Final Report for VA Wine Board

PROJECT TITLE:
Revisiting Pierce's Disease in Virginia Commercial Vineyards. (#449036)

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OBJECTIVES:

1. Conduct an expanded Pierce's Disease (PD) survey of VA vineyards, using a very sensitive technique quantitative polymersase chain reaction (qPCR) to determine the current status of PD distribution in the state.
2. Plant a trial of PD-resistant varieties at an experimental vineyard in SPAREC to evaluate their performance in VA.
3. Use qPCR to monitor the movement of *Xylella fastidiosa* (*Xf*), causal agent of PD, within an infected vine over the course of a season.
4. Develop new Extension materials for PD in VA, as well as a standard diagnostic procedure for PD at VT Plant Disease Clinic.

I. Activities Performed

Objective 1) Conduct an expanded survey of VA vineyards using a very sensitive technique, quantitative PCR (qPCR), to determine the current status of PD distribution in VA

We collected 398 grape petiole samples (7 leaves and petioles each) from all VA wine regions in conjunction with our grapevine virus survey (Table 1). Many grape varieties were collected (Fig. 1). Samples were collected from Pierce's Disease (PD) symptomatic vines and also randomly from the second row, third panel and first vine of a variety in a vineyard. (An attempt was made to collect a random sample from every variety in each vineyard.) Samples were shipped to Virginia Cooperative Extension's Plant Disease Clinic overnight, and stored at -80°C until processed for *Xf* testing. We have tested 341 of the samples.

We proposed to use a quantitative real-time PCR (qPCR) (4), which would be expected to be more sensitive than the ELISA-assay method that was used in the previous survey (6). We also compared seven DNA extraction kits and a grape DNA extraction method commonly used by several grape research groups in order to identify the extraction method best at eliminating PCR reaction inhibitors. Inhibitors are commonly present in grape tissue and are problematic in PCR reactions; this results in less sensitivity and reliability in detection tests and can also result in false negative results in PCR and qPCR tests. We also developed a multiplex qPCR protocol using published primer sequences that target the *rimM* gene in *Xf* (3, 4) and an endogenous grape gene, resveratrol (5). The endogenous grape gene target acts as a monitor of the qPCR reaction (whether the reaction is working as expected) and eliminates the chance for a false negative reaction due to inhibitors and/or operator error.

To compare the sensitivity of *Xf* detection tests approximately 60 grape petiole samples, consisting of a majority of *Xf*-positive and some *Xf*-negative samples were extracted using the DNA extraction method determined best at eliminating grape tissue PCR-inhibitors and a grape DNA extraction method commonly used by grape research groups. DNA extracted with both methods was then tested with the multiplex qPCR protocol we optimized for *Xf* detection. The 60 petiole samples were also tested with the Agdia™ *Xylella fastidiosa* ELISA, which was used in the previous PD VA survey (6).

We also planned to test previously collected and DNA-extracted grape petiole samples (approximately 400) from 136 different VA vineyards that were used in a previous grapevine virus study for *Xf*. These samples were collected based on symptom expression of grape virus and extracted using the "commonly used grape extraction method". We have tested 150 of these samples using a somewhat modified version of multiplex qPCR testing method, which reduced the negative effect of inhibitors present in the DNA extraction.

Objective 1 Results)

We optimized a multiplex quantitative PCR (qPCR) test using two published primer sets that respectively target the 16S rRNA processing proptein *rimM* in *Xylella fastidiosa [Xf] (3, 4)* and an endogenous grape gene, resveratrol synthase I mRNA (5). To test the sensitivity and efficiency of the multiplex qPCR test we obtained *Xylella fastidiosa* subsp. *fastidiosa* genomic DNA (*Xff)* from the American Type Culture Collection (1) and quantified it using a Nanodrop™ spectrophotometer. The *Xff* DNA was then diluted into grape (healthy grape negative for *Xf)* DNA extracted using the kit we identified as best at eliminating PCR inhibitors. The dilution series was a half-log dilution with 3 replicates per dilution starting with 1 nanogram (ng) *Xff* down to 0.3 femtograms (fg) *Xff*. The standard curve of the dilution series shows that the qPCR test is nearly 100% efficient and extremely sensitive at detecting *Xff* (Fig. 2). Additionally, the multiplex qPCR with the endogenous grape gene target provides high confidence in test results, since it acts as an alert to prevent any "false negative".

A pairwise test using two pair-wise comparisons (t-test and Wilcoxon singed Rank) for three *Xf* extraction/*Xf* testing methods showed that the probability of *Xf*-detection was significantly better at removing PCR-inhibitors present in grape petiole tissue using the DNA extraction kit we identified from among several kits/methods when used in the multiplex qPCR test *(*$P<0.05$). This was in comparison to a commonly used DNA extraction method used by several grape research groups. However, regardless of which DNA extraction method was used, the multiplex qPCR test was shown to be significantly more sensitive at detecting *Xf* compared to the ELISA test used in the previous Virginia PD survey ($P<0.05$) [Fig. 3] (6).

Survey samples tested were collected from all Virginia Wine Regions (Fig. 4) and represented reasonably diverse the grape varieties commonly grown in Virginia (Fig. 1). Survey results show Pierce's Disease to be widespread throughout VA (Fig. 4). The only region in which we did not detect PD was in the Heart of Appalachia Region; however, this was a region from which few samples were collected and which is low risk for PD, according to a 2008 risk assessment map for PD based on 8-year average weather data (2). Of

the 341 samples tested 41% tested positive for PD (Fig. 6). Of the samples testing positive for PD (138 samples) 32% were collected as random samples and 64% were collected as "suspected" for PD (i.e. were symptomatic for the disease when collected) (Fig. 7A). Sixty-eight percent of the randomly collected samples that tested positive for PD showed no symptoms of PD and 32% had symptoms of marginal leaf scorch (Fig. 7B). Of the samples collected as "suspect" (i.e. symptomatic when collected) 52% tested positive for PD and 48% tested negative for PD (Fig. 7C).

The 150 previously collected and extracted samples collected for the virus survey have all but two tested negative for *Xf* using the modified multiplex qPCR test. However, DNA from the older samples were extracted using suboptimal method, and based on the comparison of extraction methods we conducted, it is not surprising to see poor testing results.

Table 1. Number of samples collected in each Virginia Wine Region.

VA Wine Region	**Number of Samples Collected**
Central VA	115
Northern VA	92
Blue Ridge Highlands	48
Southern VA	41
Chesapeake Bay	38
Shenandoah Valley	26
Hampton Roads	19
Eastern Shore	14
Heart of Appalachia	3

Figure 1. Distribution of varieties in survey and PD breakdown by variety. (Note that varieties consisting of less than 4 samples are not included in this chart.)

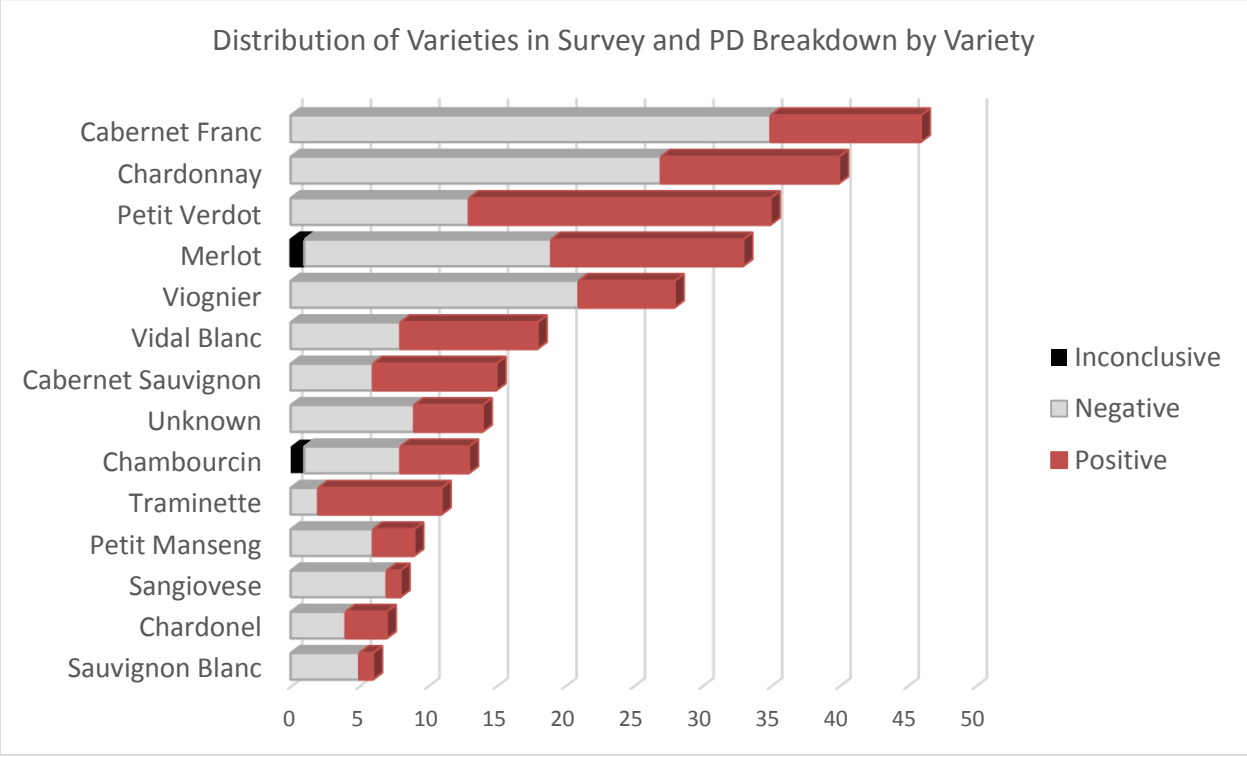


Fig. 2. Standard curve of *Xff* genomic DNA diluted in grape DNA. Threshold cycles (Ct) are plotted against the log of the genomic *Xff* DNA dilution series: Each standard was run as 3 replicates, starting with 1 nanogram *Xff* DNA and diluted in half-log dilution series down to 9.9 femtograms [fg] *Xff* DNA. The standard curve shows nearly 100% efficiency and a detection level down to 9.9 fg *Xf* DNA. (*Xf* was detected down to 1 fg in all 3 replicates; however, there was a high standard deviation among replications below 9.9 fg [omitted from standard curve].)

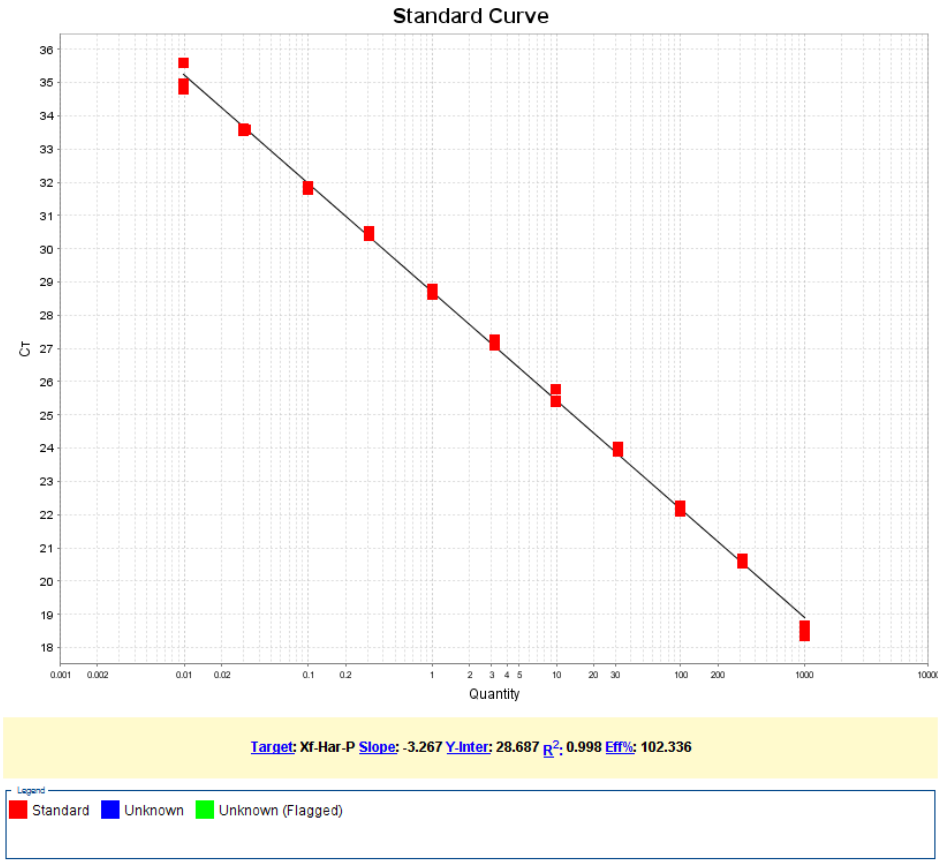


Fig. 3. Pairwise tests showed that DNA extracted using the method identified as best at eliminating PCR inhibitors when used in the qPCR multiplex test had a significantly higher probability of detecting *Xf* than DNA extracted using a commonly used grape-extraction method when used in the qPCR multiplex test ($P<0.05$). However, pairwise tests also showed that when used in the qPCR multiplext test, DNA extracted using the commonly used extraction method had a higher probability at detecting *Xf* than the ELISA test ($P<0.05$).

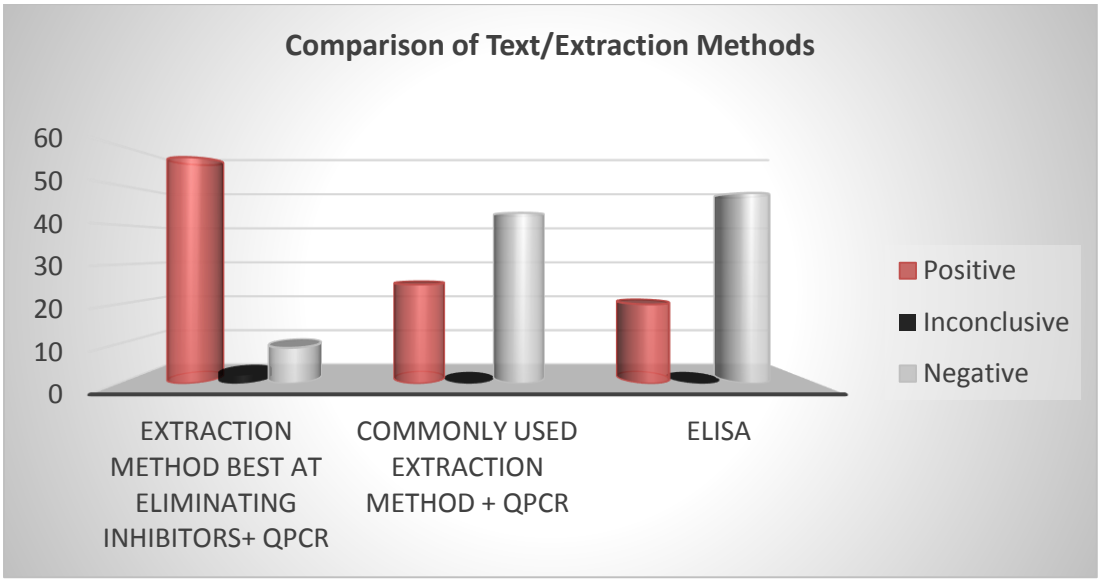


Fig. 4. PD survey results by VA wine region.

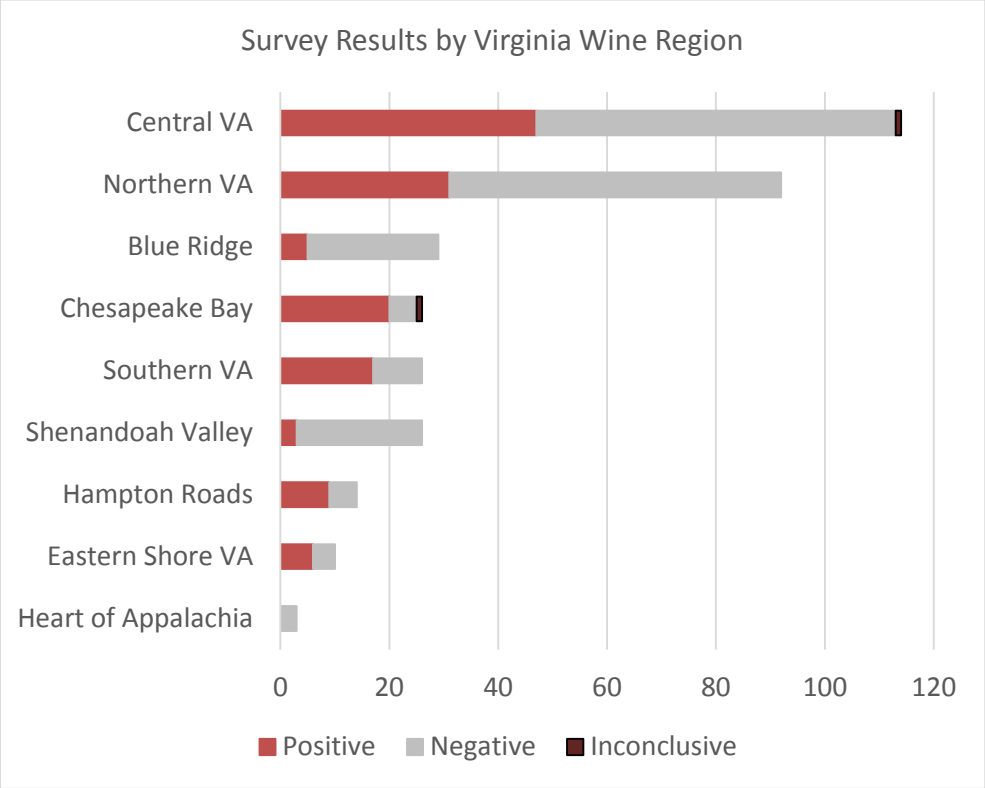


Fig. 5. Distribution of survey samples by variety and PD breakdown by variety. (Note that varieties consisting of less than 4 samples are not included in chart.)

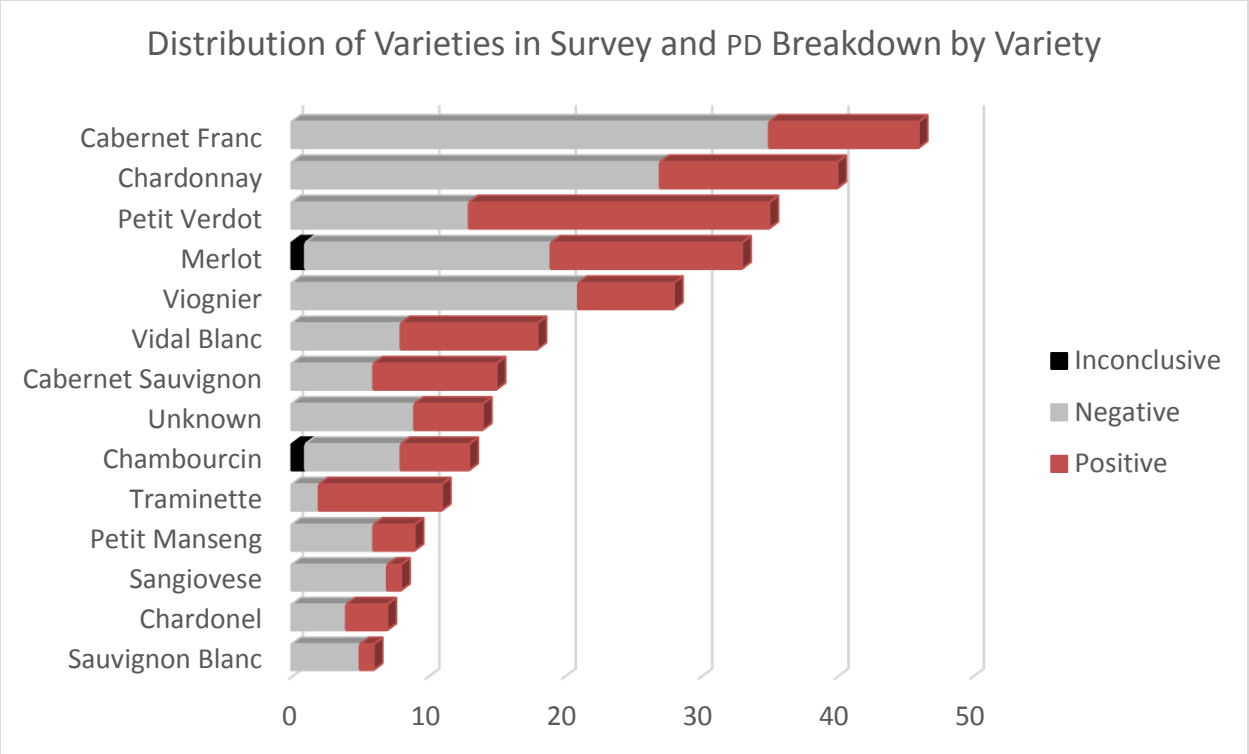


Fig. 6. Overall breakdown of survey samples (341 samples tested) for Pierce's Disease (PD).

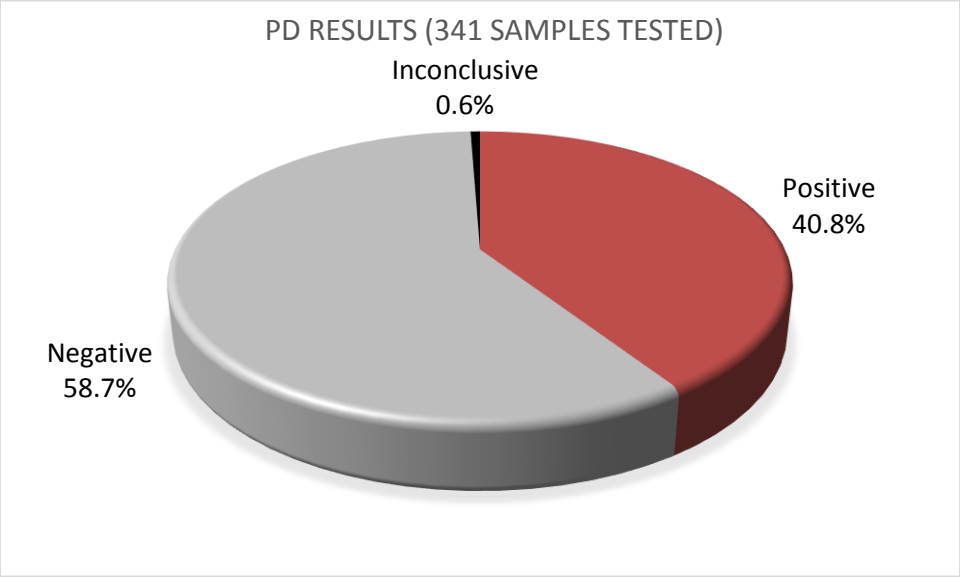
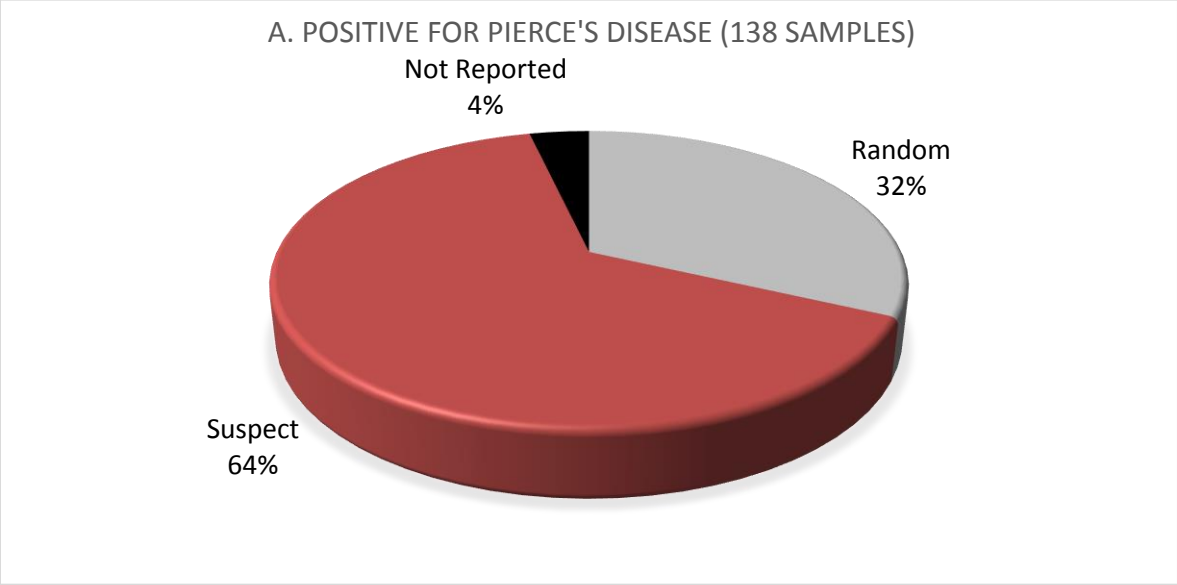
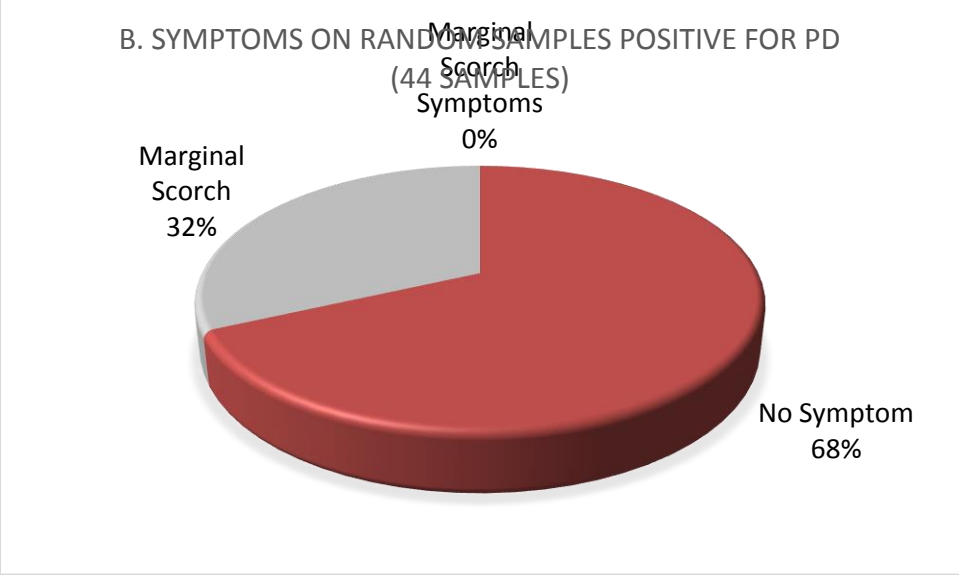
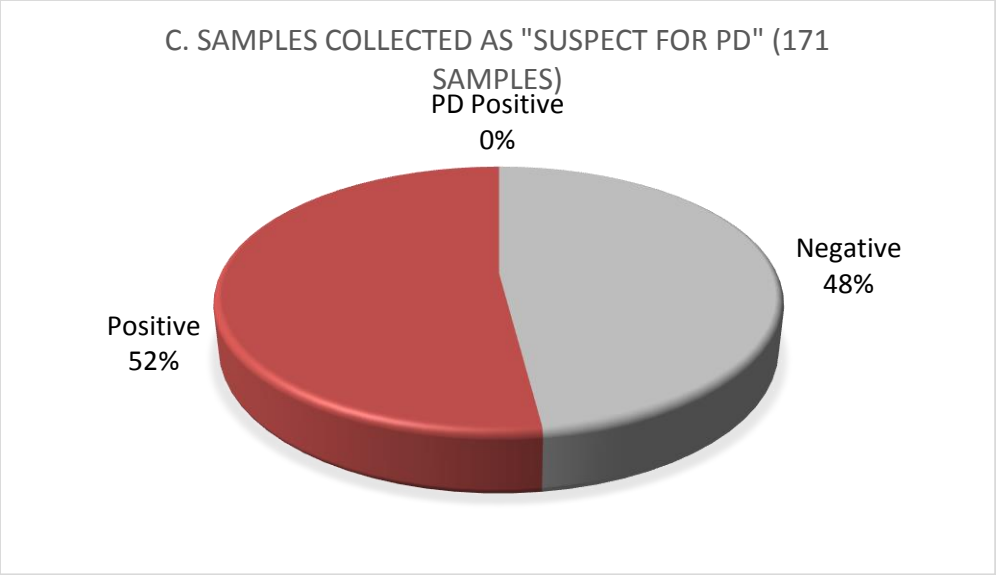


Fig. 7a, 7b and 7c. Breakdown of positive for PD samples among those collected as random samples and those collected as suspected (i.e. symptomatic) for PD. Thirty-two percent of PD positives were samples collected as random samples and 64% were suspected (i.e. symptomatic for PD); 4% of the survey samples were not reported as suspect or random (A). A breakdown of the samples collected randomly and testing positive for PD (44 samples) reveals that 68% showed no symptoms, yet tested positive for PD (B). Of the 171 samples collected as "suspect" for PD (i.e. symptomatic) 52% tested positive for PD and 48% tested negative for PD (C).






C. SAMPLES COLLECTED AS "SUSPECT FOR PD" (171 SAMPLES)
PD Positive
0%
Negative
48%
Positive
52%

Objective 2) Plant a trial of PD-resistant varieties at an experimental vineyard in SPAREC to evaluate their performance in VA

Four varieties (Blanc du Bois, Lenoir, Lomanto and one that is currently to be determined) were tested for PD using the multiplex qPCR test at the end of the growing season.

An experimental vineyard is located at the Southern Piedmont AREC in Blackstone, VA. It was originally planted as a variety trial; however, due to an outbreak of PD, there are a total of 14 empty panels, each with a capacity for 4-5 vines. We replanted these panels with PD-resistant vines to examine their performance under VA growing conditions. Varieties in the Table were selected based on PD and other disease resistance, wine quality and winter hardiness characteristics.

Table 1. PD-resistant varieties suitable for wine production under VA growing conditions

Name	Disease resistance	Wine characteristics
Blanc Du Bois	PD, downy mildew	White, can be dry or sweet with citrus and apple flavor
Lenoir (Black Spanish)	PD	Red, dry with plum and blackberry flavor, also good as a table grape
Lomanto	PD, mildews (not specified)	Red, dry or off-dry wine with black cherry flavor

Following the original design of the vineyard, these vines were planted in a completely randomized design with five replications, with each panel representing a replication. Visual disease assessment for PD and other common grape diseases (e.g. downy mildew, powdery mildew, black rot, and Phomopsis cane and leaf spot) was conducted at mid-season (2-3 weeks after bloom), and at the end of the season (1-2 weeks prior to harvest). In addition, a sample that consists of 20 random leaf and petiole tissues were collected from basal nodes of canes on each vine at the end of the season, and processed for qPCR-detection of *Xf*. The disease assessment data was analyzed using a linear mixed model where varieties will be treated as a fixed effect and replications will be treated as a random effect.

Results

In 2013, there was an outbreak of downy mildew at the tested location. Our preliminary data showed that both Lenoir and Lomanto were susceptible to down mildew. Detection of *Xf* resulted with positives in all varieties, indicating these varieties are infected with PD pathogen. Note: the infection by *Xf* on these varieties has been discussed in the past, and we are confirming the suspicion. Thus, when a grower is planning to plant PD resistant varieties, it is recommended to plant far apart from other varieties.

Objective 3) Use qPCR to monitor the movement of *Xf* within an infected vine over the course of a season

We have identified seventeen panels that consist of ten varieties at Blackstone vineyard that have been showing symptoms of PD in the past three consecutive years. We have selected four varieties (Cabernet Franc, Petit Manseng, Vidal Blanc, and Viognier) that are commonly grown in VA. We selected two vines from each variety, and petiole samples were collected at four stages of grape development: 1) pre-bloom (3-4 weeks after bud break); 2) at fruit set (2-3 weeks after bloom); 3) at veraison; and 4) near harvest (1-2 weeks prior to harvest). These vines were trained with a vertical shoot positioning system with two trunks (cordons), and samples were taken from four locations per cordon (lower center, lower end, upper center, upper end). Each sample consisted of 20 petioles randomly taken from the target area, and samples were shipped overnight to the VT Plant Disease Clinic for processing and qPCR analysis.

Results

Preliminary results showed that 1) we were able to detect *Xf* at a few weeks after bud break; however, the titer did not increase until very late in the season (i.e., we were not able to detect afterwards until the near-harvest sampling time) and 2) we have detect *Xf* more in upper part of the canopy. These findings may indicate 1) transmission of Xf within a vineyard may not be common since vectors (Sharpeshooters) tend to come in to vineyards in early summer in VA, and 2) if there is a vector that comes in late in the season, and feed on upper part of the canopy, it may spread *Xf*, but probably it is not a major mean of spread since *Xf* require a certain amount of time to spread within a vine.

Objective 4) Technology transfer plan: Develop new extension materials for PD in VA, as well as a standard PD protocol for the VT Plant Disease Clinic

We plan to publish results from objectives 1-3 as a summary in a new Extension factsheet when the study is complete. In addition, results will be used to develop new recommendations for PD management that are tailored to VA wine grape growers. The results will be presented at grower-oriented meetings (VVA meetings, vineyard meetings, etc), scientific conferences (APS conferences), and published in a peer-reviewed journal, such as Plant Disease. In addition, this project helps us to develop a standard PD protocol for the VT Plant Disease Clinic so that in the future, the PD samples can be processed with confidence.

Objective 4 Results

We have identified a grape DNA extraction protocol, which uses a commercially available extraction kit, that successfully eliminates inhibitors that interfere with PCR reactions, which are problematic with grape tissue. We have developed a very robust and sensitive multiplex qPCR protocol that uses an endogenous grape gene as an internal control to confirm the qPCR test is working correctly and, thus, provides very conclusive results and eliminates the chance for false negative detection results. The *Xf* detection protocol will be useful not only to the Plant Disease Clinic, but for diagnosticians and the grape research community at large. We have presented preliminary results at two conferences: The Cumberland-Shenandoah Fruit Workers Conference, Dec. 5-6, 2013, Berryville, VA and the Virginia Vineyards Association Winter Technical & Trade Show Meeting, Jan. 31, 2014, Charlottesville, VA.

II. Problems and Delays

More time and expenses were consumed on identifying a satisfactory extraction method than anticipated. Some of our vines in the variety trial did not take off due to a combination of lack of water and downy mildew. We are planning to replace these vines in 2014 season. Monitoring of *Xf* titer in infected vines produced very interesting results, but it needs to be further investigated.

III. Future Project Plans

Objective 1: Complete survey samples testing (155 of 396 samples remain to be tested). There are also approximately 60 other samples that were collected that we will also test for PD. Complete tests for the remaining approximately 250 previously extracted grape petiole samples from the previous grape virus survey work.
Objective 2: We will replace vines for the variety trial in 2014 season.
Objective 3: More detailed monitoring may be needed in 2014 season.
Objective 4: We plan to publish results from objectives 1-3 as a summary in a new Extension factsheet when the study is complete. In addition, results will be used to develop new recommendations for PD management that are tailored to VA wine grape growers. The results will be presented at grower-oriented meetings (VVA meetings, vineyard meetings, etc), scientific conferences (APS conferences), and published in a peer-reviewed journal, such as Plant Disease.

IV. Funding Expended To Date

We have utilized all the original allocation as of 28 January 2014. We have requested additional funds in January 2014, for sequencing of some of *Xf* we found in our survey to determine whether there is regional variations among our *Xf* isolates.

1. Wells et al. *Xylella fastidiosa* subsp. *fastidiosa* (ATCC® 700964D-5™).
2. Anas, O., Harrison, U. J., Brannen, P. M., and Sutton. 2008. The effect of warming winter temperatures on the severity of Pierce's disease in the Appalachian mountains and Piedmont of the southeastern United States. Plant Health Progress.
3. Doddapaneni, H., Yao, J. Q., Lin, H., Walker, M. A., and Civerolo, E. L. 2006. Analysis of the genome-wide variations among multiple strains of the plant pathogenic bacterium Xylella fastidiosa. BMC Genomics 7:(01 September 2006).
4. Harper, S. J., Ward, L. I., and Clover, G. R. 2010. Development of LAMP and real-time PCR methods for the rapid detection of Xylella fastidiosa for quarantine and field applications. Phytopathology 100:1282-1288.
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6. Wallingford, A. K., Tolin, S. A., Myers, A. L., Wolf, T. K., and Pfeiffer, D. G. 2007. Expansion of the Range of Pierce's disease in Virginia. Online. Plant Health Progress DOI 10.