CHARACTERIZATION AND IDENTIFICATION OF PIERCE'S DISEASE RESISTANCE MECHANISMS: ANALYSIS OF XYLEM ANATOMICAL STRUCTURES AND OF NATURAL PRODUCTS IN XYLEM SAP AMONG *VITIS*

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ABSTRACT
Grapevine genotypes differ in their susceptibility/tolerance to Pierce's disease (PD). This may be related to the concentration and presence or absence of chemical compounds in the xylem sap and/or due to anatomical features of the xylem. Here we report on a three-pronged comparative approach investigating various grapevine species ranging in PD tolerance. Results from *in vitro* xylem sap assays indicate a broad range of *Xylella fastidiosa* (*Xf*) growth responses in both planktonic growth and biofilm formation. Investigations into *Xf* population dynamics in the stem tissues of greenhouse grown plants, confirm large differences in the size of *Xf* populations between susceptible and tolerant genotypes. Microscopic investigations into xylem vessel occlusions document the differences in occlusion percentages as well as the kinds of occlusions prevalent among four different *Vitis* genotypes.

INTRODUCTION
Experimental, as well as anecdotal, information indicate a considerable range in tolerance to PD among grapevine genotypes. It appears that a number of *Vitis* as well as *Muscadinia* species evolved mechanisms allowing them to tolerate infection by *Xf*. More precisely, while it is often thought that many wild genotypes evolved tolerance mechanisms, it is also possible that it is the induction of a deleterious response by *V. vinifera* genotypes that renders them more susceptible than a number of other genotypes which may not respond to a challenge by *Xf*. Understanding of the causes of the mechanisms responsible for differential sensitivity is a critical component of crop improvement. The rich diversity of grapevine genotypes tolerant to PD can and is being utilized to serve as a source for PD resistance for breeders. While PD resistant species have been identified (Mortensen et al., 1977; Kirvanek and Walker, 2004), the mechanisms of resistance have not been identified. Breeding of resistant genotypes is likely the most sustainable means of combating PD. In order to generate highly PD tolerant grape cultivars, knowledge of the kind and function of resistance mechanisms is paramount. This research was initiated to investigate host-pathogen interactions and to screen for mechanisms of PD resistance in a range of *Vitis* species. It appears that multiple mechanisms of PD resistance mechanisms are present in wild genotypes and/or genotypes utilized in the southeastern USA. *Xf* is xylem limited and appears to kill vines by inducing or creating vessel blockage leading to disease (Goodwin et al 1988a, 1988b) and may also involve the production of toxins (Lu et al., 2004; Matthews et al., 2004). While the importance of the physical and/or the chemical environment in the xylem is unclear, xylem-related factors are undoubtedly involved in host-pathogen interactions and the mechanisms of host tolerance. Therefore, this project focuses on: 1) host-pathogen interactions using comparative analyses of *Xf* population dynamics among a group of grapevine genotypes; 2) examination of xylem anatomical factors using microscopic approaches; and 3) investigation of xylem sap chemistry by employing bioassays that will be followed by analytical approaches.

OBJECTIVES
1. Determine the effect of xylem sap collected from various grape genotypes with differential sensitivity to PD on *Xf* colony number and biofilm formation.
2. Evaluate *Xf* population dynamics in 20 grape genotypes.
3. Examine xylem structure of selected grape genotypes using SEM.

RESULTS
Objective 1
Xylem sap extracted from 14 grape genotypes was used in a bioassay to determine if there are differences in *Xf* growth characteristics associated with xylem sap source (Figures 1 and 2). The sap extracted from the field-grown plants was filter sterilized and inoculated with *Xf* ('Stags Leap') and incubated at 28°C. The number of colony forming units was evaluated using plating and biofilm formation was assessed by the crystal violet method. CFU counts and biofilm formation are summarized in Figures 1 and 2. A large range in terms of *Xf* growth and biofilm formation was found in response to xylem

sap from different grape genotypes. Both, planktonic growth and biofilm formation were influenced by the source of xylem sap. Xylem sap from some genotypes like 8909-17 (*V. rupestris* x *V. arizonica*) and 9621-67 (*V. rupestris* x *V. arizonica*) suppressed *Xf* growth very strongly, while *Xf* flourished in sap from various genotypes including *V. rufotomentosa*, *V. nesbitiana*, and *V. tiliifolia.* The differences in *Xf* growth characteristics indicate that xylem sap composition is genotype specific and that there are xylem sap compositional aspects that strongly influence *Xf* growth. However, it is still not clear if the differences are due to the presence of inhibitory and/or the absence of growth promoting compounds.

Objective 2

Twenty grape genotypes were grown under greenhouse conditions to investigate host-pathogen interactions. Leaf and petiole samples from bottom, middle and top third of each plant were collected from *Xf* inoculated and control (water inoculated) plants at 34, 77, and 113 days post inoculation. Stem samples were collected at the last sampling only. These samples are being used to determine *Xf* populations in the different plant fractions using quantitative ELISA. Kirvanek and Walker (2004) reported that stem *Xf* numbers were highly correlated with field PD performance and suggested that it would be a useful tool to predict PD resistance. Populations of *Xf* in the stems varied greatly (Figure 3). The predicted average *Xf* populations in infected 8909-19, 9621-94 (both *V. rupestris* x *V. arizonica*), 'Chardonnay', 'Metallique' (*V. rupestris*), and *V. aestivalis* plants were larger than 250,000 cells per 0.1g of stem tissue. Most other genotypes did not exhibit *Xf* populations beyond a positive detection threshold determined by the mean plus three standard deviations from samples collected from water-inoculated control plants.

Objective 3

Petioles samples collected 113 days post inoculation from *V. vinifera* (Chardonnay), *V. smalliana*, *V. arizonica*, and *V. rufotomentosa* genotypes were examined by SEM to investigate if there are differences in the xylem vessel occlusion pattern between genotypes. Preliminary results of this ongoing investigation are summarized in Table 1. Vessel occlusion was classified into four categories: occlusion by tyloses, *Xf* aggregates, gum, or a filamentous net (Figure 4). In addition, we differentiated here between completely occluded and partially occluded vessel elements. In general, xylem vessel occlusion was less in *V. smalliana* and *V. arizonica* than in *V. vinifera* and *V. rufotomentosa*. Tyloses formation in *V. rufotomentosa* appeared to be more pronounced than in the other genotypes while the presence of vessels completely occluded by *Xf* aggregates was more prominent in Chardonnay.

CONCLUSIONS

1. Xylem sap from a number of grapevine genotypes considered tolerant to PD supports *Xf* growth to varying degrees. Both planktonic growth and biofilm formation are responsive to the source of the xylem sap. Information on the distinct responses of *Xf* growth to xylem sap source allows for the selection of suitable representative genotypes for detailed investigations into xylem sap composition.
2. Large genotypic variations exist among the examined grape genotypes in respect to stem *Xf* populations.
3. Petiole xylem vessel occlusion differs between susceptible Chardonnay and tolerant *V. smalliana*, *V. arizonica* and *V. rufotomentosa*. However, even within the tolerant genotypes there appear to be differences in the number of vessels occluded and the type of occlusions present.

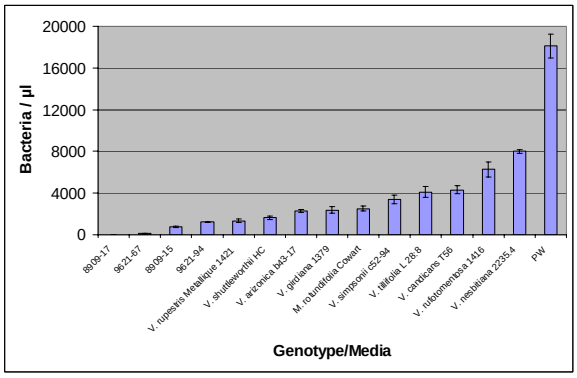

Figure 1. To determine the effect of xylem sap on colony formation, each mixture was further diluted and 100 µl diluted mixture was plated onto PW solid media for colony development. Plates were incubated at 28°C for 14 days. Bacterial count was based on the numbers of colonies per plate and a bacterial density per µl was then calculated.

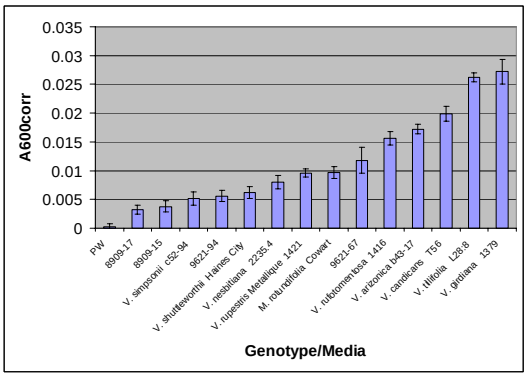

Figure 2. To determine the effect of xylem sap on biofilm formation, the same microfuge tubes after removal of aliquots for plating on solid media were rinsed several times with ddH_2O Crystal followed by an addition of 150µl of 1% incubation, violet each tube. After 15 min of Crystal violet solution was removed and microfuge tubes were rinsed 3 x with ddH_2O. After elution with 95% ethanol, absorbance was read at 600 nm wavelengths.

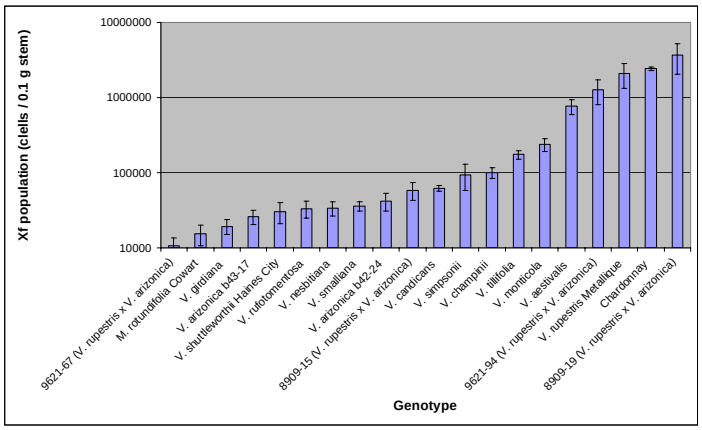

Figure 3. Predicted mean number of *Xf* bacteria per 100 µg of stem tissue in 20 different grape genotypes. Samples were taken from the bottom, middle, and top third of the stem 113 days after inoculation. Results reported represent mean values across all stem-locations within a stem. Bacterial populations were predicted based on a standard calibration curve. Error bars represent standard error of the mean.

Table 1. Characterization of xylem vessel occlusion in petioles collected from four different genotypes 113 days after inoculation with *Xf*.

Vitis	Occl	*Xf* aggregate		Matrix		Tyloses		Filament net		Total	
		Avg	SE	Avg	SE	Avg	SE	Avg	SE	Avg	SE
		% vessels completely or partially occluded									
vinifera	cplt.	6.0	3.1	8.7	1.8	6.0	2.3	0.7	0.7	21.3	1.8
	part	9.3	4.7	6.0	2.0	5.3	3.3	5.3	3.5	26	8.7
smalliana	cplt.	0		0.7	0.7	5.3	4.4	0.7	0.7	6.7	4.1
	part	0		1.3	0.7	4.0	2.0	8.0	2.0	13.3	3.5
arizonica	cplt.	0		0		0		1.3	1.3	1.3	1.3
	part	0		2.7	1.3	3.3	1.8	4.7	1.8	10.7	1.3
rufotomentosa	cplt.	0		3.3	2.4	17.3	9.0	6.7	3.5	27.3	12.7
	part	0		3.3	3.3	6.0	3.5	10.7	2.9	20.0	4.2

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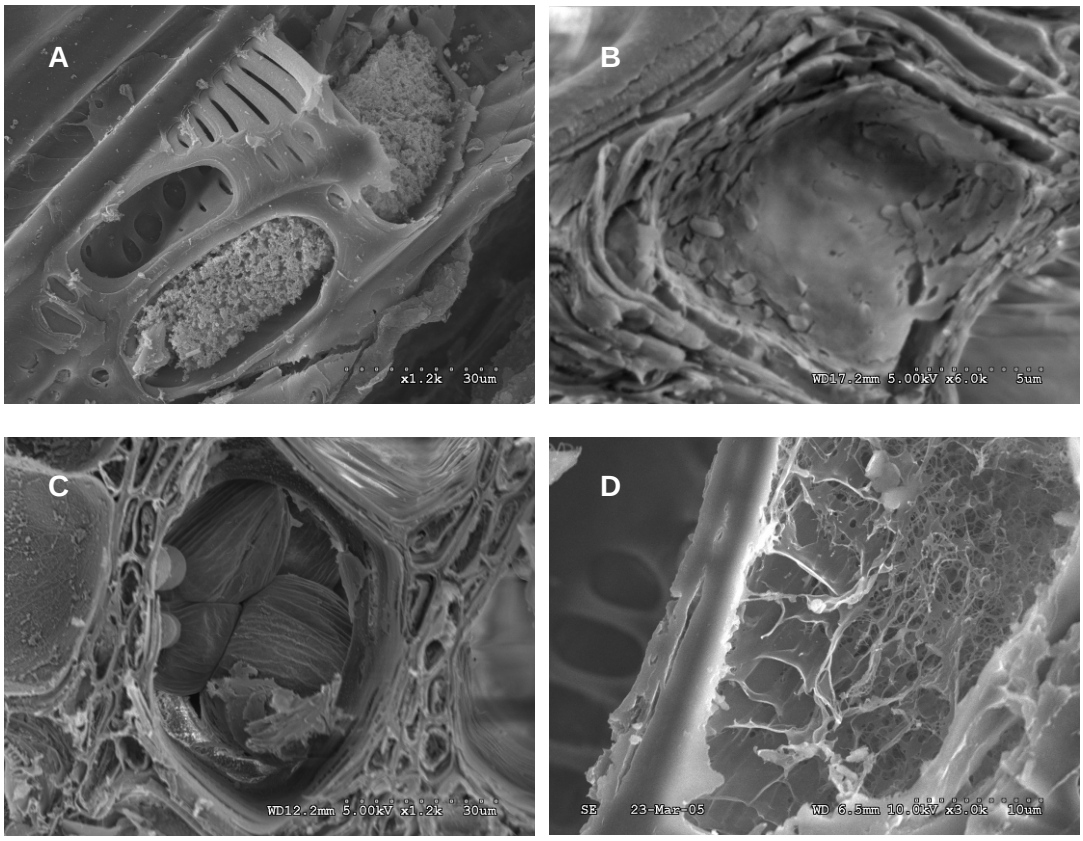

Figure 4. SEM images of complete xylem vessel occlusions by a large bacterial aggregate (A), matrix embedded bacteria (B), tyloses (C), and a filamentous network (D).