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RESEARCH PAPER

Leaf scorch symptoms are not correlated with bacterial populations during Pierce's disease

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Abstract

***Xylella fastidiosa* (*Xf*) is a xylem-limited bacterium that lives as a harmless endophyte in most plant species but is pathogenic in several agriculturally important crops such as coffee, citrus, and grapevine (*Vitis vinifera* L.). In susceptible cultivars of grapevine, *Xf* infection results in leaf scorch, premature leaf senescence, and eventually vine death; a suite of symptoms collectively referred to as Pierce's disease. A qPCR assay was developed to determine bacterial concentrations *in planta* and these concentrations were related to the development of leaf-scorch symptoms. The concentration of *Xf* in leaves of experimental grapevines grown in the greenhouse was similar to the concentration of *Xf* in leaves of naturally infected plants in the field. The distribution of *Xf* was patchy within and among leaves. Some whole leaves exhibited severe leaf-scorch symptoms in the absence of high concentrations of *Xf*. Despite a highly sensitive assay and a range of *Xf* concentrations from 10^2 to 10^9 cells g^{-1} fresh weight, no clear relationship between bacterial population and symptom development during Pierce's disease was revealed. Thus, high and localized concentrations of *Xf* are not necessary for the formation of leaf-scorch symptoms. The results are interpreted as being consistent with an atiology that involves a systemic plant response.**

Key words: Bacterial wilt, disease resistance, pathogenesis, water deficits.

Introduction

A number of bacterial and fungal plant pathogens have evolved to inhabit plant xylem vessels. For example, species of the Gram-negative bacterium *Ralstonia* and fungi *Verticillium* and *Fusarium* cause what are commonly referred to as wilt diseases. *Xylella fastidiosa* (*Xf*), a xylem-limited Gram-negative bacterium, causes diseases such as coffee leaf scorch, citrus variegated chlorosis, almond leaf scorch, and Pierce's disease (PD) in grapevine (Hopkins and Purcell, 2002). For all of these organisms pathogenesis is, or was, thought to result from their accumulation within xylem vessels leading to vascular occlusion and water deficit. In all cases, this hypothesized mechanism of pathogenesis has been the subject of controversy, usually coming into conflict with the hypothesis that disease symptoms result instead from 'phytotoxins' acting directly on plant tissue or eliciting a plant response. In reference to *Fusarium*, Beckman (1987) referred to this as 'the great debate'.

During *Xf* pathogenesis, vascular occlusion is primarily attributed to *Xf* bacteria and their associated gums (reviewed in Hopkins, 1989; Purcell and Hopkins, 1996). In general, the vascular occlusion hypothesis predicts a positive correlation between symptom severity and pathogen concentrations. Earlier studies correlated high virulence of *Xf* strains, or high susceptibility of host plants, with high *Xf* populations at the point of inoculation (10^7–10^8 CFU g^{-1}) and *Xf* movement away from the point of inoculation (Hopkins, 1985*b*; Fry and Milholland, 1990; Hill and Purcell, 1995). In a comparative study of grape hybrids, Krivanek and Walker (2005) correlated *Xf* concentration with susceptibility, as predicted. However, *Xf* lives as a harmless endophyte in most plant species, and many resistant hosts (including species of grapevine) harbour high concentrations and support

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systemic movement of *Xf* (Baumgartner and Warren, 2005; Wistrom and Purcell, 2005). Thus, although in susceptible cultivars of grapevine *Xf* infection results in a suite of symptoms that include leaf scorch and senescence, petiole 'matchsticks', incomplete periderm development (green islands), and eventually vine death (Stevenson *et al*., 2005), no symptoms occur in the presence of high concentrations of *Xf* in some plant hosts. Together these studies demonstrate that high populations of *Xf*, along with movement, are not sufficient for pathogenesis in many plant species. A similar conclusion has been reached for other vascular pathogens (Grimault and Prior, 1993; McGarvey *et al*., 1999).

The vascular occlusion hypothesis of pathogenesis depends on the development of water deficits and, despite their central role in the *Xf* literature, plant water status has seldom been measured. Inoculation with *Xf* in both grapevine (Goodwin *et al*., 1988*b*) and *Parthenocissus quinquefolia* (McElrone *et al*., 2003) resulted in leaf-scorch symptoms that were correlated with reductions in hydraulic conductance, stomatal conductance, and leaf water potential. Thorne *et al*. (2006) did not observe reduced leaf water potential in infected and symptomatic grapevines, although stomatal closure may have obscured differences in plant water status. More importantly, that study clearly demonstrated that there are qualitative and quantitative differences between the visual symptoms resulting from experimentally imposed water deficits and *Xf* inoculation. Although water deficits are clearly a component of wilt diseases, their roles in other vascular diseases such as citrus variegated chlorosis and PD, where wilting is not generally observed, are less clear. Furthermore, the presence of low leaf water potentials is not always sufficient to conclude the cause of the low water status. Biotic stressors in general induce premature leaf senescence (reviewed in Guo and Gan, 2005), a process during which there are also decreases in leaf hydraulic conductance and leaf water potential (reviewed in Sack and Holbrook, 2006). Thus, it is possible that changes in the water relations of symptomatic leaves do not result from vascular occlusion by the pathogen *per se*.

Our knowledge of the nature of the plant–pathogen interaction and the plant immune system has evolved, and it is now understood that plants recognize and respond to pathogens in a variety of ways (reviewed in Dangl and Jones, 2001; Jones and Dangl, 2006). Many molecules that were described previously as 'phytotoxins' are now recognized to bind specific plant receptors, inducing the defence response (Deboer *et al*., 1989; Meyer and Dubery, 1993). This knowledge has guided the research of some xylem pathogens to a new nuanced understanding of the mechanisms of pathogenesis. For example, in *Verticillium*, numerous small molecules have been identified and isolated that contribute to pathogenicity and evoke many of the observed wilt symptoms (Nachmias, 1985; Meyer and Dubery, 1993; Davis *et al*., 1998; Chu *et al*., 1999; Wang *et al*., 2004). In the case of *Ralstonia*, yet to be characterized elicitors certainly play a role in pathogenesis (Pfund *et al*., 2004).

Resolving this debate is important because *Xf* pathogenesis has become synonymous with vessel occlusion in the literature about PD (e.g. Hopkins, 1989; Fry and Milholland, 1990; Newman *et al*., 2003; Krivanek and Walker, 2005) without there being a single report that demonstrates a strong and positive correlation between symptoms and concentration of bacteria. Understanding the nature of the pathogenesis clearly has important implications in designing screens for resistance or other approaches to ameliorating the effects of the disease. This is illustrated in *Verticillium* and *Ralstonia* where resistance is strongly correlated with the absence of disease symptoms, but not with reduced accumulation or movement of the pathogen (Mace *et al*., 1981; Grimault and Prior, 1993). The studies of *Xf*-resistant hosts discussed above suggest that the same situation may be present for *Xf*, and a recent study provides some evidence of an alternative mode of *Xf* pathogenicity involving toxins (Reddy *et al*., 2007). In this study, the aim was to determine the relationship between *Xf* concentration and the development of leaf-scorch symptoms during PD. To this end, a novel, robust quantitative PCR (qPCR) assay was developed to quantify *Xf in planta*. *Xf* populations were quantified and related to symptom development in both artificially inoculated, greenhouse-grown and naturally inoculated, field-grown Chardonnay (a PD-susceptible variety) grapevines. This study provides the first detailed look at *Xf* populations across leaf lamina and among leaves exhibiting various severities of symptoms, and demonstrates that there is little or no correlation between localized *Xf* concentrations and symptom development.

Materials and methods

Bacterial culture

All experiments utilized a *Xylella fastidiosa* (Temecula) strain engineered to express green fluorescent protein (Newman *et al*., 2003). Use of this strain of *Xf* facilitated microscopy studies not included in this work. *Xf* was never subcultured but instead was cultured directly from glycerol stocks frozen at –80 °C. *Xf* was cultured at 29 °C on solid PW media (Davis *et al*., 1981) supplemented with 20 μg ml^{-1} kanamycin until colonies became visible. The *Xf* was harvested by washing the plate with PWG media (Hill and Purcell, 1995) supplemented with 20 μg ml^{-1} kanamycin, centrifuged, and the concentration adjusted to approximately 4×10^8 CFU ml^{-1} (Minsavage *et al*., 1994). For the inoculation of greenhouse-grown grapevines *Xf* was used immediately.

The various *Xanthomonas* species used to test the specificity of the qPCR assay were cultured in the laboratory of Dr Pamela Ronald, Department of Plant Pathology, University of California at Davis.

Greenhouse plant material, growth conditions, and inoculation

Own-rooted *Vitis vinifera* (L.), cv. Chardonnay plants were grown from dormancy in 4 l pots filled with one-third peat, one-third sand, one-third redwood compost, with 2.4 kg m^{-2} dolomite lime in a greenhouse (30/20±3 °C; 40/70±10% RH; and natural light with a daily maximum of 1200 µmol photons m^{-2} s^{-1} PAR). The vines were pruned to two shoots, and the shoots were vertically trained to ~1.5 m. Pots were drip irrigated four times a day (at 06.00, 09.00, 14.00, and 18.00 hours) for 4 min at 7.57 l h^{-1} (2 l d^{-1}) with dilute nutrient solution (90 ppm calcium, 24 ppm magnesium, 124 ppm potassium, 6 ppm nitrogen as NH_4, 96 ppm nitrogen as NO_3, 26 ppm phosphate, 16 ppm sulphate, 1.6 ppm iron, 0.27 ppm manganese, 0.16 ppm copper, 0.12 ppm zinc, 0.26 ppm boron, and 0.016 ppm molybdenum) at pH 5.5–6.0. Inoculation was carried out by placing a 10 µl drop of the *Xf* solution described above at the base of the petiole and piercing the petiole through the drop with a sterile hypodermic needle. Each plant was inoculated twice, once on each shoot at midday. All the 10 µl was readily taken up by the plant within seconds.

Greenhouse and field sampling

In an initial experiment, leaves were harvested at 93 d after inoculation (DAI). The experiment was repeated and this time leaves were sampled at both 76 DAI and 91 DAI. Non-inoculated Chardonnay grapevines grown adjacent to inoculated vines remained healthy for the duration of the experiment, and leaves were harvested and analysed as described below as negative controls. At each sampling date, six leaves from at least five different plants were harvested, the leaf position relative to the point of inoculation was recorded, and the leaves were placed in individual humidified bags. Photographs of each leaf were taken before and after removing leaf punches. Leaf punches (1.5 cm) and 1 cm portions of petiole were taken immediately; fresh weights were recorded, and total DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Inc.) following the manufacturer's protocol. The number of individual leaf punches taken from each leaf was a function of the size of the leaf, and punches were centred on one of the five primary veins. The percentage leaf area scorched was calculated and, for simplicity, each leaf was assigned a numerical symptom severity from 1 to 5 defined as: 1=no visible leaf scorch, 2=1–20%, 3=21–50%, 4=51–70%, 5=71–100% of the leaf area scorched.

Field samples were obtained from a commercial vineyard of Chardonnay grapevines (Beringer Vineyards, Yountville, CA, USA) in September 2006. Plants were identified that exhibited all of the hallmarks of *Xf* infection, including shrivelled fruit, leaf-scorch symptoms, petiole matchsticks, and green islands. Four plants were selected for sampling, two plants exhibiting very severe symptoms, and two with more moderate symptoms, as defined by the overall extent of scorched and abscised leaves. A total of 30 leaves were harvested from multiple shoots of each plant and photographed. Leaves were placed in individual humidified bags, transported to the laboratory, and the entire length of all five primary veins and a 1 cm portion of petiole (Fig. S1 in Supplementary material available at *JXB* online) were excised, combined, and homogenized. DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Inc.) following the manufacturer's protocol.

DNA isolation and standard curves

Total genomic DNA from *Xf* grown in culture was prepared using the DNeasy Tissue Mini Kit (Qiagen, Inc.) following the manufacturer's protocol. DNA concentration (in g l^{-1}) was determined by spectrophotometry (Shimadzu UV160V). The molecular weight of the *Xf* (Temecula) genome (MW_{Xf}) was estimated as follows:

$$MW_{Xf} = \left(n_{Xf}\right)\left(660\,\text{Da base pair}^{-1}\right)$$

where n_{Xf} = the number of base pairs in an *Xf* cell = 2.52×10^6 base pairs (Van Sluys *et al*., 2003). Therefore:

$$\begin{aligned} MW_{Xf} &= \left(2.52\times10^6\,\text{base pairs}\right)\left(660\,\text{Da base pair}^{-1}\right) \\ &= 1.66\times10^9\,\text{g mol}^{-1} \end{aligned}$$

The concentration of *Xf* DNA was then expressed as the equivalent cells l^{-1} as follows:

$$Xf\ \text{cells}\,1^{-1} = \left[\left(\text{DNA conc. g}\,1^{-1}\right)/\left(1.66\times10^9\,\text{g mol}^{-1}\right)\right]\left(N_A\right)$$

where N_A = Avogadro's number. In order to produce the standard curve the genomic DNA was diluted to obtain a series at 1 $\log_{10}$ intervals.

Three DNA isolation procedures—the DNeasy Plant Mini Kit (Qiagen, Inc.), the MasterPure™ Plant Leaf DNA Purification Kit (Epicentre Biotechnologies), and a CTAB extraction method (Zhang *et al*., 1998)—were compared. In order to imitate the conditions of isolating *Xf in planta*, a solution of *Xf* at known concentration (Minsavage *et al*., 1994) was diluted to obtain a series at 1 $\log_{10}$ intervals. These were then mixed with 1.5-cm-diameter Chardonnay grape leaf discs and extracted by using the three methods mentioned above. Reproducibility of the standard curve was assessed by running the reactions on several occasions.

qPCR

In this study, *Xylella fastidiosa* (Temecula) *Elongation Factor Tu* (*EFTu*) (Accession No. NC_004556) was the target gene for qPCR. The *Xf* genome contains two copies of *EFTu*, whose nucleotide sequences share 100% identity within a strain, and are highly conserved between strains. A probe-based qPCR strategy was utilized in order to decrease the likelihood of obtaining false positives. The primers and probe were designed using the ABI Primer Express software and their sequences were as follows; forward primer *EFTu*_for-1 (5′-GGATGGTGCGATTTTAG-TATGTTCT-3'), reverse primer *EFTu*_rev-1 (5′-GGCGAGC-CAACAAAATTGTGT-3'), probe 5′FAM–3′TAMARA dual-labelled *EFTu*-1 (5′-TGATGGTCCGATGCCTCAGACTCGT-3'). The amplification was performed in a total reaction volume of 12 µl. Reactions included 4 µl of template DNA, 6 µl of TaqMan Universal PCR Master Mix (2×), 1 µM forward primer *EFTu*_for-1, 1 µM reverse primer *EFTu*_rev-1, 250 nM *EFTu*-1 probe, and sterile molecular biology grade water to a total volume of 12 µl. All PCR were performed in 96-well plates and the exact reaction cycling conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Amplification and data analysis were carried out on an ABI PRISM 7700 sequence detector system (Applied Biosystems). All primer and probe concentrations were optimized. In order to assess the presence of any inhibition, an independent reaction was run for each sample. For these internal controls each reaction was run as described above with the addition of *Xf* genomic DNA corresponding to 10^4 cells. If the sample was determined to be contaminated with inhibitory compounds it was subjected to an agarose embedding purification as described in Moreira *et al.* (1998) and both the sample and internal control were run again. Negative controls containing either no template DNA or no plant leaf DNA were subjected to the same procedure. Each sample was run in triplicate.

Results

Development of a qPCR assay for Xf in planta

A qPCR assay was designed to detect and quantify *Xf* from grapevine tissue. The highly conserved target gene, *EFTu* was chosen. Figure 1A shows a multiple sequence alignment of *EFTu* across the target region. Included are two *Xf* strains, 9a5c and Temecula, and three sequences from closely related *Xanthomonas* species. This region contains 100% sequence identity between these *Xf* strains and extensive base pair mismatching with closely related *Xanthomonas* species across both the forward and reverse primers, and the probe (Fig. 1A, shading). When total genomic DNA from *Xf* (Temecula) and the *Xanthomonas* species are used as templates for traditional PCR, utilizing the primer pair described above, no amplification of the *Xanthomonas* isolates occurred (Fig. 1B).

Three methods of isolating DNA from combinations of plant tissue and known concentrations of *Xf* were compared with dilutions of *Xf* genomic DNA isolated from culture in order to determine the most robust procedure (Fig. 2). The assay was extremely reliable, resulting in r^2 values from 0.970 to 0.998 regardless of the DNA isolation method used. The variability that was present increased with decreasing concentration as would be expected. The cycle thresholds for any given concentration of *Xf* cells were greater for all of the preparation methods than for *Xf* DNA from culture. More simply, in all cases, the presence of plant tissue resulted in a decrease in the sensitivity of the assay. The Qiagen Plant extraction method was chosen because of its low background and similar behaviour across the full range of *Xf* concentrations (Fig. 2, closed triangles). The assay was also very sensitive. Theoretically the assay was able to detect <10 cells in any reaction even in the presence of plant extract. Table 1 shows the cycle thresholds obtained for each individual run, and the mean cycle thresholds and standard deviation of these values across all runs. With standard deviations that are less than a third of a cycle, the standard curve was highly reproducible.

Relationship between Xf *population and leaf-scorch symptoms*

Xf-inoculated greenhouse-grown grapevines began exhibiting symptoms at ~60 DAI. *Xf* was detected in 94% of leaves, but only 51% of the individual leaf punch/petiole samples. Bacterial populations in leaf discs were found ranging anywhere from as little as 10^2 to as much as 10^9 cells g^{-1} tissue. *Xf* populations across leaf lamina were relatively homogenous in some leaves (Fig. 3E, Q) and extremely variable in others (Fig. 3J, N). Two of the leaves analysed were leaves whose petioles were inoculated originally. Clearly these leaves harboured much greater populations of bacteria across the entirety of the lamina (Fig. 3P, Q). The relationship between bacterial population and the severity of leaf-scorch symptoms was

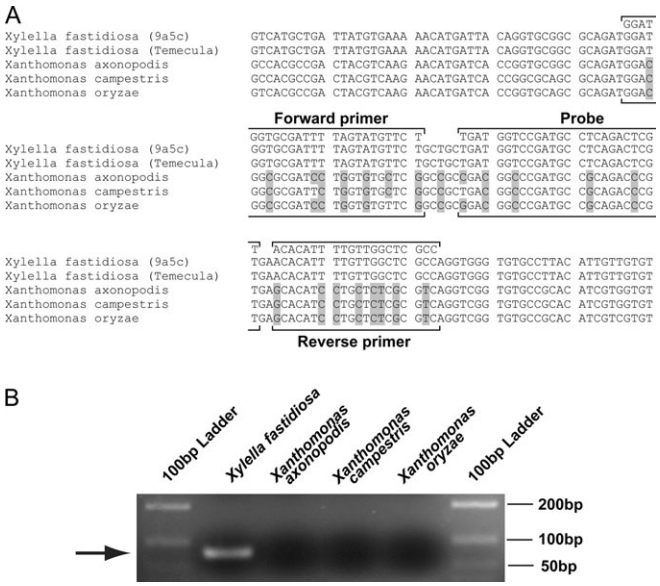
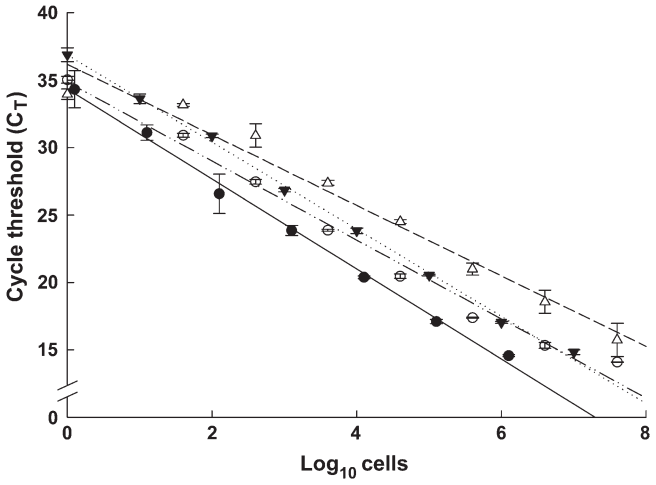


Fig. 1. (A) ClustalW multiple nucleotide sequence alignment of the target portion of *EFTu* of two *Xylella fastidiosa* strains, 9a5c and Temecula, and three *Xanthomas* species. Base pair mismatches are shaded. (B) Agarose gel electrophoresis of PCR products resulting from a reaction with the forward and reverse primers and total genomic DNA from the labelled species. The expected size of product from *Xf* is indicated by an arrow.



Preparation method	r^2	Slope	y-Intercept
Genomic DNA from culture	0.996	-3.34	34.3
Qiagen Plant Kit	0.998	-3.23	36.9
CTAB extraction	0.987	-2.93	34.9
Epicentre Plant Kit	0.970	-2.61	36.2

Fig. 2. Comparison of standard curves produced with various DNA isolation methodologies. Standard curves produced from mixed plant leaf/*Xf* DNA isolated using a standard CTAB extraction (open circles), Qiagen Plant Kit (closed triangles), or Epicentre Masterpure Plant Kit (open triangles) compared with that produced from *Xf* genomic DNA isolated from culture (closed circles). Error bars represent ±1 standard deviation.

Table 1. *Reproducibility of plant leaf/*Xf *qPCR standards*

C_T values for individual runs are means of three reactions. The coefficient of variation (CV) is expressed as a percentage.

Log_{10} cells	Cycle threshold C_T						
	Run 1	Run 2	Run 3	Run 4	Mean	SD	CV
6.3	14.3	14.3	14.3	14.4	14.3	0.05	0.3
5.3	16.7	16.6	16.7	16.6	16.7	0.06	0.4
4.3	20.0	19.8	19.8	20.3	20.0	0.24	1.2
3.3	22.7	22.4	22.6	22.5	22.6	0.13	0.6
2.3	27.2	26.7	26.9	26.7	26.9	0.24	0.9
1.3	29.9	30.1	29.4	29.6	29.8	0.31	1.0

also highly variable. For example, some of the leaves that exhibited the most severe symptoms had almost no detectable *Xf* (Fig. 3O and R), while other less symptomatic leaves harboured higher concentrations (Fig. 3C, F).

Populations were averaged across each leaf and these averages were compared with leaf-scorch symptoms. When all leaves were considered there was a weak but positive relationship between the average *Xf* population and symptom severity (Fig. 4A, r^2=0.21, P=0.056). When examined more closely this relationship was established almost entirely by the high average populations in the two inoculated leaves (Fig. 4A, circled). If these leaves were not considered, regression analysis resulted in an insignificant r^2=0.015 (P=0.073). The average population across all leaves increased significantly between 76 DAI and 91 DAI (Fig. 4B, P <0.05). Average populations increased almost 10-fold from 2.9 to 3.7 $\log_{10}$ cells g^{-1} in 15 d.

Field-grown Chardonnay grapevines were identified that exhibited all of the hallmarks of *Xf* infection, including shrivelled fruit, leaf-scorch symptoms, petiole matchsticks, and green islands (Fig. 5, white arrows). Whole plants were designated as exhibiting either severe or moderate symptoms as defined by the overall extent of scorched and abscised leaves (Fig. 5A severe, B moderate), and an equivalent number of leaves exhibiting the spectrum of symptom severities was harvested from each plant (Fig. 5C, D). Bacterial concentrations were determined for individual leaves in a combined tissue sample that included all five primary veins and a 1-cm portion of the petiole. In these field samples, *Xf* was detected in only 30% of leaves, a much lower percentage than was found in greenhouse experiments. Bacterial populations were more uniform among samples, ranging from 10^4 to 10^6 cells g^{-1} tissue (Fig. 5C, D), than among leaves in the greenhouse experiments. Comparing symptom severity and *Xf* concentration in individual leaves, regression analysis resulted in an r^2=0.09, demonstrating that similar to greenhouse experiments there was no significant relationship (P=0.115). However, when whole plants are considered, plants exhibiting severe symptoms had a higher percentage of *Xf*-positive leaves (Fig. 5E). Forty-seven percent of leaves tested positive for the presence of *Xf* in plants exhibiting severe symptoms, while only 13% of leaves tested from plants with moderate symptoms tested positive. In addition, average *Xf* populations across all leaves were greater in the plants exhibiting severe symptoms (P <0.05).

Discussion

A robust and sensitive qPCR assay for the detection and quantification of *Xf* bacteria *in planta* was used to relate *Xf* populations to leaf-scorch symptoms during PD. The results show that the nature of the *Xf* population is patchy across whole leaves. Leaves can exhibit severe leaf-scorch symptoms in the absence of high concentrations of *Xf*, and there is no apparent relationship between the severity of leaf-scorch symptoms and *Xf* concentration. These results demonstrate that localized high concentrations of *Xf* are not necessary for the formation of leaf-scorch symptoms, and suggest the involvement of an elicited plant response in *Xf* pathogenesis.

Quantifying Xf in planta

Developing the means of quantifying *Xf* from plant specimens has been an object of intense study, and a variety of approaches have been developed including immunolocalization, bacterial culture, ELISA, transgenic fluorescent *Xf*, PCR, and qPCR, all with their individual caveats. qPCR was chosen for the present study because of its superior sensitivity, relative ease in processing many samples, and ability to be adapted to an automated high throughput format. *Xf* concentrations of 10^3 cells g^{-1} fresh weight were regularly detected in this study, a level of sensitivity approximately 100 times greater than that reported for ELISA (10^5 cells g^{-1} fresh weight) (Krivanek and Walker, 2005). This provides for the quantification of *Xf* from much smaller sample sizes (e.g. <100 mg as compared with 0.5 g or more) and the ability to produce a much more detailed look at the nature of *Xf* colonization. Furthermore, the assay is highly reproducible, illustrated by coefficients of variation (CVs) in the standard curve of 1% or less (Table 1) compared with 35% on average for ELISA (Krivanek and Walker, 2005). Several qPCR assays for *Xf* have been developed in different plant species (Oliveira *et al*., 2002; Schaad *et al*., 2002; Li *et al*., 2003; Osman and Rowhani, 2006), but the majority have only employed the technology for detection, not quantification. Baumgartner and Warren (2005) is the only published report that has used qPCR in order to quantify *Xf* in grapevine but in a different species, the PD-resistant *Vitis californica*. Therefore, this is the first application of a qPCR methodology to the PD-susceptible, domesticated grapevine.

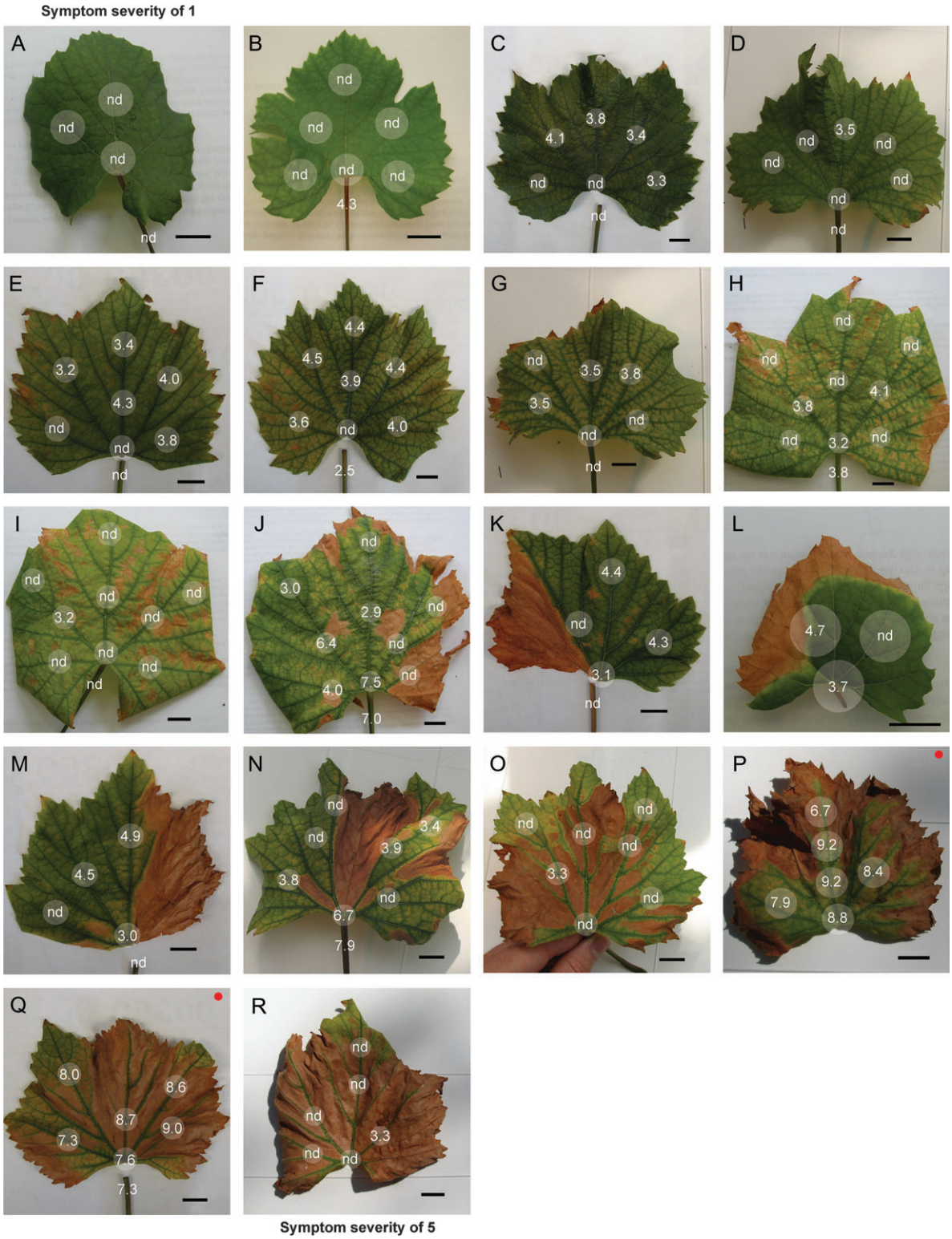


Fig. 3. (A–R) Photographs of sampled greenhouse-grown Chardonnay leaves. The leaf punches sampled are to scale (shaded), and *Xf* populations in those punches are given as $\log_{10}$ cells g^{-1} fresh weight tissue or none detected (nd). Leaves are arranged in order of increasing symptom severity from those with no symptoms (symptom severity rating of 1) to those exhibiting severe symptoms (symptom severity rating of 5). Two of the leaves analysed (P and Q) were those originally inoculated with *Xf* (indicated with red dots). Scale bars=1.5 cm.

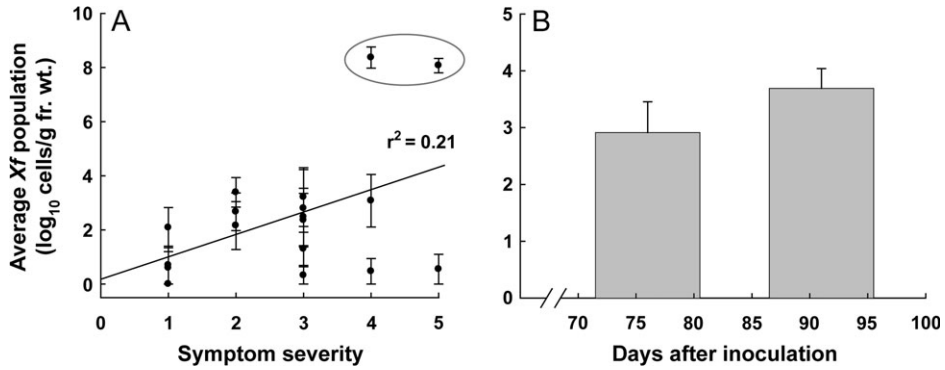


Fig. 4. (A) Average *Xf* populations ($\log_{10}$ cells g^{-1} of fresh weight tissue) in leaves with various symptom severities. Error bars represent standard error. (B) Relationship between average *Xf* populations and the date on which the samples were taken. The difference is significant (P <0.05). Error bars represent +1 standard error.

The crux of this methodology is isolating DNA template that is free from compounds having inhibitory effects on the reaction, and obtaining DNA from grapevine that is free from such compounds is notoriously difficult (Minsavage *et al*., 1994; Buzkan *et al*., 2003). In the current study, inhibition was uncommon, but also unpredictable. Inhibition occurred in some, but not other, individual leaf punch samples within the same leaf. This result should be of particular interest because the inhibition could lead to false negatives. The only solution is to evaluate every sample independently with the addition of an internal standard, as was done in this study.

The nature of Xf *colonization and false negatives*

Studies examining *Xf* in grapevine via fluorescent microscopy (Newman *et al*., 2003), by assessing a large number of field samples (Krell *et al*., 2006), as well as the study described here, all conclude that *Xf* has a patchy distribution within grapevine. Spatially variable concentrations are to be expected if the movement is dependent upon an irregular distribution of xylem conduit lengths and intervessel pit connections (Chatelet *et al*., 2006). As with the erratic inhibition discussed above, this knowledge impacts the interpretation of previously published findings and informs future experimental design. For example, many previous studies assessing *Xf* movement relied on only one or a few locations in order to evaluate *Xf* movement. This sampling approach, when used for diagnosis or for screens for resistant genotypes, may be wrought with false negatives (Fig. S2 in Supplementary material available at *JXB* online). Future studies would do better to utilize sampling methodologies that examine either a larger part of the plant, or a larger number of sampling locations in order to increase the likelihood of detecting *Xf* when it is present.

A number of previous studies in artificially inoculated grapevines demonstrate that *Xf* populations increase over time (Hopkins, 1985*b*; Fry and Milholland, 1990; Hill and Purcell, 1995); results confirmed by the current study. In the earlier studies *Xf* concentrations were often expressed in CFU ml^{-1} of extraction buffer, making direct comparisons with the present results difficult, but Hill and Purcell (1995) found populations of 10^8–10^9 CFU g^{-1} fresh weight, concentrations much greater than those detected in the majority of the samples that were positive for *Xf* in this study. This apparent discrepancy is explained by the fact that Hill and Purcell (1995) sampled at the point of inoculation, which is shown here to harbour concentrations of *Xf* (10^7–10^9 cells g^{-1} fresh weight) that are identical to those described in that work and much higher than in non-inoculated leaves. In fact, the majority of early studies quantifying *Xf* in grapevine did so by sampling at the point of inoculation, which may partly explain the common belief that high *Xf* concentrations are necessary for pathogenesis. Fry and Milholland (1990) reported populations of 10^4–10^6 CFU cm^{-1} of petiole at sites distal to the point of inoculation. Equivalent populations were regularly found in 1.5-cm-diameter leaf punch samples (see Fig. 3F, J–N).

In field samples the concentrations of *Xf* detected in positive leaves, 10^4–10^6 cells g^{-1}, were similar to those reported in the resistant alternative host *Vitis californica* by Baumgartner and Warren (2005). That both these *Vitis* species harbour similar concentrations of *Xf* illustrates that symptom expression must involve more than simply high concentrations of *Xf*. The concentrations of *Xf* in field vines were also similar to those detected in greenhouse experiments with needle-inoculated Chardonnay grapevines. As far as is known, this is the first study that has quantified *Xf* populations from naturally inoculated *Vitis vinifera* from the field.

Xf *populations and leaf scorch*

This is also the first study that correlates symptoms with the concentration of *Xf* in leaves of PD-infected plants. As discussed above, the prevailing hypothesis to explain the formation of symptoms during PD is that vascular

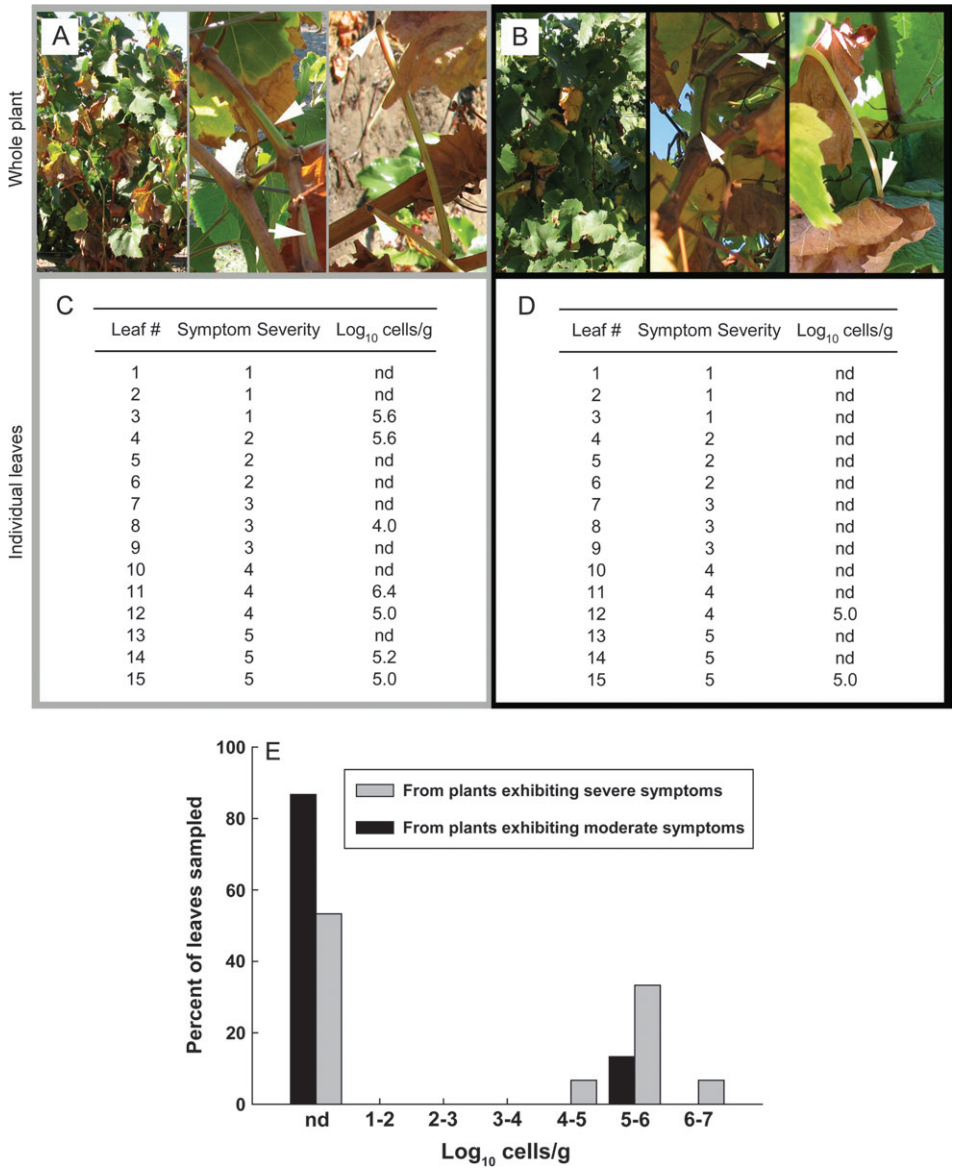


Fig. 5. *Xf* populations in field-grown Chardonnay leaves. (A, B) Representative photographs of plants exhibiting severe (A) or moderate (B) symptoms of *Xf* infection, including green islands and matchsticks (white arrows). (C, D) Symptom severity and bacterial population for leaves from plants exhibiting severe (C) or more moderate (D) symptoms. (E) Percentage of leaves with various populations of *Xf* for samples from plants exhibiting severe (grey bars) or moderate (black bars) symptoms.

occlusion, largely attributed to *Xf* bacteria and their putatively associated gums, results in localized water deficits. It is clear from this and other recent studies that leaf-scorch symptoms form in the absence of localized high concentrations of, and substantial plugging of xylem vessels by, *Xf* (Newman *et al*., 2003; Alves *et al*., 2004; Krell *et al*., 2006). Newman *et al*. (2003) showed that the percentage of colonization and occlusion by *Xf* was greater in symptomatic leaves, although overall complete occlusion of vessels was extremely rare (2–4%) even for symptomatic leaves. In other species, Alves *et al*. (2004) reported that leaves exhibiting severe symptoms contained a higher percentage of *Xf*-colonized (not necessarily occluded) vessels than those leaves exhibiting mild symptoms. Again, in these species, leaves exhibited symptoms with as little as 8–26% of vessels colonized (Alves *et al*., 2004). In addition, these authors mention that preliminary research did not demonstrate a relationship between absolute population and symptom severity. In view of the low concentrations of *Xf*, patchy distribution of *Xf*, and the low frequency of occluded vessels, it is unlikely that leaf-scorch symptoms result from the obstruction of water movement by the *Xf* bacteria themselves. These observations do not discount that at times localized water deficits could result from high concentrations of *Xf*, but certainly it appears that this

cannot account for all of the symptoms observed during pathogenesis. These observations do recall the question of the mechanisms by which *Xf* brings about PD.

Taken together, the results listed below are difficult to reconcile without the involvement of a systemic plant response in pathogenesis:

(i) the absence of a strong relationship between leaf-scorch symptoms and *Xf* concentrations in individual leaves;

(ii) a clear increase in the *Xf* population over time and in more symptomatic plants;

(iii) the presence of leaf-scorch symptoms in tissues and leaves that have undetectable amounts of *Xf*.

If the grapevine responds systemically, visible symptoms will worsen throughout the whole of the plant, irrespective of the distribution of *Xf*, and any coincidence between high *Xf* populations and leaf-scorch symptoms will break down as pathogenesis progresses. Goodwin *et al*. (1988*a*) were the first to propose that PD is in essence accelerated leaf senescence, a hypothesis that has attracted little attention. Although Goodwin *et al*. (1988*a*) attributed this senescence to water stress, several other factors, *Xf*-derived elicitors among them, could bring about the same result (reviewed in Guo and Gan, 2005). An earlier study by Hopkins (1985*a*) demonstrated that plant growth regulators known to stimulate natural leaf senescence, such as ethylene, accelerate and intensify leaf-scorch symptoms during *Xf* pathogenesis, and more recent work has provided evidence that PD symptoms may result from an ethylene-mediated plant response (Perez-Donoso *et al*., 2007). Gene expression studies suggest that ethylene-mediated plant responses are also involved in the wilt diseases discussed above (Lasserre *et al*., 1997; Dowd *et al*., 2004; Wang *et al*., 2004). This could account for leaves exhibiting leaf-scorch symptoms in the absence of *Xf* and even for these leaves to exhibit water deficit. The results of this study serve to draw attention to the need to understand better the impact of the plant's developmental context and defence responses in *Xf* pathogenesis. Serious questions remain as to how vascular pathogens may be manipulating the plant to make the xylem environment more hospitable.

Supplementary material

The Supplementary material available at *JXB* online consists of two figures: one illustrates the sampling protocol for leaf tissue from field vines; the other illustrates the hazard of false negatives when taking few and small tissue samples for *Xylella* detection. Figure S1 contains two images from photographs of experimental field vines, one of a symptomatic leaf and the second of the petiole and primary veins as they were excised from that leaf for qPCR analysis of *Xylella fastidiosa*. Figure S2 is a schematic diagram illustrating the patchy distribution of the bacteria in infected grapevines and the simple concept that the probability of false negatives in *Xylella* analyses is diminished by increased sampling.

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