

Detection of grape phylloxera (*Daktulosphaira vitifoliae* Fitch) by real-time quantitative PCR: development of a soil sampling protocol

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Abstract

Background and Aims: Quantitative PCR (qPCR) provides the basis for an efficient detection and surveillance system for phylloxera, a devastating pest of grapevines worldwide. This study aimed to develop a sample collection and handling protocol for reliable detection of phylloxera in soil by qPCR.

Methods and Results: Quantitative PCR conducted on infested soil samples stored at 10, 20 or 35°C for up to 10 days showed that extended storage did not significantly affect the rate of phylloxera detection. The limit of detection of qPCR was estimated to be 1.5 to 2 phylloxera per 200 g dry soil using samples prepared from phylloxera-infested and non-infested soil in various ratios. Comparison of phylloxera detection rate and numbers between depths, positions and vineyards, in all seasons over 2 years showed that the highest detection rate is obtained with samples taken 0–10 cm deep and 5 cm from the trunk, and that higher numbers are consistently found from mid-summer to early winter.

Conclusions: When combined with an appropriate sampling protocol, qPCR allows reliable detection of phylloxera in soil, across sites and seasons.

Significance of the Study: The sampling protocol developed in this study will facilitate the adoption of qPCR for large scale monitoring and surveillance of phylloxera by the industry.

Keywords: composite sample, early detection, high throughput, phylloxera, surveillance

Introduction

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) is a destructive pest of grapevine (*Vitis* spp.) worldwide. The insect can feed on the roots and/or leaves of most *Vitis* species, but on highly susceptible *Vitis vinifera*, this can have a severe impact on vine health and production (Benheim et al. 2012). Phylloxera is native to North America, but its economic importance became evident in the 1860s, when its introduction to the European grapegrowing areas devastated the industry (Corrie et al. 2002). In Australia, phylloxera was first introduced in 1875. In response, quarantine zones were rapidly defined to help prevent spread and assist with eradication attempts (Herbert et al. 2008). In 2006, 2% of Australian vineyards were known to be infested, all located in central and north east Victoria and south east New South Wales (Herbert et al. 2008).

Management of phylloxera worldwide relies essentially on the use of resistant or tolerant rootstocks derived from native American *Vitis* (Corrie et al. 2002). In Australia, however, 85% of commercial plantings are highly susceptible ungrafted *V. vinifera*, and management relies mainly on surveillance and prevention through quarantine regulations (Herbert et al. 2006). To assist with this, the Australian National Vine Health Steering Committee has classified Australian geographical areas into three types of zones: Phylloxera Infested Zones, where phylloxera is known to occur; Phylloxera Exclusion Zones, where current testing and historic information provide no evidence of phylloxera infestation; and Phylloxera Risk Zones, for which no evidence for phylloxera presence/absence is available. All Australian grapegrowing

areas are classified within these zones and movement of material between zones is subject to strict quarantine regulations (National Vine Health Steering Committee 2009).

Detection is a key component of any management strategy and is of critical importance for the success of surveillance and containment efforts. Typically, phylloxera abundance within infested vineyards is low; foliar symptoms that can assist in identifying potentially infested areas vary in severity with grapevine pedigree and can even be non-existent in the early years of infestation (Herbert et al. 2008, Bruce et al. 2011, Benheim et al. 2012). Phylloxera infestations are also characterised by uneven abundance and distribution of insects throughout infested vineyards (Herbert et al. 2008, Powell 2012b). Finally, phylloxera is known for its high genetic diversity, and in Australia alone, at least 83 genotypes have been characterised (Benheim et al. 2012). Therefore, reliable detection of phylloxera relies on detection methods combining high sensitivity, to achieve early detection of new infestations and limit any further spread, and high throughput, to assess large numbers of samples collected across the surveyed area and to account for uneven distribution. Methods that allow for analysis of composite samples without compromising sensitivity have the potential to reduce survey costs. Detection methods also need to be specific for phylloxera while accounting for its genetic diversity and allowing for detection of all genotypes.

A range of detection methods has been developed for phylloxera, including aerial survey, ground survey, emergence traps and molecular techniques (Benheim et al. 2012). In Australia, ground survey, in which vine roots are exposed

and examined using a magnifying glass, is the recognised method for phylloxera testing (National Vine Health Steering Committee 2009). A combination of aerial survey, to identify areas of low vigour, followed by a targeted ground survey is often used (Herbert et al. 2008). Ground surveys, however, are time consuming, require taxonomic expertise and can fail to detect infested vines (Herbert et al. 2008). The emergence trap method, which uses inverted bucket traps, is the most common alternative to ground surveys (Herbert et al. 2008). Although the emergence trap method has shown greater sensitivity than the ground survey, its efficacy is season-dependent and, like the ground survey method, is time consuming and requires taxonomic expertise. Molecular detection relies on a quantitative PCR (qPCR) assay specific to phylloxera (Herbert et al. 2008). Compared with the ground survey and emergence trap methods, qPCR is faster and can detect phylloxera at any life stage (Herbert et al. 2008). It has the additional benefits of being quantitative and particularly suitable for automation and high throughput testing (Ophel-Keller et al. 2008). Moreover, its sensitivity is slightly better than that of the ground survey method and similar to that of the emergence trap method (Herbert et al. 2008, Bruce et al. 2011, Powell 2012a).

While qPCR has some advantages in terms of sensitivity and throughput compared with more traditional detection methods, its application for detection of soil-borne pests and pathogens in soil requires the development of dedicated protocols for sample collection and handling. Soil moisture is one critical factor to be considered. When assessing methods for detection of root lesion nematode in soil, Hollaway et al. (2003) found that in dry soil, DNA did not degrade even though nematodes may have been damaged during sampling, which translated into more consistent quantification by qPCR than by the alternative Whitehead tray method based on sieving soil and recording nematode numbers per gram of soil. Herdina et al. (2004a,b) also found that DNA from dead organisms degraded rapidly in moist soil and became undetectable by qPCR in 4–8 days for fungal mycelia DNA and 14 days for nematode DNA. When evaluating sampling strategies for optimal detection of soil-borne pathogens, Heap and McKay (2004) reported that the use of composite samples helped account for the uneven distribution of some pathogens across paddocks and reduced variation in the amount of pathogen DNA per sample compared with single core samples. Based on these reports, South Australian Research and Development Institute's (SARDI) Molecular Diagnostics Centre developed a sampling strategy for detection of soil-borne pathogens by qPCR in soil, which involves collection of composite samples of multiple soil cores and recommends drying samples as soon as possible after collection to help preserve DNA (Ophel-Keller et al. 2008).

To facilitate adoption of qPCR for phylloxera surveillance by the industry, this study aimed to establish sample collection, handling and storage protocols, optimised for the reliable detection of phylloxera in soil. First, phylloxera may be damaged during sampling, thus exposing DNA to degradation and potentially compromising the chance of detection by qPCR. Therefore, the stability of phylloxera DNA during storage and handling of soil samples was investigated. Second, as phylloxera infestations are characterised by uneven distribution of insects throughout the vineyards, composite samples are likely to improve the chance of detection, but they also require a highly sensitive detection method. Herbert et al. (2008) previously estimated the sensitivity of the qPCR assay to be 20 phylloxera per 200 g of soil, using soil artificially spiked with adult phylloxera. In this study, we further assessed the sensitivity of the assay with composite soil samples and estimated the limit

of detection. Finally, sampling depth, position relative to the vine trunk and time for optimal detection of phylloxera by qPCR were established.

Material and methods

Vineyard details and infested vine selection

Three vineyards infested with phylloxera were selected in the Yarra Valley, King Valley and Rutherglen areas of Victoria, Australia. In the Yarra Valley, the vineyard was planted in 2000 with Chardonnay on own roots and diagnosed with phylloxera in 2008. The vineyard in the King Valley, planted in 1987 with Chardonnay on a mixture of own roots and Schwarzmann rootstock, was diagnosed with phylloxera in 1992. The vineyard in Rutherglen, planted in 1975 with Shiraz on own roots, was diagnosed with phylloxera in March 2013 when it was inspected for this trial. Phylloxera genotypes G1 and G4 are predominant in the Yarra Valley and King Valley areas, respectively (Powell 2012b), whereas a mixture of genotypes has been reported in Rutherglen (Powell et al. 2003). In the Yarra Valley and Rutherglen vineyards, vines were inspected by digging up and examining roots for phylloxera presence using a 10× magnifying glass before the first sampling; infested grapevines were flagged for future use. In the King Valley vineyard, vines were initially selected based on their vigour and visual inspection of the roots for galling, and phylloxera presence was completed after the first sampling.

All samples collected during this study were packaged and transported according to the current quarantine regulations (National Vine Health Steering Committee 2009) and analysed in SARDI's quarantine approved facilities.

Effect of temperature and duration of soil sample storage on phylloxera detection

For optimal preservation of DNA, soil samples should be dried as soon as possible after collection, but this is not always practicable. This experiment examined the effect of temperature on the rate of degradation of phylloxera DNA in soil samples while in storage between collection and drying. Soil samples were collected in the Yarra Valley in March 2013 and again in July 2013 from 75 known infested vines (15 sets of five vines numbered 1 to 15) using a 15 mm diameter digstick soil corer (Spurr Soil Probes, Adelaide, SA, Australia). Samples were immediately stored at 5°C and transported to the laboratory within 2 days of collection. Storage was extended for another 0, 1, 3 or 10 days at 10, 20 or 35°C before drying the soil samples prior to DNA extraction and qPCR. In March 2013, four soil cores were collected 10–15 cm from the trunk of each of the 75 vines. Then, for each set of five vines, four composite samples were prepared by pooling five cores, one from each vine in the set, giving 60 composite samples. The four composite samples from any one set of vines were stored at the same temperature (10, 20 or 35°C), and each was retrieved for analysis after 0, 1, 3 or 10 days, respectively. There were five sets of vines for each storage temperature and five replicates per storage duration at each temperature.

With the March 2013 sampling, it was not known whether the insects were still alive in the samples, therefore preserving DNA integrity, or had been killed by coring. Phylloxera has been shown not to survive freezing at –18°C for 24 h. This is actually one of the approved procedures for disinfestation of soil and plant samples (National Vine Steering Committee 2009). For this reason, in July 2013, the sampling was repeated and some samples were frozen before applying the storage treatments, to assess whether killing phylloxera by freezing

the soil affected DNA degradation. Two series of 60 composite samples were simultaneously collected from the same vines and using the same protocol as in March 2013. One series of samples was submitted to the storage treatments upon reception in the laboratory, while the other set was frozen at -20°C for 24 h prior to storage.

In both instances, samples were processed for DNA extraction and phylloxera detection by qPCR, as described later. The results were used to estimate the rate of phylloxera DNA degradation in soil samples.

Data were analysed using R software (R Core Team 2013). It was expected that DNA degradation would follow a first order equation:

$$\text{DNA}_t = \text{DNA}_0 \times \exp(-kt) + \text{error}_t \quad (1)$$

where DNA_t is the amount of DNA remaining at time t and t is the elapsed time between reception of the sample in the laboratory and drying. The logarithmic transformation of equation (1) is the linear equation (2). Simple regression theory was then used to obtain an estimate of k .

$$\ln(\text{DNA}_t) = \ln(\text{DNA}_0) - kt + \text{error}_t \quad (2)$$

Separate regressions were initially fitted for each replicate/time/temperature combination. It was found that there was no significant difference between slopes ($-k$) within a treatment but that there was a difference between the intercepts reflecting different amounts of DNA between the sets of vines. Separate intercepts were therefore allowed for data from each set of five vines. Half-life estimates were obtained using Equation (3):

$$\text{Half-life} = \ln(2)/k \quad (3)$$

Rates of decomposition (slopes) were obtained using the regression shown in Equation (2). The slopes were averaged across replicates. The differences in intercepts reflected differences between vines.

Effect of composite samples on phylloxera detection

To assess whether compositing of soil cores affects phylloxera detection, composite samples were made up in the laboratory by mixing phylloxera-free soil with phylloxera-infested soil. Bulk soil samples were taken near the trunk of infested vines in Rutherglen (phylloxera-infested soil) and from an adjacent empty paddock (non-infested soil) in March and May 2014. In both instances, soil was transported to the laboratory within 2 days of collection and dried upon arrival as described later. The bulk samples were separately homogenised with a jaw grinder (SP100x100, Labtechnics, Kilkenny, SA, Australia) prior to being used to prepare 400 g composite samples. The infested/non-infested soil ratios (mass/mass) were 300/100, 250/150, 200/200, 150/250, 100/300, 50/350 and 20/380 in March 2014 and 200/200, 100/300, 50/350, 20/380, 10/390, 5/395 and 1/399 in May 2014, with eight replicates of each. At each sampling time, there were also eight 400 g samples with phylloxera-infested soil only and another eight (five in May 2014) 400 g samples with non-infested soil only. All samples were processed for DNA extraction and phylloxera detection by qPCR, as described later.

Data were analysed using R software (R Core Team 2013). A generalised linear model with binomial errors and a logistic link function was used to analyse presence/absence data obtained with artificial composite samples.

Effect of depth, position, location and time of sampling on phylloxera detection

A multifactorial trial was designed to assess the effect of sampling depth, position relative to the trunk, location and time on phylloxera detection. Samples were collected at three depths (0–10, 10–20 and 20–30 cm), three positions (close to trunk, below dripper and between rows) and from the three selected vineyards (King Valley, Rutherglen and Yarra Valley), in autumn (March 2013), winter (July 2013), spring (October 2013) and summer (December 2013). In each vineyard, eight groups of five infested vines were identified so that the same 40 vines were sampled at each sampling date. One 30 cm deep core was taken 10–15 cm from the trunk of each vine at each position, using a 15 mm diameter digstick soil corer; each core was split into three depth sections (0–10, 10–20 and 20–30 cm); composite samples were made by pooling the corresponding position/depth sections from each of the five vines within a group, making a total of nine samples per group of five vines and 72 samples per location. Between-row samples were collected only in autumn. In December 2013, sampling was interrupted by heavy rain. As a result, all samples from the Yarra Valley, the King Valley and the first group of vines in Rutherglen (all positions and depths) were taken on the 4 December 2013; all remaining samples from Rutherglen were collected on the 14 January 2014.

To verify the trend observed in the first year of the depth/position/location/time trial, limited sampling was continued for another year. Samples were collected from the three vineyards in early (March 2014) and late (May 2014) autumn, winter (July 2014), spring (October 2014) and summer (December 2014), 0–10 cm deep, close to the trunks of the same infested vines and using the same sampling protocol as described earlier.

A separate set of samples was also collected in May 2014 to assess the effect of the sampling distance from the vine trunk on phylloxera detection. The vineyard in the King Valley was selected for this purpose as it was postulated that the overall lower incidence of phylloxera in this vineyard, as indicated by previous sampling, was more likely to reveal a distance effect if any existed. Samples were collected from the same eight groups of five vines used for the depth/position/location/time trial. Cores were collected 0–10 cm deep at 5, 10, 15, 30 and 45 cm from the trunk of each vine in a group. Composite samples were made by pooling the cores taken at the same distance from the trunk from each of the five vines within a group, making a total of eight samples per distance.

All samples were sent to the laboratory within 2 days of collection and processed for DNA extraction and phylloxera detection by qPCR as described later.

Data were analysed using R software. The sampling depth and position trial was designed with a factorial structure (three locations by three sampling positions by three depths) each with five replicates. The qPCR data were transformed using log transformation $y = \ln(x+1)$, and ANOVA was performed using the `aov` or `lm` functions in R. The results obtained with the samples collected at various distances from the trunk were analysed using a generalised linear model with binomial errors and a logistic link function.

DNA extraction and phylloxera DNA quantification

Immediately upon arrival at the laboratory, soil samples were dried overnight in a dehydration oven at 40°C , except for the storage experiment, for which samples were first submitted to extended storage prior to drying, as previously described.

Sample mass was recorded before and after drying to estimate moisture content. From each sample, DNA was extracted using the commercial DNA extraction service operated by SARDI, Adelaide (Ophel-Keller et al. 2008). The efficiency and consistency of SARDI's method to extract DNA from soil has been shown previously (Haling et al. 2011).

Phylloxera DNA was quantified by qPCR using the assay developed by Herbert et al. (2008), with forward primer 5'-AATCCGCAGGTATACGAACATC, reverse primer 5'-CGGTCTCGTCAAATTCGGA and TaqMan MGB probe 6FAM-CGACCCTCAGACAGG (Thermo Fisher Scientific, Waltham, MA, USA). This assay was previously shown to detect the main Australian phylloxera genotypes (Herbert et al. 2008). For further validation, the assay was tested against nine French and 16 American phylloxera genotypes collected between 2010 and 2012 [courtesy of Daciana Papura (Research Engineer) and François Delmotte (Scientist), INRA Bordeaux, France]. The results confirmed that a wide range of phylloxera genotypes can be detected, irrespective of their origin. The DNA extracted from soil samples was diluted 1/5 prior to PCR. All PCR were performed on a ViiA7 real-time PCR system (Applied Biosystems, Foster City, CA, USA), in 10 µL volume containing 4 µL DNA, 200 nmol/L TaqMan probe and 400 nmol/L each primer in 1× Quantitect Probe PCR master mix (Qiagen, Hilden, Germany) and with the following cycling conditions: 15 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each PCR plate included no-template controls as well as calibration standards to calculate the amount of phylloxera DNA/g sample and corresponding detection rates. Results were also converted to equivalent phylloxera number/200 g soil (hereafter referred to as phylloxera equivalent) using a calibration derived from Herbert et al. (2008).

Results

Effect of temperature and duration of soil sample storage on phylloxera detection

Across the two sampling dates, phylloxera was detected in 97% of the samples tested immediately upon arrival at SARDI (day 0) and in 75% of the samples stored for an extra 10 days after reception, irrespective of the storage temperature.

In March 2013, phylloxera was detected in 95% of the samples (CI 86–99%), irrespective of temperature and duration of storage and in 100% (CI > 95%) of samples stored for 0 and 1 day at any temperature (Table S1). Phylloxera was not detected in three samples stored for 3 days at 35°C, 10 days at 20°C and 10 days at 35°C. Replicates of these three samples, that is taken from the same set of vines and stored at the same temperature for different lengths of time, returned positive results (Table S1).

In July 2013, overall detection rate as well as the phylloxera equivalent per 200 g soil were lower than that in March 2013, whether the samples were frozen or not prior to storage (Tables S2, S3). Phylloxera was detected in 78.3% (CI 66–88%) and in 88.3% (CI 77–95%) of frozen and non-frozen samples, respectively, including all but one (non-frozen) sample processed with no further storage (Tables S2 and S3). Of the eight samples taken from the set of vines number 10 (all stored at 10°C), only one returned a low positive result, although all samples collected from that same set of vines in March 2013 returned positive results (Tables S1, S2, S3).

The overall variability in phylloxera number was large with the phylloxera equivalent per 200 g soil ranging from 1 to 8017 and from 0.08 to 829 in March and July, respectively. The average moisture content was 13 and 18% in March and July, respectively (Tables S1, S2, S3). Decomposition was observed at each temperature and regardless whether the samples had been frozen before storage (Table 1). As there was no significant difference between experiments for a given temperature, pooled decomposition rate and half-life were calculated for each storage temperature based on the two sampling events (Table 1). The half-life of phylloxera DNA was estimated to be 4.1, 3.6 and 2.6 days at 10, 20 and 35°C, respectively.

Effect of composite samples on phylloxera detection

Phylloxera was detected in all infested soil-only samples in both March and May 2014, but the mean phylloxera equivalent per 200 g was higher in March (157.4) than in May (16.5) (Table 2). No phylloxera was detected in any of the non-infested soil-only samples.

In March 2014, phylloxera was detected in all composite samples, including the ones with the least phylloxera-infested soil (20 g in 400 g total). In May 2014, phylloxera was detected in all samples containing 50 g or more of infested soil, and in 87.5, 50, 50 and 0% of samples containing 20, 10, 5 and 1 g of infested soil, respectively (Table 2). For both dates, the phylloxera equivalent per 200 g soil was directly proportional to the amount of contaminated soil in the composite samples, with higher numbers across the dilution range in March than in May.

Based on the results obtained in May 2014, it was estimated that 50% detection can be achieved when there is 0.48 phylloxera equivalent per 200 g of soil and up to 100% when there is 1.5–2 phylloxera equivalent per 200 g of soil (Figure 1).

Effect of depth, position, location and time of sampling on phylloxera detection

Position of sampling. The trial was initially designed to assess the effect of three sampling positions (close to trunk, below

Table 1. Effect of temperature on decomposition rate and half-life of phylloxera DNA in soil collected in the Yarra Valley vineyard in March and July 2013.

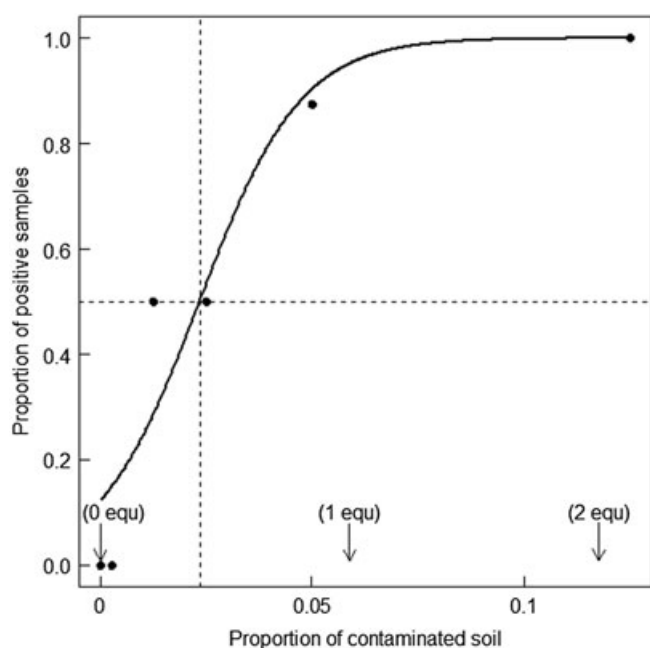
Storage temperature (°C)†	March 2013			June 2013						Pooled‡		
	Not frozen			Frozen		Not frozen						
	Slope‡	SE	T50§	Slope	SE	T50	Slope	SE	T50	Slope	SE	T50
10	-0.234	0.071	3.0	-0.193	0.112	3.6	-0.078	0.091	8.9	-0.168	0.053	4.1
20	-0.300	0.106	2.3	-0.166	0.097	4.2	-0.116	0.109	6.0	-0.194	0.060	3.6
35	-0.385	0.125	1.8	-0.190	0.093	3.6	-0.213	0.079	3.3	-0.263	0.058	2.6

†Samples were stored at 10, 20 and 35°C for 0, 1, 3 and 10 days upon reception in the laboratory or after being frozen at -20°C for 24 h. ‡Estimate of phylloxera decomposition rate. §Half-life of phylloxera in days. ¶For each temperature, pooled slope and half-life were calculated across all sampling dates/treatments. SE, standard error.

Table 2. Mean phylloxera equivalent and number of phylloxera positives in composite samples made up by mixing various amounts of phylloxera-infested and non-infested-soil collected in the Rutherglen vineyard in March and May 2014.

Amount of infested soil (g)†	March 2014			May 2014		
	Phylloxera/200 g‡	Phylloxera/200 g (fitted)	No positive§	Phylloxera/200 g	Phylloxera/200 g (fitted)	No positive
0	0	0	0	0	0	0
1		Not done		0	0.043	0
5		Not done		0.34 ± 0.21	0.21	4
10		Not done		0.26 ± 0.17	0.43	4
20	3.6 ± 1.3	8.1	8	0.88 ± 0.24	0.85	7
50	10.6 ± 2.5	20	8	8.0 ± 5.0	2.13	8
100	37 ± 12	40	8	5.4 ± 2.4	4.3	8
150	52 ± 14	60	8		Not done	
200	80 ± 28	81	8	7.6 ± 1.2	8.5	8
250	97 ± 19	101	8		Not done	
300	136 ± 24	121	8		Not done	
400	157 ± 37	161	8	16.5 ± 3.1	17.1	8

†Amount of phylloxera-infested soil in 400 g composite sample. ‡Phylloxera equivalent per 200 g of soil ± error estimates. §Number of positive samples out of eight replicates.

**Figure 1.** Estimation of the probability of detection of phylloxera in composite soil samples based on the proportion of positives amongst 400 g samples made up by mixing various amounts of phylloxera-infested and non-infested soil. Estimation was based on the results obtained from soil collected in May 2014 in the Rutherglen vineyard. Equ: phylloxera equivalent per 200 g of dry soil.

dripper and between rows) together with sampling location, time and depth on phylloxera detection. With samples collected in March 2013, both frequency of detection and phylloxera equivalent per 200 g were much lower between rows than close to the trunk and below the dripper ($P < 0.001$), irrespective of depth of sampling and vineyard (Table 3). Samples were also more difficult to collect between rows because the soil profile was drier. Subsequently, between-row samples were not collected.

Across all sampling times and depths, the frequency of detection was usually higher close to the trunk than below the dripper in the King Valley and the Yarra Valley and the opposite in Rutherglen (Figure 2). In the 0–10 cm layer of soil, the

Table 3. Effect of position of sampling on the number of phylloxera positives (out of a possible eight) in composite soil samples collected in the King Valley, Rutherglen and the Yarra Valley vineyards in March 2013.

Location	Position	No of positive samples		
		0–10†	10–20	20–30
King Valley	Trunk	3	1	3
	Dripper	3	2	4
	Between row	0	2	2
Rutherglen	Trunk	7	8	8
	Dripper	8	7	8
	Between row	3	0	0
Yarra Valley	Trunk	8	8	8
	Dripper	8	8	8
	Between row	6	4	5

†Depth of sampling (cm).

difference was not significant ($P = 0.88$) but suggested more detections near the trunk than below the dripper. Moreover, absence of detection across all replicates was observed only with samples collected below the dripper in the King Valley and more often deeper in the soil profile (Figure 2).

Location of sampling. Frequency of detection of phylloxera was consistently and significantly ($P < 0.001$) lower across seasons, positions and depths of sampling in the King Valley (0–0.5) compared with that in Rutherglen (0.625–1) and in the Yarra Valley (0.5–1) (Figure 2). Phylloxera equivalent per 200 g was also consistently and significantly lower ($P < 0.001$) in the King Valley than in the other two vineyards across all sampling depths and positions except in summer 2013, when samples collected from all locations had a low phylloxera equivalent per 200 g ($P < 0.001$, Table 4).

Time of sampling. In 2013, the frequency of detection remained similar across seasons for a given vineyard, position

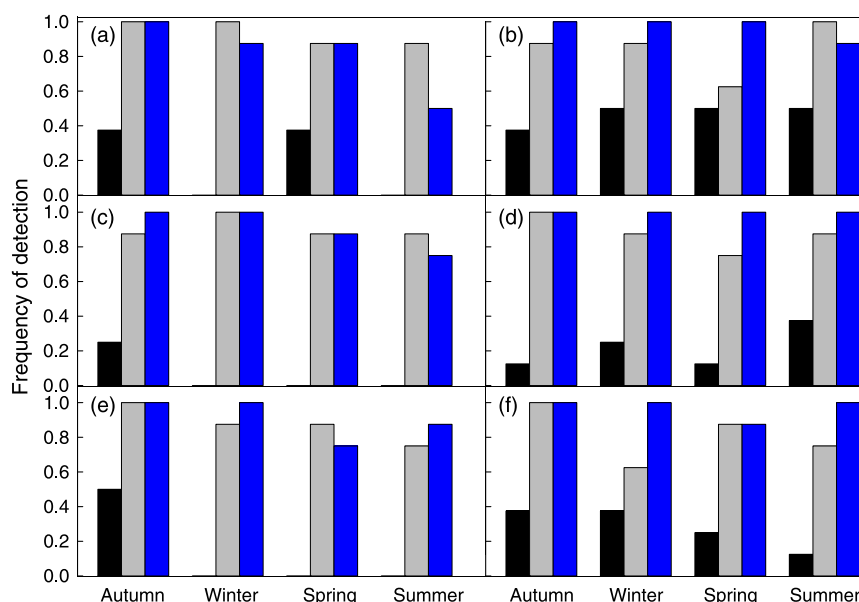


Figure 2. Frequency of detection of phylloxera (assuming all vines are infested) in composite soil samples collected (a, c, e) below the dripper and (b, d, f) close to the trunk, (a, b) 0–10, (c, d) 10–20 and (e, f) 20–30 cm deep, in the King Valley (■), Rutherglen (□) and the Yarra Valley (▣) vineyards in autumn, winter, spring and summer 2013.

Table 4. Effect of location of sampling on mean phylloxera equivalent per 200 g in composite soil samples collected in the King Valley, Rutherglen and the Yarra Valley vineyards in autumn, winter, spring and summer 2013.

Location	Mean phylloxera equivalent per 200 g soil†			
	Autumn	Winter	Spring	Summer
King Valley	6.4 ± 5.1	4.5 ± 2.2	0.16 ± 0.06	1.26 ± 0.81
Rutherglen	237 ± 40	28.5 ± 6.1	15.7 ± 4.4	89 ± 25 (3.2, 102)‡
Yarra Valley	721 ± 140	94 ± 16	3.38 ± 0.61	1.54 ± 0.37

†Mean phylloxera equivalent values (± error estimates) were estimated means from GenStat ANOVA command using results from composite samples collected across depth and position but excluding between row samples. ‡First number indicates the overall mean phylloxera equivalent per 200 g soil in samples collected in Rutherglen in December 2013 / January 2014 followed in brackets by the mean phylloxera equivalent per 200 g soil in samples collected in December 2013 and in January 2014.

and depth of sampling (Figure 2). In contrast, time of sampling had a significant effect on phylloxera numbers ($P < 0.001$), with a higher phylloxera equivalent observed in autumn (March 2013) and an overall lower phylloxera equivalent in summer in all three vineyards (Figure 3). Furthermore, the samples collected in January 2014 to finalise the summer sampling interrupted by rain in Rutherglen revealed that mean phylloxera/200 g increased from 3.2 to 102 between December 2013 and January 2014 (Table 4). This large increase in DNA concentration was associated with a moderate but not significant increase in detection rate from 67 to 88%.

In 2014, both the frequency of detection (data not shown) and phylloxera equivalent in samples collected close to the trunk and 0–10 cm deep showed the same trends as in 2013 with, in particular, a higher phylloxera equivalent in both early (March 2014) and late (May 2014) autumn (Figure 3).

Depth of sampling. With samples taken close to the trunk, 70 to 80% detection was achieved in the 0–10 cm surface layer of soil, with little additional detection in the 10–20 cm layer and even less in the 20–30 cm layer (Figure 4). A similar pattern was observed with samples taken below the dripper, with a noticeably lower overall detection rate in winter and summer, which further supports previous observations that more

detection is likely close to the trunk than below the dripper. The cumulated detection rate with samples collected between rows in autumn was lower than that of samples taken both

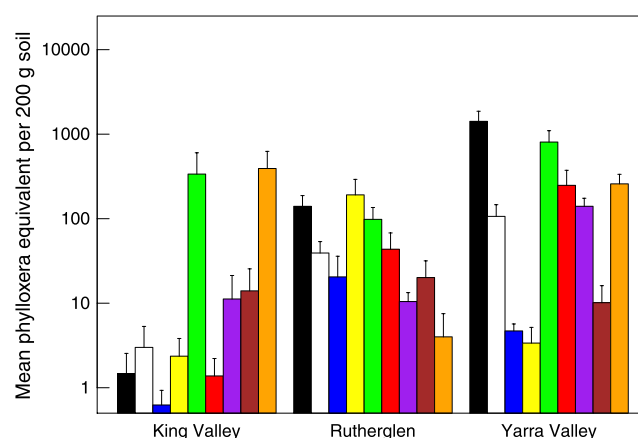


Figure 3. Mean phylloxera equivalent per 200 g soil (log y axis) across seasons over a 2-year trial in the King Valley, Rutherglen and the Yarra Valley vineyards. Means were calculated across eight composite soil samples each taken close to the trunk of five infested vines, 0–10 cm deep. Autumn 2013 (■); winter 2013 (□); spring 2013 (▣); summer 2013/14 (▤); autumn 2014 (■); late autumn (May) 2014 (▣); winter 2014 (▤); spring 2014 (▥); summer 2014 (▦).

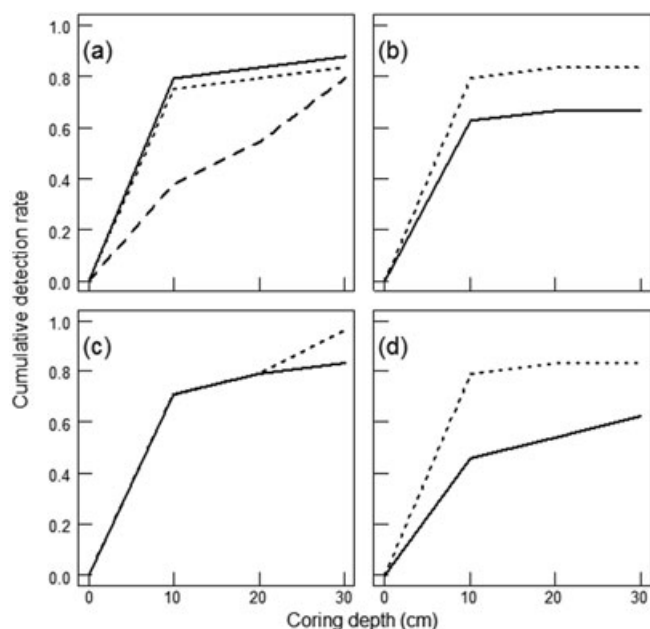


Figure 4. Effect of depth of sampling on the phylloxera detection rate in soil samples collected under the dripper (—), close to the trunk (---) or between rows (- -) of infested vines in (a) autumn, (b) winter, (c) spring and (d) summer 2013. Between-row samples were collected only in autumn. The cumulated detection rate was calculated using results obtained across three infested vineyards in the King Valley, Rutherglen and the Yarra Valley.

close to the trunk and below the dripper, with 0.37, 0.54 and 0.79 in the 0–10, 10–20 and 20–30 cm layer, respectively (Figure 4).

Distance from the trunk. The frequency of detection of phylloxera followed a negative gradient relative to the distance from the trunk, with the proportion of positive samples decreasing from 0.625 to 0.25 between 5 and 45 cm from the trunk (not shown). This gradient was not significant.

When the phylloxera equivalent was plotted against the distance from the trunk as a boxplot, there were several outliers which contributed to the overall uncertainty (Figure 5). However, a nonlinear regression of the upper quartile of the form [DNA amount = $27 \pm 4.02 \times \exp(-0.159 \pm 0.0212 \times \text{Distance})$] revealed a statistically significant effect of the distance from the trunk on the number of phylloxera detected.

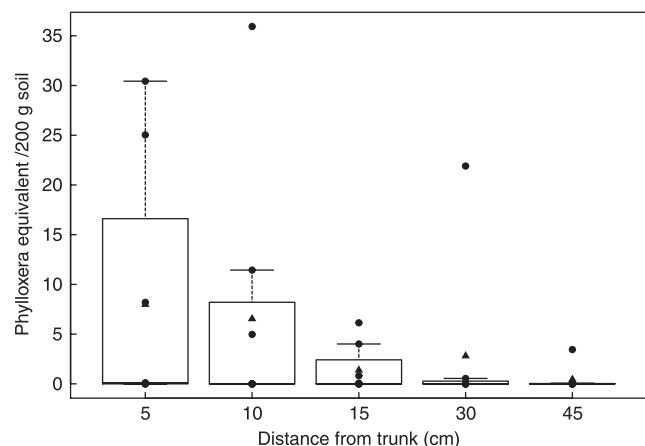


Figure 5. Mean (▲) and observed (●) phylloxera equivalent per 200 g soil in samples collected 5, 10, 15, 30 and 45 cm from the vine trunk, 0–10 cm deep in the King Valley vineyard in May 2014.

Comparison between qPCR and visual assessment. In the King Valley, the visual assessment of the vine roots for phylloxera presence was completed after the first sampling, in autumn 2013, which provided an opportunity to compare the efficiency of detection between visual and qPCR assessment. Of the 40 vines used for the experiment, phylloxera was visually identified on 11, suspected (evidence of root galling but no insect found) on six and not found on 23, with four of the eight groups supposedly not containing any infested vines (Groups 1, 2, 4 and 5; Table 5). Using qPCR phylloxera was detected in seven of the eight groups of vines, noticeably in groups 2 (1 instance), 4 (3 instances) and 5 (1 instance), in which no phylloxera was visually detected by root examination (Table 5). Group 1 did not return any positive result by visual or qPCR assessment (Table 5).

Discussion

For phylloxera detection, qPCR has been shown to be at least as sensitive as the most commonly used ground survey and emergence trap methods (Herbert et al. 2008, Powell 2012a). The absence of recommendations for collecting and handling soil samples has impeded the adoption of qPCR by the industry for routine surveillance and monitoring. In this study, we demonstrate that with simple collection and handling precautions, qPCR allows consistent and reliable detection of grape phylloxera in composite soil samples, with an estimated detection limit of 1.5 to 2 phylloxera per 200 g of soil. We also show that a higher detection rate is obtained with samples taken 0–10 cm deep within 5 cm of the vine trunk and that phylloxera can be detected all year round, but in higher numbers from mid-summer to early winter.

The integrity of DNA is of paramount importance for detection by qPCR and can be affected by sample handling and storage. Moisture has been shown to be particularly detrimental to the integrity of DNA from dead organisms in soil and can therefore affect PCR detection (Herdina et al. 2004a,b). For qPCR testing, the recommendation is to dry soil samples as soon as possible after collection (Ophel-Keller et al. 2008). In our study, samples were securely packed in the vineyards and sent for analysis without drying to limit handling, in agreement with phylloxera quarantine regulations (National Vine Health Steering Committee 2009). The average moisture content of 13% recorded in soil collected in March 2013 and 18% in July 2013 was likely to affect phylloxera DNA if samples were subjected to an extended period of storage before drying. Our estimate of phylloxera DNA half-life in soil samples of 4.1, 3.6 and 2.6 days, at 10, 20 and 35°C, respectively, is consistent with degradation rates reported for DNA of dead fungal mycelia and nematodes in moist soil (Herdina et al. 2004a,b). This finding suggests that in the soil collected for testing, most phylloxera were dead, likely as a result of the soil disturbance caused by coring. This is further supported by the absence of a significant difference between frozen and non-frozen samples. The large variability in phylloxera numbers observed between samples, however, also suggests that in some instances, the lack of detection was a consequence of the clustering of phylloxera in soil rather than of the storage conditions, which may have led to an overestimation of the degradation rate. This was particularly obvious in July 2013, when phylloxera numbers were low even before storage. Despite DNA degradation, the overall detection rate decreased only from 97% within 2 days of collection to 75% after 12 days, regardless of storage temperature.

Collection of composite samples is recommended for soil-borne pathogen detection as it minimises the effect of spatial

Table 5. Comparison of phylloxera detection results in eight groups of five vines in the King Valley vineyard as determined by visual and quantitative PCR assessment.

Vine group	Visual assessment†	qPCR assessment§																	
		0–10‡			10–20			20–30			0–10			10–20			20–30		
		Autumn			Winter			Spring			Summer								
1	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
2	0	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–			
3	3	–	–	–	–	+	+	–	–	+	–	–	–	–	–	–			
4	0	–	–	+	+	–	–	+	–	–	–	–	–	–	–	–			
5	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			
6	5	+	–	+	+	+	+	+	–	–	+	+	–	–	–	–			
7	1.5	+	+	+	+	–	+	–	–	–	+	+	+	–	–	–			
8	4.5	+	–	–	+	–	–	+	+	+	+	+	–	–	–	–			
Total	–	3	1	3	4	2	3	4	1	2	4	3	1						

†The roots of the five vines within each group were visually inspected; each vine was given a score of 1 when phylloxera was visually identified, 0.5 when phylloxera was suspected and 0 when no phylloxera was found, giving a maximum score of 5 per group of vines. ‡Sampling depth (cm). §qPCR results (+ for positive, – for negative) are for composite samples taken 0–10, 10–20 and 20–30 cm deep, close to the vine trunk in autumn, winter, spring and summer 2013. qPCR, quantitative PCR.

variation between samples (Ophel-Keller et al. 2008). For phylloxera, the use of composite samples also has the potential to increase both the coverage of the survey and its reliability by accounting for the uneven distribution of phylloxera across a vineyard. Compositing, however, needs to be implemented carefully in order to avoid detrimental consequences on detection. Our estimate of a limit of detection of 1.5 to 2 phylloxera per 200 g composite soil sample indicates that the true limit of detection of the assay is likely lower than the 20 phylloxera per 200 g soil published previously, based on results using spiked soil samples (Herbert et al. 2008). Our results demonstrate that a sampling protocol based on composite samples is appropriate for phylloxera detection by qPCR. It also means that the cost of a qPCR-based survey can be significantly reduced without compromising detection by combining soil cores into one sample. This is an important observation as cost is considered by most authors as the main impediment to qPCR use for routine phylloxera surveillance (Herbert et al. 2008, Powell 2012b).

Reliable detection of phylloxera relies on several parameters, amongst which location and time of sampling have been shown to be important. With the ground survey and emergence trap methods, phylloxera detection is highly dependent on seasonal abundance and activity in soil, and both methods have shown lower efficiency in winter (Benheim et al. 2012, Powell 2012a). Our results for phylloxera detection by qPCR show that both detection rate and number are lower in the King Valley compared with that in the Yarra Valley and in Rutherglen. Such variations between vineyards are not uncommon and could be linked to environmental factors, as well as to the phylloxera and vine genotypes (Benheim et al. 2012). In addition, the first phylloxera detection dates back to 1992 in the King Valley, 2008 in the Yarra Valley and 2013 in Rutherglen. This suggests that the infestation is much older in the King Valley vineyard than in the other two vineyards, which could contribute to a lower detection rate and number. Despite these differences, the three vineyards showed similar trends over 2 years. Higher phylloxera numbers were found from late summer to early winter, which is consistent with previously described seasonal variations in phylloxera abundance (Benheim et al. 2012, Powell 2012a). Despite this large variation in number, detection rate within each vineyard did not

change significantly across seasons. This contrasts with previous work, which reported that qPCR had a lower detection efficiency in winter (Powell 2012b). The greater detection efficiency observed in our study may be due to the use of composite samples, which accounts better for uneven distribution across vineyards.

Phylloxera location in an infested vineyard is an important parameter for designing a sampling strategy and is intimately related to its physiology and life cycle. For instance, because of its feeding habits, phylloxera is usually found on or in close proximity to vine roots (Benheim et al. 2012). This suggests that targeting vine roots when collecting soil samples for phylloxera testing is likely to increase the chance of detection. In addition, in warm soil (late spring and summer), a higher phylloxera population level is usually found on the soil surface (Benheim et al. 2012). Phylloxera, however, is also known to move down the soil profile in adverse conditions (Powell 2012a) and in Australia has been found as deep as 1 m below the soil surface (Benheim 2012). When comparing sampling position and depth in this study, we found that samples collected close to the trunk and below the dripper give a similar result, with an overall slightly better detection close to the trunk, whereas samples collected between rows had both a lower frequency of detection and a lower phylloxera number. This difference may indicate that more vine roots were present in the soil close to the trunk and below the dripper than between rows, therefore increasing phylloxera detection. A qPCR assay to check for the presence of vine roots in the soil samples is being evaluated, which could be used as an internal control to add reliability to the phylloxera qPCR results. We also found that most of the detection was achieved within the 0–10 cm surface layer of soil. These findings were not affected by seasons and only slightly by the vineyard, which suggests that over the survey period, phylloxera position in the soil profile did not vary greatly, even though its abundance changed with the season.

Conclusion

Based on the results of this study, a protocol is proposed for collection and handling of soil samples for phylloxera detection by qPCR. It is first recommended to collect composites of soil cores. The low limit of phylloxera detection by qPCR indicates that compositing samples up to 400–500 g, which is the maximum

size of samples that SARDI's Molecular Diagnostic Centre can process, will not detrimentally affect detection rate. In addition, the ability to detect new infestations is more likely to be determined by the chance of an individual core containing one phylloxera rather than by the limit of detection of the assay. Cores should be taken 0–10 cm deep and 5 cm from the vine trunk. Sampling below the dripper could be a suitable alternative, but some vineyards are not irrigated, which would make it difficult to base recommendations on the dripper position. While detection can be achieved all year round, it is recommended to sample from late summer to early winter to take advantage of the higher number of phylloxera and to avoid periods of the year when phylloxera could move down the soil profile because of adverse conditions. Once collected, samples need to be stored in cool conditions and delivered for testing within a few days of collection. Precautions need to be taken to avoid exposure to high temperature for a prolonged period of time, including during sampling and transport. This sampling protocol does not require any particular expertise and can be implemented by vineyard owners.

Although quarantine measures have been mostly efficient at containing phylloxera to a few Phylloxera Infested Zones since its first introduction in Australia, new outbreaks can still occur, and a few have been reported in recent years (Powell 2012b). Early detection and surveillance therefore remain a high priority to protect the Australian grapevine industry. With the low limit of detection and the ability to detect a wide range of phylloxera genotypes demonstrated in this study, the potential for high throughput combined with the fast and easily implemented composite soil sampling strategy developed here, qPCR stands out as the technique of choice for early detection and rapid management intervention. A cost/efficiency comparison between qPCR, ground survey and emergence trap methods is underway to fully assess the three methods. An added benefit of the qPCR method is that the same DNA extracts can be used to monitor other soil borne pests and pathogens of grapevines, such as nematodes, where suitable assays are available.

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Supporting information

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Table S1. Phylloxera equivalent per 200 g detected by quantitative PCR in soil samples collected in the Yarra Valley vineyard in March 2013 and stored at 10, 20 and 35°C for 0, 1, 3 or 10 days immediately after arrival at the laboratory.

Table S2. Phylloxera equivalent per 200 g detected by quantitative PCR in soil samples collected in the Yarra Valley vineyard in July 2013 and stored at 10, 20 and 35°C for 0, 1, 3 or 10 days immediately after arrival at the laboratory.

Table S3. Phylloxera equivalent per 200 g detected by quantitative PCR in soil samples collected in the Yarra Valley vineyard in July 2013 and frozen at -20°C for 24 h upon arrival at the laboratory, before being stored at 10, 20 and 35°C for 0, 1, 3 or 10 days.