Plant Pathology (2019) Doi: 10.1111/ppa.13027

Assessment of Pierce's disease susceptibility in *Vitis vinifera* cultivars with different pedigrees

E. Deyett[a†], J. Pouzoulet[b†], J.-I. Yang[c], V. E. Ashworth[a], C. Castro[d], M. C. Roper[d] and P. E. Rolshausen[a*]

[a]*Department of Botany and Plant Sciences, University of California Riverside, 900 University Ave, Riverside, CA 92521, USA;* [b]*Unité Mixte de Recherche, 1287 Ecophysiologie et Génomique Fonctionnelle de la Vigne (EGFV), INRA, Institut des Sciences de la Vigne et du Vin, 33883 Villenave d'Ornon, France;* [c]*Department of Plant Pathology and Microbiology, National Taiwan University, Taipei 106, Taiwan; and* [d]*Department of Microbiology and Plant Pathology, University of California Riverside, 900 University Ave, Riverside, CA 92521, USA*

Pierce's disease (PD) of grapevine is caused by the bacterium *Xylella fastidiosa*. In this study, an integrated approach was applied to assess PD susceptibility among different *Vitis vinifera* cultivars that incorporated disease severity, bacterial pathogen abundance and loss of stem xylem hydraulic conductivity. It was hypothesized that levels of PD susceptibility in *V. vinifera* can be attributed in part to the host anatomical features that are shaped by its pedigree background. Two popular wine grape cultivars were initially selected from the *occidentalis* group, Merlot and Cabernet Sauvignon, and one from the *orientalis* group, Thompson Seedless. The more recently bred table grape cultivar Scarlet Royal, that has mixed pedigree parentage, was also included. PD susceptibility was compared to the known PD resistant b43-17 *V. arizonical candicans* wild grape species from North America. The data indicated that Thompson Seedless was ranked as the most susceptible to PD because it significantly exhibited the most severe disease symptoms at 12 weeks post-inoculation and hosted the highest *X. fastidiosa* titre of the cultivars, and lost over 90% of its stem hydraulic conductivity. In contrast, the other three cultivars displayed less susceptibility to PD. The way in which the xylem anatomy could impact PD susceptibility in *V. vinifera* cultivars is discussed, together with how grape pedigrees and their cognate centre of domestication may have influenced xylem anatomical features. This work provides a reference framework to further test the hypothesis that *V. vinifera* cultivars with wide xylem vessels may be more prone to PD decline.

Keywords: grapevine, host resistance, host susceptibility, Pierce's disease, *Vitis vinifera*, *Xylella fastidiosa*

Introduction

Pierce's disease (PD) is a severe vascular disease of grapevine caused by the Gram-negative bacterium *Xylella fastidiosa* (Wells *et al.*, 1987) and costs $104 million in annual losses to the Californian grape industry (Tumber *et al.*, 2014). This disease was first described in California vineyards in the late 19th century by Newton Pierce (Pierce, 1892) and its bacterial causal agent probably originated from Central America (Nunney *et al.*, 2010). The pathogen is transmitted by a number of xylem-feeding insects (Redak *et al.*, 2004). The recent introduction of the invasive glassy-winged sharpshooter (GWSS) to southern California altered the epidemiology of PD and increased disease incidence to epidemic proportions (Purcell & Saunders, 1999).

Xylella fastidiosa is limited to the xylem tissue of its plant host and migrates both acropetally and basipetally through the xylem, in part by using type IV pili-mediated motility (Meng *et al.*, 2005). The xylem network is a collection of xylem vessels that are interconnected both horizontally and vertically by pit membranes, which are composed of primary plant cell wall. The pathogen subsequently spreads to new connected vessels by breaching the pit membrane barrier in a cell wall-degrading enzyme-dependent manner (Perez-Donoso *et al.*, 2010). The bacteria can multiply to high population numbers in susceptible hosts and these bacterial aggregates and biofilms can occlude xylem vessels (De La Fuente *et al.*, 2008). In addition, there are several plant-derived vascular occlusions (i.e. tyloses, gums and pectin gels) that occur in infected vines that further impede plant hydraulic conductivity, with tyloses being the predominant vascular occlusion associated with PD (Sun *et al.*, 2013). At the whole plant level, PD symptomology manifests into scorched leaves, irregular periderm development, irregular petiole abscission, raisining of berries, canopy stunting and vine death (Varela *et al.*, 2001).

PD resistance has only been identified in several wild grape species endemic to the Americas including *V. arizonical/candicans* and *Muscadinia rotundifolia*, whereas

*E-mail: philrols@ucr.edu
†Authors contributed equally to the work.

all *V. vinifera* genotypes from Eurasia are susceptible to this disease (Fritschi *et al.*, 2007; Riaz *et al.*, 2018). Three major pedigree lineages have been recognized within the cultivated grapevine *Vitis vinifera*; *occidentalis*, the small-berried wine grapes of western Europe; *orientalis*, the large-berried table grapes of West Asia; and *pontica*, the intermediate type from the basin of the Black Sea and eastern Europe (Aradhya *et al.*, 2003). Field observations and greenhouse experiments indicated that there were varying degrees of susceptibility to PD among the genetic pool of cultivated grapevine (Purcell, 1974; Rashed *et al.*, 2011), but none of these studies linked PD susceptibility to *V. vinifera* cultivar pedigree. Because cultivated grapevines evolved under different climates and were domesticated under different practices, populations developed different anatomical traits and strategy regarding water transport, which is illustrated by striking differences in xylem morphology (Pouzoulet *et al.*, 2014, 2017a).

It is hypothesized here that domestication of grapevine and selection for specific phenotypic traits have shaped PD susceptibility levels in *V. vinifera*. This hypothesis was initially tested by selecting grape cultivars for which there was previous knowledge on wood anatomy and specifically vessel diameters (Pouzoulet *et al.*, 2014, 2017a). These included two wine grape cultivars from the *occidentalis* group, Merlot and Cabernet Sauvignon, and two table grape cultivars, Thompson Seedless (also known as Sultanina, an ancient cultivar used for breeding of many commercial seedless table grapes) from the *orientalis* group and Scarlet Royal, a recently released cultivar from the USDA breeding programme (2006) with mixed pedigree parentage that includes *orientalis* (e.g. Sultanina) and *pontica* (e.g. Muscat of Alexandria, Emperor). The susceptibility of these cultivars was compared to the PD resistant b43-17 *V. arizonica/candicans* grape genotype from North America. Host susceptibility was measured in *in planta* bioassays as a function of disease severity and *X. fastidiosa* abundance, and these data were integrated with hydraulic conductivity for selected *V. vinifera* cultivars. This work provides a benchmark for PD susceptibility levels for some of the most widely planted table and wine grape cultivars. It also warrants additional research efforts to test a greater number of *V. vinifera* cultivars from different genetic pools and collect information on their vascular anatomical features in order to validate the stated hypothesis. Ultimately, this research may aid in the development of tools to assess and predict PD susceptibility for cultivated grapevine cultivars and facilitate recommendations to industry stakeholders with regards to the planting of PD-tolerant grape cultivars when confronted with high PD pressure.

Materials and methods

In planta evaluation of PD susceptibility

The four commercial *V. vinifera* cultivars used in this study were obtained from the Foundation Plant Service at UC Davis, and included two wine grapes (Merlot selection #06 and Cabernet Sauvignon selection #31) and two table grapes (Scarlet Royal selection #01 and Thompson Seedless selection #02A). These cultivars were selected because of their different pedigrees (Aradhya *et al.*, 2003) and also because a substantial amount of data had been accumulated on plant vascular anatomy as it relates to susceptibility to another xylem-dwelling fungal pathogen, *Phaeomoniella chlamydospora* (Pouzoulet *et al.*, 2014, 2017a). The PD-resistant b43-17 *V. arizonica/candicans* type (Krivanek *et al.*, 2006), a wild PD-resistant grapevine found in North America, was also included as a reference. Cuttings of *V. arizonica/candicans* cuttings were kindly provided by A. Walker (Department of Viticulture and Enology, UC Davis, California, USA).

One-bud cuttings were rooted and potted into a mixture of soil and sand (1:1) amended with 5 g of controlled release fertilizer (Scotts Osmocote Classic, N-P-K: 14-14-14). A single shoot per grape cutting was trained vertically onto a stake. Inoculations were performed as follows: *X. fastidiosa* subsp. *fastidiosa* strain Temecula 1 was cultured on PD3 solid medium at 28 °C for 7–10 days. Cells were harvested from PD3 plates and suspended in 1× phosphate-buffered saline (PBS) after which cell suspensions were adjusted to $OD_{600\ nm} = 0.25$ (approximately 10^8 CFU mL^{-1}). Plants were inoculated on both sides of the stem with 10 μL of cell suspension of *X. fastidiosa* between the first and second nodes at the base of the shoot by mechanical needle inoculation as previously described (Hill & Purcell, 1995). Control plants were needle-inoculated with 10 μL PBS. Twenty-four plants were used per genotype and half inoculated with *X. fastidiosa* and the other half with PBS. Thus, a total of 120 plants were used for each replicate. The experiment was replicated twice in two separate years. For each trial, all plants were kept under the same greenhouse conditions with the same watering regime over the 12-week incubation period. All vines were visually rated by the same scientist at 12 weeks post-inoculation on a PD symptom severity scale of 0–5 (Fig. 1) adapted from Guilhabert & Kirkpatrick (2005).

Detection and quantification of *X. fastidiosa in planta*

Three petioles were harvested per vine and pooled to quantify *X. fastidiosa* abundance *in planta*, starting at the first leaf with PD symptoms sampling downward. When no PD symptoms were observed (i.e. in the negative controls) the three petioles were harvested randomly and pooled. Petioles were kept at −20 °C until further processing for quantitative PCR (qPCR) analysis. The frozen petioles from each treatment were lyophilized for 36 h with a FreeZone 2.5 L benchtop freeze dry system (Labconco). Samples were then finely ground at room temperature using a MM300 grinder (Retsch; 45 s, 25 oscillations per second) in a 35 mL stainless-steel grinding jar (Retsch) with 20 mm stainless steel balls. Ground tissue was incubated at 65 °C for 10 min in a heat block with hexadecyltrimethylammonium bromide (CTAB; Sigma-Aldrich) extraction buffer. Total DNA was extracted from the 100 mg petiole tissue using a DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's instructions, with the exception of using CTAB extraction buffer as the lysing buffer. The DNA for all samples was stored at −20 °C before use. DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) according to the manufacturer's protocol.

A set of primers targeting a 90 bp fragment of the internal transcribed spacer (ITS) region of the ribosomal rRNA genes of *X. fastidiosa* was developed using PRISE 2 software (Huang *et al.*, 2014). *Xylella fastidiosa* has two copies of the ribosomal

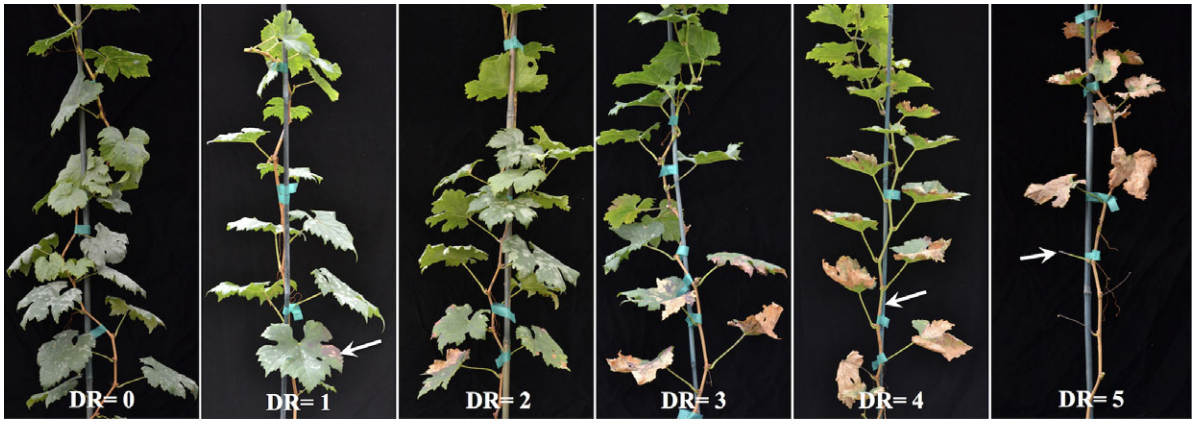

Figure 1 Disease rating (DR) scale used to evaluate Pierce's disease (PD) severity. 0 = no disease; 1 = 1–2 leaves showing initial PD symptoms with leaf scorching (white arrow); 2 = 3–4 leaves showing PD symptoms; 3 = <50% of the leaves expressing PD symptoms; 4 = >50% of the vines expressing PD symptoms including formation of green islands (white arrow); 5 = vines are dead or dying showing matchstick petioles (white arrow).

operon (Simpson *et al.*, 2000). The designed primers were XfITSF6 (5′-GAGTATGGTGAATATAATTGTC-3′) and XfITSR6 (5′-CAACATAAACCCAAACCTAT-3′). Amplification reactions were performed using a CFX96 Real-Time PCR cycler and CFX MANAGER v. 3.1 software (Bio-Rad) using default settings for amplification curve analysis. Reaction conditions (primer concentrations, annealing temperature) were optimized for amplification selectivity and PCR efficiency according to the MIQE guidelines (Bustin *et al.*, 2009). Reactions proceeded in a final volume of 25 µL and reaction mixtures contained 12.5 µL of Quantitect SYBR Green Master Mix reagent (QIAGEN), 0.4 µM of each primer, and 2 µL of DNA template. The cycling programme consisted of 95 °C for 15 min; 40 cycles of 55 °C for 15 s, 72 °C for 45 s, 95 °C for 15 s; and a melt curve analysis of 65–95 °C (0.5 °C increments every 5 s) to verify the amplicons' identity. In all qPCR plates, a standard curve was generated from a DNA solution from *X. fastidiosa* cultured on PD3 solid medium. Limit of detection and limit of quantification was determined by running absolute quantities of *X. fastidiosa* from pure cultures as previously described by Pouzoulet *et al.* (2017b), using *X. fastidiosa* DNA amounts ranging from 4.2 ng to 1.7 fg. Specificity of the qPCR method was also validated against several bacterial strains. This included the grapevine strain, *X. fastidiosa* subsp. *fastidiosa* Temecula 1; the almond strain, *X. fastidiosa* subsp. *multiplex* M12 (provided by Dr Bruce Kirkpatrick, UC Davis); and the oleander strain, *X. fastidiosa* subsp. *sandyi* Ann-1 (provided by Dr Donald Cooksey, UC Riverside). All three *X. fastidiosa* strains were cultured on PD3 solid medium at 28 °C for 7–10 days prior to DNA extraction. A closely related organism to *X. fastidiosa*, *Xanthomonas campestris* pv. *campestris* 0198-18 (provided by Dr Donald Cooksey, UC Riverside), was also included. In addition, several phylogenetically diverse bacteria were tested (*Bacillus megaterium*, *Pantoea agglomerans*, *Pseudomonas syringae*, *Agrobacterium tumefaciens* and *Pseudomonas fluorescens*) representing taxonomic groups of bacteria associated with grapevines (Deyett *et al.*, 2017). *Xanthomonas campestris* was cultured on yeast extract-dextrose-$CaCO_3$ agar (YDC) at 28 °C for 2 days before DNA extraction. All the other bacterial strains were propagated on Luria-Bertani (LB) medium at 28 °C for up to 2 days before DNA extraction. Absolute quantification of *X. fastidiosa* DNA by qPCR from total DNA extracted from plant samples was done by using standard DNA solution from *X. fastidiosa* cultured on PD3 solid medium. Nontemplate controls were included in each plate and each sample was tested in triplicate. For each reaction, the identity of the amplicon was verified through its melt-curve profile. The average quantity (fg of *X. fastidiosa* DNA) of the three replicates was standardized by the amount of total DNA input, and used for further statistical analysis.

Measurement of stem hydraulic conductivity

Hydraulic conductivity was determined on three of the total five grape genotypes that displayed contrasting symptom severity and included b43-17 *V. arizonica/candicans* (resistant), Thompson Seedless (highly susceptible) and Merlot (moderately susceptible). At the end of the experiment (12 weeks post-inoculation), following the recording of disease ratings, 10 plants were sampled for each grape genotype (30 samples in total), with five for the PBS control vines and five for the *X. fastidiosa*-inoculated vines. The day before shoot sampling, plants were watered to saturation to assure maximum stem hydration. Petioles were cut with a safety razor blade and wounds were sealed immediately with cyanocrylate glue (Gorilla Glue Company). Subsequently, shoots were cut at the basal end and immediately immersed in water. The apical part of the stem above the eighth node was cut off under water to avoid embolism. Each stem fragment that was further used to measure hydraulic conductivity was cut under water to 15 cm in length from the basal end. Stem hydraulic conductivity (K_h; kg m^{-1} s^{-1} MPa^{-1}) was determined by connecting the 15 cm long stem to a tubing system with filtered (0.2 µm), degassed 20 mM KCl solution flowing from an elevated source, through the stem, and into a reservoir on a balance (±0.1 mg; Denver Instrument P-214; Sartorius) that was interfaced with a computer to record flow rate, allowing the calculation of conductivity (Sperry & Tyree, 1988). The system was corrected for stem passive water uptake by beginning and ending each conductivity measurement with a background measurement (Torres-Ruiz *et al.*, 2012). Stem hydraulic conductivity (K_h) was calculated as $K_h = F \times L/dP$, where F is the flow rate (kg s^{-1}), L is the stem length (m), and dP is the driving force (MPa). The head pressure was 1.2 kPa. Immediately after K_h

was measured, the stem fragment was infiltrated with a solution of safranine O (0.1%) until the dye was allowed to cross along the stem fragment (appearance of a red cloud at the tip of the fragment being placed under water). Then the excess dye was flushed by dH_2O until no more stain efflux could be seen. Seventy micrometre-thick cross-sections of the middle part of the stem were sliced with a microtome, and micrographs of the specimens were obtained as previously described (Pouzoulet *et al.*, 2017a). Trans-sectional xylem area was determined with a stereomicroscope (M165C, Leica) and LAS v. 4.2 software (Leica) and was used to calculate the sapwood-specific hydraulic conductivity (K_s; kg m^{-1} s^{-1} MPa^{-1}). Vessel diameter data were obtained as described by Pouzoulet *et al.* (2014). In parallel, high contrast micrographs showing the vessel infiltrated with dye were obtained by subtracting the green from the blue filter using IMAGEJ v. 1.48 (https://imagej.nih.gov/). Diameter data from stained vessels were collected and used to calculate theoretical stem conductivity (K_{th}) using the Hagen–Poisseuille equation as described by Santiago *et al.* (2004).

Statistical analysis

Statistical analyses were conducted using R v. 3.4.2 (http://www.R-project.org/). Kruskal–Wallis rank sum statistical testing was used to determine statistical differences between PD rating, *X. fastidiosa* quantity, hydraulic conductivity and grapevine genotypes. A pairwise Wilcoxon test was used to calculate the pairwise statistical differences between the five grapevine genotypes and the visual PD ratings, hydraulic conductivity or *X. fastidiosa* quantities. *P*-values were corrected with the false discovery rate method.

Results

To validate the *X. fastidiosa* detection assay, primers were evaluated for specificity and reproducibility using real-time qPCR SYBR Green technology. Primers consistently showed robust efficiencies and R^2 values as defined by MIQE guidelines (Bustin *et al.*, 2009). The assay was able to accurately quantify *X. fastidiosa* DNA amounts ranging from 4.2 ng to 8.4 fg, and the limit of detection (LOD) of the assays was 16.8 fg of *X. fastidiosa* DNA (Fig. S1a). The *X. fastidiosa* genome size is 2.68 Mb and has two copies of the ribosomal operon (Simpson *et al.*, 2000). Considering that the average molecular weight of a base pair is 650 Da, the LOD of this assay represents approximately six *X. fastidiosa* genomes, and 12 copies of the target DNA sequence. Melt curve profiles indicated all amplicons had a single melting peak at 75.2 ± 2 °C, further verifying specific binding to the primers, and the presence of other by-products, such as primer dimers, were not observed through melt curve analysis including from plant samples (Fig. S1b). Amplicons were not detected for closely related and off-target bacteria that commonly associated with grapevines. The assay could not segregate the three *X. fastidiosa* subspecies tested from one another (data not shown), indicating that this qPCR was not specific to *X. fastidiosa* subsp. *fastidiosa*.

Vitis vinifera vines inoculated with *X. fastidiosa* exhibited characteristic PD symptoms that initiated with scorching of the basal leaves, close to the point of inoculation (Fig. 1). As the disease severity progressed, leaf scorching expanded to the apical part of the vine with appearance of matchstick petioles (i.e. leaf drop with only petiole attached) and uneven wood lignification causing formation of green islands. Nonparametric Kruskal–Wallis testing detected a significant effect with grape cultivar ($P < 0.0001$) and trial ($P < 0.01$). Post hoc analyses confirmed that b43-17 *V. arizonica/candicans* was resistant to PD with very few isolated leaves developing typical symptoms. Among the *V. vinifera* cultivars tested, Thompson Seedless was significantly more susceptible to PD than the other three cultivars (Fig. 2). The PBS-inoculated negative controls did not develop typical PD symptoms.

The results of the PD ratings were further supported by quantification of *X. fastidiosa* titre by qPCR (Fig. 3). *Xylella fastidiosa* was not detected in any of the PBS-inoculated grape genotypes. Kruskal–Wallis nonparametric testing detected a significant effect of grape cultivar ($P < 0.0001$) and trial ($P < 0.0001$). The PD-resistant b43-17 *V. arizonica/candicans* displayed the lowest amount of pathogen DNA. Among the *V. vinifera* cultivars, Thompson Seedless significantly harboured the highest *X. fastidiosa* DNA amount but no significant statistical differences were found between the cultivars Merlot, Scarlet Royal and Cabernet Sauvignon.

No significant effect of *X. fastidiosa* inoculation on hydraulic conductivity (K_s) was measured for the resistant b43-17 *V. arizonica/candicans* in comparison to the cognate PBS control (Fig. 4). In contrast, a significant decrease in K_s was measured in the *X. fastidiosa*-infected Merlot and Thompson Seedless with a reduction of 62%

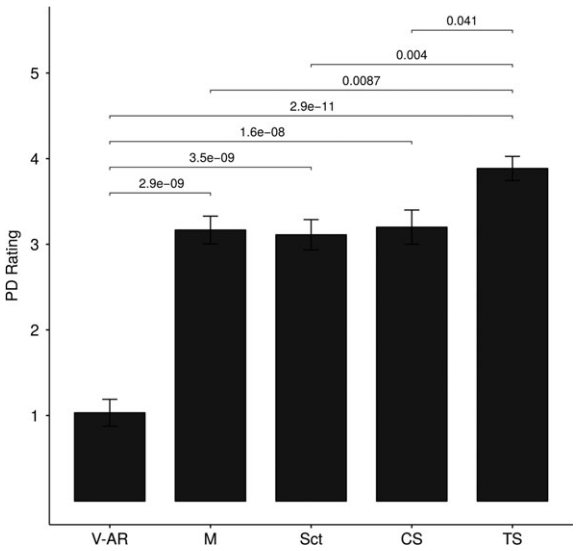

Figure 2 Mean disease rating for individual grape genotypes 12 weeks post-inoculation with *Xylella fastidiosa*. V-AR, b43-17 *Vitis arizonica/candicans*; M, *Vitis vinifera* 'Merlot'; Sct, *V. vinifera* 'Scarlet Royal'; CS, *V. vinifera* 'Cabernet Sauvignon'; TS, *V. vinifera* 'Thompson Seedless'. Bars represent standard errors.

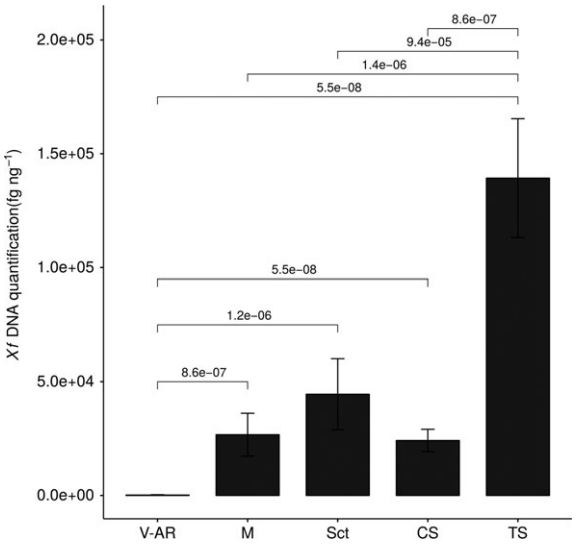

Figure 3 Mean *Xylella fastidiosa* quantification (*X. fastidiosa* fg DNA per ng of plant DNA) in petioles of individual grape genotypes 12 weeks post-inoculation. V-AR, b43-17 *Vitis arizonica/candicans*; M, *Vitis vinifera* 'Merlot'; Sct, *V. vinifera* 'Scarlet Royal'; CS, *V. vinifera* 'Cabernet Sauvignon'; TS, *V. vinifera* 'Thompson Seedless'. Bars represent standard errors.

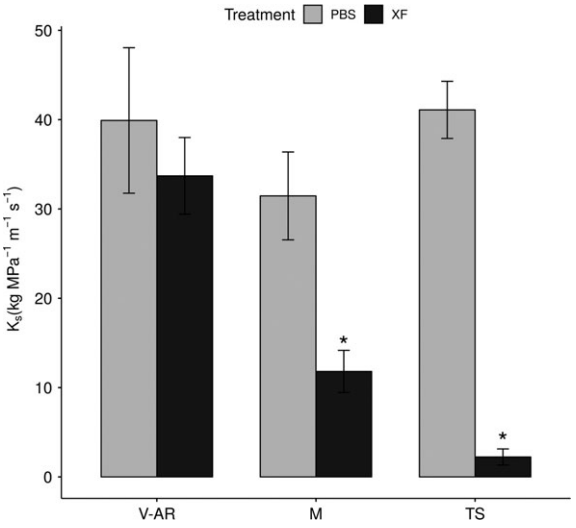

Figure 4 Specific stem hydraulic conductivity K_s (kg MPa^{-1} m^{-1} s^{-1}) measured for three grapevine genotypes, b43-17 *Vitis arizonica/candicans* (V-AR), *Vitis vinifera* 'Merlot' (M) and 'Thompson Seedless' (TS), 12 weeks post-inoculation with phosphate-buffered saline (PBS) and *Xylella fastidiosa* (XF). * indicates statistically significant differences ($P < 0.05$; $n = 30$).

and 94%, respectively. Thompson Seedless was also the most susceptible based on disease rating (Fig. 2) and harboured the highest levels of *X. fastidiosa* (Fig. 3). K_s values were not significantly different across the three genotypes following PBS inoculation. To further assess the effect of *X. fastidiosa* on stem hydraulic conductivity, the functional vessels within stem samples were visualized following a dye vacuum infiltration method (Fig. 5). The results confirmed that xylem vessel function was compromised in grapevines with strong PD symptoms, as a significant decrease in staining of conductive xylem vessels was observed, probably due to vascular occlusion. Finally, significant correlations ($P < 0.05$) were found between predicted (K_{th}) and measured (K_s) values of water conductivity (Fig. 6), supporting the conclusion that differential loss in K_s of genotype was driven by the number of vessels remaining functional following *X. fastidiosa* establishment.

The data suggested that the progression of PD symptoms as observed in the *in planta* bioassay (Fig. 1) from DR 2 (3–4 leaves showing PD symptoms) to DR 3 (<50% of leaves with PD symptoms) was the critical point for both *X. fastidiosa* titre and hydraulic conductivity (Fig. 7). Overall, *X. fastidiosa* abundance increased with appearance of disease symptoms but DR 3 was associated with the highest detectable *X. fastidiosa* DNA amount for all grapevine genotypes. Interestingly, the detection of *X. fastidiosa* DNA did not correlate with disease rating in a linear fashion because pathogen detection was compromised in plants with severe symptoms (with a DR of 4 and 5). A sharp decrease in hydraulic conductivity was measured as the disease progressed from a visual rating of 2 to 3 and the pathogen titre increased.

Discussion

PD is a multifaceted pathosystem and manifestation of disease in susceptible *Vitis* genotypes is associated with systemic movement of the bacterium and extensive occlusion of the vascular system. Breeding programmes focused on evaluating PD resistance of wild *Vitis* species and introgressing resistance into existing susceptible *V. vinifera* cultivars are well established (Krivanek & Walker, 2005; Fritschi *et al.*, 2007; Riaz *et al.*, 2018). However, experiments designed to comprehensively assess PD susceptibility in *V. vinifera* cultivars have been limited in scope. This study has used published protocols and developed new tools to fully evaluate *V. vinifera* cultivars with different pedigree backgrounds for PD susceptibility. The qPCR detection method amplified the three *X. fastidiosa* subspecies tested. *Xylella fastidiosa* subsp. *pauca* was not included in the validation assay because it is a quarantine pathogenic organism in the US. Other detection methods for *X. fastidiosa* have used TaqMan probes (Harper *et al.*, 2010) and ELISA (Krivanek & Walker, 2005) with a LOD of 10 copy numbers of DNA targeted sequence and about 10^4 CFU mL^{-1}, respectively. Initially a TaqMan probe was developed for the assay in the present study, but this was switched to SYBR Green technology because the sensitivity levels were comparable and the latter allowed processing of a large volume of samples at lower cost. Likewise, Luvisi *et al.* (2017) adapted the TaqMan primer sets developed by Harper *et al.* (2010) to a SYBR Green assay for screening *X. fastidiosa* subsp. *pauca* from olive samples

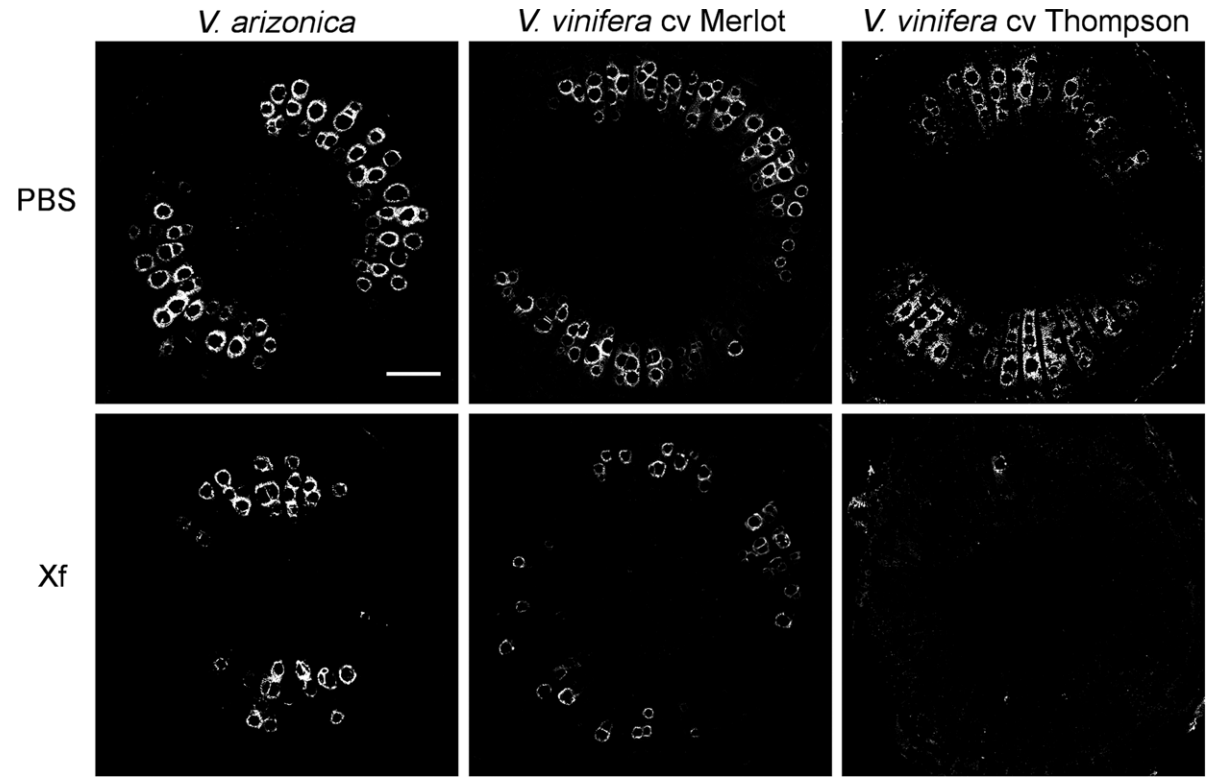

Figure 5 High contrast micrographs obtained following a dye injection method of three grapevine genotype stems, b43-17 *Vitis arizonica/candicans*, *Vitis vinifera* 'Merlot' and 'Thompson Seedless', 12 weeks post-inoculation with phosphate-buffered saline (PBS) and *Xylella fastidiosa* (Xf). Note the white conducting xylem vessels that become stained following dye vacuum infiltration. Scale bar 500 μm.

and showed similar sensitivity between the two methods. The present assay is quick, specific, more sensitive than an ELISA, and more cost-effective than TaqMan qPCR assays while displaying similar LODs (Harper *et al.*, 2010), deeming it a reliable and reproducible quantification method for *X. fastidiosa in planta*.

The results indicate that the bacterium moved systemically and achieved high population numbers in all *V. vinifera* cultivars, albeit at significantly different levels across *V. vinifera* cultivars. *Xylella fastidiosa* was also able to colonize b43-17 *V. arizonica/candicans* at a distance from the point of inoculation and the manifestation of PD symptoms was isolated to a few leaves. The bacterial population was low in b43-17 *V. arizonica/candicans* relative to the susceptible cultivars, which is similar to previous reports (Krivanek & Walker, 2005; Fritschi *et al.*, 2007). The classification of *V. vinifera* cultivar susceptibility based on *X. fastidiosa* DNA load was also supported by Rashed *et al.* (2011). Pathogen abundance strongly correlated with disease symptom severity, and based on those criteria it was established that Thompson Seedless was highly susceptible whereas Merlot, Cabernet Sauvignon and Scarlet Royal were moderately susceptible. The data also showed that severe PD symptoms do not correlate with high pathogen titre and supported the results of Gambetta *et al.* (2007). This suggests that the optimum time to quantify *X. fastidiosa in planta* and to show differences in disease susceptibility is when plants display mild to average symptom severity.

The basis of what dictates PD susceptibility versus PD tolerance/resistance is not well understood. PD resistance has only been identified in the pool of wild North American *Vitis* species, whereas all *V. vinifera* cultivars endemic to Eurasia are susceptible. In b43-17 *V. arizonica/candicans* the sequence analysis of the *PdR1* locus indicates that it contains putative candidate resistance genes belonging to the leucine-rich repeat resistance gene family (Riaz *et al.*, 2018). However, the biological function of the protein(s) encoded by *PdR1* has, to the authors' knowledge, not been characterized. In wild *Vitis* genotypes, host chemical, physical and anatomical traits appeared to be key determinants of the PD resistance. Xylem sap extracted from the PD-resistant *V. champinii* and *V. aestevalis* did not support viable *X. fastidiosa* populations when compared to PD-susceptible *V. vinifera*, suggesting that xylem sap chemistry could potentially affect bacterial viability *in planta* (Hao *et al.*, 2016). The xylem sap of *V. vinifera* also contains significantly higher soluble sugars and free amino acids than PD-tolerant *V. rotundifolia*, suggesting that *V. vinifera* xylem sap provides a more nutritionally favourable environment for *X. fastidiosa* to multiply and move systemically. In addition, plant defence-related proteins, β-1,3-glucanase, peroxidase and oxygen-evolving

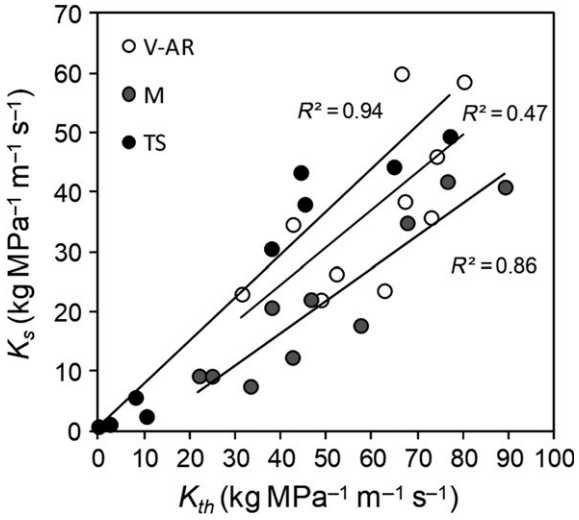

Figure 6 Relationship between theoretical specific hydraulic conductivity K_{th} (kg MPa^{-1} m^{-1} s^{-1}) determined via xylem morphological data coupled with dye injection experiment and specific hydraulic conductivity measured K_s (kg MPa^{-1} m^{-1} s^{-1}). Data represents measurements obtained from the three grapevine genotypes, b43-17 *Vitis arizonica/candicans* (V-AR), *Vitis vinifera* 'Merlot' (M) and 'Thompson Seedless' (TS), 12 weeks post-inoculation with phosphate-buffered saline and *Xylella fastidiosa*. A significant correlation was measured between K_{th} and K_s for b43-17 *V. arizonica/candicans* ($P < 0.05$, $n = 10$), *V. vinifera* 'Merlot' ($P < 0.001$, $n = 10$) and 'Thompson Seedless' ($P < 0.001$, $n = 10$).

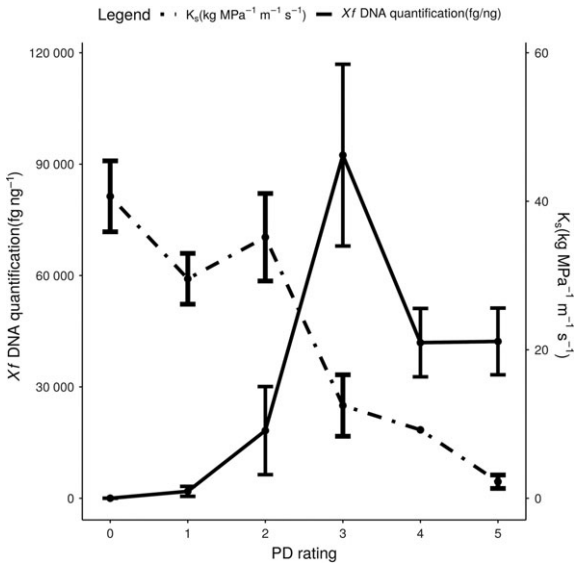

Figure 7 Relationship between Pierce's disease (PD) rating and *Xylella fastidiosa* DNA quantification (fg *Xylella fastidiosa* DNA per ng of plant DNA) and plant hydraulic conductivity measured as K_s (kg MPa^{-1} m^{-1} s^{-1}). Bars represent standard errors.

enhancer protein, were more abundant in the PD-resistant species, suggesting it can mount a more effective defence response to *X. fastidiosa* invasion (Basha *et al.*, 2010).

Pit membrane chemical composition is also hypothesized to influence pathogen movement *in planta* because the bacterium must breach this barrier in a cell wall-degrading enzyme-dependent manner to colonize new neighbouring xylem vessels. The susceptible *V. vinifera* 'Chardonnay' and 'Riesling' pit membranes have higher amounts of fucosylated xyloglucans and weakly methyl esterified homogalacturonan, whereas these ratios were inverted in the PD-resistant *V. arizonica* × *V. rupestris* and *V. vinifera* × *V. arizonica* (Sun *et al.*, 2011). It is reasonable to speculate that the chemical composition of the pit membranes in the b43-17 *V. arizonica/candicans* vines used in the present study share similar chemical characteristics with the related PD-resistant *V. arizonica* hybrids described by Sun *et al.* (2011), which may explain the restricted development of PD symptoms observed in b43-17 *V. arizonica/candicans*.

Xylem vessel occlusion via production of host-produced tyloses and gels in response to wounding or infection by xylem-dwelling pathogens is a conserved mechanism in plants to wall off compromised vessels (Beckman & Roberts, 1995; Pouzoulet *et al.*, 2017a). As the disease progresses, more vessels become occluded, which compromises plant hydraulic functions, and those functions are hindered even more under water deficit (Choi *et al.*, 2013). Sun *et al.* (2013) showed that over 60% of the vessels were occluded in the *X. fastidiosa*-infected *V. vinifera* 'Thompson Seedless' compared to less than 30% in different resistant genotypes *V. arizonica* × *V. rupestris* and *V. vinifera* × *V. arizonica*. The data mirror their results in that K_s was reduced by 62% and 94% in Merlot and Thompson Seedless, respectively, but b43-17 *V. arizonica/candicans* maintained hydraulic function following pathogen ingress.

Xylella fastidiosa moved systemically in all *V. vinifera* cultivars tested, yet differences in PD severity were significant among some of the cultivars. This suggests that factors independent of systemic movement influence the degrees of susceptibility observed across *V. vinifera* cultivars. *Vitis vinifera* 'Sylvaner' is described as PD tolerant and interestingly, has smaller vessel diameters than susceptible *V. vinifera* cultivars, indicating that the plant vascular system anatomy could be a determinant of level of PD susceptibility (Chatelet *et al.*, 2011). Vessels with larger diameters also harbour a greater number of tyloses, which may be linked to PD symptom severity (Sun *et al.*, 2013). Similar results were found for the fungal wilt disease of grapevine caused by the xylem-dwelling fungus *P. chlamydospora*, where grapevine cultivars with wider xylem vessel diameters were less efficient at restricting and compartmentalizing *P. chlamydospora* movement and that number of tyloses correlated with vessel size (Pouzoulet *et al.*, 2017a). The authors concluded that xylem vessel diameter influences timing of vessel occlusion, a key feature to compartmentalization in response to a xylem-dwelling pathogen, and that the density of xylem vessels above 120 μm correlated with increased disease susceptibility. Based on these criteria, the decreasing order of susceptibility to the fungal wilt

disease *P. chlamydospora* was classified as follows: Thompson Seedless, Cabernet Sauvignon and Merlot (Pouzoulet *et al.*, 2017a), which is comparable to the PD susceptibility levels presented in this study. From this, it is speculated that xylem vessel diameter size is a key determinant to effective defence against *X. fastidiosa* infection and cognate degree of susceptibility to PD in *V. vinifera*.

In conclusion, the cultivated table grape variety Thompson Seedless with the *orientalis* pedigree was highly susceptible to PD, whereas the *occidentalis* wine grape cultivars Merlot and Cabernet Sauvignon displayed lower susceptibility to the disease. Interestingly, the recently bred table grape cultivar Scarlet Royal showed similar PD susceptibility levels to Merlot and Cabernet Sauvignon, perhaps due to introgression of multiple genetic backgrounds with contrasting pedigrees. Future experiments should be conducted with an expanded set of cultivated *V. vinifera* cultivars from various pedigree lineages as well as its wild ancestor *V. sylvestris* in order to determine if traits of xylem morphology are a reliable marker to predict PD susceptibility. This could help breeding programmes with the selection of plant genetic materials with disease-tolerant traits. In addition, trials should evaluate how those phenotypes identified in a semicontrolled environment hold under field conditions when subjected to standard cultural practices (e.g. cultivars grafted on different rootstocks, irrigation regimes).

Acknowledgements

The authors would like to acknowledge the USDA National Institute of Food and Agriculture Hatch Projects 233883 for funding support. They would also like to thank the Foundation Plant Services at the University of California, Davis, and Dr Andrew Walker in the Department of Viticulture and Enology at the University of California, Davis, for providing the plant material used in this study.

References

Aradhya MK, Dangl GS, Prins BH *et al.*, 2003. Genetic structure and differentiation in cultivated grape, *Vitis vinifera* L. *Genetics Research* **81**, 179–92.

Basha SM, Mazhar H, Vasanthaiah HK, 2010. Proteomics approach to identify unique xylem sap proteins in Pierce's disease-tolerant *Vitis* species. *Applied Biochemistry and Biotechnology* **160**, 932–44.

Beckman CH, Roberts EM, 1995. On the nature and genetic basis for resistance and tolerance to fungal wilt diseases of plants. *Advances in Botanical Research* **21**, 35–77.

Bustin SA, Benes V, Garson JA *et al.*, 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55**, 611–22.

Chatelet DS, Wistrom CM, Purcell AH, Rost TL, Matthews MA, 2011. Xylem structure of four grape varieties and 12 alternative hosts to the xylem-limited bacterium *Xylella fastidiosa*. *Annals of Botany* **108**, 73–85.

Choi HK, Iandolino A, da Silva FG, Cook DR, 2013. Water deficit modulates the response of *Vitis vinifera* to the Pierce's disease pathogen *Xylella fastidiosa*. *Molecular Plant–Microbe Interactions* **26**, 643–57.

De La Fuente L, Burr TJ, Hoch HC, 2008. Autoaggregation of *Xylella fastidiosa* cells is influenced by type I and type IV pili. *Applied and Environmental Microbiology* **74**, 5579–82.

Deyett E, Roper MC, Ruegger P, Yang J, Borneman J, Rolshausen PE, 2017. Microbial landscape of the grapevine endosphere in the context of Pierce's disease. *Phytobiomes* **1**, 138–49.

Fritschi FB, Lin H, Walker MA, 2007. *Xylella fastidiosa* population dynamics in grapevine genotypes differing in susceptibility to Pierce's disease. *American Journal of Enology and Viticulture* **58**, 326–32.

Gambetta GA, Fei J, Rost TL, Matthews MA, 2007. Leaf scorch symptoms are not correlated with bacterial populations during Pierce's disease. *Journal of Experimental Botany* **58**, 4037–46.

Guilhabert MR, Kirkpatrick BC, 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. *Molecular Plant–Microbe Interactions* **18**, 856–68.

Hao LY, Zaini PA, Hoch HC, Burr TJ, Mowery P, 2016. Grape cultivar and sap culture conditions affect the development of *Xylella fastidiosa* phenotypes associated with Pierce's disease. *PLoS ONE* **11**, e0160978.

Harper SJ, Ward LI, Clover GRG, 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. *Phytopathology* **100**, 1282–8.

Hill BL, Purcell AH, 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and 4 other plants. *Phytopathology* **85**, 1368–72.

Huang YT, Yang JI, Chrobak M, Borneman J, 2014. PRISE2: Software for designing sequence-selective PCR primers and probes. *BMC Bioinformatics* **15**, 317.

Krivanek AF, Walker MA, 2005. *Vitis* resistance to Pierce's disease is characterized by differential *Xylella fastidiosa* populations in stems and leaves. *Phytopathology* **95**, 44–52.

Krivanek A, Riaz S, Walker MA, 2006. Identification and molecular mapping of *PdR1*, a primary resistance gene to Pierce's disease in *Vitis*. *Theoretical and Applied Genetics* **112**, 1125–31.

Luvisi A, Aprile A, Sabella E *et al.*, 2017. *Xylella fastidiosa* subsp. *pauca* (CoDiRO strain) infection in four olive (*Olea europaea* L.) cultivars: profile of phenolic compounds in leaves and progression of leaf scorch symptoms. *Phytopathologia Mediterranea* **56**, 259–73.

Meng YZ, Li YX, Galvani CD *et al.*, 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *Journal of Bacteriology* **187**, 5560–7.

Nunney L, Yuan XL, Bromley R *et al.*, 2010. Population genomic analysis of a bacterial plant pathogen: novel insight into the origin of Pierce's disease of grapevine in the US. *PLoS ONE* **5**, e15488.

Perez-Donoso AG, Sun Q, Roper MC, Greve LC, Kirkpatrick B, Labavitch JM, 2010. Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa*-infected grapevines. *Plant Physiology* **152**, 1748–59.

Pierce NB, 1892. The California vine disease. A preliminary report of investigations. *US Department of Agriculture. Division of Vegetable Pathology Bulletin 2*.

Pouzoulet J, Pivovaroff AL, Santiago LS, Rolshausen PE, 2014. Can vessel dimension explain tolerance toward fungal vascular wilt diseases in woody plants? Lessons from Dutch elm disease and esca disease in grapevine. *Frontiers in Plant Science* **5**, 253.

Pouzoulet J, Scudiero E, Schiavon M, Rolshausen PE, 2017a. Xylem vessel diameter affects the compartmentalization of the vascular pathogen *Phaeomoniella chlamydospora* in grapevine. *Frontiers in Plant Science* **8**, 1442.

Pouzoulet J, Rolshausen PE, Schiavon M *et al.*, 2017b. A method to detect and quantify *Eutypa lata* and *Diplodia seriata*-complex DNA in grapevine pruning wounds. *Plant Disease* **101**, 1470–80.

Purcell AH, 1974. Spatial patterns of Pierce's disease in Napa Valley. *American Journal of Enology and Viticulture* **25**, 162–7.

Purcell AH, Saunders SR, 1999. Glassy-winged sharpshooters expected to increase plant disease. *California Agriculture* **53**, 26–7.

Rashed A, Daugherty MP, Almeida RPP, 2011. Grapevine genotype susceptibility to *Xylella fastidiosa* does not predict vector transmission success. *Environmental Entomology* **40**, 1192–9.

Redak RA, Purcell AH, Lopes JRS, Blua MJ, Mizell RF, Andersen PC, 2004. The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. *Annual Review of Entomology* **49**, 243–70.

Riaz S, Huerta-Acosta K, Tenscher AC, Walker MA, 2018. Genetic characterization of *Vitis* germplasm collected from the southwestern US and Mexico to expedite Pierce's disease-resistance breeding. *Theoretical and Applied Genetics* **131**, 1589–602.

Santiago LS, Goldstein G, Meinzer FC *et al.*, 2004. Leaf photosynthetic traits scale with hydraulic conductivity and wood density in Panamanian forest canopy trees. *Oecologia* **140**, 543–50.

Simpson AJG, Reinach FC, Arruda P *et al.*, 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* **406**, 151–7.

Sperry JS, Tyree MT, 1988. Mechanism of water stress-induced xylem embolism. *Plant Physiology* **88**, 581–7.

Sun QA, Greve LC, Labavitch JM, 2011. Polysaccharide compositions of intervessel pit membranes contribute to Pierce's disease resistance of grapevines. *Plant Physiology* **155**, 1976–87.

Sun Q, Sun YL, Walker MA, Labavitch JM, 2013. Vascular occlusions in grapevines with Pierce's disease make disease symptom development worse. *Plant Physiology* **161**, 1529–41.

Torres-Ruiz JM, Sperry JS, Fernandez JE, 2012. Improving xylem hydraulic conductivity measurements by correcting the error caused by passive water uptake. *Physiologia Plantarum* **146**, 129–35.

Tumber KP, Alston JM, Fuller KB, 2014. Pierce's disease costs California $104 million per year. *California Agriculture* **68**, 20–9.

Varela LG, Smith RJ, Phillips PA, 2001. *Pierce's Disease. Agricultural and Natural Resources Publication 21600*. Oakland, CA, USA: University of California.

Wells JM, Raju BC, Hung HY, Weisburg WG, Mandelcopaul L, Brenner DJ, 1987. *Xylella fastidiosa* gen. nov., sp. nov. Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. *International Journal of Systematic Bacteriology* **37**, 136–43.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Performance of the *Xylella fastidiosa* qPCR assay. (a) The calibration curve displayed and associated PCR efficiency (i.e. 104.6%) using serial dilution of *X. fastidiosa* DNA. Decreasing amounts of bacterial DNA displayed are 4.2 ng, 420 pg, 42 pg, 4.2 pg, 420 fg, 84 fg, 16.8 fg, 8.4 fg and 1.7 fg. An amount of DNA was considered quantifiable when the standard deviation associated with its measurement was below 33% (SD Cq < 0.41, $n = 5$). Amounts of DNA for which the standard deviation was above 33% (SD Cq > 0.41, $n = 5$) are displayed as unquantifiable. The lowest limit of quantification was 84 fg *X. fastidiosa* DNA. The limit of detection (*), determined as the minimum amount of bacterial DNA being detected with a probability superior or equal to 95%, was 16.8 fg. (b) Example of melt profiles of amplification products observed with *X. fastidiosa* assay. Note the presence of a unique peak when *X. fastidiosa* DNA is used as template. Note the absence of amplification when nontemplate control (NTC) or host DNA are used as template. RFU, relative fluorescent unit.