most cells in the stem appear to be alive. This suggests that in resistant genotypes in-stem tissue movement and multiplication are impaired as separate or co-dependent phenomena, which doesn't seem to be the case in petioles. Presumably the process of movement of *Xf* from one infected vessel to other adjacent vessels involves the degradation of pit membrane. This degraded plant material is apparently a source of considerable nutrition to *Xf*. That is, those grape varieties that are most easily digested by *Xf* will be both more easily invaded and support more extensive multiplication by *Xf*.

FUNDING AGENCIES

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