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Australian Grape and Wine Authority

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SOUTH AUSTRALIAN RESEARCH AND DEVELOPMENT INSTITUTE

Sampling strategies for sensitive, accurate and cost effective detections of Phylloxera for quantifying area freedom status


Phylloxera adults, nymphs and eggs (Agriculture Victoria, Rutherglen)


Galls on grapevine roots (Agriculture Victoria, Rutherglen)

FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **PGI 1201**

Principal Investigator: **Vinehealth Australia**

Research Organisation: **Vinehealth Australia**

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Acronyms:

AGWA	Australian Grape and Wine Authority
DEDJTR	(Victorian) Department of Economic Development, Jobs, Transport and Resources
DPI NSW	Department of Primary Industries New South Wales
NVBC	National Vine Biosecurity Committee
PBCRC	Plant Biosecurity Cooperative Research Centre
PIRSA	Primary Industries and Regions South Australia
SARDI	South Australian Research and Development Institute
SDQMA	Subcommittee (of Plant Health Australia) on Domestic Quarantine and Market Access
SNPHS	Subcommittee (of Plant Health Australia) on National Plant Health Surveillance
SPHD	Subcommittee (of Plant Health Australia) on Plant Heath Diagnostics

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1. ABSTRACT

A rapid, cost-effective and accurate soil sample protocol for detection of phylloxera by quantitative PCR (qPCR) was developed. Composite soil samples collected in a grid pattern should be handled and transported to avoid heating above 20°C, reach the laboratory within 48 hours of collection and dried within 24-48 hours, to limit phylloxera DNA degradation. Detection limit of qPCR was estimated at 1.5 to 2 phylloxera per 200g dry soil. Highest phylloxera detection rate and numbers were obtained with samples taken 0-10cm deep and as close to the vine trunk as possible (within 10cm), and higher numbers were consistently found from mid-summer to early winter.

2. EXECUTIVE SUMMARY

Grape phylloxera (*Daktulsphaira vitifoliae*) is a devastating soft-bodied insect pest of grapevines worldwide, destroying *Vitis* species (commercial grapevines and ornamental vines) by feeding on their roots. Phylloxera is native to eastern North America, first affecting native European *Vitis vinifera* in the late 19th century. In Australia, grape phylloxera is currently confined to parts of Victoria and New South Wales. Commercial vine plantings in Australia are predominantly own-rooted *Vitis vinifera*, highly susceptible to attack by phylloxera (Vinehealth Australia, 2017). A lack of available chemical or biological controls for phylloxera means that pulling out infested vines and replanting with new vines grafted onto phylloxera-resistant rootstock is the only control option. The cost of grafted vine material is approximately 3 times that of own-rooted vine material ($4.20 versus $1.40 per vine).

Preventing movement of phylloxera to currently uninfested areas is therefore of utmost importance to the Australian wine and grape industries. To prevent the spread of phylloxera from infested areas, each state has documented their restrictions and allowable movements of phylloxera 'risk vectors' in Plant Quarantine Standards. These 'movement conditions' are underpinned by the National Phylloxera Management Protocol (National Vine Health Steering Committee, 2009) and must be adhered to by all stakeholders.

Maintaining whole or part-state area freedom from phylloxera and minimising spread out of infested areas requires accurate surveillance to determine where phylloxera is and is not. Typically, phylloxera infestations are characterised by uneven distribution. The only currently endorsed surveillance method for phylloxera is the dig method involving root inspection (National Vine Health Steering Committee, 2009). This method is resource and skill intensive and is limited to times of the year when phylloxera are closest to the soil surface and the level of soil moisture is suitable for digging. As a result, surveillance has most often been a regulators' domain and not undertaken by industry to the extent it should be. This is particularly pertinent given biosecurity risk is ever heightening with recent increasing phylloxera detections inside the Maroondah Phylloxera Infested Zone (PIZ) in Victoria and increasing movement of people, machinery and equipment between winegrowing regions and states.

This project was part of a larger PBCRC-funded project (#2061) concluding in March 2018, entitled 'Sampling strategies for sensitive, accurate cost effective detection for phylloxera for quantifying area freedom status'. The aim was to develop a fast, accurate, cost-effective phylloxera surveillance method for use by regulators and growers alike, using a qPCR method for phylloxera detection (Ophel-Keller et al., 2008) underpinned by a strong soil sampling protocol. It is expected that this DNA method, once endorsed by Plant Health Australia's Subcommittees (SDQMA, SPHDS and SNPHS) will be used as part of an integrated approach to phylloxera surveillance of vineyards, alongside the emergence trap and dig methods.

A range of parameters were evaluated in this study to determine their impact on the ability to accurately detect the relative quantity of phylloxera DNA in any given infested vineyard. This would then inform the development of a field sampling protocol for soil sample collection and handling. Parameters evaluated in this study included compositing of soil core samples, time of year of sample collection, effect of vine roots on likelihood of phylloxera DNA detection, soil core depth and position around the vine, and sample storage time and temperature.

The highest phylloxera DNA concentrations in different winegrowing regions, corresponded with times of peak phylloxera abundance in autumn months. Phylloxera DNA was however detected year-round with similar frequencies of detection, unlike the capability of other phylloxera surveillance methods. Phylloxera DNA was found to start decomposing at the time of sample collection but could confidently be detected down to 1.5-2 phylloxera equivalent per 200g dry soil. Highest detection rates and quantity of phylloxera DNA were found from soil cores containing *Vitis* DNA, and collected at a depth of 0–10cm, within 10cm from the vine trunk. The recommended field collection of soil samples involved taking cores in a grid pattern from vines located in every third row at every fifth panel. Compositing of 5 soil cores into a single sample did not affect detection rate of phylloxera DNA. The impact of spatial variation between samples was minimised by the compositing, allowing for increased coverage of the surveyed area by sampling more vines and increasing reliability of the test result by accounting for uneven distribution of phylloxera in a vineyard.

To minimise phylloxera DNA decomposition in the soil samples once collected, samples need to be delivered for testing within a few days of collection, and stored between 10-20°C (not frozen) during transport and at the laboratory, pre-drying.

A range of communication activities were undertaken as part of this project including a number of presentations, articles and grower field days. Further updates to industry will be forthcoming at the conclusion of the related PBCRC (#2061) project, once endorsement is gained for the DNA tool as an additional phylloxera primary surveillance method.

3. BACKGROUND

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 detected in 1875. In response, quarantine zones were rapidly defined to help prevent spread and assist with eradication attempts (Herbert et al., 2008). Currently, phylloxera is confined to the declared PIZs in Victoria (North East, Maroondah, Nagambie, Mooroopna, Upton and Whitebridge) and New South Wales (Sydney region and Albury/Corowa).

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:

1. Phylloxera Infested Zones (PIZs), where phylloxera is known to occur;
2. Phylloxera Exclusion Zones (PEZs), where current testing and historic information provide no evidence of phylloxera infestation; and
3. Phylloxera Risk Zones (PRZs), 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 have 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 only endorsed 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). A potential additional bonus is that the same extracted DNA sample can be used to monitor other soil microbes.

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, the 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 200g 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.

This project was conducted alongside another significantly larger project entitled ‘Sampling strategies for sensitive, accurate cost effective detection for Phylloxera for quantifying area freedom status PBCRC (#2061)’. A final project report due mid-2018 will extend findings from the trials undertaken herein and complete endorsement of the DNA tool as an additional phylloxera primary surveillance method.

Much of the content of this report has been transcribed from the peer-reviewed journal article Giblot-Ducray et al. (2016) published on the findings from this project.

4. PROJECT AIMS AND PERFORMANCE TARGETS

The objectives and planned outputs of this project are outlined below.

Project objectives of the original agreement

1. Determine the number of soil samples needed for at least 95 per cent confidence that phylloxera is not present and to achieve this in a cost-effective way.
2. Establish the number of vines and the number of samples from a vine that need to be factored into the method.
3. Assess the impact of different soils and locations on the sampling efficacy.
4. Establish guidelines to improve confidence and efficacy.
5. Assess the impact of time taken transporting the sample from the field to the processing lab on sample degradation.
6. Validate the method in different environments:
    a. Surveying across three regions (Yarra Valley, King Valley and Rutherglen).
    b. Seasonal influence - sampling at different times of the year.
    c. Impact of the moisture content of the sample.
7. Assess whether practitioners can accurately follow and perform the proposed sampling method.

As the project developed, project objectives were refined and revised to those below upon which this report will focus:

Revised project objectives

1. Assess the impact of soil type, sample depth and distance from the vine trunk on the sampling efficacy.
2. Assess the impact of time taken transporting the sample from the field to the processing lab on sample degradation.
3. Determine the limit of detection of the qPCR for phylloxera.
4. Validate the field sampling method in different environments:
    a. Surveying across three regions (Yarra Valley, King Valley and Rutherglen).
    b. Sampling at different times of the year.
    c. Sampling in vineyards of varying known levels of phylloxera.
    d. Impact of vine root on likelihood of phylloxera detection.
5. Determine the density of sampling required per hectare for accurate and cost-effective detection.
6. Assess whether practitioners can accurately follow and perform the proposed sampling method.
7. Determine the relative difference in phylloxera density undervine compared to in the headland area.

Planned project outputs of the original agreement

Outputs and activities 2012–13

Year 1	Output	Target Date	Activities
A	Research plan and trial sites finalised.	28/02/2013	Prepare a research plan and identify trial vineyards in Yarra Valley, King Valley and Rutherglen, Victoria. Establish an industry advisory group of 6-8 people representing grapegrowers, winemakers, WGGA, state and/or regional industry associations. Convene a meeting between project team, industry advisory group and collaborators outlining project objectives and timelines.
B	qPCR assay for phylloxera	30/06/2013	Polymerase chain reaction (qPCR) assay to assess sample integrity of phylloxera DNA following collection and transportation of infested soil samples.
C	Data set demonstrating the impact of storage conditions and time on the ability to detect phylloxera in soil samples	30/06/2013	Collect at least 120 soil samples from a phylloxera infested vineyard in the Yarra Valley, Victoria. Soil samples to be collected at 0-20cm deep from beneath the dripper, trunk and mid-row from five locations. Each treatment to have five replicates. Soil samples collected will be stored at 10, 20 or 35^{o}C for 2, 3, 5 or 12-14 days respectively. qPCR assay to be used to establish the impact of temperature and sampling time on detection of phylloxera DNA
D	A preliminary data set from the first season of field trials reporting how the ability to detect phylloxera in soil samples is likely to be affected by the number of vines sampled; the number of samples from a vine; the location of the samples within a vineyard; the timing of collection; and the soil type.	30/06/2013	Collect at least 432 soil samples from phylloxera infested vineyards in Yarra Valley, King Valley and Rutherglen, Victoria. Soil samples to be collected at 3 depths (0-10cm, 10-20cm and 20-30cm) beneath the dripper, trunk and mid-row. 8 x 5 vine groups (40 vines) will be sampled in each vineyard. Industry advisory group to review results and adjust project plan for second year, if required.
E	Progress report including a review of the project	30/06/2013	In consultation with GWRDC, conduct a review of the project and make recommendations for future work. (Stop/go review point)

Outputs and activities 2013–14

Year 2	Output	Target Date	Activities
A	A preliminary data set from the first two seasons of field trials reporting how the ability to detect phylloxera in soil samples is likely to be affected by the number of vines sampled; the number of samples from a vine; the location of the samples within a vineyard; the timing of collection; and the soil type.	30/06/2014	Collect at least 864 soil samples from phylloxera-infested vineyards in Yarra Valley, King Valley and Rutherglen, Victoria. Soil samples to be collected at 3 depths (0-10cm, 10-20cm and 20-30cm) beneath the dripper, trunk and mid-row. 8 x 5 vine groups (40 vines) will be sampled in each vineyard. Industry advisory group to review results and adjust project plan for third year, if required. An extension plan developed for third and fourth year of project

Outputs and activities 2014–15

Year 3	Output	Target Date	Activities
A	A data set from the first three seasons of field trials reporting how the ability to detect phylloxera in soil samples is likely to be affected by the number of vines sampled; the number of samples from a vine; the location of the samples within a vineyard; the timing of collection; and the soil type.	30/06/2015	Collect at least 864 soil samples from phylloxera-infested vineyards in Yarra Valley, King Valley and Rutherglen, Victoria. Soil samples to be collected at 3 depths (0-10cm, 10-20cm and 20-30cm) beneath the dripper, trunk and mid-row. 8 x 5 vine groups (40 vines) will be sampled from each vineyard. Develop a preliminary sampling protocol based on analysis of the data from the first three seasons. Industry advisory group to review results and adjust project plan for third year, if required.
B	Information on progress towards developing phylloxera sampling protocols available to the wine industry through at least one extension mechanism.	30/06/2015	Prepare and submit at least one industry article on current research and likely outcomes.

Outputs and activities 2015–16

Year 4	Output	Target Date	Activities
A	A full data set and its analysis from four seasons of field trials reporting how the ability to detect phylloxera in soil samples is likely to be affected by the number of vines sampled; the number of samples from a vine; the location of the samples within a vineyard; the timing of collection; and the soil type. Preliminary phylloxera sampling protocol available for review by the industry advisory group	31/12/2015	Collect at least 432 soil samples from phylloxera-infested vineyards in Yarra Valley, King Valley and Rutherglen, Victoria. Soil samples to be collected at 3 depths (0-10cm, 10-20cm and 20-30cm) beneath the dripper, trunk and mid-row. 8 x 5 vine groups (40 vines) will be sampled from each vineyard. Develop a sampling protocol based on analysis of the data from four seasons
B	Sampling protocol ready for extension to industry. Extension of project outcomes through at least four extension mechanisms.	30/6/2016	Complete sample processing and data analysis. Prepare sampling protocol for distribution. Hold at least three industry workshops on project outcomes. Submit at least two industry journal articles and at least one scientific paper to a refereed journal for publication. Produce material for distribution through the GWRDC Innovators Network.

5. METHODS

Methods outlined in section 5 reflect work required to meet the revised project objectives stated in section 4.

5.1 Vineyard details, infected vine selection and soil sample handling

The majority of the trials undertaken to develop the field sampling methodology for soil sample collection reported herein, were conducted on three different Victorian vineyards infested with phylloxera. Vineyard details are reported in Table 1.

Table 1. Phylloxera infested vineyards used in the soil sampling methodology trials.

Vineyard region	Soil type*	Variety	Rootstock	Planted	Phylloxera detection year
Yarra Valley	Restricted duplex soil with hard topsoil (sandy loam to clay loam over clay)	Chardonnay	Own roots	2000	2008
King Valley	Deep well-structured soil (sandy loam to loam topsoil grading to a well-structured subsoil)	Chardonnay	Own roots/ Schwarzmann	1987	1992
Rutherglen	Non-restrictive duplex soil with hard topsoil (hard setting sandy loam to clay loam over well -structured clay subsoil)	Shiraz	Own roots	1975	2013

*Generic soil type descriptions provided only (Dry and Coombe, 2004).

The three vineyards varied in soil type and phylloxera genotype. 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 10x magnifying glass before the first sampling, and infested grapevines flagged for future use. In the King Valley, 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 triple-bagged according to the current quarantine regulations (National Vine Health Steering Committee, 2009), transported into SA under quarantine permits and analysed in SARDI's quarantine approved facilities.

5.2 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. These experiments examined the effect of temperature on the rate of phylloxera DNA degradation in soil samples while in storage between collection and drying.

The first trial compared the effect of core types and temperature on the rate of decomposition of phylloxera DNA. Freezing at -18ºC for 24 hours has been shown to kill phylloxera, so the experiment was repeated and half the samples were frozen before applying the storage treatments. This second experiment aimed to assess whether killing phylloxera by freezing the soil affected DNA degradation.

Samples were processed for DNA extraction and phylloxera detection by qPCR, as described in 5.7. The results were used to estimate the rate of phylloxera DNA degradation in soil samples.

Data were analysed using R software (R Development Team, 2014). It was expected that DNA degradation would follow a first order equation:

$$DNA_t = DNA_0 \times \exp(-kt) + error_t \quad (1)$$

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. Equation 1 can be fitted after taking logarithms:

$$\ln(DNA_t) = \ln(DNA_0) + kt + error_t \quad (2)$$

Separate regressions were initially fitted for each replicate/treatment/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 the sets of 5 vines.

March 2013

Soil samples were collected in the Yarra Valley in March 2013 from 75 known infested vines (15 sets of five vines numbered 1 to 15) using two types of sampling tools as a comparison, either a 15mm diameter dig stick soil corer (Spurr Soil Probes, Adelaide, SA, Australia) or a hammer corer (>25mm diameter, collecting approximately three times the sample volume of the dig stick).

For each sampling tool, four soil cores were collected 10–15cm 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 per core type.

Samples were immediately stored at 5^{o}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^{o}C before drying the soil samples prior to DNA extraction and qPCR. The four composite samples from any one set of vines were stored at the same temperature (10, 20 or 35^{o}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.

June 2013

In June 2013, a series of 60 composite samples were collected using the same protocol as in March 2013, but using the dig stick corer only. Half the samples were frozen at -20^{o}C for 24 hours before applying the storage treatments (10, 20 or 35^{o}C for 0, 1, 3 or 10 days), to assess whether killing phylloxera by freezing the soil affected DNA degradation. The other samples were stored in a cool room at 4^{o}C prior to analysis.

For the remainder of the project, a 14mm diameter, 100mm AccuCoreB soil corer (SARDI) was used.

5.3 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. The bulk samples were separately homogenised with a jaw grinder (SP100x100, Labtechnics, Kilkenny, SA, Australia) prior to being used to prepare 400g 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 400g samples with phylloxera-infested soil only and another eight (five in May 2014) 400g 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.

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

The distribution of phylloxera in the soil around the vine was investigated to optimise the sampling strategy, assessing the effect of sampling depth, position relative to the trunk, location and time on phylloxera detection.

Samples were initially collected at three depths (0–10, 10–20 and 20–30cm), 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 (September 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 30cm deep core was taken 10–15cm from the trunk of each vine at each position, using a 15 mm diameter dig stick soil corer; each core was split into three depth sections (0–10, 10–20, 20–30cm); 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), 0–10cm deep, close to the trunks of the same infested vines and using the same sampling protocol as described above.

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 0–10cm deep at 5, 10, 15, 30 and 45cm 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 in section 5.7.

Data were analysed using R software (R Development Team, 2014). 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.

5.5 Effect of the presence of vine root on phylloxera detection

A *Vitis* qPCR assay to check for the presence of vine roots in the soil samples became available in 2015 for evaluation. To test whether this assay could be used as an internal control to add reliability to the phylloxera qPCR results, it was used on DNA extracted from a range of vineyard soils as part of other experiments in this project. Results of vine root spatial distribution (position and depth) between vineyards and different times of the year were analysed in conjunction with phylloxera detection levels.

The qPCR measures the amount of DNA in a sample, expressed in Cycles to Threshold (Ct) of PCR - a relative value that represents the cycle number (or number of duplications) at which the amount of amplified DNA reaches the threshold level of detection. In practice the duplication is close to, but not 100% efficient. Using exact duplication as an approximation, two samples that differ by one Ct unit would therefore differ by a factor of 2 in root DNA concentration. An estimate of the amount of vine root is therefore proportional to

$$R = 2^{-Ct}$$

The lack of calibration restricts the interpretation of the Ct values – R is therefore in arbitrary units.

5.6 Effect of sampling density on the precision of phylloxera detection

To optimise the sampling strategy, the effect of vineyard soil sampling density was investigated by assessing the relative differences in phylloxera detection from the collection of two offset sets of 8 composite samples of 40 cores each (referred to as ‘A series’ and ‘B series’) with results comparing standard rate sampling density versus double rate sampling density over a 200 panel area. Due to a lack of data in the July 2014 sampling on which to be confident with the results, the experiment was repeated in October 2014, also adding in evaluation of 120 cores.

Due to the insufficient number of samples collected in July 2014 on which to be confident with the results, the experiment was repeated in October 2014 on two vineyards. In the second experiment, 15 comparative samples of 75 cores each were collected in triplicate in an offset pattern across the vineyard ('A series', 'B series' and 'C series'). This enabled a comparison of the detection frequency using 15, 30 or 45 comparative samples per 375 panel area.

An additional sampling density trial was undertaken in February 2016 to further validate the sampling strategy and reported in the PBCRC report, entitled 'Sampling strategies for sensitive, accurate cost effective detection for phylloxera for quantifying area freedom status'.

5.7 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, processing for DNA extraction and phylloxera detection by qPCR was undertaken. 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'-AATCCGCAGGTTATACGAACATC, reverse primer 5'-CGGTCTCGTCAAATTTCGGA and TaqMan MGB probe 6FAM-CGACCCTCAGACAGG (Thermofisher, 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 1x Quantitect Probe PCR master mix (Qiagen, Hilden, Germany.) and with the following cycling conditions: 15 min at 95ºC followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Each PCR plate included negative 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 per 200g soil (hereafter referred to as phylloxera equivalent) using a calibration derived from Herbert et al. (2008).

The amount of phylloxera present was described both qualitatively and quantitatively. The qualitative description of phylloxera was expressed on a detected or non-detected basis as a percentage of samples where some phylloxera was recorded. This detection percentage is not analogous to the accuracy of the qPCR test itself. Due to the ability of the qPCR to accurately detect phylloxera down to a trace level, a low detection rate was less likely due to failure of the qPCR test to detect the phylloxera when present, but rather, absence of phylloxera in the soil sample.

The quantitative amount of phylloxera in a soil sample, tested by qPCR, was typically expressed as phylloxera equivalents per 200g of dry soil. This test quantifies the total phylloxera DNA present, both from whole or partial, alive or dead phylloxera, of all growth stages, including eggs.

6. RESULTS AND DISCUSSION

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

Results:

March 2013

Phylloxera was detected in 95% of the composite samples (CI 86–99%), irrespective of temperature and duration of storage and in 100% (CI > 95%) of composite samples stored for zero and one day at any temperature (Table S1). Phylloxera was not detected in three samples – one of five replicates stored for ten days at 20°C, one of five replicates stored for three days at 35°C and one of five replicates stored for ten days at 35°C (Table S1).

The mean number of phylloxera per composite sample was not significantly different from both corer types (Table 2). The decomposition rate was higher in the samples from the dig stick but the difference was not statistically significant (

Table 3, Figure 1).

There was a trend for increasing phylloxera DNA decomposition with increasing storage temperature but this effect was not statistically significant (p = 0.301) (Table 4, Figure 2).

Table 2. Effect of corer type on phylloxera numbers from each core type in March 2013.

Corer type	Mean number phylloxera per 200g soil	P-value
Dig stick	542 ± 195	1.000
Hammer corer	547 ± 195	

Table 3. Decomposition rates from samples collected by hammer corer and dig stick in March 2013.

Core type	Decomposition rate	Half-life (days)
Dig stick	-0.31 ± 0.09	2.26
Hammer corer	-0.21 ± 0.09	3.26

Table 4. Effect of storage temperature on decomposition rate of phylloxera DNA pooled across two corer types in March 2013.

Storage temperature (°C)	Decomposition rate	Half-life (days)
10	-0.234 ± 0.07	3.0
20	-0.300 ± 0.11	2.3
35	-0.385 ± 0.13	1.8

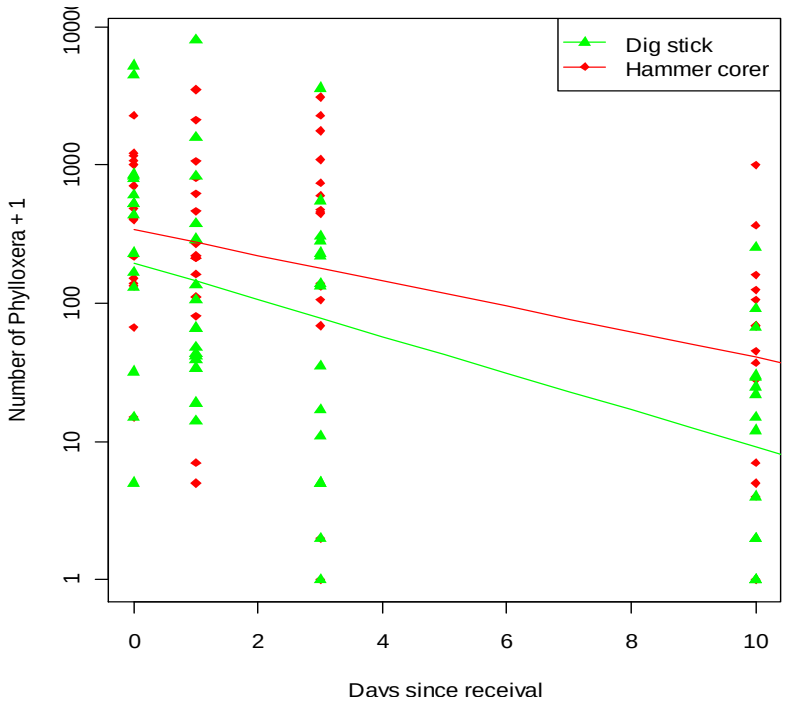


Figure 1. Effect of corer type on decomposition rate of phylloxera DNA in March 2013.

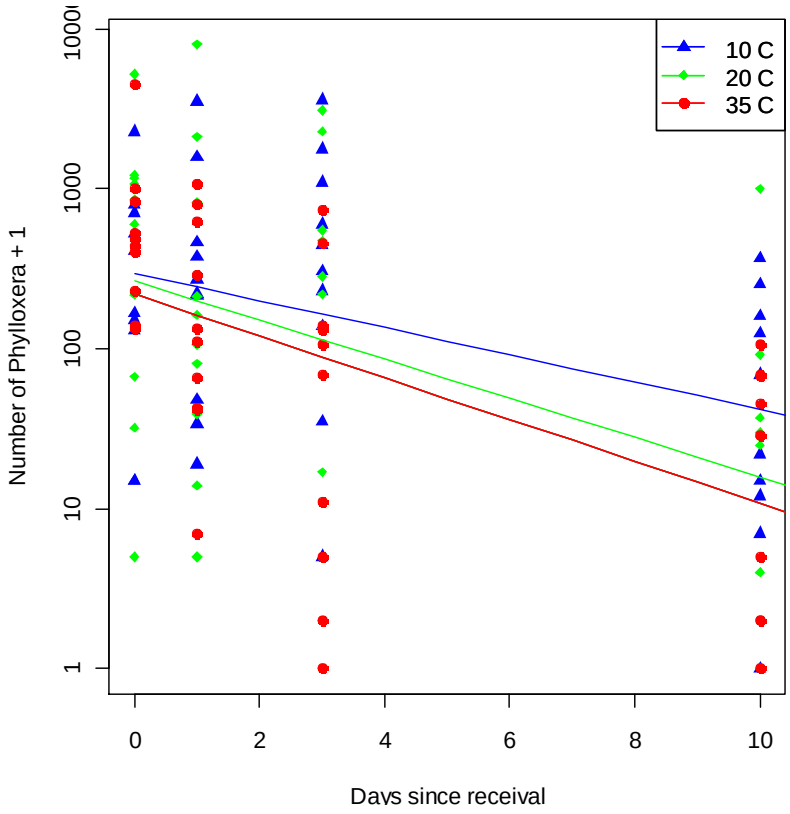


Figure 2. Decomposition rates of phylloxera DNA with different storage temperatures in March 2013. Results from the two corer types have been combined.

Discussion:

March 2013

Decomposition of phylloxera DNA from the time of sample collection to processing was anticipated, and considered more likely with a greater duration between sample time to analysis than the effects of sample temperature during storage or transportation. This was reflected in the relative inconsistency of detection of phylloxera DNA within increasing sample storage duration.

The measured half-life of two to three days was an important factor to consider, regarding timeliness of sample transport from the field to the laboratory, and capability of laboratory sample throughput. The greatest degradation of phylloxera DNA as sample storage temperature increased is an important factor to consider with respect to a future field sample packaging protocol.

The hammer corer was found to be completely impractical in the field for soil sample collection compared to the dig stick due to the sizeable effort required to extract the soil samples. Use of the hammer corer was discontinued beyond the May 2013 sampling time, and due to the relative similarity in the amount of phylloxera DNA obtained using both corer types, was not expected to negatively affect DNA detection thereafter.

Results:

June 2013

Overall phylloxera DNA detection rate and phylloxera equivalent per 200g soil were lower in June 2013 (53.45 unfrozen, 115.95 frozen) compared to March 2013 (542.32 unfrozen). Phylloxera was detected in 78.3% (CI 66–88%) in unfrozen soil samples (Table S3) and in 88.3% (CI 77-95%) of frozen samples (Table S3). For both unfrozen and frozen samples, phylloxera DNA detection was lowest at both 10°C and 35°C, compared to 20°C, and when sample storage was longest at ten days (Tables S2, Table S3). Frozen samples showed a large range of detection of DNA equivalents (Table S3).

Analyses were performed on the means of DNA equivalents (after logging) across composite replicates. Decomposition of phylloxera DNA was generally higher in frozen compared to unfrozen samples, irrespective of storage temperature, resulting in generally a shorter half-life for frozen samples (Table 5). Decomposition increased with increasing storage temperature in unfrozen samples (Table 5).

Prior to exposing the frozen and unfrozen soil samples to the various storage temperatures, the amount of phylloxera DNA measured (on a log scale) is shown in Figure 3 by the intercepts of the slopes on the y-axes. The intercepts were subject to an analysis of variance (classified by temperature and freezing) and showed no effect of either factor (data not shown). The freezing therefore did not affect the measurement of the initial amount of phylloxera DNA present.

Table 5. Average decomposition rate of phylloxera DNA from unfrozen and frozen composite soil samples in June 2013.

Storage temperature (°C)	Unfrozen		Frozen	
	Decomposition rate	Half-life	Decomposition rate	Half-life
10	-0.078 ± 0.09	8.9	-0.193 ± 0.11	3.6
20	-0.116 ± 0.11	6.0	-0.166 ± 0.10	4.2
35	-0.213 ± 0.08	3.3	-0.190 ± 0.09	3.6

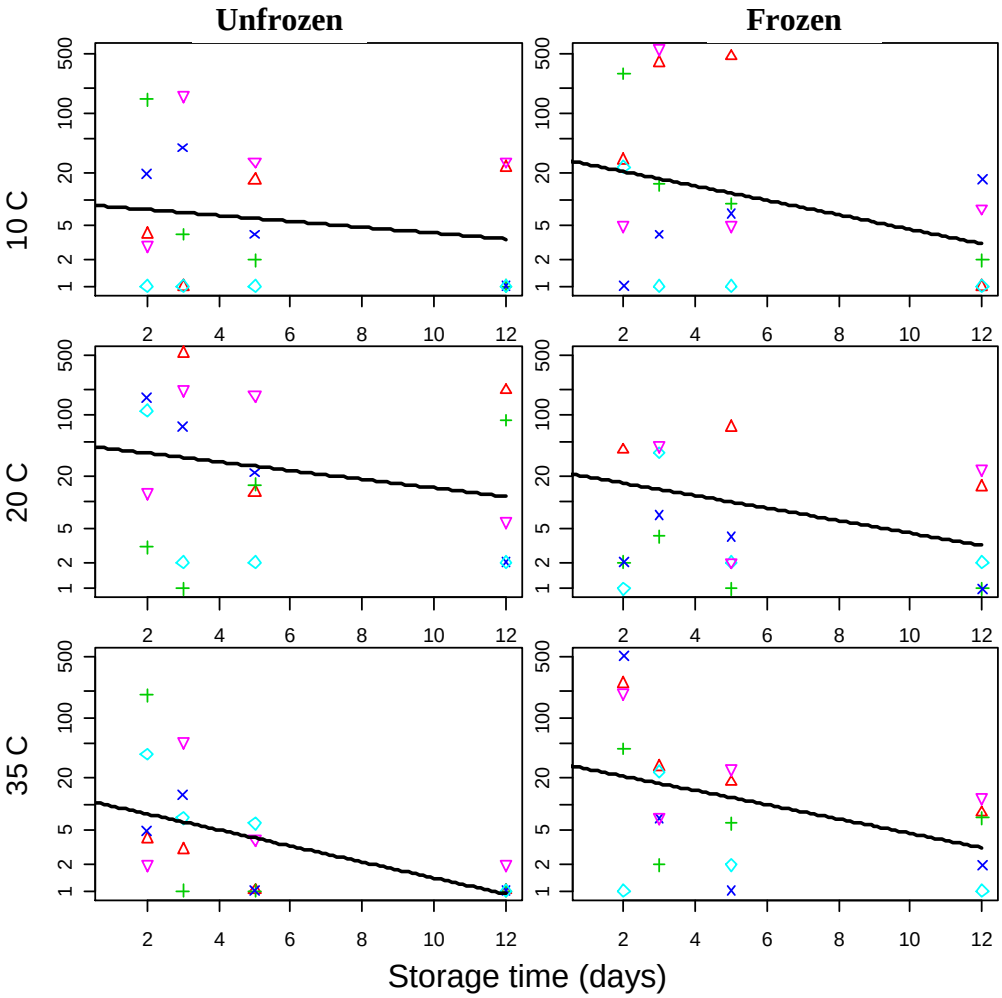


Figure 3. Effect of storage time on the amount of phylloxera DNA for frozen and unfrozen samples in June 2013. The line was fitted to the means of the 5 replicates.

Discussion:

June 2013

Repeating the March 2013 sampling in June 2013 was useful in being able to compare the relative detection rates and amount of phylloxera DNA. The lower detection rates and amount of phylloxera DNA in June are indicative of the movement of phylloxera down the soil profile in the cooler months and therefore indicative that winter should be identified as a non-preferred sampling time for phylloxera surveillance.

Overall results:

Across the two sampling dates, phylloxera was detected in 98% of the samples tested immediately upon arrival at SARDI (day 0) and in 73% of the samples stored for an extra ten days after receival, irrespective of the storage temperature.

The overall variability in phylloxera number was large with the phylloxera equivalent per 200g soil ranging from 0.42 to 8017 and from 0.08 to 2308 in March and June, respectively. The average moisture content was 13.4% and 18.6% in March and June, respectively (Table S1, Table S2, Table S3). Decomposition was observed at each temperature and regardless whether the samples had been frozen before storage (Table 4, Table 5).

Overall discussion:

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) to stabilise the DNA within the sample. In this 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 13.4% recorded in soil collected in March 2013 and 18.6% in July 2013 was likely to affect phylloxera DNA if samples were subjected to an extended period of storage before drying.

The general range of two to four days of phylloxera DNA half-life in soil samples and decreasing pattern with increasing storage temperature 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, at least some of the phylloxera were dead, possibly as a result of the soil disturbance caused by coring, or perhaps due to sample packaging. This is further supported by the absence of a significant difference between frozen and unfrozen samples. Despite DNA degradation, the overall detection rate decreased only from 98% within two days of collection to 73% after twelve days, regardless of storage temperature.

Both the March and June 2013 experiments helped to identify suitable field sampling times and storage conditions of the samples both during transportation to the laboratory and at the laboratory prior to analysis. Although freezing of soil samples upon arrival at the laboratory did not appear to reduce the amount of phylloxera detected compared to unfrozen samples, freezing did contribute to a higher degradation of phylloxera DNA in the soil samples irrespective of storage temperature. This will be an important consideration when maintaining sample integrity for potential sample retesting.

To limit phylloxera DNA decomposition, soil samples should ideally remain at approximately 10-20°C during transport and reach the laboratory within 48 hours of collection. Upon receival at the laboratory, these samples should be dried within approximately 24-48 hours and stored unfrozen at approximately 10-20°C pre-drying.

The aim is therefore, to get the soil sample to the laboratory and dried as soon as possible, so that it is in a stable state to limit DNA degradation.

6.2 Effect of composite samples on phylloxera detection

Results:

Mean phylloxera DNA equivalents were higher in March 2014 than in May 2014 for all comparable composite infested samples and no phylloxera was detected in any of the non-infested soil samples (Table 6). In March 2014, phylloxera was detected in all infested composite samples. In May 2014, phylloxera was detected in all replicates of composite 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 6). For both sampling times, the phylloxera DNA equivalent per 200g soil was directly proportional to the amount of contaminated soil in the composite samples (Table 6). Based on the results obtained in May 2014, it was estimated that 50% detection is likely when there is 0.48 phylloxera equivalent per 200g of soil and up to 100% detection when there is 1.5–2 phylloxera equivalents per 200g of soil (Figure 4).

Table 6. 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 Rutherglen vineyard in March and May 2014.

Amount of phylloxera infested soil (g) in 400g composite sample	March 2014		May 2014	
	Phylloxera equivalents per 200g	Number positive samples out of 8 replicates	Phylloxera equivalents per 200g	Number positive samples out of 8 replicates
0	0	0	0	0
1			0	0
5			0.34 ± 0.21	4
10			0.26 ± 0.17	4
20	3.6 ± 1.30	8	0.88 ± 0.24	7
50	10.6 ± 2.50	8	7.98 ± 5.05	8
100	36.8 ± 12.00	8	5.44 ± 2.38	8
200	80.4 ± 28.00	8	7.59 ± 1.17	8
400	157.4 ± 37.00	8	16.53 ± 3.13	8

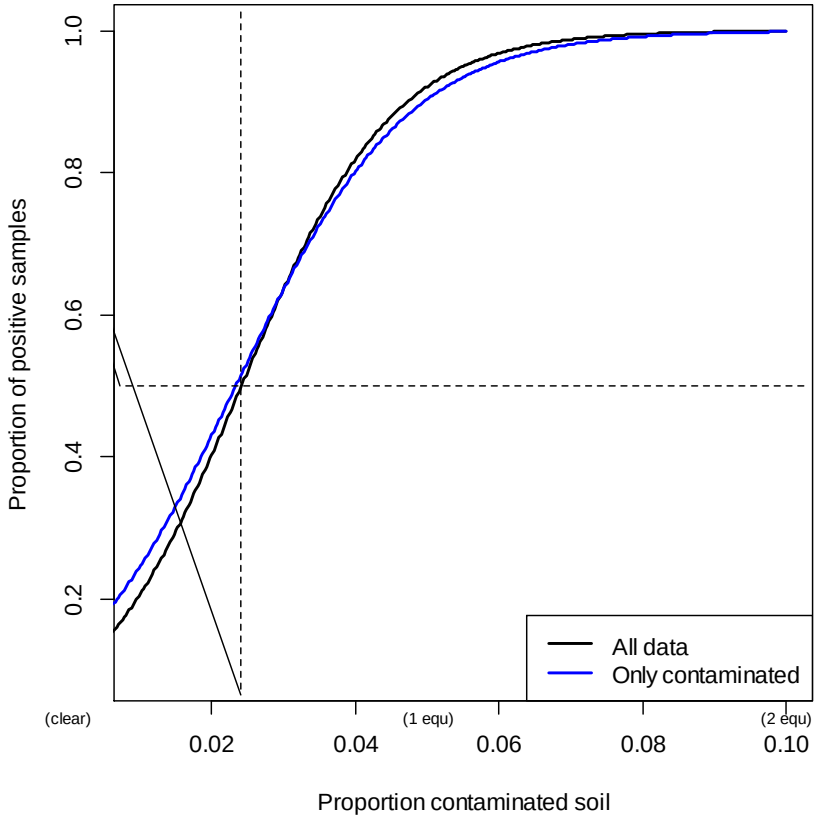


Figure 4. Estimation of the probability of detection of phylloxera in composite soil samples based on the proportion of positives amongst 400g samples made up by mixing various amounts of phylloxera-infested and non-infested soil. Estimation was based on the results obtained from soil collected in May 2014 in the Rutherglen vineyard. Equ = phylloxera equivalent per 200g of dry soil.

Discussion:

Collection of composite samples is recommended for soil-borne pathogen detection as it minimises the effect of spatial 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. The estimate of a limit of detection of 1.5 to 2 phylloxera per 200g composite soil sample from this study indicates that the true limit of detection of the assay is likely lower than the 20 phylloxera per 200g soil published previously, based on results using spiked soil samples (Herbert et al. 2008). Results in this study 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).

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

Results:

Both frequency of detection and the amount of phylloxera (equivalents per 200g) were much lower between rows (mid-row) than close to the trunk and below the dripper (p<0.001), irrespective of depth of sampling and vineyard location (Table 7), for samples collected in March 2013.

Position

Across all sampling times of the year 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 5). In the 0–10cm layer of soil, the difference was not significant (p=0.88) but suggested more detections near the trunk than below the dripper. Moreover, apart from mid-row samples, absence of detection across all replicates was observed only with samples collected below the dripper in the King Valley (Table 7) and more often deeper in the soil profile (Figure 5).

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 45cm 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 6). 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. The optimal position for sampling is as close to the trunk as possible, but within 10cm (Figure 6).

Vineyard location

Frequency of detection of phylloxera was consistently and significantly (p<0.001) lower across time of the year, positions and depths of sampling in the King Valley (0–0.5) compared to Rutherglen (0.625–1) and the Yarra Valley (0.5–1) (Figure 5). Phylloxera equivalent per 200g was also significantly lower (p<0.005) in the King Valley in autumn, winter and spring 2013, compared to the other two vineyards (Table 8).

Time

In 2013, the frequency of detection remained similar across time of the year for a given vineyard, position and depth of soil sampling (Figure 5). In contrast, time of sampling had a significant effect on phylloxera numbers (p<0.001), with highest phylloxera DNA equivalent observed in autumn (March 2013) and lower phylloxera DNA equivalent generally in spring and summer in all three vineyards (Table 8).

In 2014, both the frequency of detection (data not shown) and phylloxera equivalent in samples collected close to the trunk and 0–10cm 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 7).

Depth

With soil samples taken close to the trunk, 70 to 80% phylloxera DNA detection was achieved in the 0–10cm surface layer of soil, with little additional detection in the 10–20cm layer and even less in the 20–30cm layer (Figure 8). A similar pattern was observed with soil samples taken below the dripper, with a noticeably lower overall phylloxera DNA detection rate in winter and summer. More detection is therefore likely closer to the trunk than below the dripper. The cumulated DNA phylloxera detection rate with soil samples collected between rows in autumn was lower than that of samples taken both close to the trunk and below the dripper (Figure 8) in 2013. Soil samples were more difficult to collect between rows because the soil profile was drier. Subsequently, between-row samples were not collected after 2013.

Table 7. 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	Depth (cm)		
		0-10	10-20	20-30
King Valley	Trunk	3	1	3
	Dripper	3	2	4
	Mid-row	0	2	2
Rutherglen	Trunk	7	8	8
	Dripper	8	7	8
	Mid-row	3	0	0
Yarra Valley	Trunk	8	8	8
	Dripper	8	8	8
	Mid-row	6	4	5

Table 8. Effect of location of sampling on mean phylloxera DNA equivalent per 200g in composite soil samples collected in the King Valley, Rutherglen and the Yarra Valley vineyards in March (autumn), June (winter), September (spring) and December (summer) 2013.

Location	Mean phylloxera DNA equivalent per 200g soil[1]			
	Autumn	Winter	Spring	Summer
King Valley	6.4 ± 5.1	4.5 ± 2.2	0.2 ± 0.1	1.3 ± 0.8
Rutherglen	237.0 ± 40.0	28.5 ± 6.1	15.7 ± 4.4	89.0 ± 25.0 (3.2, 102.0)[2]
Yarra Valley	721.0 ± 140.0	94.0 ± 16.0	3.4 ± 0.6	1.5 ± 0.4

[1] 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.

[2] First number indicates the overall mean phylloxera equivalent per 200g soil in samples collected in December 2013 and January 2014 followed in brackets by the mean phylloxera equivalent per 200g soil in samples collected in December 2013 from vines 1-5 and in January 2014 from the other 35 vines).

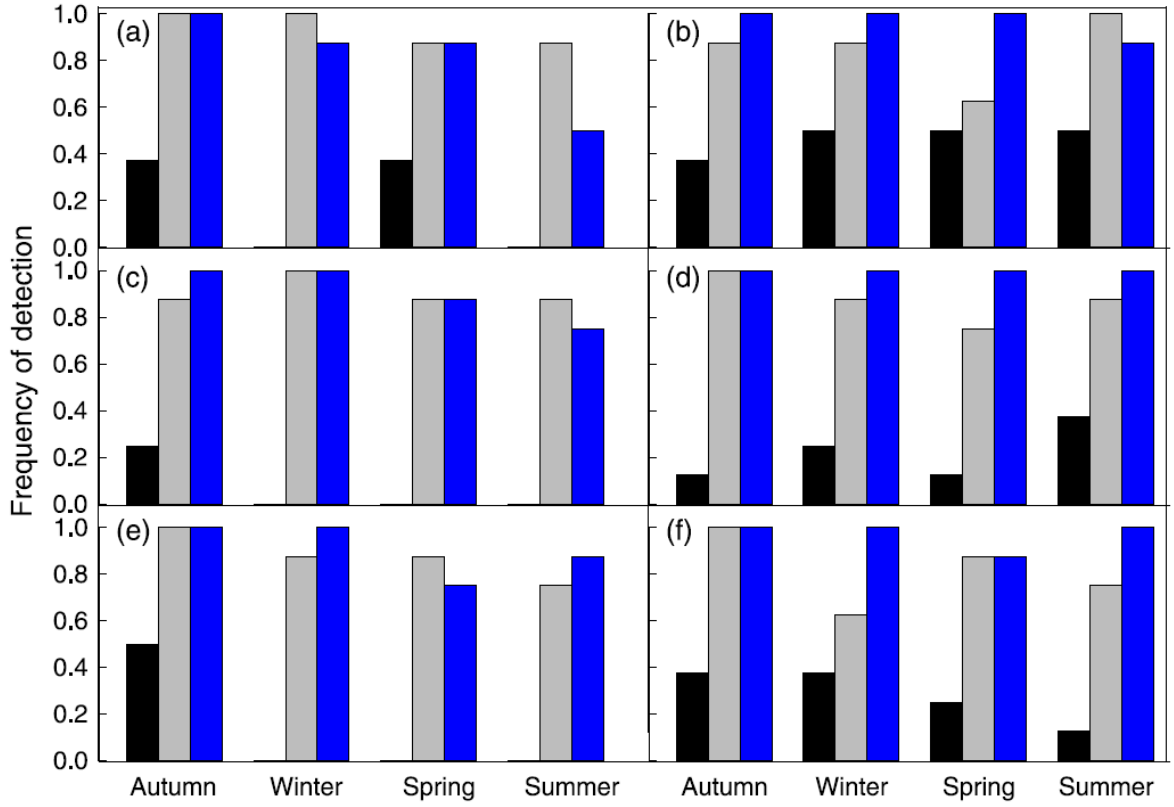


Figure 5. Frequency of detection of phylloxera (assuming all vines are infested) in composite soil samples collected (a, c, e) below the dripper and (b, d, f) close to the trunk, (a-b) 0-10, (c, d) 10-20 and (e, f) 20-30cm deep, in the King Valley (■), Rutherglen (■) and the Yarra Valley (■) vineyards in autumn, winter, spring and summer 2013.

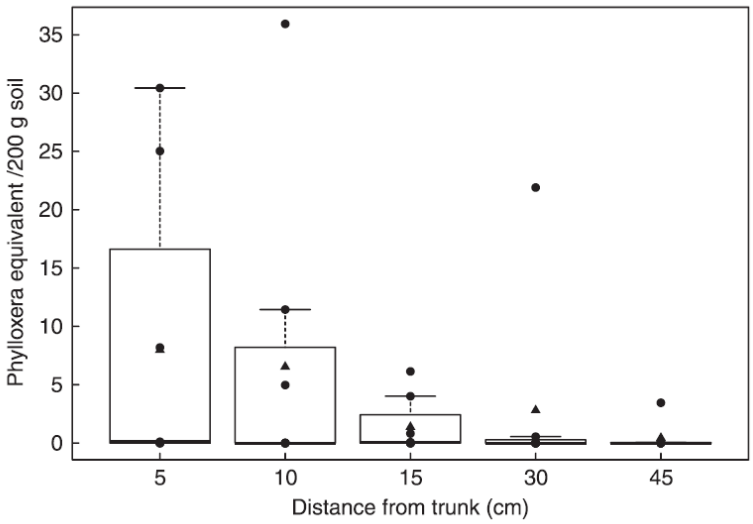


Figure 6. Mean (▲) and observed (●) phylloxera equivalent per 200g soil in samples collected 5, 10, 15, 30 and 45cm from the vine trunk, 0-10cm deep in the King Valley vineyard in May 2014.

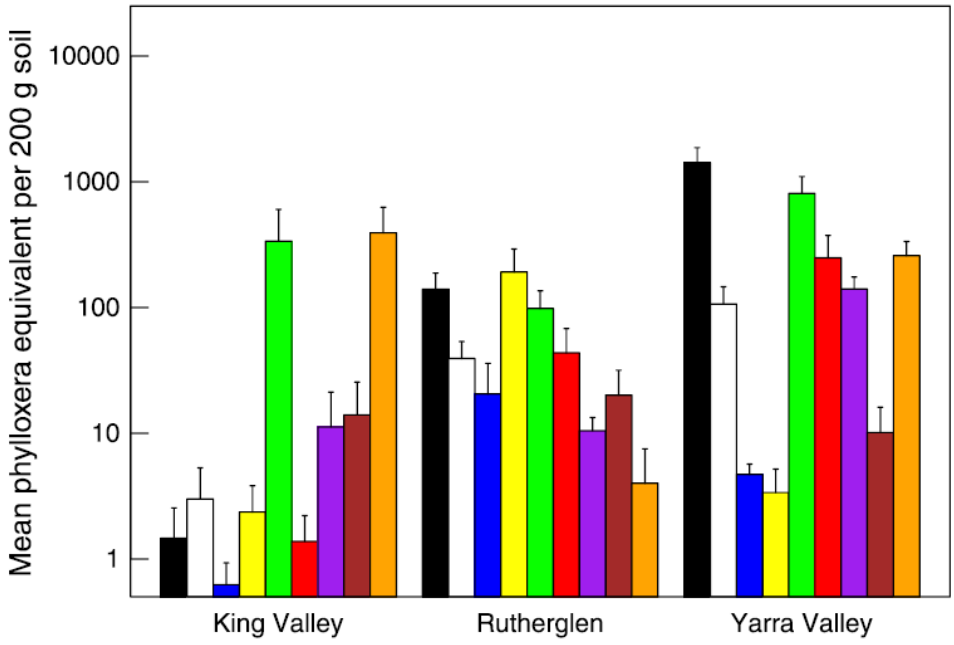


Figure 7. Mean phylloxera equivalent (log y axis) across seasons over a 2-year trial in the King Valley, Rutherglen and the Yarra Valley vineyards. Means were calculated across eight composite soil samples each taken close to the trunk of five infected vines, 0-10cm deep. March (autumn) 2013 (■); June (winter) 2013 (□); September (spring) 2013 (■); December (summer) 2013 (■); March (autumn) 2014 (■); May (late autumn) 2014 (■); July (winter) 2014 (■); October (spring) 2014 (■); December (summer) 2014 (■).

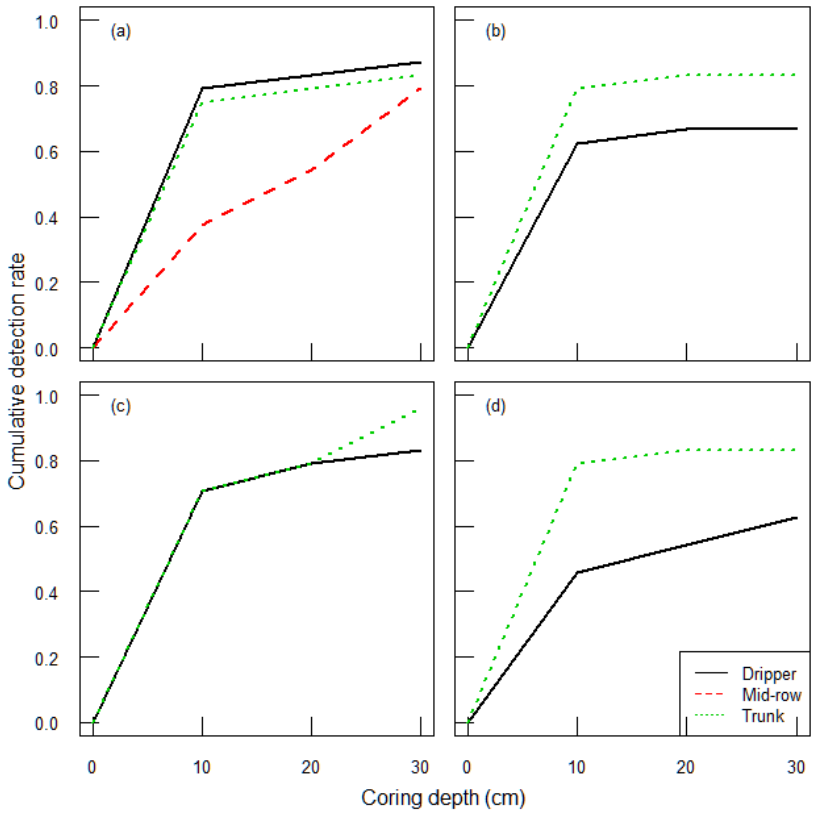


Figure 8. Effect of depth on phylloxera detection rate in soil samples collected under the dripper (—), close to the trunk (---) or between rows (- -) of infected vines in autumn (a), winter (b), spring (c) and summer (d) 2013. Between row samples were only collected in autumn. The cumulated detection rate was calculated using results obtained across three infested vineyards in the King Valley, Rutherglen and the Yarra Valley.

Discussion:

Reliable detection of phylloxera in soil samples relies on several parameters, amongst which vineyard location and time of the year have been shown to be important. With the dig and emergence trap surveillance 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). Results in this project for phylloxera detection by qPCR show that both detection rate and the amount of phylloxera are lower in the King Valley compared to that in the Yarra Valley and in Rutherglen. Such variations between vineyards are not uncommon and could be linked to environmental factors (including soil type), as well as to the phylloxera and vine genotypes (Benheim et al., 2012). As reported in Table 1, phylloxera detection dates of the sampled vineyards show that the King Valley infestation is considerably older than the other two vineyards, which could contribute to a lower detection rate and amount of phylloxera, as vine roots may have been destroyed by phylloxera which have then moved from affected vines in search of fresh vine roots. The most plausible explanation for lower phylloxera levels in the King Valley vineyard, however, is the phylloxera numbers were low (or nil) on the

rootstock component of the block, and these rootstock rows were not omitted from the sampling.

Despite these differences in detection rate and amount of phylloxera, the three vineyards showed similar trends in relative frequency of phylloxera detection in terms of sampling time, over 2 years. 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. Higher phylloxera DNA equivalent were found from late summer to early winter, peaking in autumn, which is consistent with previously described seasonal variations in phylloxera abundance (Benheim et al., 2012, Powell, 2012a).

Determining the most likely location of phylloxera in an infested vineyard is an important parameter for designing a sampling strategy for surveillance and is intimately related to phylloxera 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). In addition, in warm soil (late spring and summer), a higher phylloxera population level is usually found in the top soil (Benheim at 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 one metre below the soil surface (Buchanan, 1990) as cited by Benheim et al. (2012).

When comparing sampling position and depth in this study, soil samples collected close to the vine trunk (within 10cm) and below the dripper give a similar level of phylloxera DNA equivalents, with an overall slightly better detection close to the trunk. Soil samples collected between rows (mid-row) had both a lower frequency of detection and a lower phylloxera DNA equivalent. This difference likely indicates that more vine roots were present in the soil close to the vine trunk and below the dripper than between rows, therefore increasing phylloxera detection. Highest phylloxera detection was achieved within the top 0–10cm of soil. This finding was not affected by time of the year and only slightly by the vineyard location, 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.

6.4 Effect of the presence of vine root on phylloxera detection

Results:

Large variability in the quantity of *Vitis* root DNA was found among the soil, with a coefficient of variation of 47% (data not shown). The highest Ct values were found in the Rutherglen soil samples and there was a decline in Ct values in the Yarra Valley samples between March and December (Figure 9). The higher the Ct value, the greater the amount of duplication of DNA required for detection and therefore the lower the amount of vine root DNA present. The display of the means based on estimated vine root DNA showed very significant differences ($p<0.000$) between

vineyard locations and between the sampling times in the Yarra Valley (Figure 10). There was a significant effect of distance from the trunk on Ct values, indicative of an exponential decrease in vine root DNA with increasing distance from the vine trunk (Table 9). A large reduction of *Vitis* DNA was found in soil cores taken 30cm and further away from the vine trunk, with significantly lower vine root DNA at the 45cm distance compared to samples taken between 5-15cm from the vine trunk (Table 9). For the King Valley, soil samples collected between 5-10cm depth were found to have significantly higher ($p<0.05$) estimated vine root DNA compared to that at the 0-5cm depth (Table 10). Although an increase in estimated vine root DNA at the lower depth was also seen at the other two vineyards, this difference was not significant. The King Valley had substantially higher estimated vine root DNA levels compared to the Yarra Valley and Rutherglen, but only significant ($p<0.05$) at the 5-10cm sampling depth (Table 10). The effect of distance from the vine into the headland on the distribution of *Vitis* root DNA was also studied. Ct values were much larger in samples one metre into the headland, indicating an estimated amount of vine root DNA decreased to effectively zero post one metre from the edge of vine plantings (

Figure 11, Figure 12).

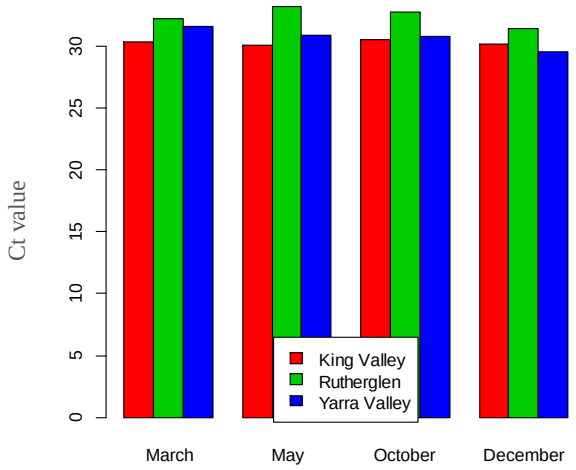


Figure 9. Ct root values for King Valley, Yarra Valley and Rutherglen vineyards at each sampling time in 2013.

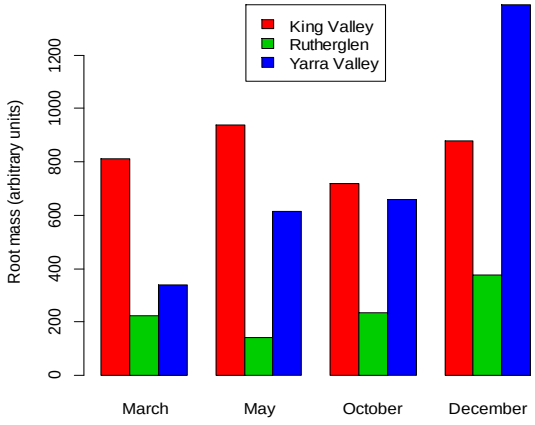


Figure 10. Estimated root DNA (arbitrary units) present in the King Valley, Yarra Valley and Rutherglen vineyards at each sampling time in 2013.

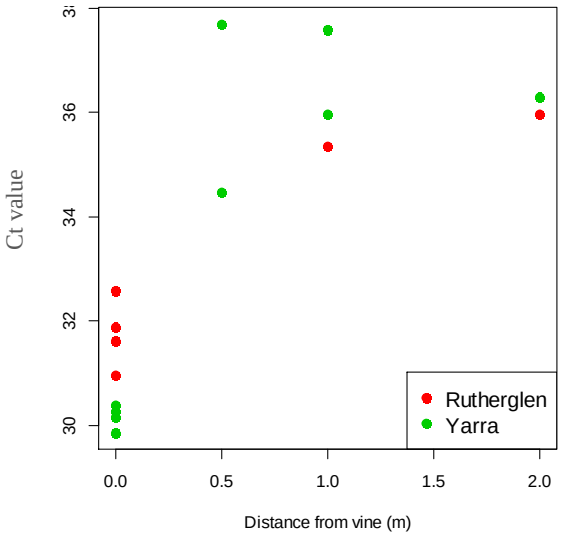


Figure 11. Effect of increasing distance into the headland on root DNA Ct values from soil samples collected in the Yarra Valley and Rutherglen vineyards in 2013.

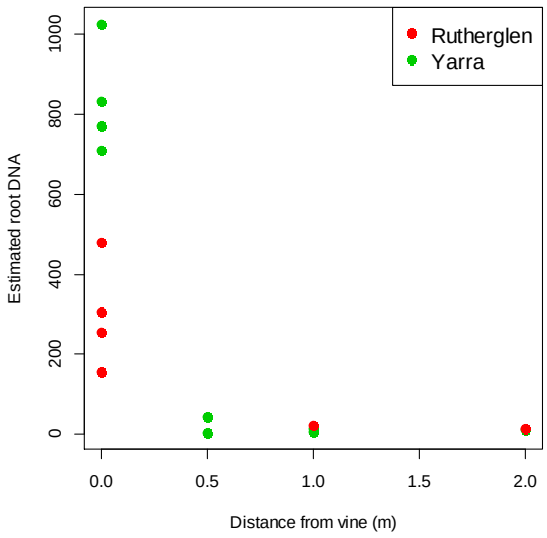


Figure 12. Effect of increasing distance into the headland on estimated root DNA (arbitrary units) from soil samples collected in the Yarra Valley and Rutherglen vineyards in 2013.

Table 9. Mean amount of estimated root DNA (arbitrary units) from vine trunk from soil samples collected in March 2014 in the Yarra Valley, King Valley and Rutherglen. Distances with the same letter are not significantly different at the 95% level.

Distance from vine trunk (cm)	5	10	15	30	45	LSD
Vine root DNA	952[a]	971 [a]	940 [a]	664 [ab]	566 [b]	347

Table 10. Comparison of Ct and estimated vine root DNA (arbitrary units) between 0-5cm and 5-10cm soil depth from samples collected in 2013 in the Yarra Valley, King Valley and Rutherglen. Ct and estimated vine root DNA at the same soil depth with the same letter are not significantly different at the 95% level.

Vineyard location	Ct at 0-5cm soil depth	Ct at 5-10cm soil depth	Estimated vine root DNA at 0-5cm soil depth	Estimated vine root DNA at 5-10cm soil depth
King Valley	31.61 [b]	29.55 [b]	337.5 [a]	1318.9 [a]
Rutherglen	33.30 [a]	31.77 [a]	119.8 [a]	350.5 [b]
Yarra Valley	33.41 [a]	31.02 [a]	95.1 [a]	478.3 [b]
LSD	1.52		389	

Discussion:

Given the requirement of phylloxera for vine roots as a food source, targeting these roots when collecting soil samples for phylloxera testing has been shown to increase the chance of detection of phylloxera DNA equivalent, despite the high variability likely in the amount of vine root DNA. Vine root density was found to be highest in soil cores collected within 15cm of the vine trunk. The vine root assay will be utilised by SARDI as an internal control to add reliability to the phylloxera qPCR results.

In 2013, vine root density was found to be higher in cores taken at 5-10cm depth compared to 0-5cm depth. This result was not surprising given the relatively common occurrence of surface crusting, higher surface soil temperature and rapid drying out at 0-5cm compared to 5-10cm in vineyards. Therefore, theoretically soil samples for phylloxera DNA analysis could be targeted at 5-10cm soil depth rather than 0-10cm depth. However, this smaller depth is not very practical and would require partitioning cores and more cores to be taken to ensure a minimum sample volume is supplied for analysis.

The spread of vine roots into the headland in the Yarra Valley and Rutherglen vineyards was found to be limited to approximately one metre when sampled at a soil depth of 10cm. This finding implies that there is only a very low risk of spreading phylloxera from soil located more than one metre from the edge of the vine rows. This finding may of course vary given the relative vine vigour and particulars of a site or vineyard. There is a possibility that vine roots could have been present in the headland area at deeper than 10cm soil depth under the headland (not measured). However, the deeper the vine roots the lower the risk of spreading phylloxera, with limited contact to phylloxera risk vectors, including footwear, clothing, machinery and equipment.

6.5 Effect of sampling density on the precision of phylloxera detection

Results:

July 2014

A summary of the detection frequency in the July 2014 trial is given in Table 11. There were 4 samples in which phylloxera was detected in the A series and 3 in the B series. There was one sample detected by B Series but not by the A Series. Doubling the sampling density in this trial increased the detection rate from 44% to 63%.

Table 11. Comparison of phylloxera detections using two different sampling densities in a Yarra Valley vineyard in July 2014.

Vineyard 1				
	A series	**B series**	**Average**	**Combined**
Number cores/composite sample	5	5	5	5
Number of composite samples	8	8	8	16
Number of detections	4	3	3.5	5
Detection %	50%	38%	44%	63%

October 2014

Phylloxera detection rates in October 2014 were lower for all three series compared to detections in July 2014 (Table 12) from the same vineyard. The combined data which was triple the sampling density increased the detection rate by almost a factor of 3. However, the actual number of detections was very low. The data from Vineyard 2 had a high detection rate and the effect of tripling the sampling density increased the detection rate from 31% to 47% (Table 12).

Combining data from both vineyards indicated that tripling the sampling density increased the phylloxera detection rate from 19% to 33%. There was an apparent differential effect of increasing sampling density between the two vineyards.

Table 12. Comparison of phylloxera detections using three different sampling densities at two Yarra Valley vineyards in October 2014.

Vineyard 1					
	A series	**B series**	**C series**	**Average**	**Combined**
Number cores/composite sample	5	5	5	5	5
Number of composite samples	15	15	15	15	45
Number of detections	2	1	0	1	3
Detection %	13%	7%	0%	7%	20%
Vineyard 2					
	A series	**B series**	**C series**	**Average**	**Combined**
Number cores/composite sample	5	5	5	5	5
Number of composite samples	15	15	15	15	45
Number of detections	6	4	4	4. 7	7
Detection %	40%	27%	27%	31%	47%
Combined Vineyard 1 and 2					
	A series	**B series**	**C series**	**Average**	**Combined**
Number cores/composite sample	5	5	5	5	5
Number of composite samples	30	30	30	30	90
Number of detections	8	5	4	5. 7	10
Detection %	27%	17%	13%	19%	33%

Overall discussion:

Across the sampling density trials in July and October 2014, the largest gain in phylloxera detection by increasing sampling density was found in the vineyard with a very low phylloxera detection rate.

Setting a sampling density for surveillance is a balance between cost (and hence area covered), time and ability to detect phylloxera when it is present. The primary purpose of landscape phylloxera surveillance is not to quantify how much phylloxera is present, but rather ascertain where phylloxera is and is not, for area freedom status. In surveillance, neither the frequency nor the distribution patterns of

phylloxera are known in advance. As long as the chosen sampling density has a relatively high chance of detecting phylloxera if it is there, and the time taken to collect the sample is ‘reasonable’ for the result, there is a greater chance that the method will be adopted by a wide range of users for surveillance purposes.

The current endorsed phylloxera surveillance method samples at a density of approximately 1 in 45 vines (assuming 3 vines per panel and sampling every fifth panel in every third row). Confirmation of appropriate sampling density for surveillance will be further explored through the PBCRC project.

6.6 Sample reconciliation

A range of individual trials were completed as part of the project. Actual soil samples collected are reconciled in Table 13. Each soil sample reported represents a composite sample made up of at least five individual cores packaged together in an individual sample bag and sent to SARDI’s Molecular Diagnostics Centre for analysis. Sample numbers reported in Table 13 therefore differ to those reported in the original planned project outputs, which we understand to have been reported as individual soil cores.

Table 13. Actual soil sample number collected across the project.

Sampling trial	Timing	Composite sample number	Total composite sample number
Storage temp, storage time, core type	Mar-13	120	240
Storage temp, storage time, storage2	Jul-13	120	
Depth, position, site, time	Mar-13	216	792
Depth, position2, site, time	Jun-13	144	
Depth, position2, site, time, weight	Sep-13	144	
Depth, position2, site, time	Dec-13	144	
Depth2, position3, site, time	Mar-14	24	
Depth2, position3, site, time	May-14	24	
Depth2, position3, site, time	Jul-14	24	
Depth2, position3, site, time	Oct-14	24	
Depth2, position3, site, time	Dec-14	24	
Depth2, position3, site, time	Mar-15	24	
Lab dilution trial (various levels of infected vs non-infected soil)	Mar-14	72	142
Lab dilution trial (various levels of infected vs non-infected soil)	May-14	70	
Validation trial. Sampling done in duplicate (40 vs 80).	Jul-14	16	115
Retesting negative samples from validation trial	Jul-14	9	
Validation trial. Sampling done in triplicate (40, 80, 120).	Oct-14	90	
Nematode sampling – VIC	Oct-14	N/A	12
Nematode sampling – SA	Oct-14	12	
Depth trial (0-5cm and 5-10cm vs 0-10cm) (split vs routine)	Mar-14	24	24
Distance from trunk trial (5/10/15/30/45cm)	May-14	24	24
Headland vs within row phylloxera density	Dec-14	28	28
Visual inspection (dig) vs DNA at King Valley only)	Mar-15	40	40
TOTAL COMPOSITE SOIL SAMPLE NUMBER COLLECTED			**1417**

7. OUTCOMES AND CONCLUSION

This study met the target of producing a protocol for the collection and handling of soil samples for phylloxera detection by qPCR, herein, termed ‘Soil sampling and handling protocol for phylloxera DNA analysis by qPCR’. A range of parameters were evaluated in this study to determine their impact on the ability to accurately detect the relative quantity of phylloxera DNA in any given infested vineyard. This would then inform the development of a field sampling protocol for soil sample collection and handling. Parameters evaluated in this study included compositing of soil core samples, time of year of sample collection, effect of vine roots on likelihood of phylloxera DNA detection, soil core depth and position around the vine, and sample storage time and temperature.

Phylloxera DNA could confidently be detected down to 1.5-2 phylloxera equivalent per 200g dry soil. This detection limit was 10-13 times higher than that reported in a previous study (Herbert et al., 2008) for qPCR, and therefore, validated the qPCR method for accurate and sensitive phylloxera DNA detection. Although the DNA surveillance method showed that the frequency of detection of phylloxera DNA was similar across all sampling times in a year, the ideal time of sample collection when the quantity of phylloxera DNA is highest, was in the three autumn months. This corresponds to the time of the year when phylloxera are at their most abundant in the upper soil layers and therefore most easily detected when sampling depth is relatively shallow.

The recommended field collection of soil samples involved taking cores in a grid pattern from vines located in every third row at every fifth panel. Compositing 5 soil cores into a single sample was found not to detrimentally affect detection rate of phylloxera DNA. This process allowed for increased coverage of the surveyed area by sampling more vines and increased reliability of the test result by accounting for uneven distribution of phylloxera in a vineyard. Compositing the soil samples using a user-friendly 15mm diameter soil corer, returned a sample size of around 150-200g for SARDI’s Molecular Diagnostic Centre to process, as opposed to collecting the same volume of soil as a single soil core, requiring a significantly larger instrument.

Given the requirement of vine roots as a food source for phylloxera, phylloxera DNA was more often detected from soil cores containing *Vitis* DNA, and collected at a depth of 0–10cm, within 10cm from the vine trunk. This finding was therefore indicative of the location of the highest concentration of vine feeder roots supporting phylloxera insect populations. Sampling below the dripper had the potential to be a suitable alternative to sampling adjacent to the vine trunk. However, as some vineyards are dry grown and others have inconsistent dripper spacing in a block, these factors would increase difficulty in creating standard sampling recommendations around dripper position.

While detection of phylloxera is shown to be possible all year round using qPCR, soil sampling is recommended from late summer to early winter to take advantage of the peak phylloxera populations in the three months of autumn. Once collected, soil samples must be transported at between 10 and 20°C and delivered for testing within 48 hours of collection, as decomposition of phylloxera DNA is considered to begin at the time of sample collection. Precautions need to be taken to avoid exposure to high temperature for a prolonged period of time, including during sampling and transport and to ensure that all quarantine requirements for transporting samples are met. Once at the laboratory, samples must be dried within approximately 48 hours of arrival and stored at 10-20°C, not frozen.

One of major benefits of this DNA soil sampling protocol for growers and regulators is that it does not require any particular expertise in the field and can therefore be easily implemented. Other benefits include timely results due to the high throughput automated testing at SARDI's Molecular Diagnostic Centre and high accuracy with the low limit of detection. In addition, the DNA surveillance method is cost-effective as the soil samples can be taken without employing an expert and samples can be composited with a recommended 8 composited samples per hectare of vineyard, without reducing accuracy. 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.

With recent increasing phylloxera detections inside the Maroondah Phylloxera Infested Zone in Victoria and increasing movement of people, machinery and equipment between winegrowing regions and states, the importance of a fast, accurate phylloxera surveillance method for use by regulators and growers alike is of utmost importance to protect the Australian grape and wine industries from phylloxera. This qPCR method for phylloxera analysis and associated soil sampling and handling protocol, stands out as a key surveillance method for early detection and minimising the spread of phylloxera.

This protocol will further enable the National Viticulture Biosecurity Committee (NVBC), Victorian Viticulture Biosecurity Committee and Vinehealth Australia, to achieve their strategic goals and safeguard our $40.2 billion wine industry from the devastating impacts of phylloxera. In addition, state regulators will be able to use this protocol for surveillance and enforcement of state biosecurity legislation and regulations. Finally, this protocol will empower growers to have an active involvement in managing the health of their vines and regional surveillance for phylloxera to support area freedom status. This all bodes well for minimising the impact of endemic strains of phylloxera on grape and wine assets, the early detection of an incursion of an exotic phylloxera strain, and provides a mechanism to share the responsibility for the management of phylloxera in Australia to be shared by industry and government.

8. RECOMMENDATIONS

The project team:

1. Recommends that the following protocol (Figure 13) (subject to further validation via the broader PBCRC (#2061) project) for collection of soil cores from a vineyard, or vineyard block, be submitted to, and becomes nationally endorsed by, Plant Health Australia's SDQMA and SNPHS as part of the broader PBCRC project.
2. Recommends that this protocol be adopted in conjunction with other primary surveillance methods for phylloxera (dig and emergence trap) in an integrated approach for detection, to ensure area freedom.
3. Recommends that following a comprehensive review of the National Phylloxera Management Protocol through the NVBC, this protocol be incorporated.
4. Endorses the inclusion of a comparative study to determine the relative effectiveness of this protocol against the two other primary phylloxera detection methods (dig and emergence traps) within the broader PBCRC (#2061) project.
5. Recommends the development of in-field qPCR diagnostic capability (negating the need to send soil samples to the laboratory).
6. Recommends further work to explore the opportunity of a single integrated diagnostic test that can detect the presence of phylloxera, and determine the amount and strain of phylloxera in a sample.
7. Recommends development of complementary methods to enable us to more effectively target the deployment of this protocol in regional and state surveillance programs.
8. Recommends this report be read in conjunction with the final report of the PBCRC project (#2061) that will be externally available on 30 June 2018 with deliverables in the coming year noted in Table 14.

Soil sampling and handling protocol for phylloxera DNA analysis by qPCR

Resources

- 14mm diameter x 100mm long AccuCore soil corer
- Screwdriver
- Sample bags, courier bags and box or other container for triple bagging
- Bar-coded labels (supplied by laboratory)
- Cable ties
- Items to disinfest soil corer

Method

- Adopt a grid sampling method in the vineyard, sampling a vine every 3rd row / 5th panel. This frequency equates to approximately 40 vines per hectare with standard 2.75m row spacing x 1.8m vine spacing.
- Collect one soil core per vine and place 5 consecutive soil cores in a single sample bag.
- You will have approximately 8 labelled sample bags with 5 cores in each per hectare of vineyard.

To take a soil sample:

1. Stand facing the vine. Place the tip of the corer on the surface of the soil as close to the trunk as possible (within 10cm from the vine trunk).
2. Push the corer into the soil – twisting slightly as you go if you hit vine roots. *If you hit a large root that you are unable to get through when inserting the corer, you may have to remove the corer and take a sample in a different position around the vine trunk.*
3. Twist the corer clockwise 180° and then back anticlockwise 180°.
4. Remove the corer from the soil, twisting as you go to ensure that the soil remains in the corer cavity (especially important in sandier soils). *Ensure your core completely fills the corer cavity with soil.*
5. Place the tip of the corer into a plastic sample bag labelled with the correct vine and row position. Carefully use a screwdriver to scrape all soil from the corer cavity into the sample bag.
   Note – the sides of the corer cavity are sharp, complete this step carefully to avoid cutting your fingers or piercing the sample bag with the screwdriver.
6. Repeat steps 1 – 5 for the collection of each of the 5 core samples to be composited together as a single soil sample. Use a cable tie to tightly seal the composite sample bag.

- Once you have collected all soil samples, place each composite soil sample bag in an individual courier bag, and roll up lengthways to close, removing as much air as possible. Seal well.
- Place all sealed courier bags in a box or additional container as the triple-bagged layer prior to transporting to the laboratory.
- Prior to leaving the vineyard, all samples must be accompanied by appropriate quarantine documentation.

Figure 13. Soil sampling and handling protocol for phylloxera DNA analysis by qPCR.

Table 14. PBCRC project (#2061) deliverables.

	Activity
1	External experts to review protocol and supporting material
2	Undertake an import risk analysis for the movement of soil samples from a PRZ or PIZ to a PEZ in South Australia, and also of the potential of movement of strains of phylloxera with a PIZ.
3	Consultation with SARDI as to business delivery model for DNA analysis, identify options and perform SWOT for each
4	Consultation and briefing with NVBC – seek endorsement to take to PHC sub - committees
5	Establish relevant criteria / requirements that must be met to obtain endorsement by PHC sub-committees (DMQA and SNPHS)
6	Prepare and lodge submission for PHC sub- committee and provide any additional information on request
7	Undertake a case study to demonstrate the application of the protocol for surveillance of an area larger than a vineyard 'block'. This will provide an opportunity to demonstrate the method to end users and also provide practical information to include in submissions for endorsement. For research conducted to date, the protocol has only been used on a block basis, so this applies the learnings from the research but to a larger scale for surveillance and area freedom.
8	Refine end user / stakeholder analysis and profile to better understand motivators/barriers for adoption of new method
9	Identify delivery model options for each end user and select preferred based on SWOT analysis and other defined metrics
10	Develop communication and engagement plan for delivery
11	Develop collateral to support extension / education for end users
12	Consultation with State regulators, NVBC, and other relevant bodies to facilitate the integration of the protocol into protocols and regulations – this will require the development of supporting briefs and collateral
13	Investigate the regulatory and legal framework and systems to support mandatory reporting of positive samples (for particular states or management zones)
14	Implementation of communication and engagement plan
15	Roll out selected delivery model
16	Monitor uptake, usage and conduct survey of end users – provide recommendations to improve system

9. APPENDICES

9.1 Supplementary results

Table S1. Phylloxera DNA detection in soil samples collected in the Yarra Valley in March 2013 and stored at 10, 20 and 35°C for 0, 1, 3 or 10 days after arrival at SARDI laboratory. qPCR results were converted to number of phylloxera equivalents per 200g soil.

Replicate	Storage time (days)	Storage temperature (°C)					
		10		20		35	
		Moisture content (%)	Phylloxera equivalent per 200g soil	Moisture content (%)	Phylloxera equivalent per 200g soil	Moisture content (%)	Phylloxera equivalent per 200g soil
1	0	16.7	14	14.5	5216	14.3	4493
2	0	16.4	527	19.1	853	14.0	524
3	0	12.5	166	14.9	4	14.3	436
4	0	12.5	130	11.5	603	12.8	230
5	0	13.7	796	10.6	31	13.5	837
1	1	15.1	18	13.2	38	5.9	41
2	1	12.7	375	16.3	105	14.0	134
3	1	14.3	33	10.6	13	12.8	65
4	1	15.2	1588	9.3	8017	11.4	42
5	1	16.0	47	10.2	825	18.9	288
1	3	15.1	4	16.0	218	13.2	1
2	3	11.8	306	15.2	133	13.7	10
3	3	14.0	34	14.3	283	9.6	138
4	3	13.0	229	10.0	550	14.0	4
5	3	11.8	3583	12.2	16	14.5	0
1	10	14.0	0.42	13.5	0	15.1	0
2	10	11.8	253	15.6	24	14.9	28
3	10	13.7	14	12.8	3	10.4	1
4	10	12.5	10	12.0	91	14.3	66
5	10	12.8	21	13.7	29	13.0	1
AVERAGE		13.8	407	13.3	853	13.2	367

Table S2. Phylloxera DNA detection in soil samples collected in the Yarra Valley in June 2013. Samples were stored at 10, 20 and 35°C for 0, 1, 3 or 10 days immediately after arrival at SARDI. qPCR results were converted to number of phylloxera equivalents per 200g soil.

Replicate	Storage time (days)	Storage temperature (°C)					
		10		20		35	
		Moisture content (%)	Phylloxera equivalent per 200g soil	Moisture content (%)	Phylloxera equivalent per 200g soil	Moisture content (%)	Phylloxera equivalent per 200g soil
1	0	19.6	3	16.4	829	16.7	3
2	0	17.1	143	18.2	2	20.8	181
3	0	15.7	19	19.7	163	16.7	4
4	0	19.1	0	20.6	113	20.3	37
5	0	16.4	2	19.4	12	19.4	1
1	1	19.2	0.26	14.3	531	19.6	2
2	1	17.6	3	22.2	0	25.0	0.08
3	1	14.9	39	19.1	73	15.2	12
4	1	19.4	0	17.6	1	19.7	6
5	1	15.9	160	19.7	200	19.0	52
1	3	18.2	16	14.7	12	16.7	0
2	3	15.8	1	22.6	15	19.2	0
3	3	20.0	3	19.4	21	15.6	0
4	3	20.3	0	20.0	1	19.2	5
5	3	17.0	27	19.4	170	17.9	3
1	10	26.9	23	16.1	197	9.3	0
2	10	19.4	0	23.8	87	21.4	0.16
3	10	17.6	0	20.7	1	20.0	0
4	10	22.2	0	31.4	1	22.0	0
5	10	18.5	27	15.2	5	22.9	1
AVERAGE		18.5	24	19.7	84	18.9	16

Table S3. Phylloxera DNA detection in soil samples collected in the Yarra Valley in June 2013. Upon arrival at SARDI, samples were frozen at -20°C for 24 hours before being stored at 10, 20 and 35°C for 0, 1, 3 or 10 days. qPCR results were converted to number of phylloxera equivalents per 200g soil.

Replicate	Storage time (days)	Storage temperature (°C)					
		10		20		35	
		Moisture content (%)	Phylloxera equivalent per 200g soil	Moisture content (%)	Phylloxera equivalent per 200g soil	Moisture content (%)	Phylloxera equivalent per 200g soil
1	0	19.2	28	24.0	39	20.8	245
2	0	4.1	288	21.0	1	18.2	43
3	0	21.3	0.28	17.1	1	19.7	527
4	0	12.0	23	19.0	0.48	17.1	0.40
5	0	20.0	4	17.0	2308	11.7	192
1	1	16.7	389	22.6	1453	19.2	26
2	1	18.0	14	20.0	3	18.5	1
3	1	17.0	3	14.7	6	20.0	6
4	1	18.2	0	19.4	36	17.5	23
5	1	23.1	556	17.3	44	20.6	6
1	3	17.9	459	21.4	73	19.6	17
2	3	19.0	8	19.4	0.36	16.7	5
3	3	18.8	6	23.8	3	18.6	0
4	3	15.7	0	20.7	1	17.4	1
5	3	23.3	4	17.4	1	19.7	25
1	10	14.0	0	17.5	14	20.0	7
2	10	18.3	1	16.0	0	14.3	6
3	10	19.3	16	15.6	0.18	18.0	1
4	10	15.7	0	20.7	1	13.3	0
5	10	18.8	7	17.3	23	18.8	11
AVERAGE		17.4	94	18.8	209	17.8	47

9.2 Communication

The following communication activities have been undertaken as part of this project, representing a broad range of activities:

Activity	Number
Presentations – 6th International Phylloxera Conference	1
Presentations - PBCRC Science Exchange and meetings	5
Presentations - other	4
Regional field days with growers	5
Vinehealth Australia media releases	3
Vinehealth Australia newsletter articles	5
Feature article in industry journal	1
Peer–reviewed journal article*	1
Web page (http://www.vinehealth.com.au/projects/dna-testing-early-accurate-detection/)	1

Attendance by growers at various samplings throughout the project and at field days specified above, allowed the project team to observe and measure the ability of growers to accurately follow and perform the proposed soil sampling protocol. Similar detection levels were measured in matched samplings completed by growers and the project team on these occasions (data not presented), thereby indicative of successful following of the protocol.

Further updates to industry will be forthcoming at the conclusion of a related PBCRC (#2061) project in mid-2018, as endorsement is gained for the 'Soil sampling and handling protocol for phylloxera DNA analysis by qPCR' as an additional phylloxera primary surveillance method.

*Giblot-Ducray, D., Correll, R., Collins, C., Nankivell, A., Downs, A., Pearce, I., Mckay, A. C., and Ophel-Keller, K. M. (2016) Detection of grape phylloxera (Daktulosphaira vitifoliae Fitch) by real-time quantitative PCR: development of a soil sampling protocol. Australian Journal of Grape and Wine Research, 22: 469–477.

9.3 Intellectual Property

As a result of this project, the valuable information gained is the protocol termed ‘Soil sampling and handling protocol for phylloxera DNA analysis by qPCR’.

The qPCR method rests with SARDI but other intellectual property generated regarding the aforementioned protocol, rests as per the Project Agreement.

9.4 References

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9.5 Staff

The following staff have been engaged on the project:

Institution	Staff
Vinehealth Australia	Alan Nankivell (to 15 Feb 2016) Inca Pearce (from 16 Feb 2016) Andrew Downs (to 6 Dec 2014) Brendan Tully Matthew Edge Suzanne McLoughlin (from 25 July 2016)
SARDI	Dr Kathy Ophel-Keller Dr Alan McKay Dr Danièle Giblot-Ducray Russell Burns
University of Adelaide	Dr Cassandra Collins

Dr Ray Correll of Rho Environmetrics was engaged to provide statistical expertise to the project team.

9.6 Project Management

Oversight of this project has been undertaken via a project team as follows:

• Vinehealth Australia	Alan Nankivell (to 15 Feb 2016) Inca Pearce (from 16 Feb 2016) Andrew Downs (to 6 Dec 2014) Suzanne McLoughlin (from 25 July 2016)
• SARDI	Dr Kathy Ophel-Keller Dr Alan McKay Dr Danièle Giblot-Ducray
• University of Adelaide	Dr Cassandra Collins
• PIRSA (Biosecurity SA)	Geoff Raven
• DEDJTR	Greg King
• DPI NSW	Chris Anderson
• Plant and Food Research (NZ)	Grant Smith
• Rho Environmetrics	Dr Ray Correll

During the course of the project twenty-one project team meetings were held, all in Adelaide, South Australia. The general format was a face to face meeting of the project team in conjunction with those team members based interstate dialling in. Results of completed trials were discussed, including issues arising and progress against objectives. Consensus was gained from the group through discussion, prior to any changes made to trial methodology.

Meetings were held on 22 February 2013, 11 April 2013, 8 May 2013, 31 July 2013, 25 September 2013, 20 November 2013, 13 February 2014, 10 April 2014, 18 June 2014, 10 September 2014, 18 November 2014, 18 February 2015, 10 March 2015, 23 April 2015, 6 August 2015, 29 October 2015, 15 December 2015, 8 January 2016, 6 July 2016, 14 December 2016 and 3 January 2017.

During some meetings, additional personnel were present, including from AGWA (Adrian Loschiavo, Keith Hayes) and PBCRC (Jo Luck).

9.7 Budget Reconciliation

Statement submitted via AGWA's Clarity Investment Management System.