***Vitis* Resistance to Pierce's Disease Is Characterized by Differential *Xylella fastidiosa* Populations in Stems and Leaves**

A. F. Krivanek and M. A. Walker

Department of Viticulture and Enology, One Shields Ave., University of California, Davis 95616.

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ABSTRACT

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The pattern of *Xylella fastidiosa* infection in resistant and susceptible grapevines representing a diverse selection of *Vitis* spp. was characterized through measurements of *X. fastidiosa* bacterial movement and accumulation in artificially inoculated greenhouse-grown grapevines. A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was optimized for quantification of *X. fastidiosa* populations and tested on known amounts of *X. fastidiosa* added to grape tissue extracts. Predicted versus known *X. fastidiosa* concentrations proved to be highly correlated ($R^2 = 0.99$). Populations of *X. fastidiosa* in stem internode, stem node, petiole, and leaf blade samples from the genotypes in this study were measured at 12 weeks postinoculation using the optimized ELISA procedure. Samples from each plant part were taken at eight positions along the inoculated shoots. Systemic infection was detected in both susceptible

and resistant genotypes. Resistant genotypes were characterized by significant differences in *X. fastidiosa* populations between stem internodes and leaves (1.0×10^6 and 1.1×10^7 cells/g of sample, respectively). In contrast, the susceptible genotypes were characterized by high mean *X. fastidiosa* populations in both stems and leaves (5.6×10^7 and 4.8×10^7 cells/g, respectively) the latter of which were not significantly different from the resistant genotypes. A high correlation ($R^2 = 0.97$) between stem *X. fastidiosa* numbers to previously characterized field Pierce's disease (PD) performance indicates that the quantitative ELISA measurements of *X. fastidiosa* in greenhouse-grown grapevines should be a useful tool for predicting PD resistance under field conditions.

Additional keywords: Blanc Du Bois, Chardonnay, Chenin blanc, D'Arpa, D8909-15, Roucaneuf, standard calibration curve, Tampa, *V. aestivalis* subsp. *smalliana*, *V. arizonica*, *V. shuttleworthii*, *V. simpsonii*, *V. vinifera*, Zehnder 71-50-1.

The pathogen *Xylella fastidiosa* is the causal agent of Pierce's disease (PD) in grape and numerous other diseases in many economically important crops. Descriptions of *X. fastidiosa* infection and accumulation in grapevines have been made in a number of studies; however, little is known about the complete pattern of bacterial infection in field-resistant genotypes. Several studies have examined *X. fastidiosa* infections in susceptible *Vitis* bunch grape cultivars through evaluation of bacterial accumulation in petiole and leaf samples (19,37) or in petiole, leaf, and stem samples (13,14,41). Two microscopy studies evaluated the extent of vessel occlusions (which often are associated with *X. fastidiosa* infection) in resistant genotypes, but were restricted to leaves and petioles of field-evaluated grapevines (24,26). Only two studies have examined *X. fastidiosa* accumulation within stem, petiole, and leaf samples of both resistant and susceptible genotypes (9,20). The later study was conducted on naturally infected plants, which would have resulted in multiple inoculations via insect vectors, confounding conclusions about systemic infections. These studies had a relatively narrow focus because only one or two resistant genotypes were evaluated and they were restricted to the muscadine grape species, *Muscadinia rotundifolia*.

The purposes of this study were threefold. The first was to develop an optimized enzyme-linked immunosorbent assay (ELISA) procedure capable of accurately and reliably measuring quantitative differences in *X. fastidiosa* populations within infected grapevines. Previous studies have used ELISA to detect *X. fastidiosa* in grape (13,25,31,41) and in other host plants (11,16,17,36,38,40).

To our knowledge, only one study has utilized ELISA to quantify *X. fastidiosa* populations (37), using mean absorbance readings to measure *X. fastidiosa* in petioles. However, absorbance values often vary greatly from one evaluation to another, making significant differences difficult to obtain. The optimized ELISA procedure presented here addresses this problem by utilizing a standard calibration curve to normalize the absorbance data and compensate for day-to-day procedural variation.

The second purpose of our study was to use ELISA to determine if *X. fastidiosa* populations vary significantly among different plant parts, and verify whether systemic *X. fastidiosa* infections can occur within several PD field-resistant *Vitis* bunch grape genotypes. The final purpose was to determine the best plant part and position to sample in order to most effectively differentiate susceptible from resistant genotypes at 12 weeks postinoculation. The resistant genotypes chosen for our study, fully described in a companion article (21), are a genetically diverse array of *Vitis* bunch grape genotypes that survive in high-PD-pressure regions of North America. Therefore, insights into the pattern of bacterial accumulation in the resistant grapevines should be broadly applicable to the *Vitis* genus and should help in the understanding of resistance to PD.

MATERIALS AND METHODS

Plant material and inoculation. The six field-resistant and two field-susceptible *Vitis* genotypes utilized in this study were previously described in a companion article (21). The genotypes Blanc Du Bois, Roucaneuf (SV12.309), Tampa, Zehnder 71-50-1, and D8909-15 are resistant to PD under field conditions (10,28-30). D'Arpa, previously described as "moderately resistant," was the only resistant genotype in this study having a lower level of field resistance (J. A. Mortensen to United States Department of

Corresponding author: M. A. Walker; E-mail address: awalker@ucdavis.edu

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Agriculture–Agricultural Research Service Clonal Germplasm Repository, Davis, CA, *personal communication*). The sources of resistance for these genotypes were *Vitis aestivalis* subsp. *smaliana*, *V. simpsonii*, *V. arizonica*, *V. berlandieri*, *V. cinerea*, and *V. shottii*. The two field-susceptible genotypes evaluated were *V. vinifera* Chenin blanc and Chardonnay. Previous studies found Chardonnay to be one of the most susceptible genotypes and Chenin blanc one of the least susceptible genotypes, with mild symptoms and lower mortality rate relative to other *V. vinifera* cultivars (34,35,37). The genotypes were propagated clonally from green cuttings, grown under greenhouse conditions, and inoculated with the Stags Leap *X. fastidiosa* isolate as previously described (21).

Experimental design. Four to five *X. fastidiosa*-inoculated replications were used per genotype. The individual potted plants of each genotype served as the experimental unit and the plants were distributed randomly on greenhouse benches in a completely randomized design. Two to four water-inoculated replicates of each genotype served as controls. Due to the unequal number of replications, the statistical design was treated as unbalanced.

Sampling method. Grapevines were sampled 12 weeks post-inoculation by taking 0.5-g sections of internode, node, petiole, and leaf blade from points nearest to the point of inoculation (POI), 10 and 20 cm below the POI and 10, 20, 30, 40, and 50 cm above the POI. The internode lengths across genotypes were approximately the same, although some plants had nodes between selected points that were not sampled. Samples were placed into AGDIA bags (AGDIA, Elkhart IN) with a phosphate-buffered saline (PBS), 0.05% Tween, and 2% soluble polyvinylpyrrolidone (PVP-40) buffer (31), at a volume-to-sample weight ratio of 10 ml:1 g. Samples were lightly crushed with a hammer and ground using a Homex 6 mechanical homogenizer (Bioreba, Longmont, CO), and the resulting extract was stored at -20°C .

Optimized ELISA method. Whole immunoglobulin (IgG) antiserum was raised against *X. fastidiosa* in a rabbit (supplied by B. Kirkpatrick, Department of Plant Pathology, University of California, Davis). The IgG was purified using a protein A affinity column (5 ml of HiTrap protein A, Amersham Biosciences) and horseradish peroxidase (HP) was conjugated to the IgG (39).

A standard concentration of *X. fastidiosa* bacteria in suspension was prepared for the ELISA positive controls. *X. fastidiosa* was grown at 29°C on plates of solid periwinkle wilt medium (6) and actively growing colonies were washed from the medium with sterile ddH_2O . The *X. fastidiosa* suspension was adjusted with ddH_2O to an absorbance at 600 nm (A_{600}) of 0.25, and three replicates of a 10-fold serial dilution (10^{-1} to 10^{-6}) were used to estimate *X. fastidiosa* concentrations via standard culture plating). Colonies were counted after 10 days and the concentration of the original dilution was calculated. A suspension with 6.5×10^7 CFU/ml was divided into 300- μl aliquots and stored at -20°C .

A double-antibody sandwich ELISA procedure (4) was used for *X. fastidiosa* quantification in plant samples with the following modifications. In each step, volumes of 200 μl per well were employed. The IgG was optimized to a concentration of 10 $\mu\text{g}/\text{ml}$ in 0.05 M sodium carbonate buffer (pH 9.6), similar to a concentration previously reported (31), and used to coat wells of polystyrene microtiter plates (AGDIA). Plates were covered with an empty plate, wrapped in plastic wrap, incubated at 37°C with agitation on an Lab-Line Orbital Titre Plate Shaker 4625 (Lab-Line Instruments, Inc. Melrose Park IL), removed after 1 h, and washed four times with PBS + 0.05% Tween (PBST). Dilutions of bacteria added to healthy stem extract at concentrations of 6.5×10^6 , 3.25×10^6 , 6.5×10^5 , 3.25×10^5 , 6.5×10^4 , and 3.25×10^4 CFU/ml were added to duplicate wells. Stem extract alone and PBST alone also were included in duplicate wells as controls. Plates were covered, incubated for 1.5 to 3 h, and washed as before. The HP conjugate was added to wells at a concentration of 1:400 parts PBST with 1% bovine serum albumin (immunoassay

quality). The conjugate concentration was optimized previously to achieve the most linear absorbance curve for the *X. fastidiosa* dilution series as addressed in the discussion. The conjugate was not added to the two wells where only PBST had been added previously. Plates were incubated as above for 1 h and washed six times. A 3,3',5,5'-tetramethylbenzidine substrate buffer system (8) was used and reactions were stopped with 100 μl of 2 M sulfuric acid within 15 to 25 min. Absorbance measurements were made at A_{450} using a Finstrument Microplate Reader (MTX Lab Systems, Vienna, VA). The computer program "DeltaSoft" (Mac version 3; BioMetallics, Princeton, NJ) was used to organize the output data. Background absorbance from the two wells with PBST was subtracted from all wells. The standard calibration curve derived from the dilution series was used to convert absorbance values to cells-per-milliliter concentrations using the DeltaSoft 3 program and by choosing the log/log cubic polynomial with smoothing at $P = 0.5$ and J weighting from experimental data. Positive samples with a coefficient of variation (CV) $>20\%$ between duplicates were repeated. All predicted values were reported as cells per milliliter. However, because the ratio of buffer volume to sample weight was 10:1, the cells-per-milliliter concentrations equate to 1/10th the cells per gram of sample.

Statistical analyses. The ELISA procedure was evaluated prior to analysis of experimental samples. Optical absorbance readings from an *X. fastidiosa* dilution series run on 7 days over a 3-week period were assessed. These values were converted to cells per milliliter by using a paired dilution series on a plate run on the same day. The mean absorbance, predicted cells per milliliter, and the natural log of predicted cells per milliliter were calculated for each separate dilution over the 7 days, along with corresponding standard deviations, standard errors, and CVs. Estimates were regressed on to the known concentrations to assess accuracy via the R^2 coefficient.

Statistical comparisons of *X. fastidiosa* populations in plant samples were analyzed with the MIXED procedure of SAS (version 8; SAS Institute, Cary, NC) in a repeated measures analysis. The REML method of parameter estimation in the MIXED procedure was chosen because it allows for missing data in a repeated measures design, whereas the GLM procedure requires balanced data (7,42). The MIXED procedure also manages the potential violation to assumptions of uncorrelated errors and homogeneous variances due to the repeated samples (22,42), unlike in a typical split-plot design using the GLM procedure. In order to compare water-inoculated with *X. fastidiosa*-inoculated plants, the control ELISA readings were converted to theoretical concentrations. The water-inoculated samples also were used to calculate the conservative positive threshold within each plant part, determined by the mean plus three standard deviations. Interactions between factors were included in each model. When interactions were significant, the simple effects (main effect within each level of the other factor) were evaluated using the SAS "slice" option. For significant simple effects, a post-hoc mean separation was performed using Tukey-Kramer's honestly significant difference (HSD) test. The first-order autoregressive covariance structure was chosen for modeling the covariance among repeated samples after testing for the best fit by the Bayesian criterion value. Due to the unbalanced nature of the design, the Satterwaith method was used for estimating degrees of freedom. Residuals were plotted and analyzed for normal distribution visually and via the W statistic (W) of the Shapiro-Wilk test.

Two major analyses were run. First, the effect of plant part on *X. fastidiosa* populations in samples at 30 cm above POI was tested. The experimental unit was the individual potted plant of each genotype and was treated as a random factor. Fixed-factor treatments were inoculation, plant part, genotype, and field PD status (the reported field PD performance). Field PD status had two levels (resistant genotypes as a group and susceptible genotypes as a group). Genotype, nested within field status, had eight

levels (six resistant and two susceptible individual genotypes). Plant part treated as the repeated measures (within subject) factor had four levels (stem internode, stem node, petiole, and leaf blade). Inoculation had two levels (*X. fastidiosa*- and water-inoculated). In a second analysis, the effect of sample position on internode *X. fastidiosa* populations was tested. Sample position was the repeated measures factor with eight levels (–20 cm, –10 cm, POI, +10 cm, +20 cm, +30 cm, +40 cm, and +50 cm). Field PD status and genotype levels were the same as in the first analysis of plant part effects on *X. fastidiosa* populations.

A field PD status rank was assigned to each genotype based on performance in previous studies so as to assess the reliability of *X. fastidiosa* population measurements for determining field resistance. The field-resistant genotypes Blanc Du Bois, Roucaneuf, Tampa, Zehnder 71-50-1, and D8909-15 were given a rank of 1, the moderately field-resistant D’Arpa a rank of 2, the moderately field-susceptible Chenin blanc a rank of 3, and the field-susceptible Chardonnay a rank of 4. The mean *X. fastidiosa* concentrations of each genotype were plotted onto their respective field PD status rank and correlation was measured with the R^2 coefficient.

RESULTS

Evaluation of optimized ELISA method for bacterial quantification. Absorbance readings from the standard dilution series run independently over 7 different days are presented in Table 1. The mean CV of the entire dilution series was 35.3%, indicating a relatively high level of day-to-day variation in absorbance readings from the procedure. The series mean CV of the predicted cells-per-milliliter concentrations at 14.2% was lower than the mean CV for absorbance readings. The accuracy of the ELISA procedure for predicting cell concentrations proved to be high because the regression of mean predicted cells-per-milliliter concentrations on known CFU-per-milliliter concentrations of each dilution yielded a regression coefficient of $R^2 = 0.991$. A conservative positive threshold for the dilution series was calculated at 3.1×10^4 cells/ml. For further improvements, the predicted bacterial concentrations were natural log transformed and the resulting mean CV of the natural log (predicted cells per milliliter) concentrations was a low 1.27%. The transformed data also proved to be accurate because the mean natural log (predicted cells per milliliter) and the mean natural log (known CFU per milliliter) concentrations were highly correlated ($R^2 = 0.99$) (Fig. 1).

Water-inoculated controls and positive threshold determination. In order to determine the effect that different genotypes and plant parts have on background ELISA values, the effects of plant part, genotype, field PD status, and the corresponding interactions were tested on samples taken at 30 cm above POI. Initial analyses on samples of both water-inoculated control plants and bacteria-inoculated plants using the sample bacterial concen-

tration as the dependent variable indicated that the residuals were not normally distributed. Thus, the data were transformed to their natural log and 8 outliers of 219 data points were removed ($W = 0.992$, $P = 0.08$). The mean absorbance values for internode, node, petiole, and leaf blade samples from the water-inoculated control plants were 0.045, 0.050, 0.053, and 0.091, respectively, and converted to theoretical mean natural log (cells per milliliter) concentrations of 9.86, 9.91, 9.94, and 10.41, respectively. Interactions of inoculation–genotype, inoculation–plant part, and inoculation–genotype–plant part all were highly significant ($P < 0.0001$). The statistical simple effects of genotype, plant part, and the genotype–plant part interaction were tested within the two inoculation levels (*X. fastidiosa* and water) using the SAS slice option. Within water-inoculated control plants, the effects of genotype, plant part, and the plant part–genotype interaction were not significant (Table 2). All pairwise comparisons among samples as measured by Tukey–Kramer’s HSD showed no significant variation associated with genotype or plant part of the water-inoculated control plants. However, because of greater variation in leaf blade samples, the study-wide conservative positive thresholds for positive samples were calculated separately for stem internode, node, petiole, and leaf blade at 11.09, 11.30, 10.84, and 12.51 natural log (cells per milliliter), respectively.

Fig. 1. Plot of known and predicted bacterial concentrations in a *Xylella fastidiosa* (Xf) dilution series. Bacterial concentrations were predicted from absorbance values using a standard calibration curve. Absorbance readings were measured via seven independently run enzyme-linked immunosorbent assay procedures. Standard deviation error bars represent sample variation.

TABLE 1. Enzyme-linked immunosorbent assay absorbance measurements and predicted bacterial concentrations derived from the absorbance values from a *Xylella fastidiosa* dilution series of known bacterial concentrations added to healthy grape stem extracts^x

Measured and predicted ^y	Known <i>X. fastidiosa</i> dilutions in healthy stem extract (CFU/ml)						
	0	32,500	65,000	330,000	650,000	3,300,000	6,500,000
Measured absorbency							
Mean	0.045	0.077	0.105	0.335	0.613	1.993	2.632
Standard error	0.008	0.013	0.015	0.048	0.074	0.206	0.208
Standard deviation	0.021	0.034	0.041	0.126	0.195	0.545	0.550
CV	46.9	43.8	38.9	37.5	31.8	27.3	20.9
Predicted cells/ml							
Mean	17,470 ^z	41,231	69,341	334,629	718,923	3,777,822	6,230,441
Standard error	1,732 ^z	3,304	3,459	10,030	36,926	142,166	179,800
Standard deviation	4,584 ^z	8,742	9,150	26,536	97,697	376,137	475,707
CV	26.2	21.2	13.2	7.9	13.6	10.0	7.6

^x Measurements were from the standard dilution series run independently over 7 different days.
^y CV = coefficient of variation.
^z Negative control samples have theoretical concentrations based on measured absorbance values.

Bacterial distribution. Populations of *X. fastidiosa* in each plant part and at each position of the individual genotypes are presented in Figure 2. The stem samples (Fig. 2A and B) of the resistant genotypes generally had bacterial populations above the

mean value for water-inoculated controls of stem internode and node samples, but at levels between 10.0 and 12.0 natural log (cells per milliliter) (2.2×10^8 and 1.6×10^5 cells/ml). The mean *X. fastidiosa* populations within all petiole samples of the

TABLE 2. *F* table of the genotype, plant part, and the genotype–plant part interaction main effects on *Xylella fastidiosa* populations evaluated within each inoculum level^z

Main factor effects	Inoculum	Numerator df	Denominator df	<i>F</i> value	<i>P</i> > <i>F</i>
Genotype	Water	6	36.7	0.4	0.8732
Plant part	Water	2	89.4	0.1	0.9061
Genotype–plant part	Water	30	71.7	1.17	0.2916
Genotype	<i>X. fastidiosa</i>	7	41.1	31.31	<0.0001
Plant part	<i>X. fastidiosa</i>	3	91.9	41.15	<0.0001
Genotype–plant part	<i>X. fastidiosa</i>	31	74.3	17.96	<0.0001

^z Samples were taken 12 weeks postinoculation at 30 cm above the point of inoculation.

Fig. 2. Mean *Xylella fastidiosa* (Xf) bacteria in **A**, internode, **B**, node, **C**, petiole, and **D**, leaf blade for eight genotypes. Samples were taken from eight positions relative to the point of inoculation after 12 weeks. Genotypes Chardonnay and Chenin blanc are field susceptible, D'Arpa is moderately field resistant, and D8909-15, Tampa, Blanc Du Bois, Roucaneuf, and Zehnder 71-50-1 are field resistant. Bacterial populations are natural log transformed concentrations of the mean population of cells per milliliter of plant extract as determined from enzyme-linked immunosorbent assay absorbance readings using a standard calibration curve. Error bars represent standard error of the mean.

resistant genotypes (Fig. 2C) taken from 10 cm below the POI to 50 cm above the POI were above the mean values and conservative positive threshold level for petiole samples, and ranged from 11.0 to 15.2 natural log (cells per milliliter) (6.0×10^4 to 4.0×10^6 cells/ml). These data demonstrate that *X. fastidiosa* multiplies within and systemically infects resistant *Vitis* bunch grape genotypes as well as susceptible genotypes under the described screening conditions.

Plant part effect on bacterial populations. Initial evaluations of *X. fastidiosa* populations across the four plant parts of the resistant status (resistant genotypes as a group) indicated high populations in leaf blades, but low populations in stems sampled at the same position. This result was typified by the resistant genotype Tampa, which manifested classic leaf scorch symptoms (Fig. 3).

In order to evaluate the effect of plant part, genotype, and field PD status on *X. fastidiosa* populations, samples from *X. fastidiosa*-inoculated plants at 30 cm above the POI were analyzed. The interaction of plant part–field PD status was highly significant ($P < 0.0001$). This interaction is reflected in Figure 4A, where the difference between resistant and susceptible status decreases as the sample changes from stem internode to stem node to petiole to leaf blade. Populations of *X. fastidiosa* in the internode and node samples from the resistant genotypes (1.0×10^5 and 1.6×10^5 cells/ml, respectively) were not significantly different from each other ($P = 0.59$ HSD), yet both had significantly lower bacterial populations ($P < 0.0001$ HSD) than the petioles ($1.3 \times$

10^6 cells/ml) at the same position. Internodes, nodes, and petioles of the resistant genotypes had significantly lower *X. fastidiosa* populations than the leaf blades (1.1×10^5 cells/ml) at the same position ($P < 0.0001$, $P < 0.0001$, and $P = 0.008$ HSD, respectively). In contrast, the mean *X. fastidiosa* populations among the internodes, nodes, petioles, and leaf blade samples of the susceptible genotypes (5.6 , 6.0 , 9.8 , and 4.8×10^5 cells/ml, respectively) were not significantly different ($P > 0.05$ HSD).

The *X. fastidiosa* populations between resistant versus susceptible genotypes were significantly different ($P < 0.0001$ HSD) in three of the four plant parts sampled, but the leaf-blade samples were not different ($P = 0.1452$ HSD). The largest difference in *X. fastidiosa* populations between the resistant genotypes as a group and the susceptible genotypes as a group occurred within stem internodes (Fig. 4A). The mean *X. fastidiosa* concentration in internode samples from the resistant genotypes was 60-fold lower than in the internodes from the susceptible genotypes. The plant part–genotype interaction also was significant ($P < 0.0001$) (Fig. 4B). Among the individual resistant genotypes, only D8909-15 and D'Arpa had significantly different internode *X. fastidiosa* populations ($P = 0.007$ HSD) (with 2.0×10^4 and 3.0×10^5 cells/ml, respectively). D'Arpa was significantly lower than Chardonnay ($P = 0.0001$ HSD) and all resistant genotypes except D'Arpa had significantly lower ($P < 0.0001$ HSD) internode *X. fastidiosa* populations than both Chenin blanc and Chardonnay. In contrast, within leaf blades, only Blanc Du Bois had

Fig. 3. A, Leaf scorch and B, differential bacterial populations in leaf and stem in a *Xylella fastidiosa* (Xf)-inoculated replicate of the field-resistant genotype Tampa. Leaf scorching was similar to PD symptoms typical of susceptible genotypes, with zones of chlorosis progressing into necrotic zones at the peripheral margins of the infected leaves. Sample positions are relative to the point of inoculation indicated by the arrow and tag on the plant. Photograph and samples were taken 12 weeks postinoculation. Ruler marks are at 5-cm intervals.

significantly lower bacterial populations than Chardonnay ($P = 0.009$ HSD).

Position effect on bacterial populations and extent of infection. To achieve normally distributed residuals, the cells-per-milliliter concentration data from internode samples at all positions of the *X. fastidiosa*-inoculated plants were transformed to their natural log and 6 outliers of 289 data points were removed, resulting in a normal distribution of the residuals ($W = 0.993$, $P = 0.24$). After testing for main effect interactions, the sample position-field PD status interaction proved to be significant ($P = 0.0275$) and, therefore, the simple effect of PD status was tested within each of the eight position levels. Field PD status had a significant effect ($P < 0.0001$) on internode *X. fastidiosa* populations at all positions. The F statistic indicated that the greatest variation in internode *X. fastidiosa* populations caused by field PD status was at 40 cm above POI, with a 100-fold difference in *X. fastidiosa* populations between the two (Table 3).

The interaction of position-genotype (nested within field PD status) was significant ($P = 0.004$), as were the simple effects of genotype within all position levels ($P < 0.0001$). The F statistic indicated that the greatest variation in internode *X. fastidiosa* populations caused by genotype was at 40 cm above POI, as was found in the position-field PD status interaction. Based on internode samples at 40 cm above the POI, all resistant genotypes had significantly lower *X. fastidiosa* populations than both Chenin blanc and Chardonnay ($P < 0.0001$ HSD) (Table 4). The reliability of using *X. fastidiosa* population measurements for determining field resistance was tested by regressing the internode *X. fastidiosa* population data (40 cm above the POI) onto the field PD status rank levels, and the two measures were highly correlated ($R^2 = 0.97$).

DISCUSSION

The ELISA procedure optimized in this investigation reliably can measure quantitative differences in *X. fastidiosa* populations within grapevine and is sufficiently sensitive for *X. fastidiosa* detection. The accuracy of the procedure for quantification of *X. fastidiosa* populations was demonstrated by the high correlation ($R^2 = 0.99$) of predicted to known bacterial concentrations added to healthy plant extracts. The reliability of the ELISA procedure was demonstrated by the low within-procedure variation (CV 1.27%). Such performance is equivalent to that of a real-time polymerase chain reaction (PCR) assay reported for *X. fastidiosa* in citrus (32). A major contributor to the low CV was the use of a standard calibration curve (derived from a dilution series on each ELISA plate) to convert absorbance values to *X. fastidiosa* concentrations. This conversion compensated for day-to-day procedural variation in absorbance readings. In comparison, experimental variation (which includes ELISA variation plus plant-to-plant *X. fastidiosa* population variation across experimental replicates) had typical CVs ranging from 30 to 100% and 2 to 15% for untransformed and transformed concentrations, respectively. The highest accuracy and precision in the conversion of absorbance values to cell per milliliter was achieved when the absorbance readings for healthy extract controls and the highest concentration of the dilution series were maintained in the range of 0.040 to 0.110 and 2.2 to 3.2, respectively. This helped to keep the entire dilution series within the linear portion of the ELISA response curve, which is essential for quantitative analysis of the ELISA data (3). Finally, the sensitivity of this procedure at 4.0 to 7.0×10^4 cells/ml of plant extract was similar to previous ELISA procedures (1,13,25,31) when using a conservative standard positive threshold calculated as in other studies (5,39). Our procedure was approximately 200- to 400-fold less sensitive than that reported for a standard PCR detection assay (25) or culture plating (12), and 1,000- to 10,000-fold less sensitive than that reported for nested PCR (2,33) and quantitative PCR (32). It is important to

note that, although the positive threshold for this procedure is higher than in many other *X. fastidiosa* detection protocols, it is sufficiently lower than the mean 1.0×10^5 cells/ml concentration of bacteria within stem internodes of the resistant genotypes evaluated in this study. In addition, the ability of ELISA to quantitatively measure *X. fastidiosa* concentrations was critical for distinguishing resistant and susceptible genotypes.

The result that systemic infections can occur within PD-resistant *Vitis* bunch grape genotypes was consistent with field evaluations that detected marginal leaf necrosis in PD-resistant grapevines (15,18,20,23,24). However, the inoculations by native sharpshooter vectors likely occurred at multiple points and times in the field and, therefore, the results were not conclusive evidence that systemic *X. fastidiosa* infection occurs in PD-resistant

Fig. 4. Plant part effect on mean *Xylella fastidiosa* (Xf) populations **A**, within the field-resistant Pierce's disease (PD) status (resistant genotypes as a group) and the field-susceptible PD status (susceptible genotypes as a group) and **B**, within the individual genotypes after Xf inoculation. Samples were taken at 30 cm above the point of inoculation after 12 weeks. Bacterial populations are natural log transformed concentrations of the mean population of cells per milliliter of plant extract as determined from enzyme-linked immunosorbent assay absorbance readings using a standard calibration curve. Error bars represent standard error of the mean.

genotypes. One greenhouse study (9), conducted under controlled conditions and with a single point inoculation, did measure low level systemic infections as far as 50 cm distal to the POI in resistant grapevines; however, only two resistant genotypes were evaluated and both were muscadine (*Muscadinia rotundifolia*) grape cultivars. The resistant *Vitis* bunch grape genotypes in our study were chosen to represent a range of genetic diversity, such that conclusions can be broadly applied to the genus *Vitis*. Systemic infections measured at distal samples from the POI also are supported in a companion article (21), where classic leaf scorch symptoms consistently were manifested on leaves of the *X. fastidiosa*-inoculated resistant genotypes and mean scorched leaf area ranged from 26 to 57%.

The optimized ELISA procedure was critical in measuring quantitative differences in *X. fastidiosa* populations across different plant parts in the resistant genotypes. It is possible that the large differences between bacterial populations from leaves and stems of resistant genotypes would not be observed in infected grapevines under field conditions; however, our data are consistent with previous field studies. In one evaluation, the highly susceptible *V. labrusca* Schuyler was compared with the *M. rotundifolia* Carlos under heavy PD pressure in a Leesburg, FL vineyard (20), and both cultivars allowed accumulation of *X. fastidiosa* in leaf veins and petioles, whereas *X. fastidiosa* accumulation was low in the stems of the field-resistant Carlos. Milholland (24) observed *X. fastidiosa* in xylem vessels of leaf veins and petioles of the scorched leaves of a resistant *M. rotundifolia* cultivar. In a greenhouse evaluation conducted by Fry (9), *X. fastidiosa* populations within stems of the highly susceptible *V. vinifera* French Colombard (when evaluated at 8 weeks

postinoculation) were up to 10,000-fold greater than in the stems of two resistant *M. rotundifolia* genotypes. In comparison, a separate evaluation of petiole-inoculated grapevines in the same study showed a <10-fold difference in petiole *X. fastidiosa* populations.

Differences in *X. fastidiosa* population levels between stems and leaves of resistant grape genotypes suggest that the mechanism of resistance to *X. fastidiosa* is localized within stem xylem and not fully functional or, possibly, absent in the xylem of petioles and leaf blades. This localized resistance suggests a host-pathogen interaction leading to a plant part-specific response within the resistant genotypes. A second hypothesis is that a more constitutive resistance mechanism is present, based on nutritional or structural difference between the two plant parts or between resistant and susceptible grapevines. A functioning resistance mechanism in the stem of the resistant genotypes may reduce the probability of *X. fastidiosa* bacteria access into petioles and leaf blades distal to the POI in this experiment and would bias downward the measured mean of leaf and petiole *X. fastidiosa* populations. Bacterial populations in the leaves and petioles of resistant genotypes reached high levels, but not as high as the *X. fastidiosa* populations in the susceptible genotypes; however, under equal opportunity of infection, these populations may equal those in the leaves and petioles of susceptible genotypes. Further study is needed to test these hypotheses.

The conclusions of this research have direct implications on future studies of PD resistance. The first conclusion is that low-level infections and systemic spread of *X. fastidiosa* within xylem tissues are characteristic of *X. fastidiosa* infections in field-resistant genotypes. Highly sensitive but qualitative detection procedures such as standard and nested PCR would not be useful for

TABLE 3. Mean enzyme-linked immunosorbent assay absorbance values and mean *Xylella fastidiosa* populations for the field-resistant status (resistant genotypes as a group) and field-susceptible status (susceptible genotypes as a group) at each of eight sample positions relative to the point of inoculation (POI)^y

Field Pierce's disease status	Position (cm) ^z	Rep no.	Mean absorbance	Mean cells/ml	Natural log (cells/ml)	Standard error
Resistant	-20	27	0.042	31,729	10.09	0.13
Resistant	-10	27	0.068	84,082	10.68	0.19
Resistant	POI	28	0.091	130,273	11.04	0.20
Resistant	+10	25	0.070	76,657	10.86	0.17
Resistant	+20	26	0.060	60,233	10.69	0.15
Resistant	+30	27	0.077	100,716	10.83	0.21
Resistant	+40	28	0.074	69,490	10.64	0.17
Resistant	+50	25	0.067	65,285	10.66	0.18
Susceptible	-20	9	1.009	2,608,612	13.86	0.53
Susceptible	-10	9	1.391	3,691,041	14.49	0.46
Susceptible	POI	9	1.613	4,256,008	14.86	0.36
Susceptible	+10	9	1.561	3,900,688	14.80	0.37
Susceptible	+20	9	1.768	4,666,888	15.00	0.36
Susceptible	+30	9	2.019	5,635,484	15.34	0.26
Susceptible	+40	8	2.270	6,711,109	15.54	0.27
Susceptible	+50	8	1.790	5,518,732	14.90	0.52

^y Samples taken from internodes 12 weeks post-*X. fastidiosa* inoculation. *X. fastidiosa* populations were determined from absorbance reading using a standard calibration curve.

^z Sample position relative to point of inoculation.

TABLE 4. Mean enzyme-linked immunosorbent assay absorbance values and internode *Xylella fastidiosa* populations for each genotype taken 12 weeks post-*X. fastidiosa* inoculation at 40 cm above point of inoculation^x

Genotype	Rep no.	Mean absorbance	Mean cells/ml	Natural log (cells/ml) ^y	Standard error
Roucanneuf	4	0.035	28,212	10.09 a	0.32
Zehnder 71-50-1	5	0.036	27,044	10.10 a	0.22
D8909-15	4	0.042	27,491	10.19 ab	0.14
Tampa	5	0.047	40,518	10.44 ab	0.29
Blanc Du Bois	5	0.084	54,281	10.78 ab	0.25
D'Arpa	5	0.187	222,737	12.06 b	0.38
Chenin Blanc ^z	3	1.916	4,971,819	15.10 c	0.65
Chardonnay ^z	5	2.483	7,754,682	15.81 c	0.17

^x *X. fastidiosa* populations were determined from absorbance readings using a standard calibration curve.

^y Results followed by different letters differ significantly ($P < 0.05$ Tukey-Kramer's honestly significant difference).

^z Field-susceptible genotypes.

PD resistance screens because a high percentage of positive samples are likely in both resistant and susceptible genotypes. A quantitative measure of *X. fastidiosa* populations such as culture plating, real time PCR, or the optimized ELISA procedure presented here is necessary to distinguish these genotypes. In addition, the ability of any detection procedure to quantify *X. fastidiosa* populations will be critical for future heritability or quantitative trait locus studies if the PD resistance trait is inherited in a polygenic manner as has been suggested (27). The second conclusion is that differential expression of the resistance trait across different plant parts makes sample selection critically important for resistance evaluations and gene expression experiments such as those using microarrays or cDNA libraries for PD resistance gene discovery.

Finally, our measurements of stem *X. fastidiosa* populations with the optimized ELISA procedure were cost effective and reflective of each genotype's past performance under field conditions. Such characteristics are key components of any screening method applied to large genetic or breeding populations. One individual can process up to 320 samples in duplicate wells in ≈ 9 h. The reduction in operating time compared with typical ELISA procedures is achieved through the use of a microtiter plate shaker and higher temperatures for reducing incubation times. Consumable materials were typically \$0.25 per sample (including duplicate wells). The procedure also has the additional advantage over culture plating in that samples do not have to be run on the same day they are collected, and can be stored at -20°C for at least several months without influencing ELISA readings (data not shown). The field-resistant genotypes, when considered as a group, had 30- to 100-fold lower *X. fastidiosa* populations in all stem internode samples than did the group of susceptible genotypes. D'Arpa, the only resistant genotype previously described as "moderately resistant" to PD under field conditions, had the highest concentration of bacteria in stem internodes (2.0×10^5 cells/ml). In addition, Chenin blanc, reported to be less susceptible to PD in the field than is the highly susceptible Chardonnay (34,35,37), had lower (although not significantly lower) internode *X. fastidiosa* populations when compared with Chardonnay. The high correlation ($R^2 = 0.97$) between *X. fastidiosa* populations in stem samples and field PD status rank indicates that rapid greenhouse-based screening for *X. fastidiosa* resistance using ELISA can be a useful method for predicting field performance of genotypes in PD resistance breeding programs.

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