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

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Abstract:
To better understand the processes contributing to disease and resistance to Pierce's disease of grape we examined the movement and multiplication of a gfp-marked strain of *Xylella fastidiosa* in the stems and petioles of Cabernet Sauvignon, Chenin Blanc, Roucaneuf and Tampa grape varieties which differ in their susceptibility to this disease. *X. fastidiosa* achieved much lower population sizes colonized fewer xylem vessels in the stem of resistant varieties compared to more susceptible varieties. In contrast, *X. fastidiosa* achieved similarly high population sizes and colonized a similar proportion of the vessels in petioles of susceptible and resistant varieties, suggesting that compared to the stem, *X. fastidiosa* is relatively unrestricted in its movement and growth within the petiole. There was not a direct relationship between the population size of *X. fastdiosa* in the stem and the proportion of vessels colonized; a much higher population size of the pathogen was observed in susceptible varieties than expected based on the proportion of vessels colonized. The high populations of *X. fastidiosa* in susceptible genotype stems were associated with both a high number of infected vessels and a much higher extent of colonization of those vessels that become infested than more resistant varieties. This suggests that the movement and multiplication of *X. fastidiosa* in the stem of grape are co-dependent phenomena. Given that intervessel movement of *X. fastidiosa* and its multiplication within a vessel are apparently linked it appears that the pathogen gains some growth benefit by the process of movement between vessels, presumably due to consumption of the pit membrane. The formation of large cellular aggregates in vessels is not required for *X. fastidiosa* to move laterally in the stem to adjacent vessels as most vessels harbored only small aggregates, especially in resistant varieties such as Roucaneuf in which some inter-vessel movement was detected. Resistance to Pierce's disease is apparently not due to inhibitory compounds that circulate through the xylem since they might be expected to operate similarly in all tissues.

Introduction:
Pierce's disease (PD), a chronic problem in the grape industry in California, is now a potentially more devastating disease due to the introduction of the glassy-winged sharpshooter, a more effective vector of the pathogen *X. fastidiosa* than native vectors. The management of this disease is difficult since the vascular colonization of grapevines by the pathogen limits its accessibility to bactericides. Many agriculturally important plants besides grape such as citrus, almond, alfalfa, coffee, as well as many ornamental plants are susceptible to diseases caused by *X. fastidiosa* (14,21).

X. fastidiosa is transmitted to new host plants during xylem sap feeding by sharpshooter vectors and then multiplies and spreads from the site of inoculation to colonize the xylem, a water transport network of vessels composed of dead, lignified cells. Vessels are interconnected by bordered pits, that allow the passage of xylem sap but block passage of larger objects due to the presence of a pit membrane (6,7,26). Cells of the pathogen multiply and attach to the vessel walls, forming biofilm-like colonies that can, when sufficiently large, occlude xylem vessels, blocking water transport (2,10,16,21,27). In susceptible plants, leaf scorching, fruit shriveling and other symptoms result with time, likely due to water stress due to xylem blockage. A much higher proportion of vessels in symptomatic tissues are colonized by *X. fastidiosa* than in non-symptomatic tissues suggesting that the disease is a progressive one associated with increasing levels of colonization of vessels (16). However, the colonization of many plants by *X. fastidiosa* is relatively restricted compared to susceptible crops such as grape, and they do not exhibit signs of disease (10,11). The population size of *X. fastidiosa* in grapevines resistant and susceptible to Pierce's disease is highly correlated with symptom expression (2,10,11,13,18). However we still lack an understanding of the process of colonization and how high populations of *X. fastidiosa* lead to symptom expression.

In order for water movement in a grape stem to be sufficiently restricted that disease develops, a large percentage of the xylem pathways must be blocked. While over 40% of the xylem vessels in a given cross-section of an infected grape stem may be infested with *X. fastidiosa* (16,27) this alone is unlikely to explain water stress. Sequential sections of grape tissue however, demonstrated that different xylem vessels are blocked in different cross-sections; the cumulative percentage of occluded vessels in one of several sections along 5 mm of a petiole was 5 times that of a single cross section (27). This suggests that the likelihood of blockage of flow through a xylem vessel will increase non-linearly with the proportion of occluded vessels as secondary and tertiary paths for water movement are blocked. While tyloses have been noted in infected grape it has been suggested that they do not limit the spread of *X. fastidiosa* and account for resistance in *V. arizonica, V. Samllina.v. rufotomentosa.*(9). Given that inoculation of grape with *X. fastidiosa* must occur at a relatively few sites on a vine, it is clear that the pathogen has the ability to move extensively both axially and radially in xylem tissues. Such movement is presumably sequential, and must take some time, explaining why the disease is "progressive" and appears only several weeks after inoculation.

While dispersion of *X. fastidiosa* through the plant apparently follows the natural course of the xylem vessels the pathogen requires a mechanism for breaching vessels connected by bordered pits since pit membranes do not readily allow passage of objects 20 nm or larger (6). Considerable evidence suggests that *X. fastidiosa* degrades pit membranes to traverse bordered pits. *X. fastidiosa* has been observed on pit membranes or near pits with breached membranes that presumably allowed bacterial movement from vessel to another. (9). *X. fastidiosa* expresses genes predicted to encode pit membrane degrading enzymes such as endoglucanases and polygalacturonases *in vitro* (1). Furthermore, a mutant blocked in production of polygalacturase was unable to move within grape and thus was avirulent (22). In addition, transgenic grapes expressing a pear polygalcturonase inhibiting protein (PGIP) exhibited more resistance to *X. fastidiosa* than did untransformed plants (1). While the progressive colonization of xylem vessels is associated with disease, the nature of this process in resistant plants compared to more susceptible plants is poorly understood.

Nearly all studies of *X. fastidiosa* colonization of grapes have focused on the petioles, with little examination of *X. fastidiosa* movement and distribution in the stems have been made. 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 (18). They speculate that a resistance mechanism based on nutritional or structural differences between resistant and susceptible types restricts the growth and movement of the pathogen in the stem xylem. Given that in resistant grapes varieties, as well as other plant species, *X. fastidiosa* can exhibit systemic infection but with relatively low populations (18) the process of movement in plants presumably is a quantitatively highly variable one. Our study was designed to examine differences in the colonization process of the stem of different grape genotypes to identify resistance mechanisms. We have exploited the ability to rapidly and quantitatively assess the presence of a gfp-marked strain of *X. fastidiosa* by fluorescence microscopy so that the process of multiplication of movement of the pathogen in plants differing in resistance could be related to each other.

Materials and Methods:

Bacterial strains and culture conditions. A gfp-marked strain of *X. fastidiosa* strain Temecula (12) was recovered from previously infected petioles of Cabernet sauvignon plants. Petioles were surface sterilized and macerated and appropriate dilutions of the macerate were plated on solidified PWG media supplemented with kanamycin (30ug/ml) as previously described (16). Cells of *X. fastidiosa* were harvest by scraping from PWG plates containing kanamycin suspended in SCB buffer and diluted to a concentration of 10^8 cells/ml in SCB buffer to yield inoculum for plant inoculation.

Plant Inoculations. Greenhouse grown *Vitis vinifera* Cabernet sauvignon and Chenin Blanc grapevines (susceptible varieties) and Roucaneuf and Tampa (resistant varieties) (18) were mechanically inoculated by needle puncture using standard procedures . Roucaneuf is a complex hybrid that includes Vitis c*inerea* and *Vitis berlandieri* and has been described as "fully-resistant" to Pierce's disease under field conditions. Tampa also is described as Pierce's disease resistant genotype. A total of 12 plants of each genotype were inoculated to enable 4 to be sampled at each of 4 times after inoculation. Self-rooted plants derived from segments of dormant vines or from green cuttings were stem inoculate with a 5ul drops of bacterial cell suspension (10^8 cells/ml) when they were about 40 cm tall and kept in a greenhouse with daytime temperatures of about 28 C. The point of inoculation on the stem was marked for further reference. Plant stems from two of each genotype were sectioned at the point of inoculation as well as at 30, 60 and 120 cm away from the point of inoculation at 6, 11, and 16 weeks after inoculation. In addition, petioles at these same distances from the point of inoculation were sampled at these same times. Stem and petiole samples each consisted of a segment 1 cm in length that was weighed and surface sterilized. *X. fastidiosa* populations were estimated by culturing as described above and as in (21)

Microscopy of plant tissues. Petiole and stem segments from plants treated as above were sectioned and prepared for microscopy by methods similar to that used by Newman et al. (16). Individual hand-sectioned stem or petiole cross sections were mounted on a

microscope slide and immersed in a 50% glycerol solution. Microscopic evaluation was done with 5X and 10 X magnifications on an AxioImager373 Epifluorescence microscope. GFP fluorescence was captured using a 505 to550-nm band pass filter for petiole cross section imagines while stem cross sections were imaged using a filter optimized for use with fluorescence isothiocyanate Deltavision FITC using a 490 to 528-nm band. Twelve sequential cross sections were prepared from tissues immediately adjacent to each of the stem or petiole segments in which *X. fastidiosa* populations were determined from dilution plating of tissue macerates. The total number of vessels in a given cross section as well as the number of infested vessels was counted. An average of about 300-400 vessels were present in each stem cross section and about 150-200 vessels were present in a typical petiole. The number of colonized vessels were averaged for each sampling location and grape genotype and normalized for the average number of total vessels per section. Multiple comparisons among treatment effects were made using a least significant difference (LSD) test. All analyses were performed using Statistica (Statsoft Inc., Tulsa, OK).

The extent of colonization of individual stem xylem vessels was also assessed by similar methods. Hand-sectioned cross sections were examined as above but at a magnification of
20 X and 40 X to enable better estimation of the number of cells present in each vessel. We distinguished between those having high levels of colonization (which we estimated to be about 100,000 cells/ vessel, those having moderate levels of colonization (about 1000 cells/vessel) or those having low colonization (less than 10 cells/vessel). About 300-400 vessels were present in each stem section of each genotype; a total of about 10,000 vessels were examined for each genotype. Statistical analysis of the data was as described above.

Results:

Colonization of petioles and stems of resistant and susceptible grape by *X. fastidiosa*.

Colonization of susceptible Cabernet Sauvignon and Chenin Blanc as well as the resistant genotypes Tampa and Roucaneuf by a gfp-marked *X. fastidiosa* strain were examined by paired culturing and epifuorescence microscopy at various times and distances from the point of inoculation so that the processes of growth and movement in the plants could be separately assessed. There were no obvious differences in over-all anatomy of the stem and petiole tissues of the resistant and susceptible varieties that were apparent by fluorescence microscopy**.** The varieties differed, however in the number and timing of appearance of tyloses in the stem tissue. While Tampa and Roucaneuf harbored abundant tyloses by as soon as 6 weeks after inoculation, the susceptible varieties produced tyloses only later (after 11 to 16 weeks after inoculation). The incidence of tyloses was greatest in stems near the point of inoculation but at least some were seen through the length of the stem tissue examined. Tyloses were more commonly seen in stem tissues than in petioles, especially in resistant grape varieties.

The incidence of infestation of stem xylem vessels by *X. fastidiosa* was directly related 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). A very low proportion of the stem vessels at sites away from the point of inoculation of the most highly resistance variety, Roucaneuf, were colonized by any cells of *X. fastidiosa* compared to that of Cabernet and Chenin Blanc (Figure 1 and 2) while Tampa exhibited an intermediate level of colonization

(Figure 2). It was apparent that near the point of inoculation the proportion of vessels that harbored any number of cells of *X. fastidiosa* was higher than at more distal sites. With increasing time since inoculation the number of vessels colonized also increased in the susceptible varieties; this effect was less pronounced in the more resistant varieties since the over-all incidence of infestation was much lower. The reduced proportion of colonized vessels in resistant genotypes, particularly at distal sites presumably reflects that reduced inter-vessel movement was possible in the resistant varieties.

In contrast to the stem tissue, petioles of all four varieties were equally well colonized by the gfp-tagged cells of *X. fastidiosa*, and the incidence of colonization was substantially higher than that of the stem tissues at a given distance form the point of inoculation (Figure 1). There was no significant difference in population of *X. fastidiosa* detected by culturing between the resistant and susceptible genotypes in the petioles. Similar results were found by Walker et al. 2004 and Hopkins 1980**.** The proportion of the total stem xylem vessels that are colonized by *X. fastidiosa* appears to be less than that of the xylem vessels in the petiole for a given variety. Thus the petioles seem to offer less resistant to movement and or multiplication of *X. fastidiosa* compared to stem tissue.

The numbers of cells in vessels of different grape varieties determined by culturing small stem segments were non-linearly related to the proportion of vessels colonized as determined by microscopic detection of a gfp-marked *X. fastidiosa*. While the population size of *X. fastidiosa* detected in the stems of Cabernet and Chenin Blanc was as much as 100-fold greater than that in Roucaneuf and Tampa only about twice as many vessels were colonized (Figure 3). Thus, a much higher population size of *X. fastidiosa* was observed in susceptible varieties than expected based on the relative number of infested vessels in a stem. This suggested that there may be substantial differences in the average population size of *X. fastidiosa* in a given infested vessel in different grape varieties.

To address whether *X. fastidiosa* not only moves into more vessels of susceptible grape varieties than resistant varieties but also multiplies more extensively in those vessels into it moves, we estimated the extent of colonization of each vessel that was infested with a gfp-marked strain. We distinguished between those stem vessels having high levels of colonization, which we estimated to be about 100,000 cells/vessel, from those having moderate levels of colonization (about 1000 cells/vessel) or those having minor colonization (10 cells or less/vessel). The colonization of each vessel in a given cross section was assessed by fluorescence microscopy for each variety at several different times and distances from the point of inoculation. A large variation in the extent of colonization of individual vessels in a given section was observed for all varieties. In all varieties the large majority of vessels harbored relatively few cells of *X. fastidiosa* (Figure 4). On average, for all varieties about 10-fold more vessels harbored at least a few cells of *X. fastidiosa* than those that harbored moderate numbers of cells. Vessels that harbored high numbers of *X. fastidiosa* cells were only observed in the most susceptible variety, Cabernet sauvignon (Figure 4). Likewise, the more susceptible varieties Cabernet sauvignon and Chenin Blanc both had much higher proportions of vessels that harbored intermediate numbers of cells of *X. fastidiosa* than the more resistant varieties (Figure 4). These differences in extent of colonization were highly statistically different between varieties in most cases (Table 2). For all varieties the proportion of vessels that harbored even low numbers of cells of *X. fastidiosa* decreased with distance from the point of inoculation. While at least some

vessels having at least a few cells per vessel were observed in samples as far as 120 cm from the point of inoculation, no vessels harbored moderate cell numbers at this sampling distance (Table 2). While the ratio of the number of vessels harboring small numbers of cells to that of vessels harboring moderate numbers of cells at a sampling distance of 60 cm was only 9.2 for the highly susceptible Cabernet sauvignon variety this ratio was 32.4 for Tampa; no vessels in Roucaneuf harbored moderate numbers of cells of *X. fastidiosa* (Table 2). Thus both the numbers of vessels colonized by *X. fastidiosa* as well as the number of cells in a given colonized vessel are generally lower in resistant grape varieties than more susceptible ones.

The recovery of lower number of culturable cells than expected in stems of resistant varieties which had similar numbers of colonized vessels as susceptible varieties could be due to a lower cell viability in the resistant plants. To test this possibility we estimated the total number of cells of the gfp-marked strain of *X. fastidiosa* microscopically based on the assumption that gfp fluorescence was persistent even in cells which had died. Given that we had made independent measures of both the incidence and extent of colonization of stem xylem vessels by *X. fastidiosa* by microscopy as well as direct measures of viable population sizes of *X. fastidiosa* by culturing of the adjacent tissue we tested the model that cells of *X. fastidiosa* had similar frequencies of viability in different grape varieties. We estimated population sizes in stems from microscopy measurements by multiplying the number of colonized vessels by the number of cells observed in a given vessel and with knowledge of the amount of plant material that had been examined by observing cross sections (28 um/section x 12 cross sections per sample). In sampling locations more proximal to the point of inoculation 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 5). At a distance of 120 cm from the point of inoculation the numbers of cells of *X. fastidiosa* estimated by microscopy were somewhat higher than the culturable populations in resistant varieties. This is likely due to sampling issues since relatively few vessels were colonized by *X. fastidiosa* at such distances (Table 3), making accurate estimates of incidence and extent of colonization difficult and subject to stochastic variation due to sampling success. It seems likely that the underestimation of total populations in Cabernet sauvignon and over-estimation of total populations in Tampa at 120 cm relative to that estimated by culturing were both due to stochasticity in sampling of such infrequent colonization events at this distal sites from the point of inoculation.

Discussion:

While differences between the internal structure of stems and petioles of grape have been previously described (10,12,14,24) it remains unclear why petioles appear to be preferred sites of development of large population sizes of *X. fastidiosa*. Stems have relatively few but large diameter xylem vessels and many smaller tracheary elements. Petiole vessels are organized in clusters that are shorter and narrower that those in the stem. Leaves and petioles contain more narrow and terminal tracheary elements and shorter conduits (24). Petioles and leaf traces also have a higher proportion of parenchyma and tracheary elements. In contrast, vessel elements in the stem internodes had scalariform lateral-wall pits and simple perforation plates. Vessel elements with helical secondary walls were predominant in leaf traces and petioles (25). The different structure of these pits in stem vessels may also be indicative of a differential susceptibility to degradation of *X. fastidiosa* that could account

for the lower incidence of colonization of stem vessels compared to those in the petiole (Figure 1). Similarly high population sizes of *X. fastidiosa* were seen in the petioles of susceptible Cabernet sauvignon and Chenin blanc varieties, as well as resistant Tampa and Roucaneuf varieties (Table 1). Similar results were obtained by Fry et al. (10), who noted comparable *X. fastidiosa* populations in petioles of *Vitis Vinifera* French Colombard (susceptible) *Vitis rotundilofia* Carlos (tolerant) and Noble grape (resistant) varieties. In their studies grape petioles were inoculated and subsequently sampled at different times after inoculation while in our experiment, stems were inoculated and the petioles were sampled at various distances from the point of inoculation, requiring *X. fastidiosa* to disperse to the distal petioles. Walker et al. also found no significant differences in *X. fastidiosa* populations in petioles and leaf blades by an ELISA method among resistant and susceptible varieties following stem inoculation (18). While Hopkins et al. (13) examined the incidence of infected vessels seasonally in a single variety moreover, he found a lower percentage of infected vessels in the stems as compared to petioles, which is consistent with our findings. It seems likely that these later "natural" infestations of the petiole were initiated with fewer cells and most likely involving fewer vessels than direct needle inoculation. Despite the differences in inoculation method, *X. fastidiosa* attained high population densities in petioles irrespective of the degree of whole- plant resistance to Pierce's disease, suggesting that resistance was not operative in leaves. Resistance to movement of *X. fastidiosa* in different grape varieties thus appears to be restricted to the stem tissue and is likely due to structural differences in the stem 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. Resistance to Pierce's disease is apparently not due to inhibitory compounds that circulate through the xylem since they might be expected to operate similarly in all tissues. Since *X. fastidiosa* was found in high numbers and high frequency within vessels of petioles, even in resistant varieties and at some distance from the point of inoculation, it appears that *X fastidiosa* follows a sinuous path up the vessels in the stem, colonizing relatively few vessels, but when it enters the petiole it can spread laterally to many more vessels and also multiply to high numbers.

The movement and multiplication of *X. fastidiosa* was quite different in stems of grape varieties varying in resistance to Pierce's disease despite any obvious structural differences in this study. In other studies resistance to the movement of *X. fastidiosa* in different grape varieties appeared also to be restricted to the stem tissues (18). Similar results were noted by Fry et al. 1990 where the multiplication and movement of *X. fastidiosa* in the stems of French Colombard, Carlos and Noble grapes were found to be similar for a few weeks post-inoculation but increasing in susceptible varieties and decreasing in resistant varieties thereafter. Both studies suggest that multiplication is inhibited in resistant varieties but successful in susceptible ones. These studies could not determine whether the lack of multiplication is the main cause of further movement or whether the lack of further movement is the primary cause of poor bacterial multiplication. While viable *X. fastidiosa* population sizes in stems of the resistant Tampa and Roucaneuf varieties was much lower than that in the susceptible Cabernet sauvignon and Chenin blanc varieties (Figure 3) the proportion of vessels harboring at least some cells of the pathogen did not differ nearly as much (Figure 2). The relatively high population size of *X. fastidiosa* in susceptible varieties was thus associated with substantially more multiplication that had occurred in those vessels that had become infested with the pathogen. Overall, *X. fastidiosa* in resistant varieties

invaded a smaller fraction of plant stem vessels and exhibited lower population densities. The finding that there is a non-linear relationship between the population size of *X. fastidiosa* in stem tissue and the proportion of vessels that are colonized clearly indicates that multiplication was unequal within vessels in the different varieties. This suggests that in resistant genotypes movement within stems and multiplication within stem vessels are both impaired and likely are co-dependent phenomena, unlike in petioles. Recent studies of the *rpfF*-dependent regulation of virulence of *X. fastidiosa* also has suggested that movement and multiplication in grape are linked (3). An rpfF mutant which is de-repressed for production of cell wall degrading enzymes such as polygalacturonases both invaded more vessels and achieved a higher population size in infected tissues than the wild-type strain (4). Importantly, the increased population size of the *rpfF* mutant (10 to 100-fold) compared to the wild type was much greater than the two to three-fold increase in the proportion of vessels that were colonized; such a non-linear relationship between multiplication and movement between vessels is similar to that seen here with a wild-type strain in different grape varieties. Presumably the process of movement of *X. fastidiosa* 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 *X fastidiosa* (5). That is, those grape varieties that are most easily digested by X. *fastidiosa* will be both more easily invaded and support more extensive multiplication by *X fastidiosa.* The *rpfF*-mediated control of virulence in *X. fastidiosa* also accounts for a larger effect of resistant varieties on multiplication than on movement. Cell density-dependent regulation of virulence in *X. fastidiosa* is mediated by the accumulation of a diffusible signal factor (DSF) produced by RpfF (16). DSF accumulation is associated with increased adhesiveness due to the expression of a variety of afimbrial adhesins, and a decreased expression of cell wall degrading enzymes such as polygalacturonases, as well as elevated expression of type IV pili associated with twitching motility. Thus it appears that at relatively low populations in a plant (vessel) *X. fastidiosa* expresses a suite of genes that are most consistent with active movement and multiplication within a plant, while when populations become higher, such virulence traits are suppressed. One can imagine that the pit membranes of resistant grape varieties are less easily degraded/breached by *X. fastidiosa* and hence provide a poor nutritional resource for multiplication. The pathogen thus would not be able to achieve such high population sizes in resistant varieties, which in turn would enable the pathogen to remain in an active “exploratory” phase of plant colonization unlike in more susceptible plants where more extensive multiplication leads to cessation of movement. Thus, in resistant varieties the pathogen, while perhaps moving to few adjacent xylem vessels due to their recalcitrance to pit degradation, would continue to attempt to move within the plant due to the low population sizes dictated by the recalcitrance of the plant to degradation. It is apparent from this study 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 through which the bacteria move.

The much higher population size of *X. fastidiosa* observed in susceptible varieties than resistant varieties than expected based on the proportions of infested vessels (Figure 2) raised the question as to whether cells in the resistant varieties may die as they age. Cells of many bacteria, including some plant pathogens can enter a viable but non-culturable state upon exposure to certain stresses. It seemed possible that the environment of the resistant varieties might constitute such a stress. Within the constraints on sampling imposed by the examination of a limited number of cross

sections of stem tissue, we found that the total population size, estimated by microscopy was quite similar to the culturable population estimated following maceration of stem segments. Given that the half-life of the gfp marker protein that we used in this study is quite long, and gfp fluorescence persists in cells even after they die (16) we would have expected more total cells estimated by counts of gfp-fluorescent bacteria than culturable cells if an appreciable proportion of the population of *X. fastidiosa* had died in the resistant grape varieties. Since the culturable populations of *X. fastidiosa* in resistant grape varieties were as much as 100-fold lower than expected based on the proportion of stem vessels infested with the pathogen, such large numbers of dead cells should have been readily detected by our microscopic sampling. Thus most cells of *X. fastidiosa* appear to be culturable within the time frames of the infections studied here.

Examination of the extent of stem vessel colonization my prove to be a useful and high throughput method to screen germplasm for resistance to Pierce's disease. While estimations of total number of cells of *X. fastidiosa* in stems, but not petioles, are predictive of the disease resistance phenotype (18) culturing of the pathogen is relatively labor-intensive, and other methods such as ELISA or quantitative PCR estimate population sizes are either not very sensitive, or suffer from background especially at low population sizes. Examination of stem segments for the proportion of infested vessels, and especially for the extent of colonization of the infested vessels may prove to be an expedient means to screen large numbers of plant genotypes as both will be expected to be low in resistant varieties.

In summary, there is apparently limited *X. fastidiosa* movement and growth within stem tissues but unlimited movement and growth within petioles of susceptible and resistant grape varieties. Low *X. fastidiosa* 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 *X. fastidiosa* 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 *X. fastidiosa* stem populations in susceptible varieties is due to both a higher incidence and intensity of vessel colonization since *X. fastidiosa* is able to degrade pit membranes, consequently multiplying more as well as subsequently invading more vessels.

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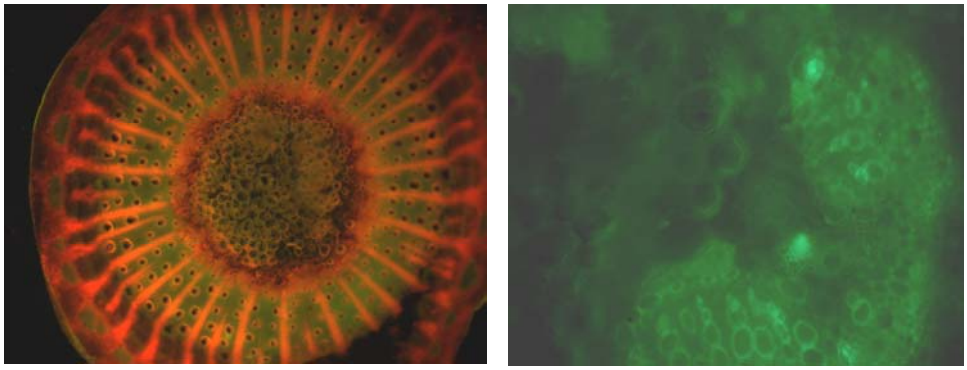
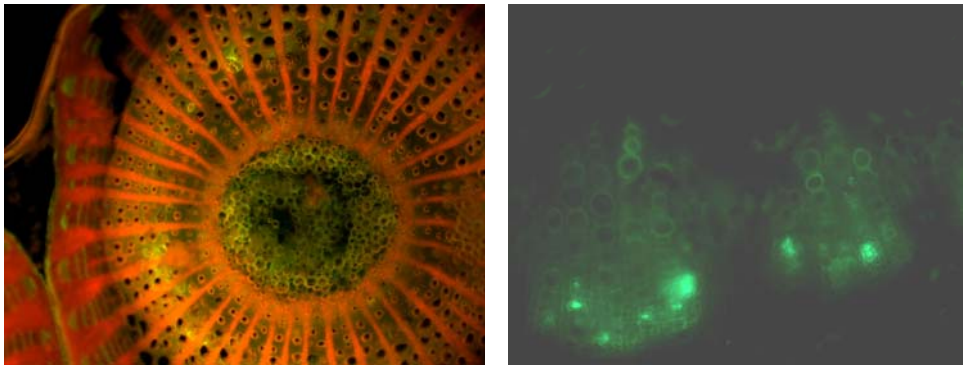
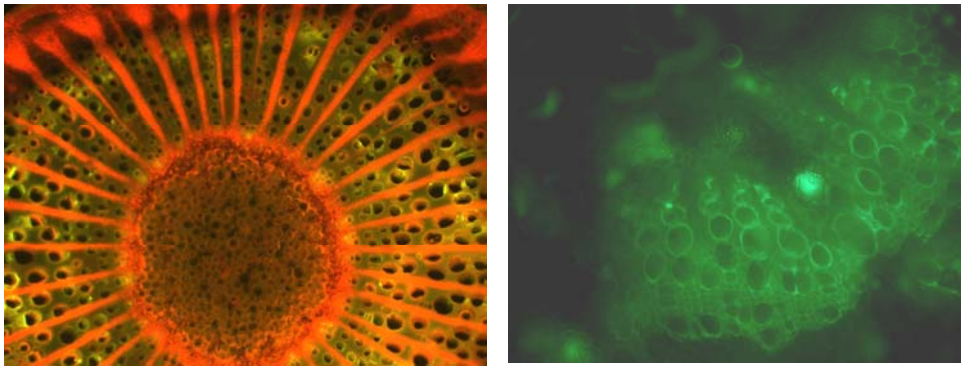
Stem **Petiole**



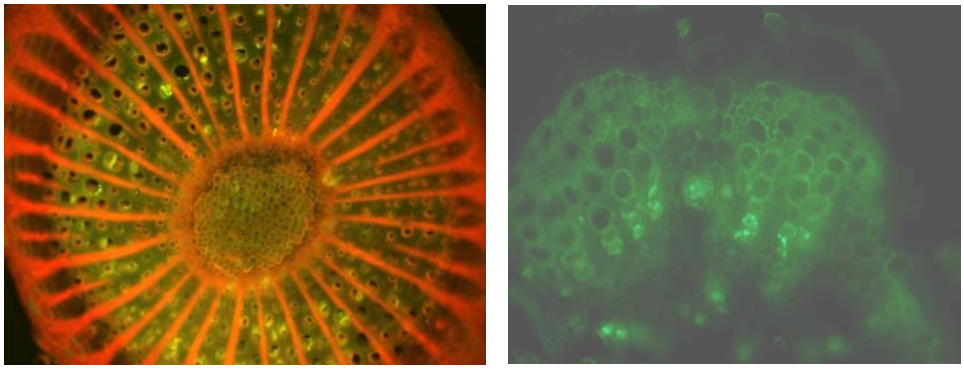
Roucaneuf



Tampa



Chenin Blanc



Cabernet sauvignon

Fig. 1

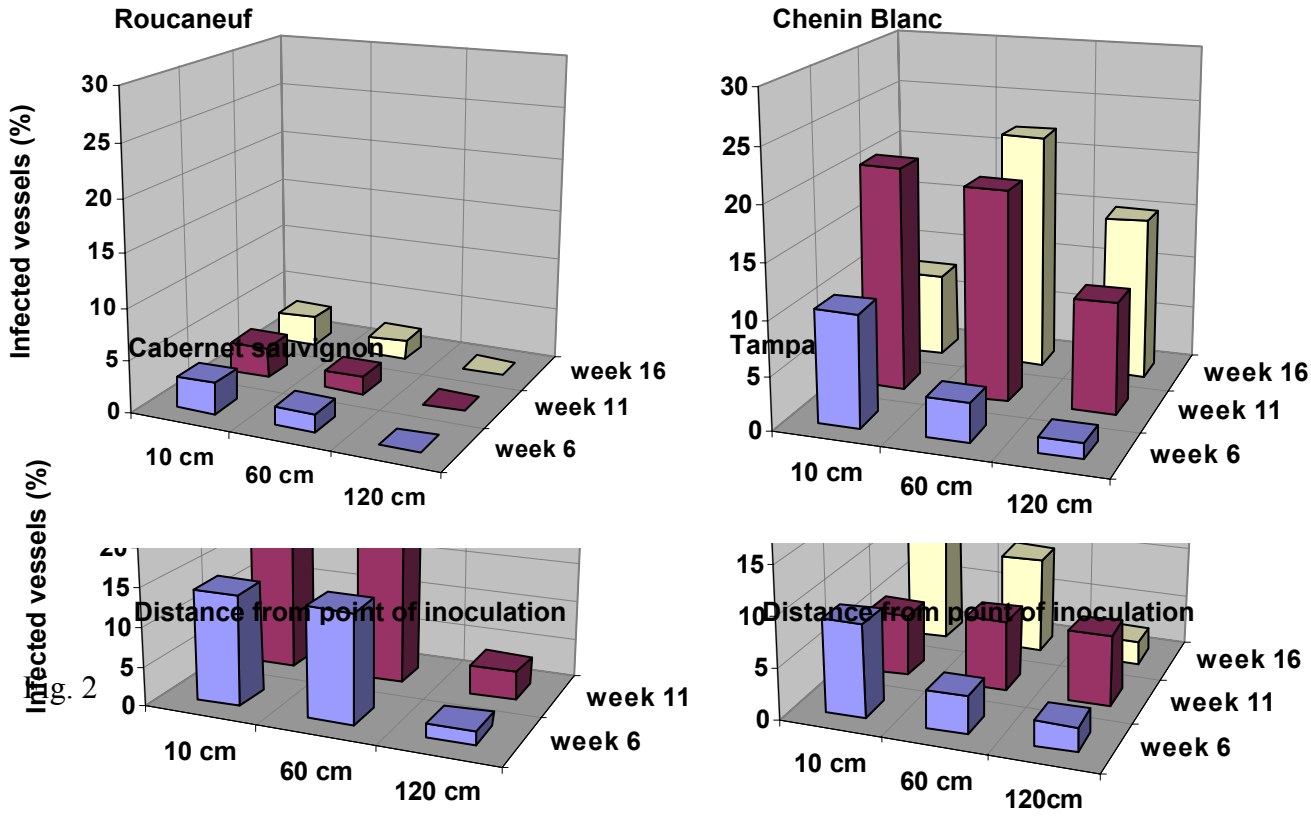

Roucaneuf
Chenin Blanc
Infected vessels (%)
30
25
20
15
10
5
0
Cabernet sauvignon
Tampa
week 16
week 11
week 6
10 cm
60 cm
120 cm
Distance from point of inoculation
120cm

Fig. 2

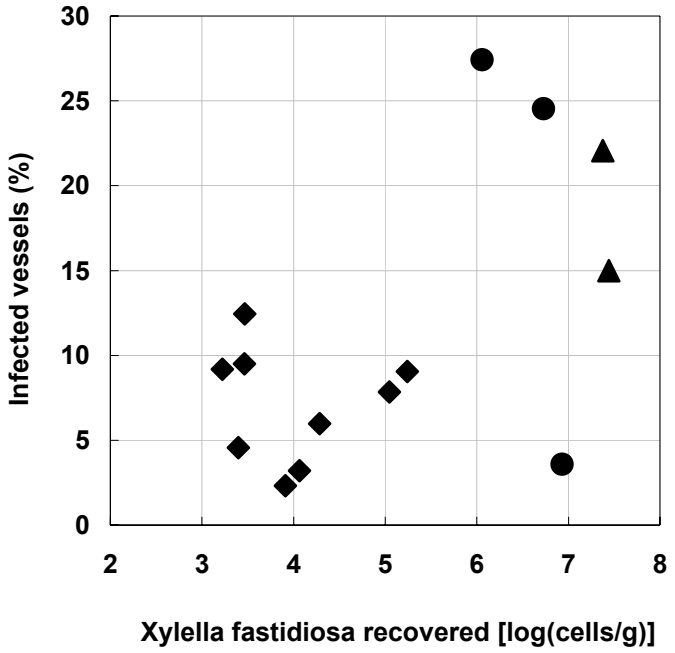

30
25
20
15
10
5
0
Infected vessels (%)
2
3
4
5
6
7
8
Xylella fastidiosa recovered [log(cells/g)]

Fig. 3

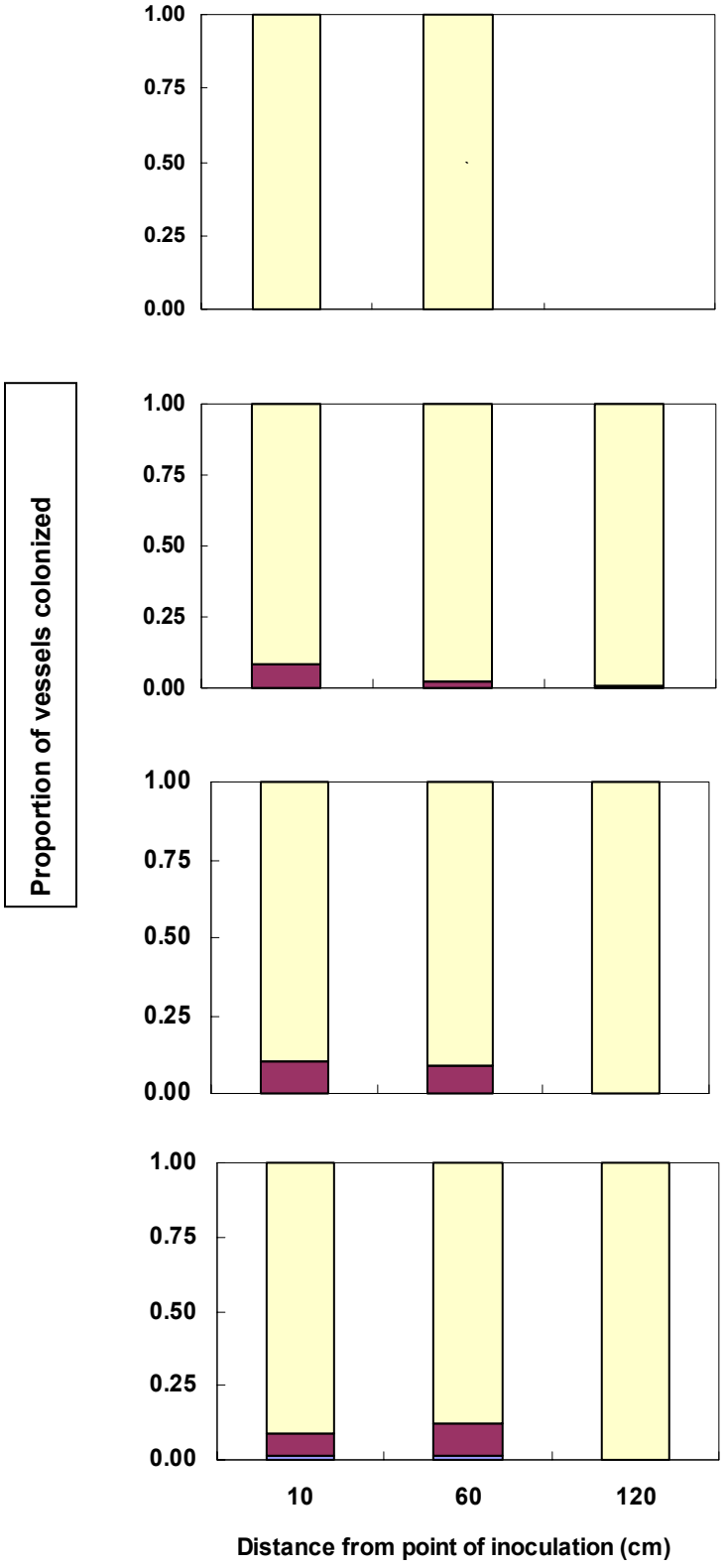

Proportion of vessels colonized
1.00
0.75
0.50
0.25
0.00
1.00
0.75
0.50
0.25
0.00
1.00
0.75
0.50
0.25
0.00
1.00
0.75
0.50
0.25
0.00
10
60
120
Distance from point of inoculation (cm)

Fig 4: From bottom grape variety Cabernet sauvignon, Chenin blanc, Tampa and Roucaneuf

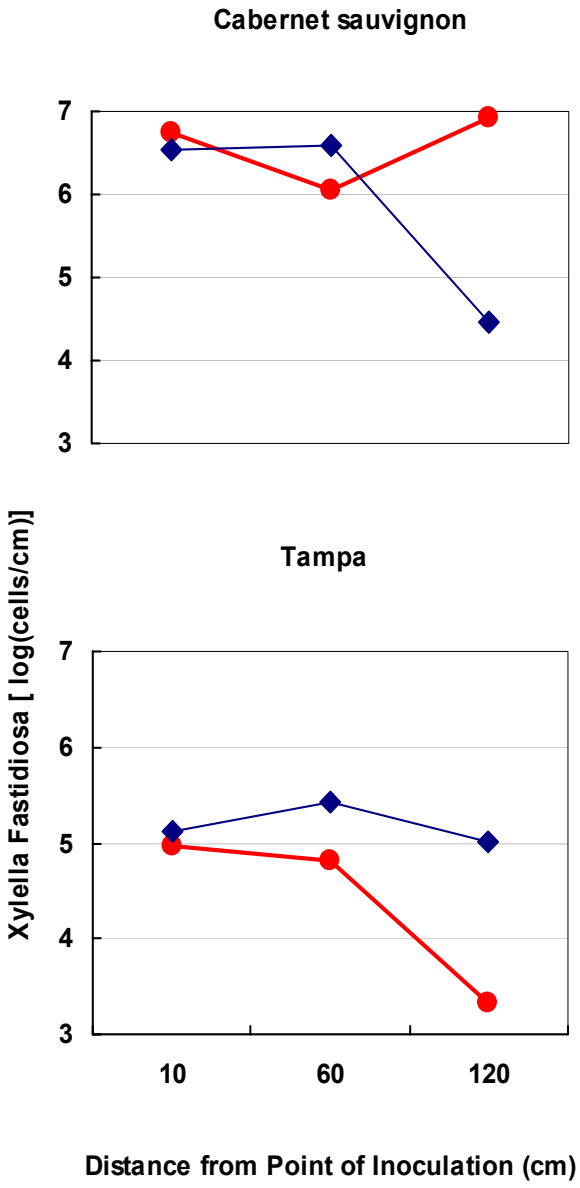

Cabernet sauvignon
7
6
5
4
3
Tampa
Xylella Fastidiosa [log(cells/cm)]
7
6
5
4
3
10
60
120
Distance from Point of Inoculation (cm)

Fig. 5

Table 1: Differences in extent of colonization of stem xylem vessels in different grape varieties determined by microscopic detection of a gfp-marked strains of *X. fastidiosa* at different distances from the point of inoculation.

	Proportion of vessels [a]		
	Distance from point of inoculation		
	10 cm	***60 cm***	***120 cm***
Low vessel colonization [b]			
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
Moderate vessel colonization [c]			
Cabernet sauvignon	5.7b	8.3b	0a
Tampa	3.4a	0.41a	0a
Chenin Blanc	3.2a	1.41a	0a
Roucaneuf	0c	0a	0a
High vessel colonization [d]			
Cabernet sauvignon	0.9b	0a	0a
Tampa	0a	0a	0a
Chenin Blanc	0a	0a	0a
Roucaneuf	0a	0a	0a

Means followed by the same letter within a column do not differ ($P<0.05$). Means followed by the same letter within a column do not differ ($P<0.05$).
b) vessels harbored few *Xf* cells (<10)
c) vessel harbored moderate number of *Xf* cells (>10 but <1000)
d) vessel harbored large number of *Xf* cells (>1000)
a) An LSD test was performed on the mean number of colonized vessels 11 weeks post-infection.

Figure legends

Figure 1: Visualization of plant colonization infected with a gfp-marked strain of Xylella fastidiosa. From bottom: Cabernet Sauvignon, Chenin Blanc (susceptible)

Tampa and Roucaneuf (resistant) stem cross sections, with corresponding petiole cross sections (right).The plants were sampled 11 weeks after inoculation.

Figure 2. Percentage of stem xylem vessels infested with at least one cell of a gfp-marked strain of *X. fastidiosa* as determined by microscopy in grape varieties differing in resistance to Pierce's disease that were sampled at different times after inoculation and at various distances from the point of inoculation.

Figure 3: Relationship between incidence of colonization of stem xylem vessels of Cabernet sauvignon (●) Tampa (♦) and Chenin Blanc (▲) by a gfp-tagged *X. fastidiosa* strain as determined by fluorescence microscopy (Y-axis) and the population size of *X. fastidiosa* determined in closely adjacent tissues by culturing of stem macerates (abscissa).

Figure 4. Proportion of the vessels of Cabernet sauvignon , Chenin Blanc , Tampa and Roucaneuf in stems sampled at various locations distal to the point of inoculation that were infested with at least some cells of a gfp-marked strain of *X. fastidiosa* that exhibited various extents of colonization by this pathogen. Yellow represent portion of vessels which had minor colonization; purple represent proportion of vessels which had moderate

Figure 5. Comparison of population sizes of a gfp-marked strain of *X. fastidiosa* in Tampa (A) and Cabernet sauvignon (B) stem segments at different distances from the point of inoculation that were estimated by culturing or fluorescence microscopy at 11 weeks after inoculation.

Summary and status of intellectual property produced during this research

None produced in this period.

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, 2007, San Diego, California

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, 2008, San Diego, California

Baccari, C. and Lindow, S.E. 2009. Assessment of the process of movement of *Xylella fastidiosa* within susceptible and resistant grape varieties. Phytopathology 99: (submitted)

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 have investigated the spatial distribution of cells of *X. fastidiosa* within susceptible and resistant grape varieties. 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* may be 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. Our finding that the petioles of all grape varieties are apparently easily colonized by *Xylella fastidiosa* while the stem tissues greatly differ in ease of colonization suggests strongly that structural difference in pit membranes or other subtle anatomical differences account for disease resistance in grape. These findings also suggest strongly that circulative factors such as composition of xylem sap can not account for differences in disease susceptibility since these fluids will be the same in both petioles and stem xylem. Our work thus helps elucidate the mechanisms of disease resistance and also aid in the process of breeding for resistant grape varieties by identifying the sites of resistance expression.