

INTEGRATED MANAGEMENT OF ESTABLISHED GRAPEVINE PHYLLOXERA

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Cover image: Phylloxera infested vines showing yellow symptoms.

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1.0. ABSTRACT

This project conducted research directed at developing tools, technologies, and practices to improve the integrated pest management (IPM) of phylloxera in Australia. The three-year program of research has delivered outputs that will be of significant benefit to growers, industry, and national plant biosecurity. These include new tools for the in-field detection of phylloxera, robust screening assays to improve rootstock selection and safeguard Australia against the spread of virulent phylloxera strains , scientifically validated disinfestation procedures, new knowledge in phylloxera temperature tolerance, and a review of phylloxera biocontrol.

2.0. EXECUTIVE SUMMARY

This project aimed to develop tools, technologies and practices to improve the integrated pest management (IPM) of phylloxera in Australia. Research fell into five broad themes that are seen as being crucial to preventing the spread of this pest: (i) enhancing early detection systems, (ii) providing region- and genotype-specific rootstock recommendations, (iii) developing improved quarantine protocols, (iv) addressing key knowledge gaps on the biology of phylloxera endemic strains, and (v) re-evaluating biocontrol options. The three-year program of research has delivered outputs that will be of significant benefit to growers, industry and national plant biosecurity.

NEW AND IMPROVED TOOLS FOR EARLY DETECTION.

The potential for using electronic noses to detect phylloxera. A commercially available electronic nose (e-nose) was evaluated for its ability to identify phylloxera-infested vine roots across different rootstocks, during early and late stages of infestation. The device failed to detect differences in leaf odours of healthy and phylloxera-infested vines. For root odours, there was evidence that the e-nose might be capable of identifying infested roots, but the level of accuracy was not acceptable for a detection tool and there were major practical limitations in the use of the e-nose (sample preparation, testing environments, sensors consistency). To explore the potential for developing future e-nose with finely tuned sensors, gas chromatography-mass spectrometry (GC-MS) analysis was conducted on leaves and excised roots of infested and phylloxera-free plants. GC-MS analysis revealed small variations in the concentrations of multiple root volatiles induced by the presence of phylloxera, which differed according to rootstock and phylloxera strain. However, no “key volatiles” indicative of the presence of phylloxera were identified, and results suggest root volatiles are unlikely to be reliably used to predict the infestation status of vines. The volatiles identified might, however, provide insight into the biochemical basis of rootstock resistance.

An industry perspective on sniffer dogs. To better understand the views of vignerons, biosecurity officers and researchers regarding the use of sniffer dogs in phylloxera detection, an online survey in which participants were asked eight multiple choice questions addressing practicality, feasibility, priority, and costs, was conducted. Out of 54 respondents, only 29% thought that sniffer dogs would likely be adopted for phylloxera detection, and 73% of respondents felt there were biosecurity concerns in using sniffer dogs. The outcome of this study triggered a stop-go milestone and led to a variation, removing further sniffer dog research from the project.

LAMP, an exciting new tool for in-field phylloxera detection and identification. LAMP is a portable molecular technology that Agriculture Victoria has recently developed for rapid and accurate diagnostics of phylloxera. The current study focused on validating and applying LAMP for in-field use. Surveillance trapping was conducted using “bucket traps” and trapped insects (including phylloxera) were processed as “crude” whole specimens, which were contaminated by plant tissue and soil. Our field-optimised methods clearly demonstrated the success of LAMP for presence/absence of phylloxera. We further evaluated the LAMP surveillance approach for detecting phylloxera on root samples dug up from infested vineyards and tested in the field. Our methods successfully detected phylloxera in eighteen positive vine samples.
LAMP appears accurate and sensitive enough to accurately diagnostics to be carried out without entomological expertise, hence could save considerable training and diagnostic time and effort. Further research should focus on incorporating LAMP protocols into larger-scale visual surveys, particularly during rezoning undertakings.

ENABLING STRAIN-RELATED ROOTSTOCK SELECTION.

Uncovering susceptibility of 5C Teleki. Six genetically diverse phylloxera strains (G1, G4, G19, G20, G30, and G38) were screened for resistance against rootstock, 5C Teleki. With the exception of G1, five strains caused nodosities on vines in pots, implying that these strains will damage 5C Teleki vines. Compared with *Vitis vinifera* (which is highly susceptible to phylloxera), reproduction of strains G19, G20 and G30 on excised roots of 5C Teleki was, however, low. Results demonstrated that 5C Teleki is tolerant to specific strains, and insects can survive and develop to reproductive adults, implying a hidden risk of phylloxera dispersal.

Superclone G38 screening and in-field studies. Field trials in a mixed-rootstock block infested by the virulent "superclone" G38 showed rootstocks 101-14 and Schwarzmann were susceptible to this strain. Standardised laboratory and glasshouse screening assays performed using the same rootstocks also indicated susceptibility to this strain. The study was important in that it validated current screening assays in terms of predicting resistance / susceptibility of rootstocks in vineyards. That rootstocks may be becoming susceptible to G38 is particularly worrying given additional findings in this project that indicate that this genotype may be able to reproduce sexually.

Should we be worried about phylloxera genetic diversity in the King Valley? Phylloxera has been known in the King Valley since the early 1990's, where previous studies found only a single strain (G4) present on vine roots. An updated and more detailed survey was conducted to better understand the genetic variation present. A total of 1400 phylloxera were collected from 18 blocks and 764 genotyped. The study revealed that the amount of genetic diversity present in the King Valley is considerably higher than previously thought, with many novel genotypes present. A total of 36 genotypes were identified, with 4 strains known from previous work and 32 new strains. A potential explanation for the high diversity is that sexual reproduction is occurring. If this is the case it is of considerable importance, as sexual reproduction of phylloxera could significantly increase the risk of resistance adaptation and long- range dispersal (reproductive winged forms) occurring in the field. Strong associations of strains with specific rootstocks were not found, and vines were frequently found to have more than one strain of phylloxera on their roots. Currently, there is a single PIZ in northeastern Victoria. Our new data has revealed several groups of genetically related phylloxera strains which appear geographically localized to regions within this PIZ. This evidence, together with the cluster of new (possibly sexually reproducing) variants appearing in the King Valley, might warrant a re-evaluation of the single northeastern PIZ to reduce the risk of novel strains being introduced into new areas. These results show that a larger state- wide survey is needed to update strain-specific distribution maps for other PIZs across Victoria, particularly where the genotypes are still unknown.

IMPROVED QUARANTINE.

Are there alternative treatments for disinfestation of footwear? Household bleach is currently recommended for disinfecting footwear and small hand-held tools against phylloxera, but this product is unfavourable for use by growers. Off-the-shelf products were reviewed and scored using a matrix that looked at availability, safety and practicality. Twenty-two products were then tested in laboratory assays using first instar G4 phylloxera in 30 or 60 second immersions, with or without a follow-on water rinse. Products that were effective were further screened across genetically diverse strains (G1, G19, G20, G30

and G38). Only Dettol (5% active ingredient Chloroxylenol) achieved 100% mortality against the six phylloxera strains tested and was deemed suitable for use in a practical setting. We can therefore recommend Dettol as a suitable substitute to bleach for disinfestation of footwear and handheld tools.

Validating fermentation treatments as a disinfestation protocol. Currently, the endorsed procedure in the National Phylloxera Management Protocols for movement of grape products to complete at least three days (72 hours) of fermentation to ensure disinfestation of phylloxera (Procedure D). The efficacy of this procedure was evaluated under laboratory conditions testing five phylloxera strains in red and wine ferments, with and without the addition of yeast, for 6, 12, 24, 48 and 72 hours. 100% mortality was achieved across all phylloxera strains when subjected to yeast fermentation of grape products lasting 48 and 72 hours. However, phylloxera survived in ferments without yeast at 48 and 72 hour time points. The current protocols are therefore only valid when yeast is added to wine ferments. Fermentation protocols in the NPMP require a review in light of these findings. New protocols should be developed and evaluated to determine mortality in naturally fermented products.

PHYLLOXERA BIOLOGY – KNOWLEDGE GAPS.

The effect of temperature on phylloxera survival. A better understanding of phylloxera strain-specific temperature tolerance and survival is important for effective quarantine and could help with predictions for phylloxera distribution under climate change. The effect of temperature on phylloxera survival, development and reproduction was studied in laboratory assays, using temperatures of 18, 22, 26 and 30°C. Survival was evaluated on excised roots (i.e. with a feeding site), and also removed from roots (without a food source) under dry and wet conditions. Results suggest that the threshold temperature for phylloxera to establish a feeding site (hence assimilate nutrients, survive and develop to adulthood) by the Australian phylloxera strains is <30°C, and that the optimal temperature to maintain a feeding site and develop to adulthood is >18°C. Survival at lower temperatures while phylloxera is not attached to a vine root was found to be longer than previously thought, with G20 strain surviving for up to 29 days at 18°C. This finding has implications for managing movement of contractor workers or vineyard machinery from PIZs to exclusion zones.

RE-EVALUATING BIOCONTROL OPTIONS.

Drawing on expert advice from North America, Europe and Australia, an **extensive review on phylloxera biocontrol** evaluated approaches including the potential release of exotic natural enemies from the pest's native range, commercially-reared predatory insects, the application of insect killing microorganisms such as nematodes and fungi, and practices to enhance native predators in vineyards. The review found 16 predatory species, of which only two attack root- feeding phylloxera. An exotic predatory syrphid fly is particularly promising and warrants further investigation. Targeted field surveys in eastern USA could search for native predators and pathogens. In Australia, predators in vineyards could be supported through habitat manipulation for conservation biocontrol. There is also considerable scope to further investigate the use of entomopathogenic nematodes and fungi as biological pesticides.

3.0 SUMMARY OF RESEARCH, OUTPUTS AND OUTCOMES

Grapevine phylloxera is the highest priority pest for the Australian wine industry. Although restricted to quarantined areas in Victoria and NSW, phylloxera has been increasing its geographic range in the last 15 years and poses a significant threat to states and regions where the vast majority of vineyards are planted with ungrafted *Vitis vinifera*. The recent spread of phylloxera has been largely attributed to poor detection rates, the slow uptake of resistant rootstocks, quarantine breakdown, knowledge gaps in our understanding of the biology and ecology of the pest, and a lack of options of phylloxera control. The main objective of this project was to conduct research that tackled each of these issues, delivering outputs that would lead to an improved integrated approach for managing endemic strains of grapevine phylloxera in Australia. The project components are presented under five themes. A general summary of the methods, results and key findings is presented below, followed by details of project outcomes as specified in the project document. Full reports for research delivered in the project is then presented in five sections corresponding to each theme.

I. NEW AND IMPROVED TOOLS FOR EARLY DETECTION

Evaluating the potential for using electronic noses in phylloxera detection.

Plants under attack by insects can emit characteristic odours, and in this component of the project we explored the possibility for detecting phylloxera infestation through odour sensing. Our study used the Cyranose 320, a commercially available electronic nose (e-nose) with demonstrated capability in detecting odours associated with insect infestation on plant material. We conducted experiments to evaluate the ability of the Cyranose to identify phylloxera-infested vine roots across different rootstocks during early and late infestation. In trials aimed at above ground (leaf) odours, the device failed to detect differences in odours of healthy and phylloxera-infested vines. In trials on root odours, we found encouraging evidence that the e-nose was capable of distinguishing odours of infested and phylloxera-free roots, but this was specific to rootstock variety and not at a level of accuracy or statistical confidence that we would consider acceptable for a detection tool. More importantly, practical limitations in terms of the preparation of root material, the necessity for controlled sampling environments, and the need to retune the e-nose (sensor drift) currently prevent this particular e-nose from being a useful detection tool for phylloxera. Newer e- nose technology with more stable sensors will potentially overcome this issue. The broadly-tuned sensors in the Cyranose 320 cast a wide net for detecting volatiles, and more targeted sensors specialized in the
detection of “key” root volatiles would most likely improve the e-nose performance and alleviate issues linked to background odours and temporal changes in sampling conditions.

To identify potential key volatiles that could be used to tailor future e-noses, we used gas chromatography-mass spectrometry (GC-MS), analysing root and leaf volatiles of susceptible, tolerant and resistant rootstocks at early and late stages of infestation. As with the e-nose, leaf volatiles showed limited usefulness for the identification of infested plants: although we found an apparent odour distinctness between the leaf volatiles of infested and phylloxera-free plants, we believe this was largely due to slightly differing ambient conditions between the two glasshouses where plants from each treatment were kept. This environmental bias is likely to overshadow changes induced by the presence of phylloxera on the plants in the field. By contrast, excised roots appeared to show clearer dissimilarities in odour composition, especially at later stage of infestation, although the roots could not

be sampled in the soil or from undamaged soil-contaminated samples. GC-MS analysis revealed small variations in the concentrations of multiple root volatiles induced by the presence of phylloxera, which differed according to rootstock and phylloxera strain. Although subtle quantitative changes were

detected, no “key volatiles” indicative of the presence of phylloxera were identified. Neither was there any visible and consistent pattern in the way concentrations of volatiles varied in response to phylloxera. The results suggest that although discernible, root volatiles are unlikely to be reliably used to predict the infestation status of vines. The compounds that make up the greatest part of the variation between the odours of infested and phylloxera-free plants were nonetheless identified for potential use in the design of specific e-nose sensors. Among these, methyl salicylate was the only volatile consistently detected in greater concentrations in the odour of infested plants of all rootstocks. Methyl salicylate is a known precursor in plant systemic defence against pathogens. The variations of different volatiles involved in responses of vines to infestation and their association with different plant defence signaling pathways should be explored to provide new insights into the genomic basis of resistance. The study will be written up and submitted as a publication in 2021.

Survey on the potential use of sniffer dogs

In previous experimental work we found some evidence that sniffer dogs might be able to detect phylloxera under controlled laboratory conditions. The results were, however, insubstantial, and required a significant amount of further research to ascertain with any confidence if this was the case. Since then, potential issues have been raised regarding cost and practicality of using sniffer dogs for in- field detection. To better understand the views of vignerons, biosecurity officers and researchers regarding the use of sniffer dogs in phylloxera detection, we designed an online survey in which participants were asked eight multiple choice questions addressing practicality, feasibility, priority, and costs associated with using sniffer dogs and sniffer dog research. Out of 54 respondents, only 29% thought that sniffer dogs would likely be adopted for phylloxera detection, and 73% of respondents felt there were biosecurity concerns in using sniffer dogs. Sniffer dogs ranked low against other research priorities. The outcome of this study triggered a stop-go milestone in the project and led to a variation removing further sniffer dog research and focusing instead on field validation of LAMP as a new detection tool.

Developing and validating LAMP as a new in-field tool for detection and identification

LAMP (loop-mediated isothermal amplification) is a new, reliable, portable molecular technology that has recently been developed for rapid and accurate in-field diagnostics of insect pests. In a previous project (Agriculture Victoria’s “Tackling Phylloxera” program) LAMP was successfully developed for phylloxera; and in the current study we focused on applying LAMP for in-field use. Surveillance trapping was conducted over two growing seasons in summer months. Phylloxera “bucket traps” were used, which trap the crawler stage (in a film of condensation) as they move from roots to canopy. Trapped insects were processed using laboratory and field-compatible methods for extracting DNA from these “crude” whole-insect phylloxera specimens, which were contaminated by plant tissue and soil. This included optimising a simple DNA extraction method (Hotshot, HS6), at different dilutions for optimal in- field use. Our study clearly demonstrated successful LAMP detection of phylloxera by bucket trapping over the two growing seasons.

We further evaluated our new LAMP surveillance approach for detecting phylloxera on root samples in infested vineyards. Vines roots were dug up and fine roots and nodules (if present) were tested using the LAMP device in the field. It was necessary to dilute HS6 DNA samples with water in the field to produce reliable amplification results and root samples collected from positive plants using an optimal dilution of Hotshot resulted in phylloxera detection from eighteen positive vine samples. The major advantage of using LAMP to detect phylloxera is that LAMP appears accurate and sensitive enough to remove the requirement for human visual detection in the field, saving considerable training and diagnostic time and effort. If LAMP were to be integrated into surveillance programs biosecurity officers could focus on locating symptomatic vines ("hot spots") and vine roots (nodosities), then employ LAMP to detect phylloxera from bucket trap samples or directly on roots, rather than have to visually detect the tiny insects themselves. The protocols we have developed for on-site LAMP assays can provide results within one hour, including DNA extractions, and are now available for easy routine use by end users, such as industry, growers, and biosecurity services. Further work on this approach would involve incorporating these LAMP detection protocols into larger-scale visual surveys to improve the accuracy of phylloxera detections carried out in the field.

II. ENABLING STRAIN-RELATED ROOTSTOCK SELECTION

Screening rootstock 5C Teleki against genetically diverse phylloxera strains.

Screening rootstocks that are resistant to phylloxera involves robust laboratory (excised roots) and glasshouse (potted vines) experiments and can also benefit from in-field trials where there are known genetic strains infesting vines. In 2015, Agriculture Victoria recommended a dozen rootstocks to the industry which had been screened against six phylloxera strains. In the intervening four years, new phylloxera strains have emerged in NE Victoria, one of which, G38, appears to be a potential 'superclone' that is highly adaptable to 'resistant' rootstocks and is known to have a leaf-infesting form. In this project, six genetically diverse phylloxera strains (G1, G4, G19, G20, G30, and G38) were screened for resistance against rootstock, 5C Teleki. With the exception of G1, five strains caused nodosities on vines in pots, implying that these strains will damage 5C Teleki vines. Compared with *V. vinifera* (which is highly susceptible to phylloxera), reproduction of strains G19, G20 and G30 on excised roots of 5C Teleki was, however, low. Results demonstrated that 5C Teleki is tolerant to specific strains, though the insects can survive and develop to reproductive adults, implying a hidden risk of phylloxera dispersal.

In-field analysis of strain G38 distribution across different rootstocks and screening for resistance under controlled conditions.

The G38 phylloxera genetic strain was detected for the first time in 2015, in a mixed-rootstock vineyard block at Whorouly, North East Victoria. A three year in-field survey was subsequently conducted in 2016- 2018 to monitor insect populations and study the performance of G38 on different rootstock cultivars. We conducted experiments to compare findings on rootstock susceptibility to G38 in these field trials with our laboratory/glasshouse screening protocols that use excised roots and potted vines. Results showed high numbers of insects feeding on rootstocks 101-14 and Schwarzmann in the field and this agreed with assays using potted vines and excised roots, where G38 was shown to be reproducing in relatively high numbers compared to own roots (*V. vinifera*). The findings clearly showed the susceptibility of 101-14 and Schwarzmann to G38 phylloxera. Importantly, this study provides evidence to

support the validity of our current laboratory and glasshouse screening assays in terms of predicting resistance / susceptibility of rootstocks in vineyards. That G38 may be showing signs of virulence on some common rootstocks is particularly worrying, especially given our findings on the increased genetic diversity of phylloxera in the King Valley, which may indicate that phylloxera may be able to reproduce sexually in Australia.

Determining phylloxera genetic diversity in the King Valley (NE Victoria).

Regionwide surveys of phylloxera genetic diversity were conducted almost two decades ago, and only included a very limited number of samples from the King Valley, where phylloxera has only been known since the early 1990's. These previous studies found very limited diversity, with only a single genotype (G04) present on grapevine roots. The current study aimed to conduct an updated and much more detailed survey of phylloxera on roots in the King Valley, with a view to understanding the genetic variation present. Over 700 phylloxera collected in the King Valley were screened for genotype identification (microsatellite variation). The study revealed that the amount of genetic diversity present in the King Valley is considerably higher than previously thought, with many novel genotypes present. A total of 36 genotypes were identified, with 4 strains known from previous work (G04, G20, G35 and G41) and 32 new strains (G84 to G115). This is a 39% increase in known phylloxera strains in Australia, to 115 strains. Given that phylloxera has only been known from the King Valley recently, and importantly that all genetic variation found involves new combinations of previously characterized genotypes, a potential explanation for the high diversity is that sexual reproduction is occurring. If this is the case it is of considerable importance, as sexual reproduction of phylloxera could significantly increase the risk of resistance adaptation and long-range dispersal (reproductive winged forms) occurring in the field.

The level of genetic variation present at individual sites varied greatly, with between 1 and 14 strains present per block. Analysis of genetic variation showed over 20% of the genetic variation was found to be localized to specific geographic areas, i.e. vineyards and blocks. Spatial autocorrelation analyses indicated that adjacent sites shared the same phylloxera strains up to 10 km apart, while more distant sites, up to 40km apart, were more likely to have different strains present. Strong associations of strains with specific rootstocks were not found, and vines were frequently found to have more than one strain of phylloxera on their roots (16% of vines examined). This finding has implications for interactions between phylloxera strains and rootstocks, and for the level of sampling required to ascertain which phylloxera strain is present on a grapevine.

Currently, there is a single PIZ in north-eastern Victoria, based on region wide phylloxera surveys conducted almost twenty years ago. Combining these previous surveys with our new data has revealed several groups of genetically related phylloxera strains which appear geographically localized to regions within this PIZ. The King Valley was previously recognized as a separate PIZ and the genetic data from the current study supports the genetic distinctiveness of the King Valley region, particularly with the prevalence of a virulent strain (G38) and the possibility of sexual reproducing forms. Likewise, the Rutherglen and Glenrowan regions appear to predominantly consist of unique strains of phylloxera, suggesting that the delimitation of the north-eastern PIZ requires re-evaluation. Rezoning the NE PIZ would obviously have major implications for the movement of equipment and produce between zones, but once done it would better reflect the underlying genetic structure of phylloxera populations within the region and would reduce the risk of novel strains being introduced into new areas.

III. IMPROVED QUARANTINE

Evaluation of new treatments for disinfestation of footwear and hand-held tools.

Household bleach is currently recommended for disinfecting footwear and small hand-held tools against grapevine phylloxera; but health and safety risks associated with chlorine, and damage caused to disinfested items, makes it unfavourable for use by growers. This study aimed to evaluate alternative off-the-shelf products for disinfestation of footwear and small tools. Product screening followed the following process: First, a review was conducted on off-the-shelf products used in insect pest/disease management and also recommendations from the wine industry; second, products were scored using a matrix that looked at availability, safety and practicality; third, products were tested in laboratory assays using first instar G4 phylloxera and solutions of recommended rates, for 30 or 60 seconds, with or without a follow-on water rinse; finally, products that were effective against G4 phylloxera were screened across genetically diverse strains (G1, G19, G20, G30 and G38). From the initial screening matrix, 22 products were considered for laboratory testing with G4 phylloxera. Of these, only Dettol (5% active ingredient Chloroxylenol) achieved 100% mortality against the six phylloxera strains tested and was deemed suitable for use in a practical setting. We can therefore recommend Dettol as a suitable substitute to bleach for disinfestation of footwear and handheld tools.

Validating fermentation treatment as a disinfestation protocol for grape products.

Fermentation of must or juice is recommended as ensuring grape products are free from live phylloxera and safe for movement. Currently, the endorsed procedure in the National Phylloxera Management Protocols is for grape products to complete at least three days (72 hours) of fermentation (Procedure D). The efficacy of this procedure was evaluated under laboratory conditions by testing survival of five root galling phylloxera strains in red and wine ferments, with and without the addition of yeast (*Saccharomyces cerevisiae*) for 6, 12, 24, 48 and 72 hours. Two of the strains used in the experiments (G19 and G38) are also known to have leaf galling forms. 100% mortality was achieved across all phylloxera strains when subjected to fermentation lasting 48 and 72 hours, when yeast is added to grape products. Fermentation was ineffective at 48 and 72 hours without addition of yeast. The current procedure that allows movement of must or juice from a PIZ or PRZ into a PEZ requires completion of at least three days (72 hours) of fermentation; this is only valid when yeast is added to wine ferments. The fermentation protocol in the NPMP requires a review in light of these findings and new protocols should be developed and evaluated to determine mortality in naturally fermented products.

IV. PHYLLOXERA BIOLOGY – KNOWLEDGE GAPS.

Investigating the effect of temperature on phylloxera survival, development and reproduction under controlled environments.

Of the 119 phylloxera strains identified in NE Victoria, relatively few have spread outside a specific NE region. The reasons for this are unclear but may be related to environmental conditions. Temperature in particular could impact on strain distribution, and a better understanding of strain-specific temperature tolerance could help with predictions for phylloxera distribution under climate change. This component of the project conducted experiments to explore the effects of temperature on phylloxera survival, development and reproduction. The study focussed on five phylloxera strains (G1, G4, G19, G20 and G30) at

temperatures of 18, 22, 26 and 30°C. Survival of the five phylloxera strains was evaluated on excised roots (i.e. with a feeding site), and also removed from roots (without a food source) under dry and wet conditions. On excised roots, temperatures were optimal for survival and reproduction across the five phylloxera strains at 22 and 26°C. At 30°C and above, temperatures were unfavourable for development and reproduction, with none of the eggs on excised roots developing to reproductive adults at 30°C for G19, G20 and G30. Results showed that phylloxera survival at lower temperatures appears to live longer than previously thought, with G20 at 18°C surviving up to 29 days while not attached to a vine root. This finding has implications for managing movement of contractor workers or vineyard machinery from phylloxera infested zones to exclusion zones especially *during and after* vintage when crawlers are above-ground.

V. RE-EVALUATING BIOCONTROL OPTIONS.

A detailed review on the potential for biocontrol in phylloxera management was undertaken and is presented as a full report. Drawing on expert advice from North America, Europe and Australia, this review evaluated different approaches including: (1) the release of exotic natural enemies from the pest's native range (Classical Biological Control), (2) the release of commercially-reared predatory insects, including mites or parasitoids (inoculation biological control), (3) the application of entomopathogenic organisms such as nematodes and fungi (inundation biological control) and, (4) attracting resident native predators such as canopy and ground-dwelling spiders and insects through the provision of 'insectary plants' to provide shelter, nectar, pollen and alternative prey (conservation biological control. The review found 16 predatory species, of which only two attack root-feeding phylloxera. A predatory syrphid fly is particularly promising and warrants further investigation. We also suggest targeted field surveys in eastern USA to search for root predators and insect pathogens (entomopathogens). Similarly, in Europe most predators were of the leaf-galling form. In Australia, similar predatory guilds occur in vineyards and could be encouraged through habitat manipulation for conservation biocontrol. There is considerable scope to further investigate the use of entomopathogenic nematodes and fungi as biological pesticides with the aim of combining multiple biocontrol approaches for phylloxera management.

4.0 BACKGROUND

Grapevine phylloxera is the highest priority pest for the Australian wine industry (Plant Health Australia 2018). Incursions and spread lead to significant economic losses, impacting on profitability for grape and wine producers. As an example, the financial impact due to recent infestations in the Yarra Valley is estimated at $1 billion (Vinehealth Australia 2019). To manage an infestation, vineyards are planted or replanted onto phylloxera resistant rootstocks, which are costly compared to own-rooted *Vitis vinifera* and can be up to $50,000 per hectare on top of lost production for up to five years (Vine Health Australia, 2019). In wine-growing communities, phylloxera infestation adds business uncertainty and restricts market access, which translates into higher compliance costs. For the individual, meeting the regulation compliance costs increases management costs by nearly 20 per cent. Although historically limited in its distribution, being restricted to quarantine areas in Victoria and NSW, phylloxera has been increasing its geographic range in the last 15 years. The pest is currently absent in South Australia, Western Australia, Queensland and Tasmania, but vines in these regions are particularly vulnerable to phylloxera, as more than 70% of vineyards are planted on the highly susceptible ungrafted *Vitis vinifera*, where infestations spread rapidly and can lead to vine death within 4-5 years.

The recent spread of phylloxera has been attributed to poor detection rates, slow uptake of resistant rootstocks, quarantine breakdown, knowledge gaps in our understanding of the biology and ecology of the pest under Australian conditions, and a lack of options of phylloxera control. Early detection is a core component of phylloxera management and is integral to the success of surveillance (for rezoning) and containment. Detection of phylloxera relies upon a surveillance system that primarily uses physical inspection of grapevine roots and grapevine foliage, and then follow up identification of suspected phylloxera by trained entomologists, using morphological and genetic methods in insect diagnostics. The surveillance process is labour intensive and costly, and ground surveys rarely detect phylloxera in its early infestation stage. The search for new early detection methods includes remote sensing, new molecular diagnostic tools, and odour-sensing tools such as sniffer-dogs and electronic noses (Henderson et al. 2010; Benheim et al., 2011; 2012).

The uptake of resistant rootstocks is key to controlling and containing phylloxera within the restricted zones (Benheim 2012; Powell et al., 2013). Recent research by Agriculture Victoria identified that phylloxera resistance or tolerance to rootstocks is dependent on insect genotype-plant genotype interactions. Recommendations for 10 resistant rootstocks (tested against 6 genotypes) were released to industry in 2015 (Powell and Krstic 2015): this information has now been incorporated into the Grapevine Rootstock Selector tool, the industry's independent resource to match rootstocks to wine regions and a means for growers to assess key factors that are key to a planting site (Wine Australia 2019). Since 2015, an additional phylloxera genotype (G38) has been identified and screening needs to be conducted against this strain, particularly as early field observations indicate this could be a 'superclone' which thrives on grafted rootstocks. Popular commercially available rootstocks that have yet to be subjected to screening trials pose a threat to the grower (yield loss) and more broadly the industry (biosecurity / spread) if they are unknowingly susceptible to particular strains. Agriculture Victoria uses internationally recognised protocols for screening commercial and newly developed rootstocks for resistance against phylloxera, using laboratory bioassays (on excised roots) and greenhouse bioassays (potted plants). Field studies validating these screening methods would provide growers and the wine industry with greater confidence with regards to rootstock selection. A comprehensive understanding of strain-specific susceptibility of rootstocks together with knowledge of the local distribution of phylloxera strains could provide a powerful strategy for controlling and containing this pest.

Quarantine regulations are guided by endorsed disinfestation protocols, which have played a major role in containing phylloxera infestations within PIZ's in parts of Victoria and South eastern New South Wales. Recent detections and spread of G1 phylloxera within the Maroondah PIZ in central Victoria have, however, raised concerns about the effectiveness of quarantine protocols. Agriculture Victoria recently validated disinfestation procedures to provide recommendations to the industry for improved protocols (Projects DED 1301 and DED 1701; Clarke, 2017; Clarke, 2018a, b). The outcomes showed that the NPMP that was initiated in 2009, contained procedures and techniques that required updating to ensure effectiveness based on i) rationality ii) timing iii) temperature limits iv) recognition of diverse strains of phylloxera and v) biology of the pest. Consequently, recommendations were made in the National Phylloxera Management Protocols (NPMP) to develop robust quarantine processes which initiated a comprehensive review, currently ongoing, of the NPMP through national and state biosecurity committees.

Of the 115 phylloxera genetic strains identified to-date, relatively few appear to have spread outside the North East Victoria phylloxera infested zone (PIZ). The reasons are commonly attributed to environmental conditions, particularly temperature and soil characteristics, which could impact on strain spread and distribution under future climate change predictions. In NE Victoria, leaf galling strains have recently shifted their distribution into the King Valley and a root galling strain (G38) that adapts well to a range of 'resistant' rootstocks has also spread further. If, as suspected, temperature ranges are pivotal to the virulence level and establishment of particular strains it is important to determine optimal temperature ranges. This knowledge would allow risk maps to be determined for current and future climate scenarios in different grape-growing regions.

Ultimately, vignerons would like to see a phylloxera management strategy that, in addition to containment, aims at actively controlling phylloxera within their vineyards. Biological control can reduce pest populations through augmenting natural enemies (i.e. releasing agents from the native range) or supporting populations of natural enemies in local environments (i.e. a conservative approach). For phylloxera, biological control has received surprisingly little attention in comparison to research on chemical control and development of phylloxera-resistant rootstock. Preliminary research under controlled conditions has shown some success with soil-borne pathogens, nematodes, and predatory mites, but in general this has stopped short of any attempt to implement and evaluate this as an IPM strategy, including research that might assist the establishment of the biocontrol agents. In short, the door to implementing biocontrol for phylloxera IPM is by no means closed, with the potential for enormous benefits to growers and Industry should a field biocontrol strategy be identified.

6.0 PROJECT AIMS

This main objective of this project was to develop an improved integrated approach for managing endemic strains of grapevine phylloxera. The project focused on five key themes:

1. New and improved tools for early detection
2. Improved rootstock selection
3. Improved quarantine
4. Re-evaluating biocontrol options
5. Addressing key knowledge gaps on the biology of phylloxera

The outputs from the project agreement aligned against the five key themes are outlined in Appendix 1.

7.0 PROJECT OUTPUTS AS SPECIFIED IN THE PROJECT DOCUMENT

Theme and short title	OUTPUT	Milestones [Yr.no]	Page number
NEW AND IMPROVED TOOLS FOR EARLY DETECTION.			
Evaluating an electronic nose for early detection of phylloxera	Technical report evaluating a commercially available e-nose [PART A]	1.4, 2.3,	18
Using GC-MS to identify key volatiles associated with infestation	Technical report on using GC-MS to investigate key volatile compounds related to infestation [PART B]	1.4, 2.3,	27
Sniffer dog survey	Survey investigating views by industry, biosecurity and researchers on the use of sniffer dogs [PART C]	1.5	36
Surveillance systems	Comparison of grower friendly surveillance systems in Section 9.4. Protocols submitted for endorsement for phylloxera surveillance.	1.5	49
LAMP as a tool for rapid in-field diagnostics	Initial field trials evaluating LAMP, and validation of LAMP for bucket trap samples.	2.2 (variation)	41
ENABLING STRAIN-RELATED ROOTSTOCK SELECTION			
Genotype mapping in the King Valley	Technical report on study developing a 'strain-specific' phylloxera distribution map for the King Valley region, including recommendations to biosecurity / industry.	1.3, 2.4, 3.2	67
Screening 5C-Teleki rootstock across phylloxera strains	Technical report screening rootstock 5C-Teleki across phylloxera strains, including updated rootstock recommendations	2.5	51
Screening G38 strain across rootstocks	Technical report detailing screening phylloxera strain, G38, against widely use d phylloxera-resistant rootstocks, including updated rootstock recommendations.	1.3, 2.5	57
IMPROVED QUARANTINE			
Alternative disinfestation procedures for footwear.	**Technical report** including recommendations provided to key biosecurity stakeholders/committees on NPMP.	1.2	84, 123
Validating fermentation procedures for disinfestation	**Technical report** including recommendations for key biosecurity stakeholders/committees on NPMP (see section 14).	2.1	93, 123
Extension	Information on alternative disseminated to industry through at least one extension mechanism.	1.2	123
Publications	Publications on improved disinfestation techniques.	3.3	123
RE-EVALUATING BIOCONTROL OPTIONS.			
Biocontrol review	Desktop review evaluating the potential for phylloxera biocontrol.	1.5	113
PHYLLOXERA BIOLOGY – KNOWLEDGE GAPS.			
Study on temperature and survival	Technical report on the effect of temperature on phylloxera survival, development and reproduction under controlled environments.	3.1	102

INDUSTRY REFERENCE GROUP			
IRG meetings	Reference group formed and two meetings held.	1.7, 2.6,	123
PUBLICATIONS			
	At least one industry publication / website update / media release on other outputs of the project in Section 14.	3.3	123
Outputs not addressed			
Data set / report on sniffer dog research	Stop/go milestone and approved variation.	2.2	41
FINAL REPORT SUBMITTED	Full final report	3.5	

A table of outputs against six-monthly milestones is presented in Appendix 1.

8.0 RESEARCH ACTIVITIES UNDER PROJECT OBJECTIVES – GENERAL METHODS

General culturing methods.

The maintenance of diverse phylloxera populations is essential for rootstock screening and other empirical studies under controlled conditions. Throughout the report, diverse phylloxera genotypes are referred to as *"strains"* based on a classification of grape phylloxera biotypes that considers the performance of phylloxera on rootstock hosts as measured by survival, development, and growth of population parameters (Forneck et al. 2016). The cultures were originally selected in 2003 based on phylogenetic analysis of microsatellite alleles and mitochondrial DNA sequences distinct to the genetic structure of phylloxera populations in Australia (Hoffmann, 2003). Seven genetic strains of phylloxera are currently maintained under quarantine - G1, G4, G7, G19, G20, G30, and G38 at Agriculture Victoria Rutherglen. The G38 strain was used in laboratory and glasshouse trials for the first time. Wild phylloxera were originally collected from roots of grafted and ungrafted *V. vinifera* in commercial vineyards in central and north-east Victoria. G4 were collected from the King Valley; G7, G19 and G30 from Rutherglen; G20 and G38 from the Buckland Valley; and G1 from the Yarra Valley (Maroondah PIZ). The identity of the phylloxera strains was confirmed at the commencement of the project and in years 2 and 3 using six nuclear DNA microsatellite markers (described in Umina et al. (2007)). In year 3, the G7 culture was found to be contaminated with G4 and was thus excluded from all data in this project. This cross- contamination (despite strict culturing protocols) highlights the necessity to regularly genotype laboratory populations to confirm purity. G7 founders (wild collected insects) will need to be collected from field material to re-establish the culture for future screening trials. G4, G20, G19 and G30 have been kept in continuous culture for many generations and we recommend collecting new wild stock to ensure insects used in the next phase of research have the genetic ability to diapause (G1 and G38 were collected from the wild before the beginning of this project).

Insects used in experiments were cultured under quarantine at 25°C on excised *V. vinifera* cv. Chardonnay roots. The roots were sourced from a clean vineyard and prepared using a protocol modified from Granett et al. (1985) and Kingston (2007). The preparation process was as follows; roots were washed with a soft brush under running water to clean soil debris; roots were excised into 5-10cm pieces; excised roots were soaked in Axiom® Plus fungicide solution (7.5 g/L) for 15 minutes; once rinsed in distilled water, and three times in ultra-pure water; air dried under the laminar flow for three hours; the end of dry roots were wrapped with cotton wool and moistened with three drops of water; two excised roots were placed in a Petri dishes (90 × 25 mm) and inoculated with phylloxera eggs or stored at 4°C until required, or at start of experiments. To maintain the cultures, phylloxera were incubated in the dark at 25 ± 3°C. Root pieces inoculated with phylloxera were cleaned of fungal contamination using an artist's sable-haired paintbrush (Art Basics Sable spotter 300-R 2/0, Japan) moistened with 80% ethanol and, the cotton wool wetted once every week to maintain viability.

RESEARCH THEME 1

NEW AND IMPROVED TOOLS FOR EARLY DETECTION

9.0 NEW AND IMPROVED TOOLS FOR EARLY DETECTION

9.1. ODOUR SENSING TECHNOLOGIES FOR EARLY DETECTION OF GRAPEVINE PHYLLOXERA

Overarching aim: This component investigated the current and future potential for odour sensing technologies for early detection of phylloxera through (i) evaluation of a commercially available electronic nose (PART A), and (ii) using gas chromatography-mass spectrometry (GC-MS) to explore the potential for developing future e-nose with finely tuned sensors (PART B).

PART A: EVALUATION OF A COMMERCIALLY AVAILABLE ELECTRONIC NOSE

Summary

A commercially available electronic nose (e-nose) was evaluated for its ability to identify phylloxera-infested vine roots across different rootstocks, during early and late stages of infestation. The device failed to detect differences in leaf odours of healthy and phylloxera-infested vines. For root odours, there was evidence that the e-nose might be capable of identifying infested roots, but the level of accuracy was not acceptable for a detection tool and there were major practical limitations in the use of the e-nose (sample preparation, testing environments, sensors consistency).

Key findings from e-nose trials:

- The e-nose could not detect infested vines based on sampling leaf volatiles.
- The e-nose could distinguish odours of infested roots from odours of phylloxera-free roots during early infestation sampling, but differences were more subtle for late infestation sampling.
- Root odours were more distinct for some rootstocks than others.
- Different phylloxera strains appear to affect root volatiles in different ways.

Background

When an insect feeds on a plant, odours associated with tissue damage are released. These odours can be characteristic of the insect species (Clavijo McCormick, Unsicker and Gershenzon 2012), which opens up the possibility of developing sensing technologies, commonly called electronic noses (e-noses), as a promising avenue for pre- and post-harvest surveillance of insect pests. Unlike tradition benchtop technology for odour analysis (gas chromatography-mass spectrometry, GC-MS), which is immobile and can take an hour or more to analyse odours, e-noses offer a rapid, cheap and portable device for odour analysis. E-noses cannot, however, identify chemical constituents of an odour (volatiles) per se; instead their suites of sensors generate an odour-specific response pattern, which can then be compared and analysed by multivariate statistical analysis or machine learning techniques with the intention of discriminating odour groups (Wilson 2018).

The Cyranose 320 (Sensigent LLC, California) is one such e-nose that has shown promise in its ability to detect and classify odours caused by insects and rots infesting plant material (Henderson et al. 2010; Li et al. 2010; Gancarz et al. 2017). The use of an e-nose involves three steps: (i) “training” sessions consisting of exposing the device to given odours, each leading to distinct patterns of activity across the e-nose’s 32 broadly tuned sensors, which are known as “smellprints”. These smellprints are

implementable in subsequent classification or recognition tasks; ii) a cross-validation step during which the quality of the smellprints obtained during training are assessed and; iii) a validation step that aims to confirm the robustness of the model and ability of the device to reliably discriminate different smells against which it was previously trained. In instances where the data are too noisy and all data must be used for training, the validation step may be replaced by statistical techniques that will give an indication of model performance.

The aim of this study was to determine whether the Cyranose 320 is able to produce distinguishable smellprints after training the device against infested and phylloxera-free grape vine odours under controlled conditions across several grape rootstocks. Classic bench-top chemical analyses (GC-MS) of samples (presented in Part B) was conducted in parallel to this study on the same samples, with the further aim of identifying key volatiles that could assist with the development of e-nose sensors for improved phylloxera detection.

Methods

Vines were purchased from Yalumba Nursery, stored at 4°C for no more than four days when they were potted in 80% horticultural soil and 20% Perlite. 128 grapevines from three rootstocks were grown in controlled-environment greenhouses for 1 year before they were used in experiments to allow the development of roots prior to phylloxera infestations. The rootstocks were chosen based on previous research evaluating levels of susceptibility to grape phylloxera infestation: *Vitis vinifera* L. is a phylloxera-susceptible rootstock, Ruggeri 140 is tolerant, while 1103P is resistant to phylloxera infestation (Powell and Krstic, 2015). *V. vinifera* rootstocks were grown with either Shiraz or Chardonnay variety grapes, while the other rootstocks were grown with Shiraz only. Drip lines were used to provide vines with consistent watering and vines were checked regularly for signs of stress or unwanted pests and pathogens. There were 16 vines were infested with the G19 strain of grape phylloxera and 56 vines were infested with the G4 strain of phylloxera. Infested vines were grown in separate greenhouses from healthy plants and the two infested treatments were kept in separate rooms. Plants were infested by uncovering soil at the roots of the vines and placing a small plastic Petri dish upside down with phylloxera eggs at the roots (Figure 1). The Petri dishes protected the eggs and ensured they were not washed away or damaged by watering before the larvae hatched.



Figure 1 Phylloxera eggs placed on filter paper strips for inoculation.

Leaf odour sampling. Two weeks post-infestation, early sampling of leaf volatiles was conducted using the Cyranose 320. Volatiles were collected by enveloping a branch with 4 or more healthy leaves in a large odour-free oven bag sealed with zip ties for approximately 1 hour to allow volatiles to accumulate. Following headspace odour equilibrium, a small incision was made in the bag through which the e-nose syringe was inserted. The plastic oven bag was pinched around the e-nose syringe to seal the bag. Sampling consisted of an initial purge to return e-nose sensors to baseline, followed by 20 seconds of sample intake when the needle of the e-nose was inserted into the bag, followed by another purge. All control plants were sampled before infested plants to avoid transferring any phylloxera from the infested vines to the phylloxera-free glasshouse. Within the healthy and phylloxera-infested groups, plants were arranged randomly in the glasshouses and sampled randomly. Sampling was repeated for late stage (8 weeks after infestation) odours using the same methodology on a second batch of grape vines grown alongside the first batch.

Root odour sampling. Root samples were collected (following leaf sampling) by removing the plants from pots. Young fibrous roots were cut from the plant in 5 cm lengths using scissors. The roots were then gently washed several times with distilled water or using fine forceps to remove soil particles, dried using paper towel and weighed to obtain at least 1 g of clean root material. Clean root material was wrapped in aluminium foil and placed in liquid nitrogen for snap freezing. The aluminium packets were transported to AgriBio from Rutherglen under dry ice and stored in a -80°C freezer prior to sampling. For sampling, roots were removed from the aluminium packets and crushed using a mortar and pestle under liquid nitrogen (Figure 2). The sample was then inserted into a glass SPME vial and reweighed before being capped with aluminium foil. The vials were left for 10 minutes to allow headspace volatiles to accumulate (equilibration) before the e-nose snout was inserted through the foil into the vial. Sampling consisted of an initial purge to return e-nose sensors to baseline, followed by 20 seconds of sample intake where the needle of the e-nose was inserted into the vial, followed by another purge.

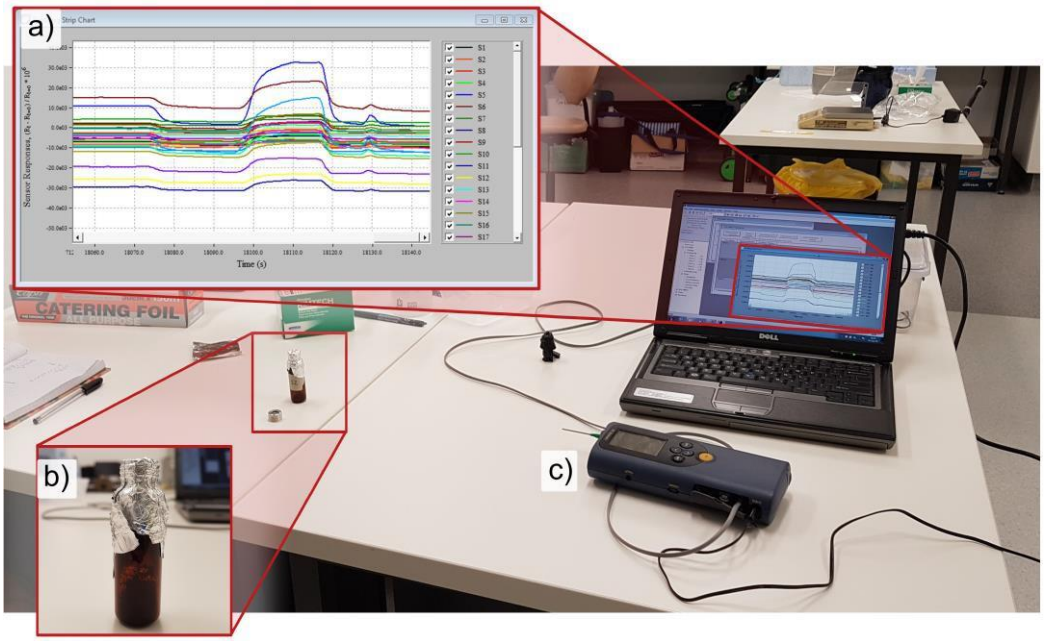


Figure 2 Set up of Cyranose 320 for root sampling, depicting a) scrolling strip chart of e-nose sensors that have reacted to infested root material, b) glass SPME vial with aluminium foil covering, and c) the Cyranose 320 e-nose device

Two-axes non-metric multidimensional scaling (NMDS) plots were created to display the e-nose data and depict differences among treatments. These plots seek to depict patterns in the data by presenting each datum as a point on a grid. Similar data points are placed in close proximity resulting in clusters of similar points. Summary NMDS plots were created featuring all treatments within tissue type (leaf or root) and sampling time (early or late). Finer-scale NMDS plots were also generated to display the differences between healthy and infested samples within rootstock treatment. Analysis of Similarities (ANOSIM) analyses were performed in parallel with each NMDS ordination in order to generate significance values to establish whether significant differences were evident among treatment groups according to the Cyranose 320 sensor data. G19 plants are included in NMDS plots but not in ANOSIM analyses as the G19 sample size was too small to be useful for this analytical method. One plant in the late G19 infested group with *V. vinifera* (Shiraz) died before sampling, as did one plant in the late G19 treatment for the Ruggeri rootstock.

Results and discussion

Early versus late samples

The e-nose trial investigating leaf volatiles with healthy and phylloxera infested grape vines showed that smell profiles produced by the phylloxera infested plants were more similar to each other than to smell profiles produced by healthy plants. In this instance, however, treatments are conflated with time of sampling so results must be interpreted with caution. Figure 3 displays an NMDS plot of data generated by the e-nose, which summarises similarities between data points on a reduced set of axes. Each point in the graph represents one replicate sample. Both early (2 weeks post infestation) and late (8 weeks post infestation) leaf samples group according to sampling date and infestation status (Figure 3 a and b),

indicating that the groups have distinct odours according to the e-nose. Both leaf subplots show a significant relationship evident in the data ($p<<0.05$) ostensibly driven by the effect of the time the sample was taken. This interpretation arises from the fact that phylloxera did not appear to infest all treatment plants equally effectively, but all the treatment plants still fall into the one distinct cluster. There is a notable lack of differentiation within infested and healthy samples, indicating that the e-nose leaf data cannot be used to distinguish rootstocks.

NDMS plots were also used to depict the similarities between roots volatile samples according to data generated by e-nose sampling (Figure 3 c and d). Each point in the graph represents one replicate sample, with points that are proximal representing data that are similar and points that are disparate representing data that are dissimilar, resulting in clustering of similar points. Root samples show evidence of clustering and separation for all treatments, both in early and late sampling, thus indicating that each group produces somewhat distinctive odours according to the e-nose. The samples cluster in accordance with both rootstock—indicated by colour—and infestation status within rootstock—indicated by the shape of the symbols. Early root sampling produced a slightly higher ANOSIM r coefficient compared to late root samples, at r=0.3781 and r=0.3150 respectively, indicating that early root samples had slightly more distinct odours according to the e-nose.

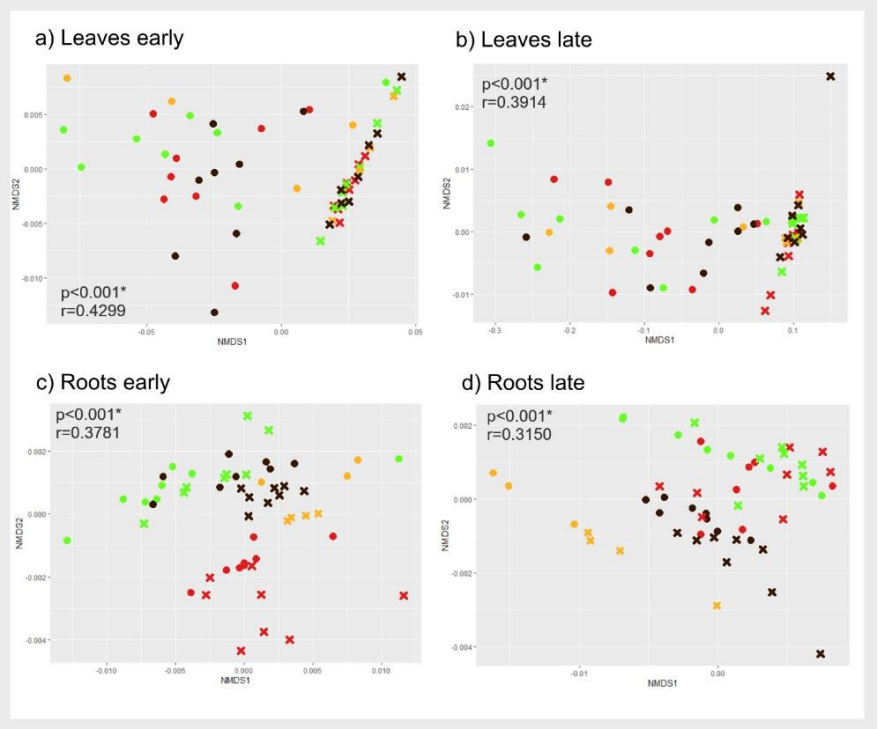


Figure 3 Early vs late sampling of leaves and roots (all rootstocks). NMDS plots of e-nose data, presenting results for a) leaves sampled early, b) leaves sampled late, c) roots sampled early, and d) roots sampled late. Red=1103P rootstock, green=Ruggeri 140 rootstock, brown=*Vitis vinifera* rootstock with Shiraz variety grapes, orange=*Vitis vinifera* rootstock with Chardonnay variety grapes. Circles=healthy plants, crosses=G4 infested plants. Significance and correlation coefficients from ANOSIM are displayed for each subplot.

E-nose results with leaf volatiles across rootstocks, early and late stage infestation.

At a finer scale, NMDS visualisations of the e-nose leaf data **show distinct patterns between healthy and infested plants which are most easily attributed to the timing of sampling** (Figures 4 and 5). Figure 4 indicates that healthy plants appear to produce odours distinct from plants in the phylloxera treatment group according to the e-nose. However, even the infested plants where no phylloxera could be found on the roots cluster distinctly with the treatment group, indicating that the groups are likely the product of sampling date rather than infestation status. Samples from the G4-infested treatments cluster separately from healthy and G19 infested treatments, including in the case of 1103P rootstock (4a) where phylloxera largely appeared to have died off prior to any evidence of root damage. G4 samples form tighter clusters than healthy plant samples, potentially indicating that the healthy plants had a higher level of variation in regard to the volatiles produced, or that they do not smell as distinct according to the e-nose. G19 patterns differ between NMDS plots and may be located together (4a) or widely separated (4d). Early and late leaf samples show very similar patterns. All rootstocks generate significant p-values from ANOSIM for the leaf data, with the exception of early sampled *Vitis vinifera* with Chardonnay grapes, though it is worth noting that Chardonnay treatments had fewer samples than other treatments. This is consistent with the NMDS results in showing that the e-nose recognises differences in the odours of the healthy and phylloxera infested plants for the various rootstocks.

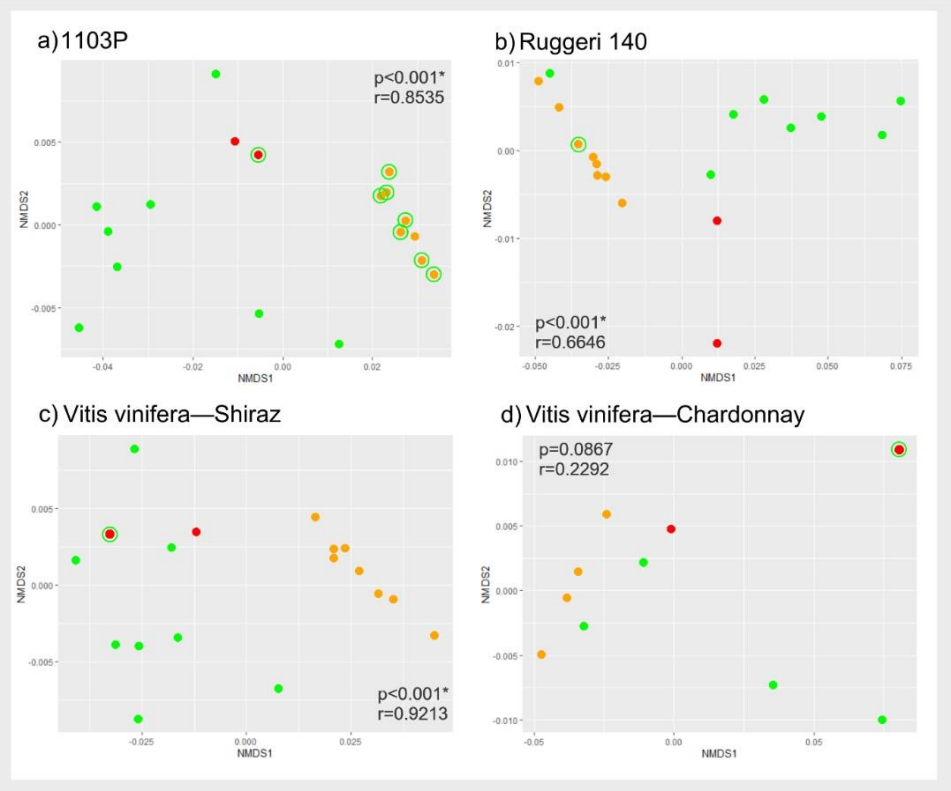


Figure 4 Healthy vs infested sampling of early leaves (all rootstocks). NMDS plots of early leaves e-nose data, presenting results for a) 1103P rootstock, b) Ruggeri 140 rootstock, c) *Vitis vinifera* rootstock, Shiraz grapes, and d) *Vitis vinifera* rootstock, Chardonnay grapes. Green=healthy plants, orange=G4 infested plants, red=G19 infested plants. Plants from the infested treatments that showed no evidence of phylloxera eggs or root nodosities upon collection of root samples are circled in green. Significance and correlation coefficients from ANOSIM are displayed for each panel.

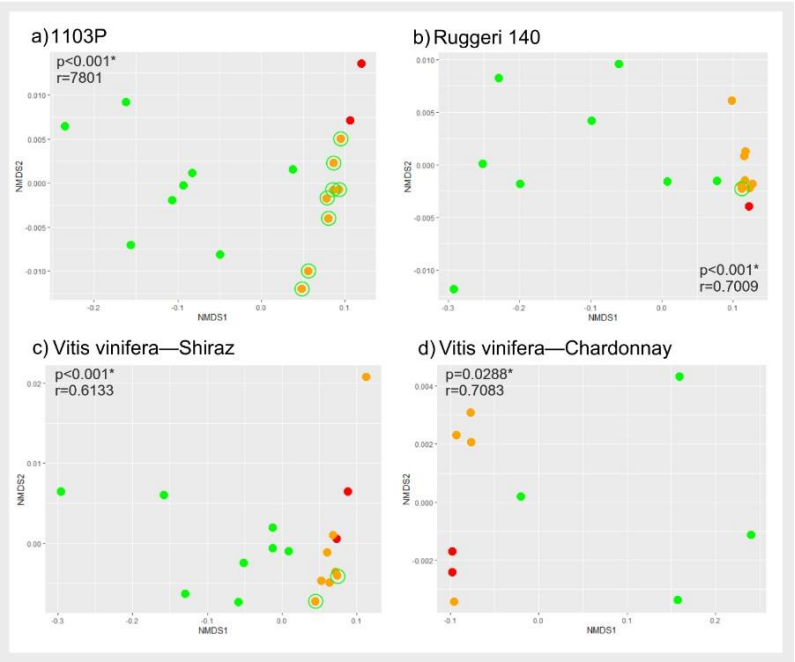


Figure 5 Healthy vs infested sampling of late leaves (all rootstocks). NMDS plots of late leaves e-nose data, presenting results for a) 1103P rootstock, b) Ruggeri 140 rootstock, c) *Vitis vinifera* rootstock, Shiraz grapes, and d) *Vitis vinifera* rootstock, Chardonnay grapes. Green=healthy plants, orange=G4 infested plants, red=G19 infested plants. Plants from the infested treatments that showed no evidence of phylloxera eggs or root nodosities upon collection of root samples are circled in green. Significance and correlation coefficients from ANOSIM are displayed for each panel.

E-nose results with root volatiles across rootstocks, early and late stage infestation

The NMDS plots of the root e-nose data depict more ambiguous grouping than the leaf plots, though **partial separation of the treatment groups is evident** in most NMDS plots (Figures 6 and 7). The groupings indicate that, according to the e-nose, healthy and infested roots produce different odours but the differences are too subtle to be separated completely. Separation appears to be **slightly more distinct in the early stage root infestation** plots than the late stage root infestation plots. G19 patterns differ between NMDS plots and may be located together (6b) or widely separated (7a), but this lack of consistency for G19 may be partially attributed to low sampling. The early stage infestation root data generates significant p-values from ANOSIM for the Ruggeri rootstock (p=0.0147) and *Vitis vinifera* rootstock with Shiraz variety grapes (p=0.0406), though the ANOSIM r coefficient is weak in both cases (r=0.2338 and r=0.1507 respectively). The ANOSIM results reflect the results seen in the NMDS plots, that during early root sampling the roots produced slightly more distinct odours for separating healthy and infested plants than late roots. The 1103P rootstock does not produce significant p-values for early or late sampling, nor does the *Vitis vinifera* rootstock with Chardonnay grapes, though the latter treatment group had fewer replicates. As 1103P is the resistant rootstock, the lack of distinction between healthy and infested plants could be the result of insects dying quickly or not feeding extensively, resulting in the infested group producing odours similar to the healthy plants. No significant p-values were obtained for the data generated from late root sampling. However, it should be noted that the p-values produced during late sampling are not radically different to those produced early, despite none of them meeting the benchmark to be considered significant.

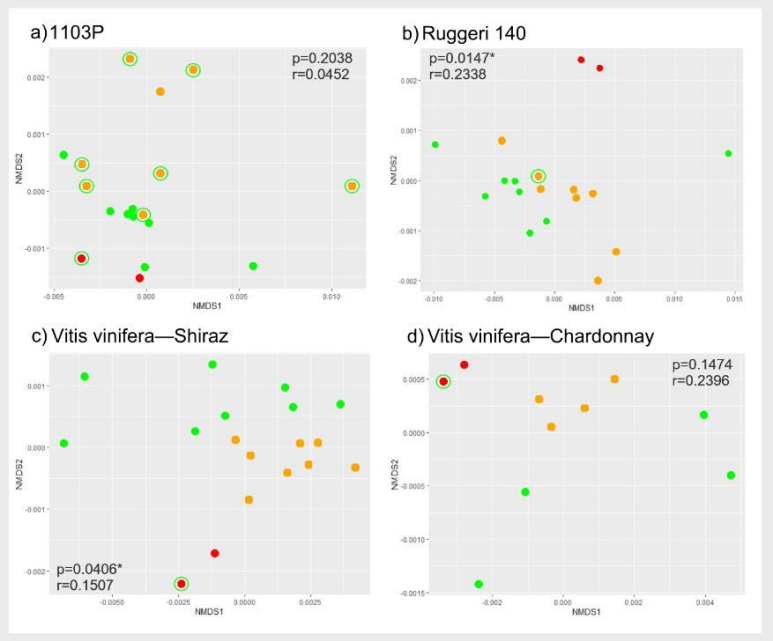


Figure 6 Healthy vs infested sampling of early roots (all rootstocks). NMDS plots of early roots e-nose data, presenting results for a) 1103P rootstock, b) Ruggeri 140 rootstock, c) *Vitis vinifera* rootstock, Shiraz grapes, and d) *Vitis vinifera* rootstock, Chardonnay grapes. Green=healthy plants, orange=G4 infested plants, red=G19 infested plants. Plants from the infested treatments that showed no evidence of phylloxera eggs or root nodosities upon collection of root samples are circled in green. Significance and correlation coefficients from ANOSIM are displayed for each panel.

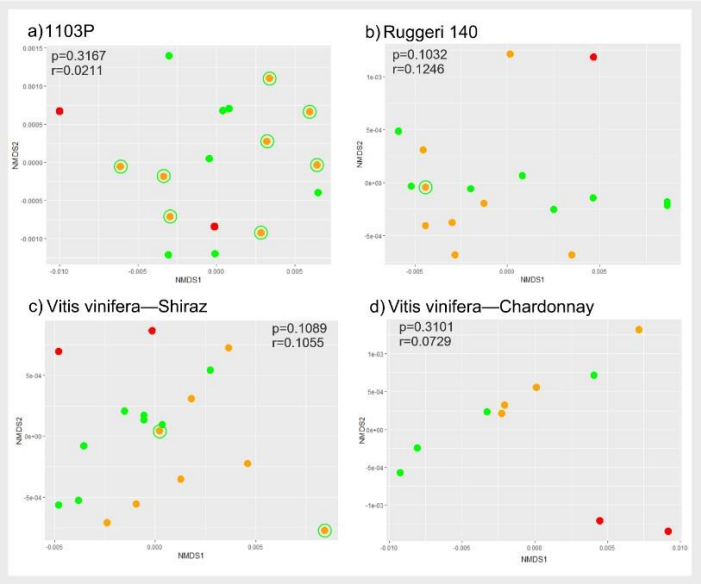


Figure 7 Healthy vs infested sampling of late roots (all rootstocks). NMDS plots of late roots e-nose data, presenting results for a) 1103P rootstock, b) Ruggeri 140 rootstock, c) *Vitis vinifera* rootstock, Shiraz grapes, and d) *Vitis vinifera* rootstock, Chardonnay grapes. Green=healthy plants, orange=G4 infested plants, red=G19 infested plants. Plants from the infested treatments that showed no evidence of phylloxera eggs or root nodosities upon collection of root samples are circled in green. Significance and correlation coefficients from ANOSIM are displayed for each panel.

Due to the nature and way the device operates, not only is the e-nose performance intricately linked to the quality of the data acquired during training, it is also heavily constrained by the sampling conditions (including background odours, moisture, temperature) under which training was undertaken. This in itself limits practicality, as the conditions during e-nose use must remain consistent with those of training, and possible changes in ambient conditions may otherwise entail the necessity to re-train the device under the new conditions before use. This limitation is problematic for conducting trials on healthy and phylloxera infested plants effectively using an e-nose in laboratory settings, as quarantine practices prohibit healthy and infested plants from being kept in one area and limits which group can be sampled at a time. The issue of the e-nose being sensitive to sampling conditions is also problematic for real world implementation of the device, as training data produced on one day cannot justifiably be used if the slightly differ from the conditions the e-nose was trained under. Under natural growing conditions where variation in plants is likely to be higher than for plants grown under glasshouse conditions, the e-nose is likely to have increased difficulty in distinguishing healthy and phylloxera infested plants.

PART B: CHEMICAL ANALYSIS (GC-MS) OF LEAF AND ROOT VOLATILES OF VINES INFESTED WITH DIFFERENT PHYLLOXERA STRAINS.

Summary

To explore the potential for developing future e-nose with finely tuned sensors, gas chromatography-mass spectrometry (GC-MS) analysis was conducted on leaves and excised roots of infested and phylloxera-free plants. GC-MS analysis revealed small variations in the concentrations of multiple root volatiles induced by the presence of phylloxera, which differed according to rootstock and phylloxera strain. However, no "key volatiles" indicative of the presence of phylloxera were identified, and results suggest root volatiles are unlikely to be reliably used to predict the infestation status of vines. The compounds that make up the greatest part of the variation between the odours of infested and phylloxera-free plants were nonetheless identified and further exploration of their biochemical pathways may provide new insights into the genomic basis of rootstock resistance.

Key Findings:

- Sampling leaf volatiles has limited potential for the detection of phylloxera.
- Excision and pulverization of vine roots was necessary to obtain exploitable root volatiles data, which has practical implications if these odours cannot be detected in intact roots.
- Changes in root odours in phylloxera infested vines were discernible only at 8 weeks after infestation.
- No consistent "key" volatiles were identified indicative of phylloxera infestation across rootstocks, apart from methyl salicylate, which is a common to metabolic pathways in plant defence (i.e. induced by other herbivorous insects).
- Substantial overlap between the odour profiles of infested and uninfested plants suggests that root volatiles do not provide the most reliable information for the detection of phylloxera.
- Different phylloxera strains altered root volatiles in different ways thus adding another layer of complexity to using odour for phylloxera detection.
- A number of volatile compounds that vary in concentrations in the presence of phylloxera were identified offering the potential to customizing e-nose sensors to be more finely tuned to the chemicals.

Background

Insect feeding can induce characteristic changes in plant odours via the activation of direct and indirect plant defense pathways (Erb and Reymond 2019). These changes in volatile emissions may be localized to the site of feeding but can also be systemically expressed in other parts of the plant. Here, leaf and root volatiles of two varieties of grapevines (Shiraz and Chardonnay) of differing rootstocks (1103P, Ruggeri 140 and *Vitis vinifera*) were analysed by GC-MS with the aim of investigating whether "key volatiles" held potential use for the detection of different strains of phylloxera. The odour profiles of uninfested (phylloxera-free) plants were compared to those of phylloxera-infested plants. Qualitative and quantitative changes in volatile emissions were examined for volatiles that were characteristic of the presence of phylloxera and could be used to customize the sensors of a portable e-nose device.

Volatiles sampling and chemical analysis

The GC-MS analysis carried out in this study was a follow up to the e-nose study (see PART A above), using the same vines / treatment groups, collecting leaf and root volatiles at 2 and 8 weeks after infestation with phylloxera once the e-nose sampling was completed. Leaf volatiles were collected by dynamic headspace sampling; a method which consists of sequestering the odours in a container and applying an air stream to entrain them through an odour trap (adsorbent filter acting as a molecular sieve trapping small organic volatile molecules). Following sampling, volatiles trapped on the filters are eluted by flushing the adsorbent phase with a small volume of organic solvent. The obtained extracts were then used for chemical analysis by Gas Chromatography coupled with Mass Spectrometry (GC-MS). Leaf volatiles collections were carried out on branchlets comprising 3-4 fully expanded leaves enveloped in odourless oven bags for 16-18 hours (see Figure 8A). More details about the sampling method and chemical analysis of leaf volatiles are provided in the appendix (Appendix 2).

In early pilot experiments, volatiles collections from live roots, either via the introduction of adsorbent filters in the soil inside pots (using a Teflon tubing extension probe), or with excised roots placed inside small glass collection vessels, were attempted using a similar approach. However, no exploitable chromatographic data could be obtained due to excessive soil background odours obscuring lower concentrations of root volatiles. As an alternative, a more commonly employed method involving root pulverization as described earlier (solid phase microextraction (SPME) was used (Hiltpod et al. 2011; Lawo et al. 2011; Velásquez et al. 2020) (Figure 8, detailed methods in Appendix 2).

The chemical profiles of leaf and root odour were visualized using ordination plots obtained from multivariate analysis (NMDS). Multivariate tests (ANOSIM) were employed to determine the significance of the statistical dissimilarly between profiles and the degree of overlap between different odour profiles. SIMPER (Similarity Percentage analysis) was used to determine the contribution of different volatiles to the dissimilarity between odour profiles. Full description of data analysis is presented in Appendix 2.

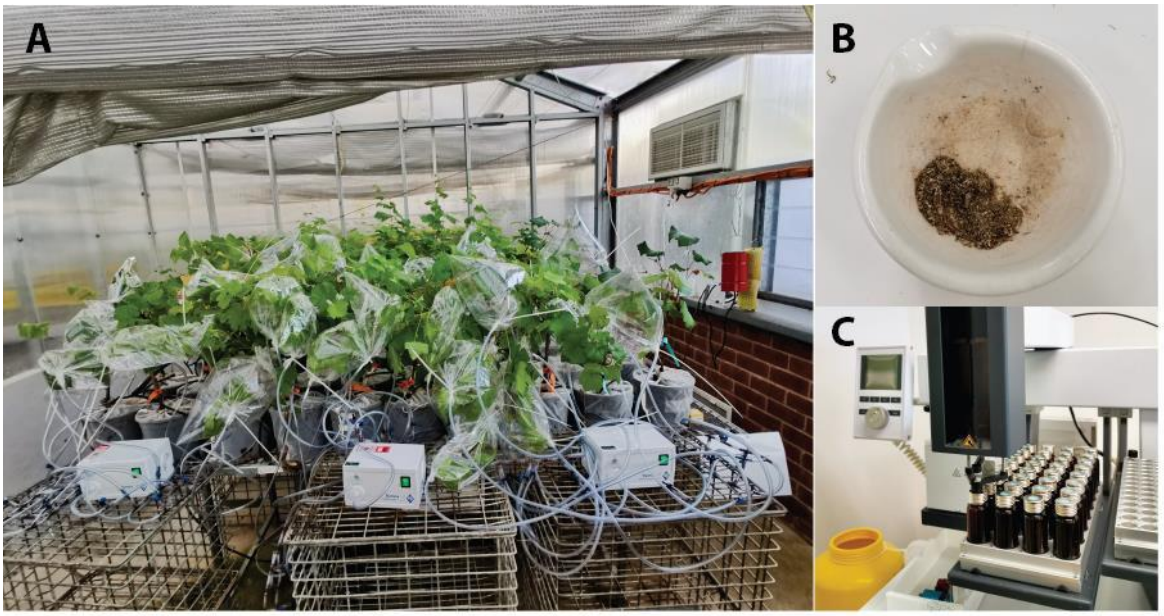


Figure 8 Photographs of A) the push-pull dynamic headspace sampling system used to collect volatiles from leaves enclosed in oven bags, using laboratory portable pumps; B) Pulverised root material used

for e-nose and SPME-GC-MS analysis; C) GC-autosampler equipped with a SPME fibre processing root samples placed in amber SPME amber vials.

Results and discussion

Significant grouping was observed in early and late samples of roots and leaves, reflecting the natural changes in odour profiles during the course of plant maturation. Therefore, to focus on the phylloxera-induced changes in chemical profiles, early and late samples were analysed separately.

Leaf and root odour profiles of grapevines from different rootstocks

The differences between odour profiles from aerial and subterranean plant modules were examined in different grapevines used in these experiments. As expected, significant differences were found in the odours of underground parts (Figure 9c-d) due to the use of different rootstocks. By contrast, aerial parts exhibited greater odour similarities (Figure 9a-b) though the influence of rootstocks on leaf volatiles emissions among plants of Shiraz vines was visible at later stages between *Vitis vinifera* (susceptible) and the rootstocks 1103P (resistant, ANOSIM $p = 0.001$, $r = 0.26$) and Ruggeri 140 (tolerant, ANOSIM $p = 0.005$, $r = 0.19$), regardless of their infestation status (Figure 9b). Leaf volatiles of the variety Chardonnay formed a different cluster from their Shiraz counterparts (pairwise ANOSIM $p < 0.001$, $r > 0.71$).

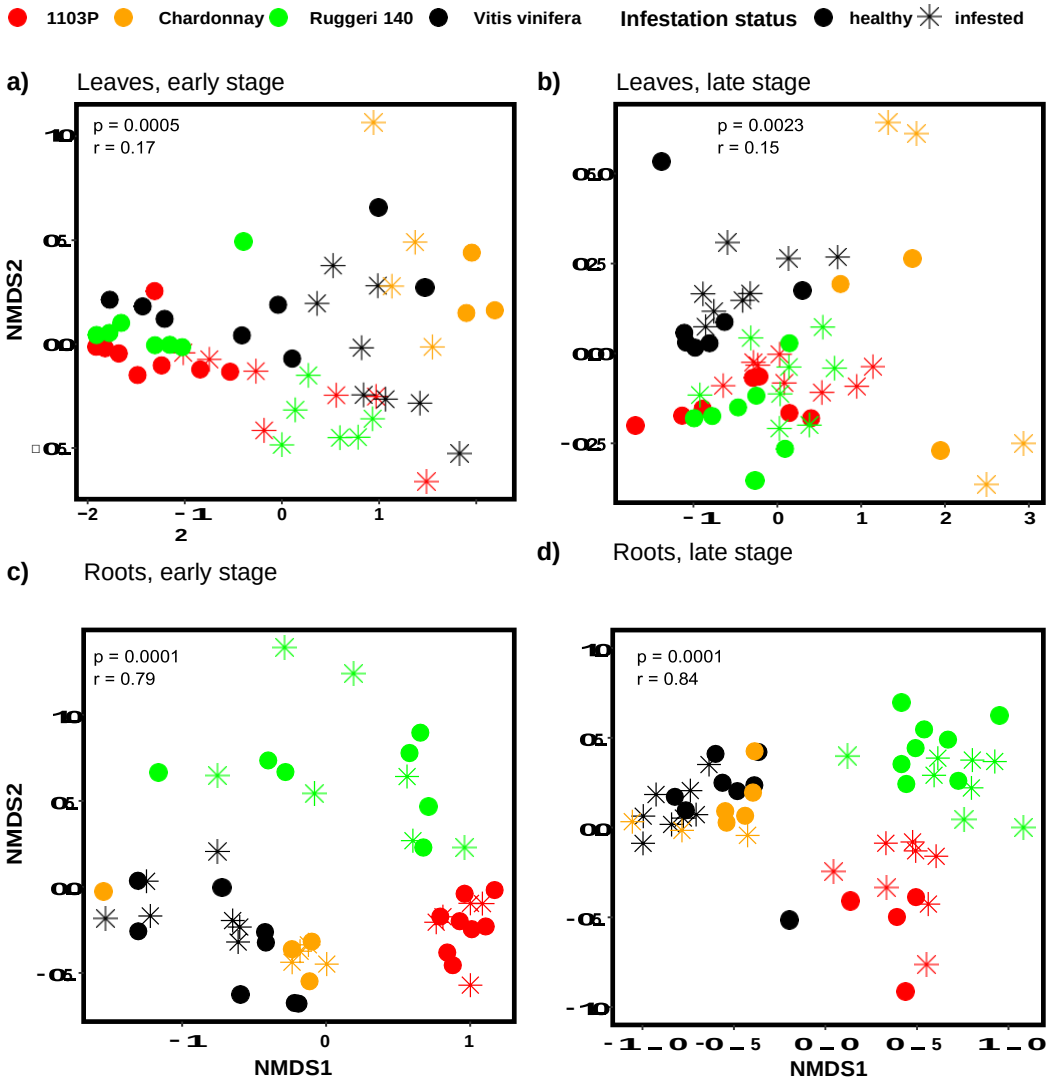


Figure 9 Spatial representation of the leaf odour profiles of individual plants: a) The clustering of points (healthy plants) and stars (infested plants) at early infestation stage depict a marked dissimilarity between the odours of infested and healthy plants. However, the absence of clear clustering among stars (infested plants) of different colours (rootstocks) suggest that this dissimilarity is most likely linked to variations in sampling conditions and day rather than to phylloxera infestation. b) This effect was not as clear at late infestation stage. *Vitis vinifera* of the variety Shiraz (black) and Chardonnay (orange) have the most distinct profile as opposed to Ruggeri 140 (green) and 1103P (red) which appear very similar (no grouping); c-d) The root volatiles profiles of different root stocks were very distinct (clear clustering) at early and late stages of infestation. The profiles of the *Vitis vinifera* rootstocks of the varieties Shiraz (black) and Chardonnay (orange) were similar (no grouping).

Points indicate "healthy" phylloxera-free plants and stars plants infested with G4 phylloxera strain. ANOSIM p values < 0.05 indicate significant differences between rootstock odour profiles (regardless of infestation status), and r values indicate the degree of overlap between rootstock odour profiles (r < 0.25: slight difference with a lot of overlap; 0.25 < r < 0.5: different with overlap; 0.5 < r < 0.75: different with little overlap and r > 0.75: highly different with no overlap).

Influence of phylloxera infestation on leaf volatiles

Marked differences were observed in leaf odours as a result of infestation with phylloxera G4 strain at early stage (Figure 10). These differences were more pronounced in Ruggeri 140 grapevines (Figure 10b) than in 1103P and *Vitis vinifera* rootstocks (Figure 10a & c). By contrast, G19-infested plants did not appear to group consistently with either G4-infested or healthy plants of all rootstocks suggesting that G19 influence leaf volatiles in different ways from the G4 strain. Interestingly, volatile emission rates from all rootstocks were considerably lower in infested plants compared to healthy plants. Due to the need to prevent phylloxera from contaminating glasshouses where healthy plants were grown, collections from healthy and infested plants were carried out on different days and in different glasshouses. Hence, ambient conditions during sampling are the likely explanations for such quantitative differences. In addition, the similarity between the odour profiles of infested plants of all rootstocks obtained from both e-nose and GC-MS data corroborate that **differences observed between the two treatments are more likely linked to environmental conditions on the day of sampling than to the presence of phylloxera** (Figure 10a).

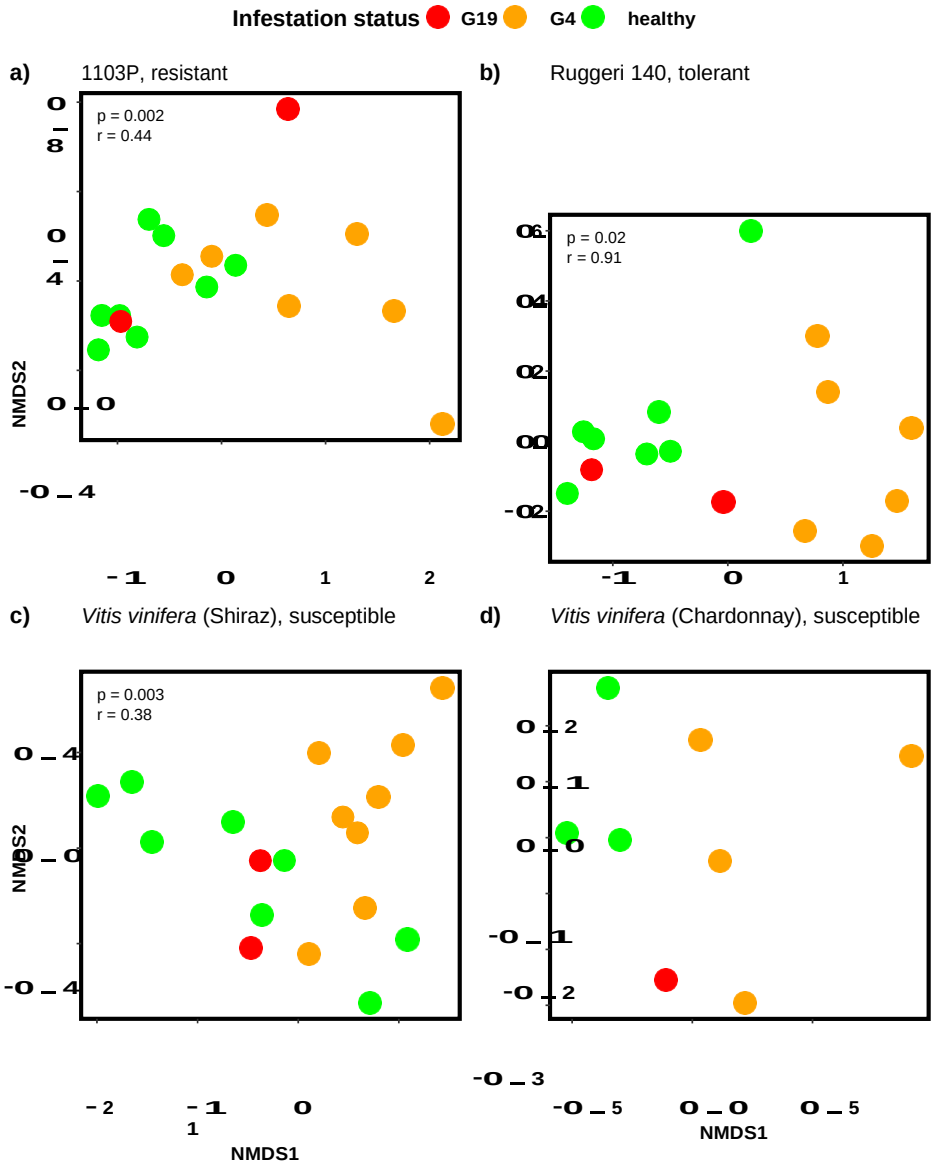


Figure 10 Visible clustering of green (healthy) and (range (G4 infested) points indicate strong dissimilarities between the leaf odour profiles of infested and phylloxera -free plants in all rootstocks 6 weeks post infestation (early stage). The red points (G19 infested) group with the green ones (healthy plants) suggesting that their odour profile is more similar to that of healthy plants. G19 plants were kept in the same glasshouse as that of G4-infested plants but in a different sub-chamber where conditions were comparable to those of the glasshouse in which healthy plants were kept. p values indicate the statistical significance of the dissimilarity between G4 infested and healthy plants and r values the degree of overlap between profiles (increasing r values indicate greater resolution between profiles).

SIMPER analysis using both compound release rates (Appendix 2; Table 1) and relative amounts indicate that the greatest part of the dissimilarity is linked to the same compounds across all rootstocks; namely, α-farnesene, α-ocimene, (Z)-3-hexenyl acetate, indole, methyl salicylate and other compounds **commonly associated with plant's response to biotic and abiotic stresses.** (Z)-3-hexenyl acetate was the only compounds of which the concentration remained constant or increased among infested and phylloxera-free plants.

GC-MS analysis of **samples collected at the later stage** (8 weeks after phylloxera infestation) were carried out on a cooler day and under overcast weather conditions, and **failed to show clear differences between phylloxera-infested and uninfested plant odours with all rootstocks** (Figure 11) confirming that odour profile changes observed at earlier stage were most likely linked to ambient conditions rather than to the presence of phylloxera.

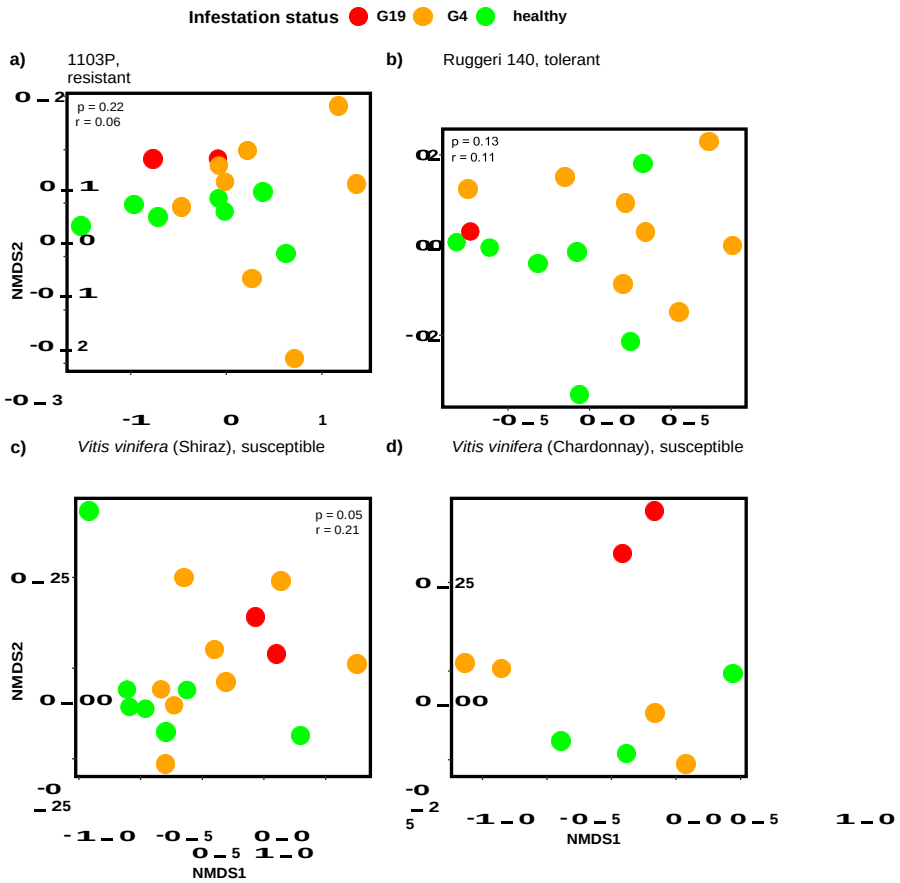


Figure 11 Strong overlap between points representing vines leaf volatiles profiles of different rootstocks indicate the absence of clear differences in the odours emanating from infested and phylloxera-free plants at 8 weeks post infestation (late stage). p values indicate the statistical significance of the dissimilarity between G4 infested and healthy plants and r values the degree of overlap between profiles (increasing r values indicate greater resolution between profiles).

Influence of phylloxera infestation on root volatiles

In contrast to e-nose results, there were **no apparent differences between the root volatiles profiles** of infested and healthy 1103P, Ruggeri 140 and *Vitis vinifera* from the Chardonnay variety, **at early stage of infection** (Figure 12a-b&d). Although differences were observed with *Vitis vinifera* (Shiraz), the chemical profiles of infested and healthy plants were not well-resolved (Figure 12c). Again, G19 infested plants did not group clearly with either G4 infested or healthy plant odour profiles. In fact, G19 infested plants appeared more similar to healthy *Vitis vinifera* (Shiraz) than to G4-infested ones.

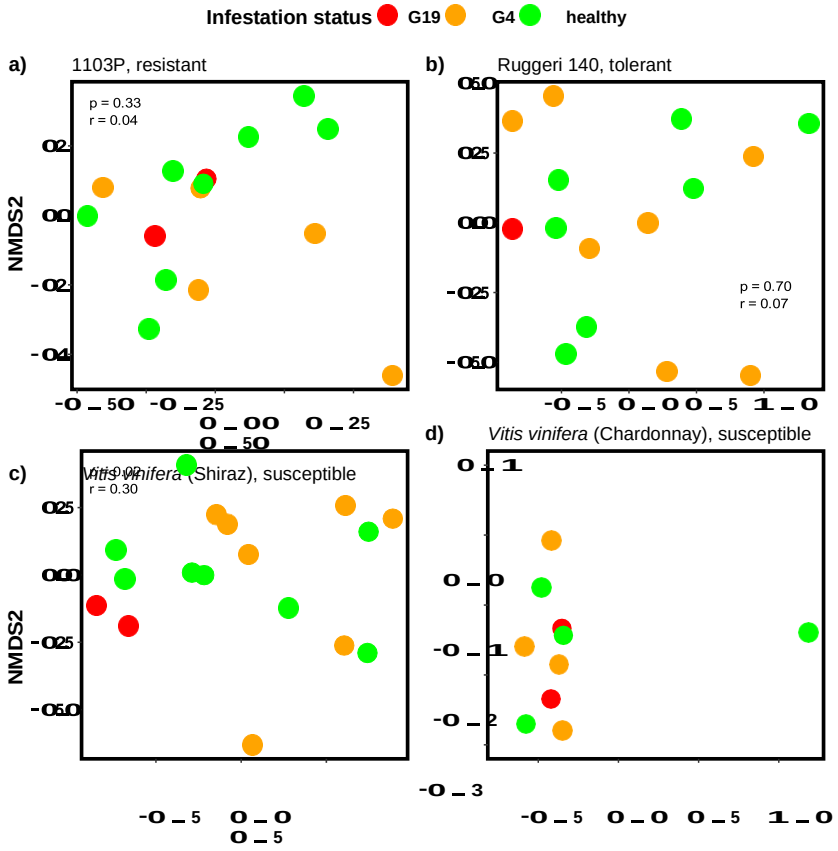


Figure 12 Ordination plots indicate no clear clustering between infested (orange: G4 and red: G19) and healthy plants (green) root odours at early infestation stage (2 weeks after infestation). p values indicate the statistical significance of the dissimilarity between G4 infested and healthy plants and r values the degree of overlap between profiles (increasing r values indicate greater resolution between profiles).

Root volatiles profiles were more discrete at later infestation stage. The separation between infested and healthy plants was visible with all, including the resistant rootstock (1103P) (Figure 13). G19 infested root odours appeared to group with those G4 infested Ruggeri 140 and *Vitis vinifera* (Shiraz) but seemed to form a different cluster in 1103P.

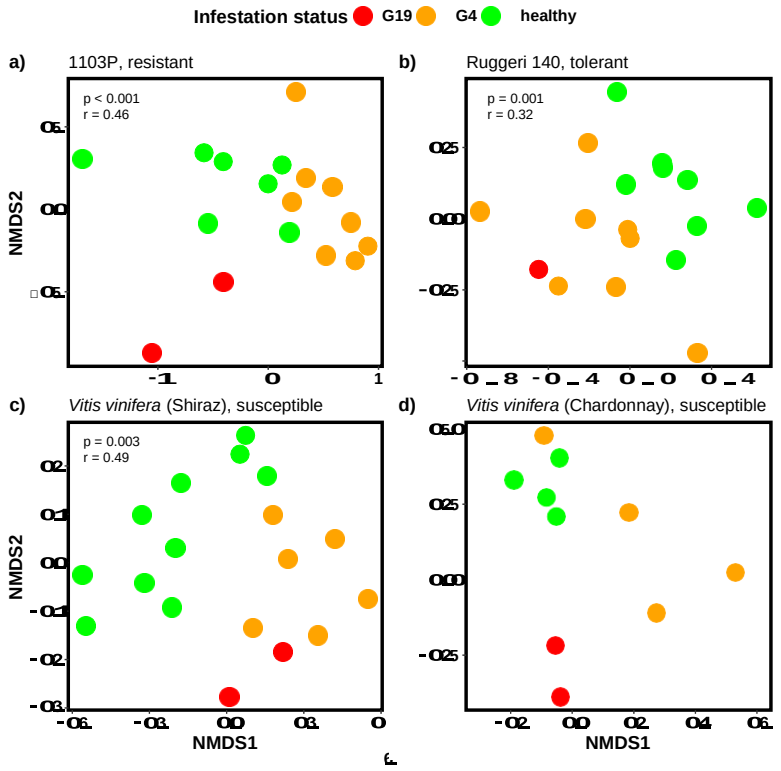


Figure 13 Separate clusters formed by green (healthy) and organge-red (G4 and G19) points indicate clear differences between the root volatiles of different rootstock 8 weeks post-infestation (late stage) p values indicate the statistical significance of the dissimilarity between G4 infested and healthy plants and r values the degree of overlap between profiles (increasing r values indicate greater resolution between profiles).

The high degree of communality of the compounds accounting for the dissimilarity between healthy and infested plants across rootstocks suggests that phylloxera infestation induces subtle quantitative changes in root compound ratios rather than the emergence of distinct plant metabolites. However, the trajectory followed by these varying compounds (i.e. increase or decrease) appear to differ depending on rootstocks. For example, the apparent decrease of benzaldehyde concentrations in infested 1103P and Vitis vinifera, contrasts with the increase of the same compound in infested Ruggeri 140. Conversely, concentrations of another predominant root volatile, myrtenol, increased in the presence of phylloxera in the 1103P rootstock odour but decreased in that of Ruggeri 140. Infested *V. vinifera* differed from other rootstocks via increased emissions of phenylethyl alcohol, benzyl alcohol, geraniol, cuminyl alcohol (p-cymen-7-ol), β-myrcene and another unidentified compound (tentatively, exo-Norbornyl alcohol, Appendix 2; Table 2). Importantly, all rootstocks exposed to phylloxera exhibited increased emissions of methyl salicylate – a compound associated with the salicylic acid defense pathway typically induced by most phloem-feeding hemipterans such as aphids, psyllids, whiteflies and scale insects. These results agree with previously published work carried out with other *Vitis* rootstocks in response to phylloxera infestation (Lawo et al. 2011). Despite feeding on parenchyma cells and not vascular tissues like other hemipterans, not only have phylloxera shown to induce the salicylic acid pathway, but it uses it in a way that suppresses jasmonic acid plant defenses to which it is highly susceptible (Eitle et al. 2019; Savoi et al. 2020). A similar example of manipulation of crosstalks between plant defense signaling pathways has also been demonstrated with mealybugs (Zhang et al. 2015). Interestingly, methyl salicylate concentrations were found to increase in all

rootstocks, including the resistant one (1103P).

Conclusion and Recommendations: E-nose vs GC-MS analysis

The results from both e-nose testing and GC-MS analysis suggest that leaf volatile analysis does not provide information that could be used for the detection of phylloxera. These two technologies for odour analysis were, however, successful at identifying changes in odours associated with root infestation in vines from different rootstock. Intriguingly, the e-nose was able to differentiate between root odours of infested and phylloxera-free plants at early stage (2 weeks after infestation) while SPME-GC-MS analysis failed to find clear differences. Conversely, the e-nose did not seem capable of discriminating between the odours of the different treatments at later stage in spite of apparent dissimilarity in root odours revealed by GC-MS analysis. No clear explanation for the apparent discrepancies between GC-MS and e-nose results is evident. On the one hand, GC-MS data clearly indicate that the chemical variations induced by the presence of phylloxera on the plants are subtle and require a careful examination of chromatograms, as well as the use of suitable statistical tools to become discernible. On the other hand, the e-nose exhibits great sensitivity to odours but also to sampling conditions. In fact, frequent recalibration (training) is recommended by the e-nose manufacturer to overcome problems caused by variations in ambient conditions that are likely responsible of recurrent “sensor drift” issues. In addition, high cross-validation scores achieved after e-nose training do not always guarantee great performances in odour classification in validation tests.

Altogether, the lack of chemical resolution between infested and phylloxera-free plant material combined with interferences inherent to the e-nose technology render phylloxera detection unachievable using the e-nose. Hence, in spite of being able to perform equally well as classic chemical analysis methods under controlled conditions, the technology did not prove advanced enough to be used as an on-site detection tool for the detection of phylloxera.

Overall, the work carried out suggests that the use of root volatiles, whether it is using an e-nose or classic laboratory-based chemical analysis for the detection of phylloxera, is at best suboptimal and made extremely unpractical by the logistics, time and labour required by the preparation of root samples for volatiles sampling. The development of sensors specific to compounds specific or influenced by phylloxera attack (e.g. methyl salicylate) could certainly contribute to overcome current limitations linked to soil contaminants, ambient odour background noise and possibly sensor drift. Ideally, the e-nose could be calibrated using standard solutions and perform quantitative measurements of the target chemicals. Perhaps, under these conditions would it be able to detect the presence of phylloxera without the need to pulverize the roots? In any case, since phylloxera-induced changes in root volatiles emissions are mostly quantitative, it appears likely that standardized conditions will be needed for the device to perform.

PART C: A SURVEY AND ANALYSIS ON FEASIBILITY OF USING SNIFFER DOGS FOR IN-FIELD DETECTION

Summary

To better understand the views of vignerons, biosecurity officers and researchers regarding the use of sniffer dogs in phylloxera detection, we designed an online survey in which participants were asked eight multiple choice questions addressing practicality, feasibility, priority, and costs. Out of 54 respondents, only 29% thought that sniffer dogs would likely be adopted for phylloxera detection, and 73% of respondents felt there were biosecurity concerns in using sniffer dogs. The outcome of this study triggered a stop-go milestone and led to a variation removing further sniffer dog research from the project.

Background

Odour-sensing tools (sniffer-dogs and electronic noses) have shown promise in other fields of detection, for example in animal and plant diseases, food spoilage, airport surveillance, explosives and in emergency response programs. For insect pests, studies have shown the capability of detection dogs to locate insects of quarantine importance such as red imported fire ants (Brooks, et al. 2003), bed bugs (Pfiester, et al. 2008) and the gypsy moth (Wallner and L. 1976). Early detection would greatly improve our ability to contain phylloxera, manage it effectively with quarantine and reduce the cost of replanting through targeted strain-specific rootstock selection. With their renowned senses of smell and proven ability to sniff out insect pests, could sniffer dogs be used to detect phylloxera?

A pilot study was conducted at Agriculture Victoria in 2017 to investigate this question (Project DED 1301: Risks and Management of Endemic and Exotic Phylloxera), having been approved by the Agricultural Research and Extension Animal Ethics Committee of Agriculture Victoria (AEC 2017-02). Two dogs were used in olfactory learning trials that required scent discrimination to find phylloxera targets. The dogs were exposed to samples of live phylloxera presented in Petri dishes and in potted plants, and then rewarded with either play, food or a combination of both, following a positive identification. Midway through the trial, one of the dogs was removed as she lost motivation and struggled with scent discrimination in a new environment. At the end of the trial, the remaining dog showed some evidence for recognising phylloxera samples at all life stages (i.e. eggs, intermediates and adults) in Petri dishes and on excised own rooted vines (*V. vinifera*).

The trial was certainly encouraging, but the methods used were limited in terms of understanding whether the dogs could identify phylloxera specifically. Since this initial 2017 study, questions regarding the practicality and safety of using sniffer dogs in vineyards were raised by industry. Sniffer dogs are expensive to train and maintain, and moreover there are biosecurity issues related to sniffer dog use on infested properties. To explore these questions further, we designed a survey aimed at capturing the views of growers, biosecurity officers and researchers.

Methods

An online survey was conducted using the web-based platform Survey Monkey. The survey contained eight multiple-choice questions regarding the use of sniffer dogs in vineyards and included an opportunity for participants to rank sniffer dog studies against other research priorities. The survey was emailed to 97 individuals across eight wine regions in Victoria. Out of 54 responses received, 77% of the

respondents were directly working in the wine industry (i.e. growers, wine makers, vineyard managers), 13% were researchers and 10% were biosecurity officers.

Results and discussion

When given a range of scenarios where sniffer dogs might be most useful for phylloxera detection, 43% of the respondents felt sniffer dogs would be useful for early detection of phylloxera across a range of activities (early detection in vineyards, inspecting machinery / personnel coming from other vineyards, monitoring spread, checking for presence or absence on roots before planting) (Figure 1). 10% of the respondents did not think sniffer dogs were of benefit for the four detection options provided. Respondents noted that dogs are a high risk of transferring phylloxera to other vineyards and should not be used.

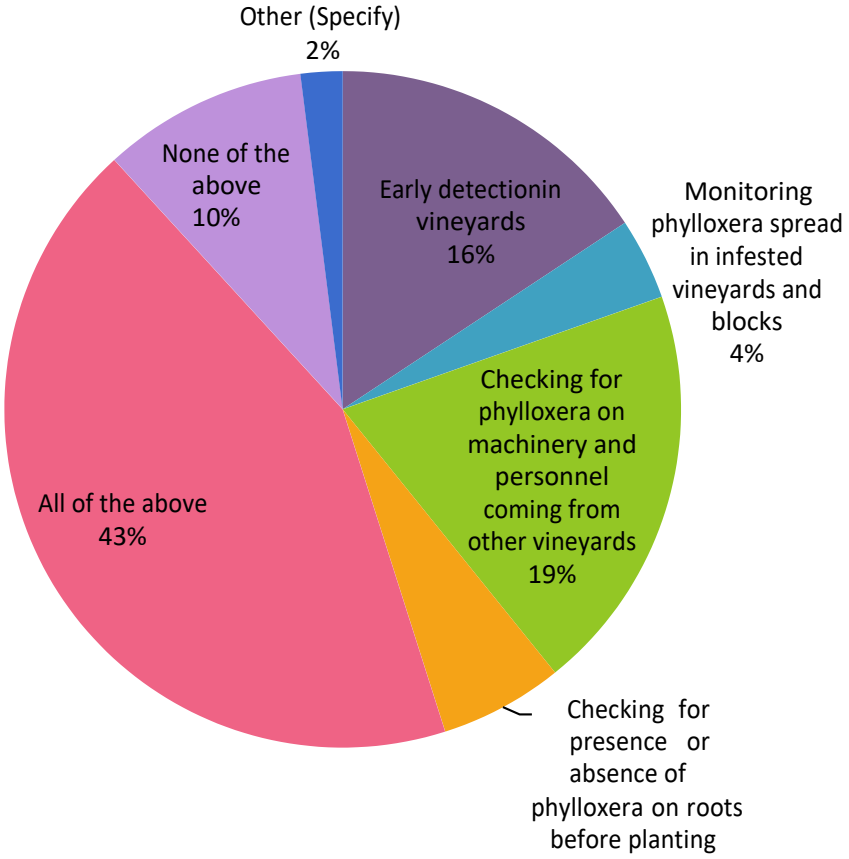


Figure 1 Scenarios for possible application of sniffer dogs in phylloxera detection posed to participants as "If sniffer dogs were to be used for phylloxera detection, when do you think they would be most useful?"

Participants were asked which phylloxera detection methods they thought will be adopted by growers and state government biosecurity teams in the next ten years, with the options: emergence trapping (bucket traps), root inspection (digging), DNA probe, sniffer dogs and remote surveillance using visual imaging technologies (e.g. drones, aircraft, satellites). This question was presented separately with a distinction between growers and the government to eliminate the expectation that either party would be providing the dogs. Thirty percent of the respondents indicated sniffer dogs were least likely to be adopted for detection by growers, and 40% by state government biosecurity teams in the future. By comparison, the majority of respondents (71% growers and 92% government) thought remote

surveillance using visual imaging technologies (e.g. drones, aircraft, satellites) for detection of infested vines was most likely to be adopted as a phylloxera detection method.

Participants were asked what they would be most concerned about when using sniffer dogs as a detection tool, with the options of: contamination, handlers having direct or indirect effects on dog alerts, disinfestation of dog and handler between blocks and vineyards, having other dogs on the property, dog welfare and cost of training and dog upkeep. Their responses highlighted a general concern that dogs could act as vectors of phylloxera and further increase quarantine risk surrounding disinfestation of dog and handler and increase policing for biosecurity personnel (73% of survey respondents). There were also concerns regarding the cost of training and upkeep of dogs (70% of survey respondents). Twenty-seven percent of respondents were concerned with having other dogs on the property, while only 12% of respondents had concerns about dog welfare. Only 25% of respondents were concerned about handlers influencing dog alerts.

When asked who should be responsible for the dogs, the majority of participants (81%) thought biosecurity teams should be the preferred responsible authorities, as opposed to researchers (41%), growers / contractors (38%), or winemakers (19%).

When asked where sniffer dogs rank against other research priorities, participants ranked research priorities as follows (Table 1):

1. Developing visual imaging (e.g. drones, aircraft, satellites) and e-sensing (E-Nose) technologies for early detection.
2. Advancing an understanding of phylloxera biology and ecology in relation to phylloxera genotypes, soil types and climate change
3. Bettering quarantine using novel disinfestation procedures
4. Screening rootstocks for genotype-specific resistance and mapping phylloxera strain distribution
5. Sniffer dogs for early detection
6. Use of biocontrol and new insecticide technology

Note: this question unfortunately placed biocontrol in the same category as “new insecticide”, the two representing widely contrasting practices; thus care should be taken in interpreting this rank.

Table 1 Survey on overall rank and score of phylloxera research priorities posed to participants as "For phylloxera management, which of the following research topics do you see as most important to you? Please rank the topics in order of importance from 1 to 6 (where 1 is most important and 6 is least important)".

Research theme	Overal Rank	Score	Total Respondents
Developing visual imaging (e.g. drones, aircraft, satellites) and e-sensing (E-Nose) technologies for early detection of grape phylloxera.	1	161	37
Developing an understanding of phylloxera biology and ecology in relation to genotypes, soil types and climate change	2	130	36
Quarantine; research into novel disinfestation procedures e.g. new products for disinfestation of footbaths	3	125	36
Screening rootstocks for genotype - specific resistance and mapping phylloxera genotypes distribution across infested zones	4	121	36
Sniffer dogs research for early detection of grape phylloxera	5	118	37
Eradication of phylloxera e.g Biocontrol and new insecticide technology	6	109	35

The main concerns for sniffer dogs in phylloxera detection were contamination and costs associated to research and dog upkeep. Dogs would have to be managed (upkeep, training, retraining, validating, operating) to ensure efficiency in early detection of phylloxera, and this would require protocols dealing with disinfestation and additional compliance procedures. Further research to validate the use of dogs in phylloxera detection would be costly, requiring more rigorous study protocols that involve larger sample sizes of dogs, and experimental design that enables researchers to determine factors such as percentage failure rate in trained dogs, timespan of learning, error rate in detection, specificity of detection (e.g. phylloxera versus other insects versus root damage), field validity (detection in vineyards, detection underground) to authenticate the results. Few respondents were concerned with handlers having direct or indirect effects on dog alerts, but research has shown that humans greatly impact the success of target detection by dogs (Lit, et al. 2011): in vineyard settings, human signals and new target odours as a result of phylloxera activity below and above ground at different times of the year are likely to sway dog alerts (Coombe 2016).

Conclusions and recommendation

The survey revealed a lack of confidence from industry and biosecurity in the use of sniffer dogs as a potential detection tool, mainly from the perspectives of practicality (contamination / biosecurity) and cost (upkeep and research). As a result of this survey, and a “stop-go” milestone within the current project, a variation in the project was proposed by the project team and approved by Wine Australia in 2020. This variation enabled research on sniffer dogs within DED1701 to be replaced by research to field- validate the new LAMP in-field molecular diagnostic tool (Section 1.3). The DNA-based technology using LAMP is accurate and quick in returning results and, applicable in field settings without requiring extensive training.

Remote surveillance scored highly as a research focus. The potential application of unmanned aerial vehicles (drones) for phylloxera detection has been evaluated in a separate Agriculture Victoria study and report (Agriculture Victoria Horticulture Development Plan 2017-2020, Agriculture Infrastructure and Jobs Fund). Research developing and evaluating remote surveillance at scale (aircraft, satellite) is ongoing.

9.2. LAMP- A NEW TOOL FOR RAPID IN-FIELD DETECTION OF PHYLLOXERA

Summary

LAMP is a portable molecular technology that Agriculture Victoria has recently developed for rapid and accurate diagnostics of phylloxera. The current study focused on validating and applying LAMP for in-field use. Surveillance trapping was conducted using "bucket traps" and trapped insects (including phylloxera) were processed as "crude" whole specimens, which were contaminated by plant tissue and soil. Our field- optimised methods clearly demonstrated the success of LAMP for phylloxera ID. We further evaluated the LAMP surveillance approach for detecting phylloxera on root samples dug up from infested vineyards and tested in the field. Our methods successfully detected phylloxera in eighteen positive vine samples. LAMP appears accurate and sensitive enough to remove the requirement for human visual detection and could save considerable training and diagnostic time and effort. Further research should focus on incorporating LAMP protocols into larger-scale visual surveys, particularly during rezoning undertakings.

Background

Current surveillance practices for phylloxera rely on visual inspection of vine roots by trained entomologists, particularly of grapevines showing signs of weakness that may be due to the presence of the insect. When suspect phylloxera are detected using these methods, they are visually examined using a hand lens (National Vine Health Steering Committee, 2009), and suspect samples sent to Agriculture Victoria Research diagnostics to confirm identity using morphological and molecular tools, to accurately confirm (or reject) the identification as phylloxera (Bruce *et al.,* 2011, Herbert *et al.,* 2008). Getting a result on whether a vineyard is infested or not can take up to two weeks. More rapid and cost-effective identification methods for phylloxera would be of considerable benefit to biosecurity officers, consultants, and the grape and wine industry.

Surveillance for phylloxera involves a grid surveying approach where vineyard blocks are searched for the presence of weak spots (stunted, yellowing vines) that is an indicator of the below-ground presence of the pest. Upon identification of weak spots, roots at vines from the 5th panel in every 3rd row are sampled and collected for identification. Quarantine measures are then implemented at once and a PIZ established within a 5km radius, after a phylloxera detection is confirmed.

Molecular technologies are useful additional diagnostic tools for detecting grape phylloxera (e.g. Herbert *et al.* 2008). LAMP (loop-mediated isothermal amplification, (Notomi *et al.*, 2000) is a relatively new molecular technology that has been developed for rapid and accurate diagnostics (Li and Cai 2011; Parida *et al.* 2008). LAMP tests are accurate, targeted assays that can be conducted using portable devices for field-based (on-site) diagnostic testing (Yaseen *et al.* 2015) and are able to handle more crude extracts compared to conventional molecular techniques, such as conventional PCR. LAMP tests can detect target DNA in less than one hour and performing LAMP assays and interpretation of results is relatively easy after brief initial training (e.g. Blacket *et al.* 2020). Recently, a LAMP assay has been developed for the detection of phylloxera (Agarwal *et al.* 2020) which includes an optimised DNA extraction procedure, Hotshot "HS6" (Zieritz *et al.* 2018), for potential LAMP in-field use on phylloxera adults, crawlers, nymphs and eggs (Agarwal *et al.* 2020).

The aim of this component was to *evaluate whether the new phylloxera LAMP assay can be used to (i) identify phylloxera collected from bucket trapping in vineyards, and (ii) detect phylloxera on infested root samples collected in the field.*

Note: This study replaced the research component involving experimental work on sniffer dogs, through a stop-go milestone detailed in the original proposal, as detailed above.

Methods

Sample collection of phylloxera by bucket traps

Insect traps for grape phylloxera were placed in the vineyards as previously described (Benheim *et al.,* 2012). Emergence ("bucket") traps are made from 4 to 5 litre translucent round plastic containers (21cm width x 18cm depth) that have been wetted with water on the internal surface (Figure 1a; Further details in Appendix 3 Part A). A fine spray of water was added to containers which were placed upside down to create a seal that encourages a build-up of humidity (condensation). Metal tent pegs were used to secure the containers in place and prevent damage or displacement of the traps by vineyard machinery, vineyard personnel and animals. Crawlers and alates moving above ground enter the containers and are trapped in the condensation.

In the first field season (2018-2019), 25 bucket traps were placed in one vineyard (Vineyard-PR) and 26 traps in a second vineyard (Vineyard-SL). After 4 weeks, insects caught in these 51 traps (including phylloxera) were collected by rinsing the inside of trap with 80% ethanol and stored in plastic tubes at 4°C. In the second season (2019-2020), 150 traps were placed in both the Vineyard-PR and the Vineyard-SL vineyards (total 300 traps) and collected after 4 weeks (Figure 1b). The inside of trap was carefully rinsed with 100% ethanol to collect any insects including phylloxera and stored in the plastic tubes at 4°C. The use of 100% ethanol in the second field season was implemented as a measure to improve the preservation of DNA within the trapped insects, as lower ethanol concentrations have been shown to be adequate for morphological preservation of insects, but suboptimal for preserving DNA (Stein *et al.* 2013).

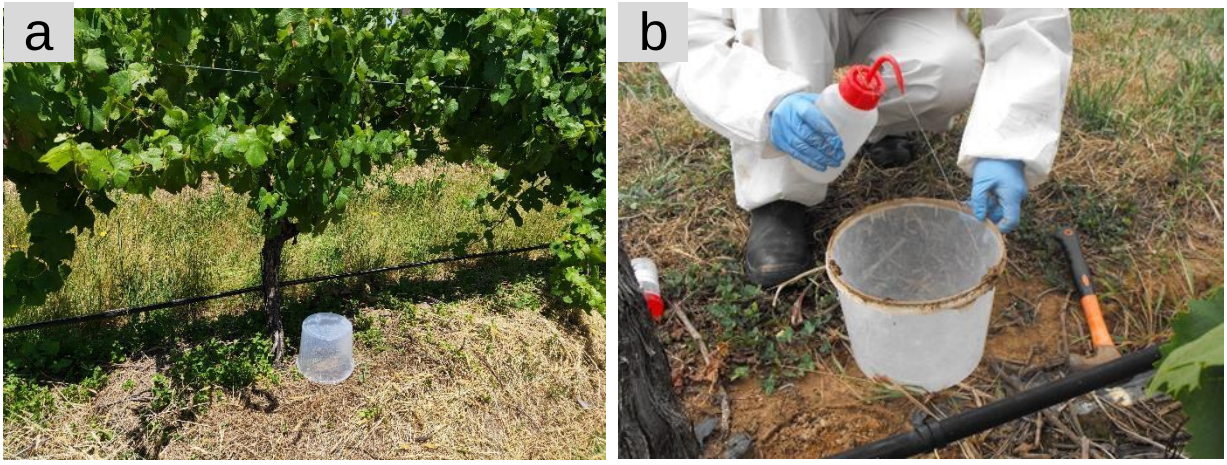


Figure 1 Collecting insect specimens from the bucket traps in the field. (a) Bucket traps containing a film of condensation (sprayed water), placed onto the soil surface on level ground using three metal pegs, and left in the field for 4 weeks; (b) Insects collected by rinsing the inner surface with ethanol to collect trapped insects in a plastic specimen container for later identification.

DNA extraction of phylloxera from ethanol preserved bucket trap samples

Bucket trap samples, collected in 80% (season 1) or 100% (season 2) ethanol, were filtered using a coffee filter placed in a funnel (Figure 2a-c). After washing samples using 100% ethanol in the laboratory, the filter and remaining residue (insects and soil) was removed from the funnel and air-dried for 5 minutes to evaporate any ethanol. The air-dried filter paper was then trimmed down to the section containing residue and placed in 2 mL microtubes (Figure 2d-e). 400 µl of HotShot buffer (containing 25 mM NaOH and T10E1 buffer) was added to the trimmed filter in the 2 mL microtube (Figure 2f). The tube was incubated at 95°C for 10 minutes (Figure 2g) followed by chilling on the ice for 1 minute, and then stored in a -20°C Freezer.

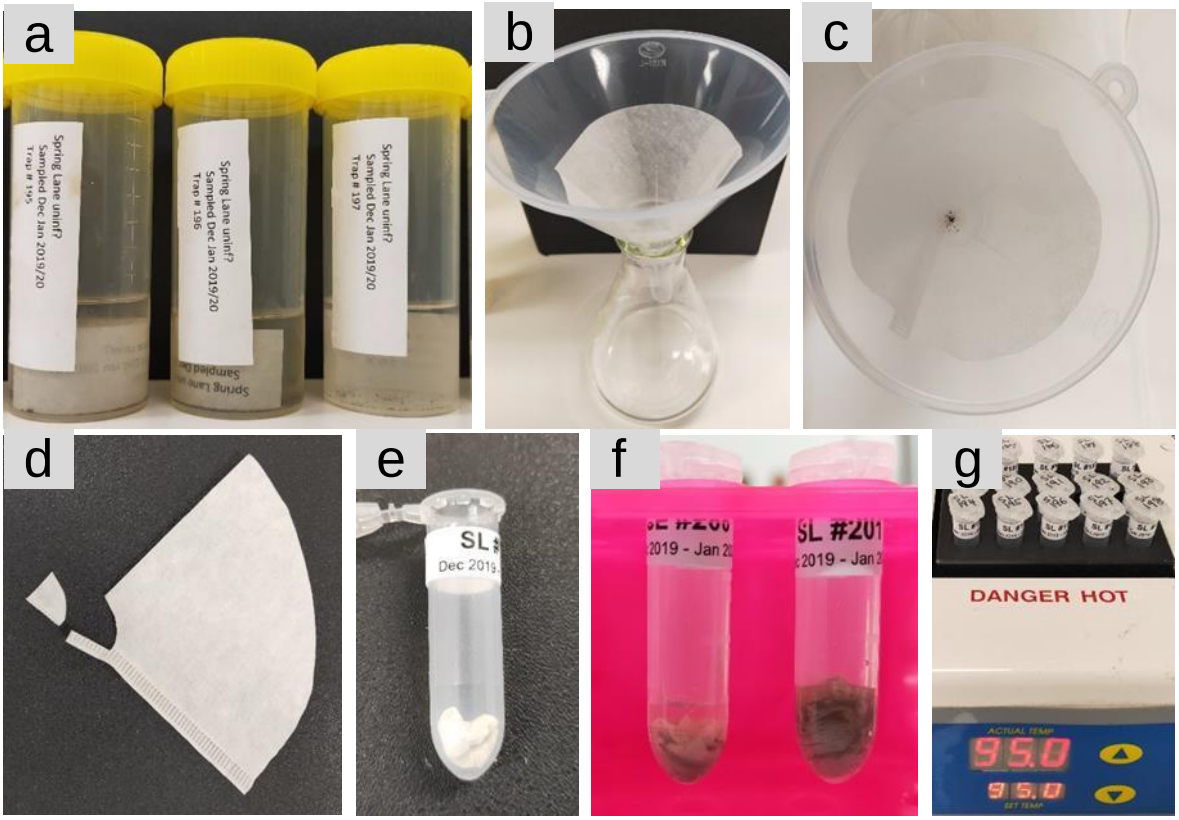


Figure 2 Collection and preparation of insect samples. (a) Insect samples collected from the bucket traps stored in 80% or 100% ethanol in plastic tubes. (b) Coffee filter placed in the funnel. (c) Insect samples collected in the residue at the bottom of the filter paper. (d) Cut section of the filter paper containing specimens. (e) Filter paper tip inside tube. (f) 400 ul of HotShot buffer added. (g) Tubes incubated at 95°C for 10 minutes, to extract DNA.

LAMP Assay

Each LAMP reaction was conducted in a total volume of 25 µL of reaction mix, following Agarwal *et al.* (2020). The LAMP reaction mix contained 15 µL of Isothermal Master Mix Iso-001 (OptiGene), 10 µL of primer master mix equivalent to 0.4 µM final concentration in reaction and 1 µL of template DNA. A positive control consisting of phylloxera genomic DNA and a no-template control were included in each run to test that the assay reagents worked well and for possible reagent contamination, respectively. To allow greater time for amplification from dilute DNA samples (see below) the LAMP assays were run at 65 °C for 35 minutes, rather than the standard 25 minutes used in Agarwal *et al.* (2020), in a portable real-time fluorometer (Genie III, OptiGene).

Evaluation of LAMP for root collected samples

Based on the LAMP results from the bucket trap detection data (see below), we randomly selected three plots which were found to be positive for phylloxera and one negative plot. We evaluated LAMP detection of phylloxera from roots in the field through harvesting small samples of fine roots, preferably with evidence of galls, by digging at the base of each vine and testing six replicate samples by LAMP in the field. To collect each sample, soil at the base of each plant was removed using a spade (Buchanan 1987, Bruce *et al.* 2011a). Exposed symptomatic root sections (with galls) were collected into six wells of an OptiGene LAMP reaction strip containing 100µl of HS6 HotShot buffer. DNA extractions of root samples were performed using the HotShot method using the Genie III (OptiGene) as an incubator in the field. During the Genie III run, the battery was charged from vehicles in the field. A series of water dilutions (10x, 25x and 100x) were made in the field from these HS6 extractions. All samples including undiluted and serial dilutions were examined by LAMP assay, as described above. After the LAMP assay, a subset of the samples was randomly selected for morphological confirmation of the presence of phylloxera by microscopy.

Results and Discussion

Detection of phylloxera in the bucket traps

In the first season (2018-19), samples were collected from 51 bucket traps in Vineyard-PR and Vineyard-SL (Figure 3). Phylloxera was detected from only a single trap (Appendix 4, Table 1).

In the second season (2019-20), a much larger number of bucket traps were deployed (Figure 4). DNA extracted from these samples was found to be positive for LAMP from 13 of the 150 traps from Vineyard-PR (Appendix 4, Table 2A), and from 3 of the 150 traps from Vineyard-SL (Appendix 4, Table 2B). The means ± s.e. of amplification time were 15.55 ± 1.23 and 13.66 ± 1.71 minutes in Vineyard-PR and Vineyard-SL, respectively, which are well within the expected LAMP amplification times for phylloxera positive samples (Agarwal *et al.* 2020).

After the LAMP assay, a subset of the samples was randomly selected for morphological confirmation of the presence of phylloxera by microscopy, with whole phylloxera still present for morphological examination post LAMP testing (Figure 5).

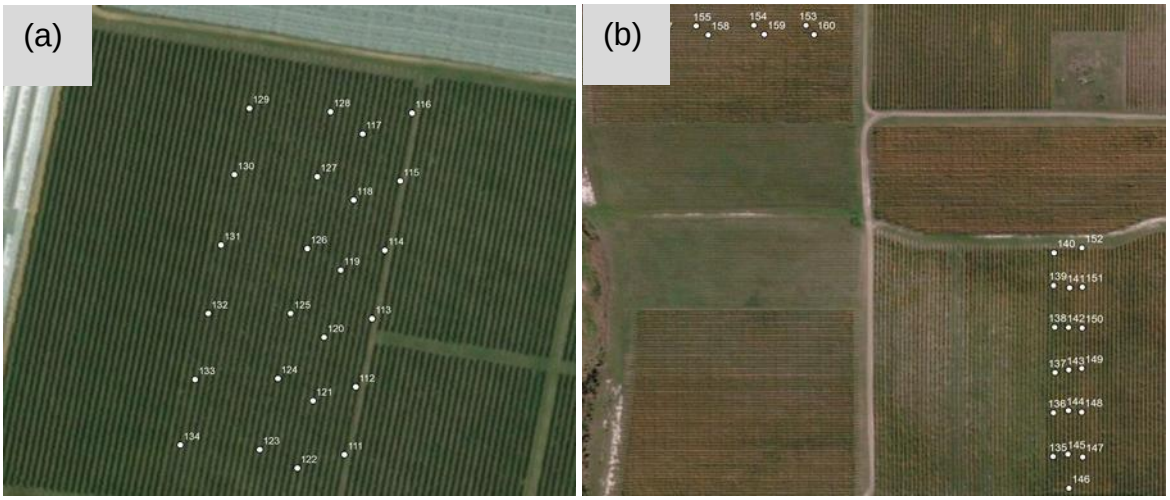


Figure 3 Vineyard with the location of bucket traps in 2018-2019. (a) 25 traps in Vineyard-PR; (b) 26 traps in Vineyard-SL.

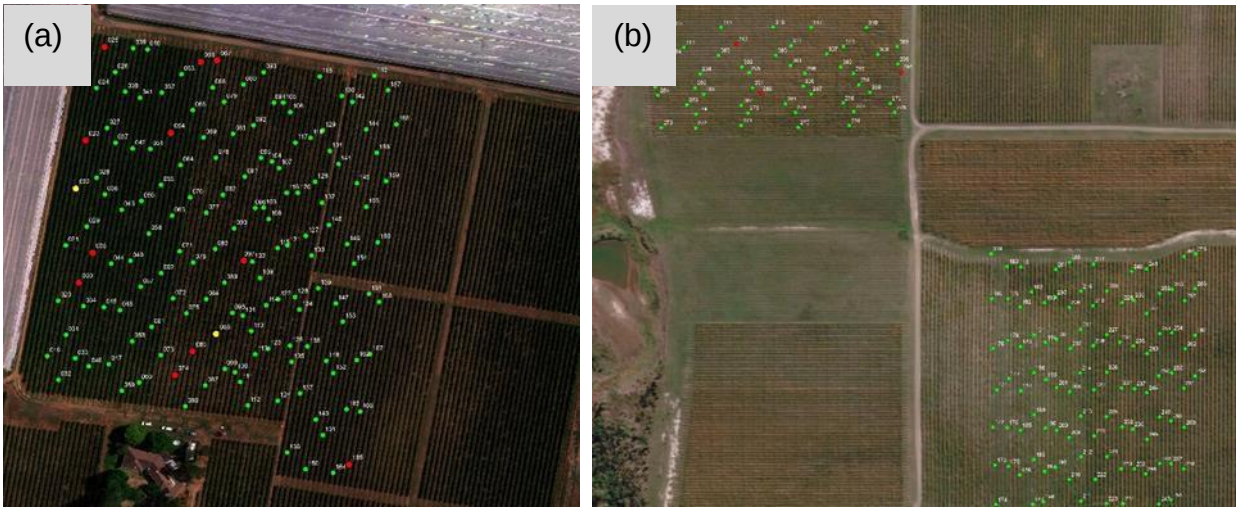


Figure 4 Vineyard with the location of bucket traps in 2019-2020. (a) Vineyard-PR; (b) Vineyard-SL. Green dots indicate bucket traps with no phylloxera detected by LAMP, red dots indicate detection of phylloxera by LAMP and yellow dots indicate weak signal of detection by LAMP (probable infestations).

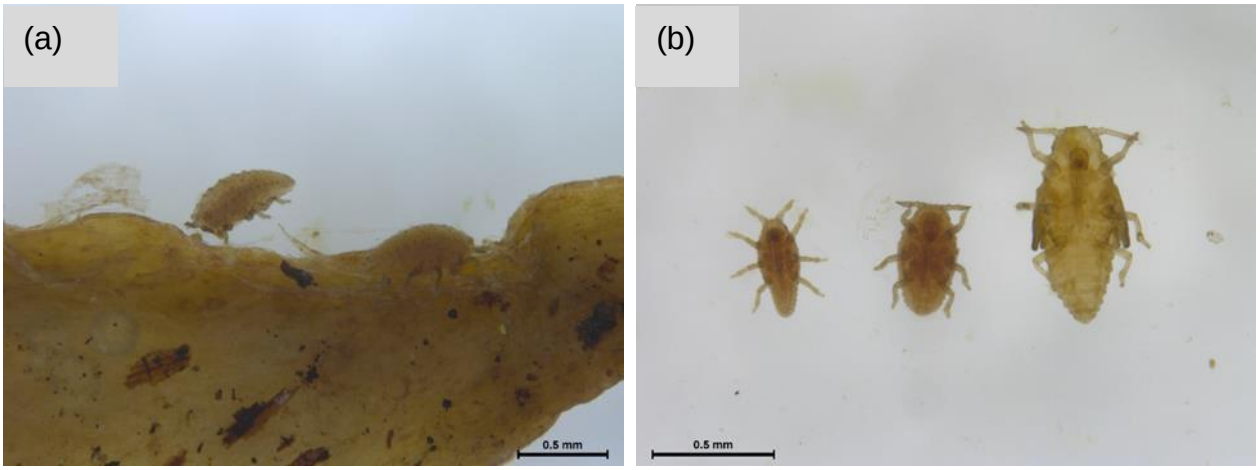


Figure 5 Image of intact phylloxera specimens following DNA extraction. (a) Phylloxera specimens remaining on the root tissue of the infested plant after DNA extraction using the HotshotHS6 method. (b) Nymphs and alate found in a sample after DNA extraction by the Hotshot HS6 method.

Validation of bucket trap data through direct collection of root samples

Six replicate root samples of three plants from phylloxera positive field plots, defined by bucket trap survey, were tested in the field by LAMP to determine if phylloxera was present on the roots (Figure 6). Positive responses for phylloxera DNA in the LAMP assays was found in most (>90%,) of the dilution samples, while they were produced a LAMP positive result in only 28% of the non-diluted samples (Table 1). The single suspected uninfested plant did not indicate any phylloxera amplification in any roots or diluted DNA assessed through LAMP (Table 1). Dilution likely reduces plant and soil contaminants which have are present in "crude" field extracted DNA samples. The optimal dilution of crude DNA extracts for use in the LAMP assay to detect phylloxera in plant or soil contaminated samples was defined in our study 25 x dilution (Table 1).

The major advantage of testing for phylloxera in suspect soil and root material samples using molecular methods is that this removes the requirement to detect and isolate the tiny insects themselves, allowing field surveys to instead focus on the far easier task of locating symptoms of phylloxera infestation, such as root nodules on grapevine plants, to be used as samples for phylloxera detection through LAMP testing.

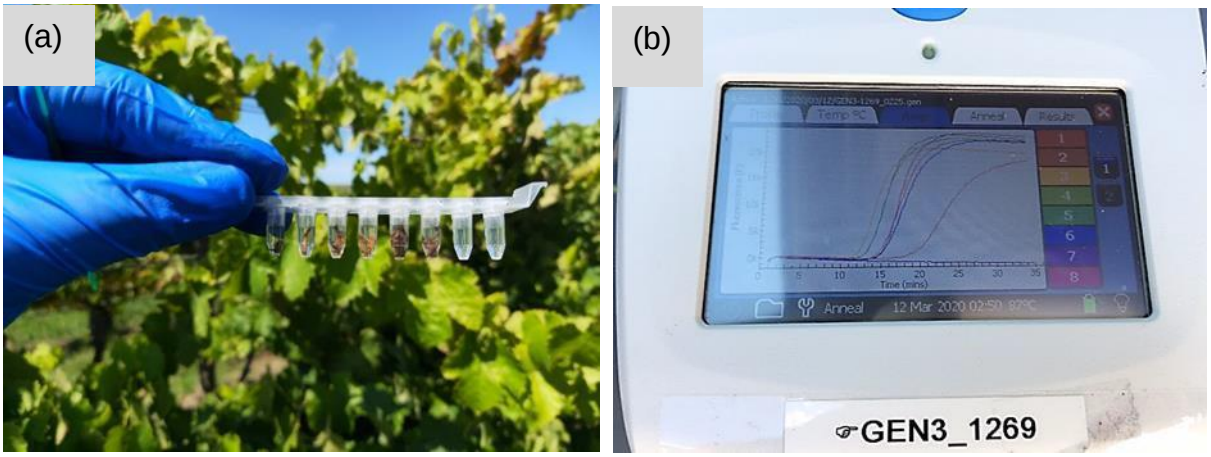


Figure 6 Field-base (on-site) LAMP assay. (a) Six root samples collected directly into 100 µl of HotShot extraction buffer. (b) Detection of LAMP amplification for phylloxera from DNA extracts in the field.

Table 1 Comparison of effectiveness of dilution ratio from field trials of three plants from phylloxera infested plots and one plant from uninfested plots.

Plant Number	Plot Number	Replicated Root	Amplification time (minutes) in dilution **			
			x 1	x 1/10	x 1/25	x 1/100
Plant #1	Plot #66	1	-	-	22.50	21.50
	(Trap positive)*	2	-	17.50	16.75	28.00
		3	-	14.25	13.75	15.25
		4	-	-	15.00	19.75
		5	-	15.75	15.25	17.50
		6	-	-	16.75	18.25
Plant #2	Plot #67	1	15.00	15.25	14.00	16.25
	(Trap positive)*	2	-	20.30	17.75	21.50
		3	-	16.00	14.50	17.00
		4	-	17.00	15.75	20.00
		5	-	18.25	16.50	21.50
		6	-	16.25	15.50	18.00
Plant #3	Plot #23	1	27.50	18.50	17.25	-
	(Trap positive)*	2	18.00	16.50	15.25	17.50
		3	20.25	16.25	15.75	17.00
		4	17.00	15.50	14.75	21.75
		5	-	19.00	20.25	20.25
		6	-	16.50	15.50	-
Plant #4	Plot #22	1	-	-	-	-
	(Trap negative)*	2	-	-	-	-
		3	-	-	-	-
		4	-	-	-	-
		5	-	-	-	-
		6	-	-	-	-
Total examined			24	24	24	24
Count			5	15	18	16
Positive (%)			20.8	62.5	75.0	66.7
Mean			19.55	16.85	16.26	19.44
Standard error (S.E.)			2.16	0.41	0.51	0.77

Annealing temperature of all the positive detected samples were between 78.9 and 79.9°C

* Based on previous field survey (Miyazaki *et al.* unpublished data)

**Amplification times (minutes) are indicated for samples detected as positive responses on LAMP run, and the samples detected as no responses are indicated as '-'.

Conclusions and recommendations

In this study we have extended our newly developed phylloxera LAMP assay for the detection of phylloxera in the presence of plant and soil contamination through evaluating in-field approaches to using LAMP. We successfully demonstrated two approaches which can be used for in-field LAMP detection of phylloxera: (1) detecting crawler stage insects captured in bucket traps; or (2) detection of insect samples associated with roots in the field. Protocols developed for on-site LAMP assays can provide results within one hour, including DNA extractions, and are now available for easy routine use by end users, such as industry, growers, and biosecurity services. Further work on this approach would involve incorporating these LAMP detection protocols into larger-scale visual surveys to improve the accuracy of phylloxera detections carried out in the field.

Our protocol of on-site LAMP assay can provide results within one hour. We have clearly demonstrated that LAMP detection of phylloxera is possible using both existing bucket trap approaches and our new field protocols for extracting phylloxera DNA from suspect root samples.

The major advantage of using LAMP to detect phylloxera is that LAMP is accurate and sensitive enough to remove the requirement for human visual detection in the field, saving considerable training and diagnostic time and effort. If LAMP were to be integrated into surveillance programs, biosecurity officers could focus on locating symptomatic vines (“hot spots”) and vine roots (nodosities), then employ LAMP to detect phylloxera from bucket trap samples or directly on roots, rather than have to visually detect the tiny insects themselves.

9.3. COMPARATIVE EVALUATION OF GROWER-FRIENDLY PASSIVE AND INTEGRATED SURVEILLANCE TOOLS.

In 2016, a comparative study of three detection approaches, emergence trapping (bucket traps), DNA probe (soil sampling) and root inspection (digging) was conducted. The methods were found to be equally effective at detecting phylloxera with evidence that an integrated strategy using all three methods could improve surveillance. The study was written up and submitted by VHA in a Final Report to the Plant Biosecurity CRC project [PBCRC no. 2016] (Pearce, et al. 2018). In this current project, no further data were collected for a comparative evaluation and for validation of this integrated approach. Detailed sampling protocols for emergence trapping and root inspection were, however, reviewed and developed under this activity and submitted to VHA in November 2018 as a component of the [PBCRC no. 2016] report (Pearce, et al. 2018).

Processes to formally endorse emergence trapping and root inspection methods, the DNA probe as sampling protocols in the NPMP have been started with state biosecurity committees.

Sampling protocols for emergence trapping (also known as bucket traps) and root inspection (also known as dig method).

Emergence traps are used to monitor phylloxera populations in blocks and vineyards. The method utilises wetted translucent buckets (220 mm diameter and 170 mm depth) with the open end placed onto a flattened soil surface 100 mm from the base of the vine. The bucket is secured with tent pegs to create an airlock that traps moisture and also stops the buckets being flipped over by people, moving animals or wind. First instars, alate or winged adults upon emergence from the soil are trapped in condensation on the container sides. The insects are collected into vials with 80% alcohol and samples examined under a low powered microscope in the laboratory.

The root inspection method is the operational procedure for conducting ground surveys for presence of phylloxera and is endorsed in the National Phylloxera Management Protocols (National Vine Health Steering Committee 2009). The method involves digging at the base of vine trunk, usually within 20cm or near irrigation drippers to expose actively growing feeder roots. A mass of fibrous roots is then collected and a piece of at least 10cm long carefully inspected for root galls and phylloxera stages attached to feeding sites, using a 10x magnification hand lens.

Additional data for comparative evaluation was not carried out in this project. The emergence trapping and root inspection methods were, however, comprehensively reviewed and submitted to VHA in November 2018 (See reviews in Appendix 3). The detailed procedures are currently being applied in i) the collection of root samples to test on LAMP and ii) for training contract workers to find phylloxera.

Recommendation

Emergence trapping is useful for growers that would like to monitor for phylloxera movement between blocks, as is common with some growers in the Yarra Valley. An integrated approach that uses LAMP to identify phylloxera from collected samples, followed by emergence trapping for monitoring spread when phylloxera is confirmed, would help growers establish disinfestation protocols in their vineyards to stop the spread between blocks.

RESEARCH THEME 2

ENABLING STRAIN-RELATED ROOTSTOCK SELECTION.

10.0. ENABLING STRAIN-RELATED ROOTSTOCK SELECTION.

10.1 RESISTANCE AND SUSCEPTIBILITY STATUS OF 5C TELEKI TO DIVERSE PHYLLOXERA STRAINS

Summary

Six genetically diverse phylloxera strains (G1, G4, G19, G20, G30, and G38) were screened for resistance against rootstock, 5C Teleki. With the exception of G1, five strains caused nodosities on vines in pots, implying that these strains will damage 5C Teleki vines. Compared with *Vitis vinifera* (which is highly susceptible to phylloxera), reproduction of strains G19, G20 and G30 on excised roots of 5C Teleki was, however, low. Results demonstrated that 5C Teleki is tolerant to specific strains, though the insects can survive and develop to reproductive adults, implying a hidden risk of phylloxera dispersal.

Background

In the early 1900s, grafting European vine varieties onto American rootstocks became the recognised viticultural practice (Hadju 2015). Sigmond Teleki (1854–1910), a wine businessman in Germany, first realized the importance of rootstock breeding for phylloxera management, and produced new rootstocks from seeds, and crossed seedlings of different vine types to produce varieties that were genetically diverse (Hadju 2015,Hajdu and Bakonyi 2006). Teleki selected traits that included resistance to phylloxera, tolerance to lime, desired vine size and graftability with scions. By 1920, Teleki rootstocks such as 125 AA Teleki-Kober, SO4 Teleki-Fuhr, 5BB Teleki-Kober, 5C Teleki, 8B Teleki and 10A Teleki were well sought-after in Europe, and were acknowledged worldwide as genetically rich propagation material (Csepregi and Zilai 1989). A decade later, 5C Teleki was a favourite rootstock due to its moderate vigour and suitability for grape varieties with small fruit set.

Phylloxera feeds on vine roots and foliage causing swellings that develop into galls on young fibrous roots (herein called nodosities) and tuberosities on older lignified roots. Nodosities and tuberosities act as nourishing and food storage organs to the insects (Forneck 2011) and as these “organs” enlarge and insects crowd at feeding sites, uptake of water and nutrients by roots is impeded. Cracks subsequently appear on the root swellings, making them susceptible to soil pathogens (Edwards, et al. 2006), eventually causing vine death. The speed of vine decline is dependent on rootstock susceptibility or tolerance to phylloxera strain. Susceptible vine varieties such as *V. vinifera* die within 4-5 years whilst tolerant rootstocks can host phylloxera populations for many years (Granett 1990).

Phylloxera performance on rootstocks is studied using feeding and reproductive behaviour trials (Forneck, et al. 2016; Eitle and Forneck 2017). Our understanding of phylloxera and rootstock interactions continues to grow with evidence of some strains causing unprecedented damage on rootstocks (Powell and Krstic 2015; Lund, et al. 2017). Seven genetic strains, G1, G4, G7, G19, G20, G30 and G38 are currently cultured at Agriculture Victoria under quarantine for research and development and are integral to screening commercially available and emerging rootstocks. In the last four years, new phylloxera strains have emerged in North East Victoria, one of which (strain G38) appears to reproduce in high numbers on rootstocks (See the study details in section 2.2). Knowledge on resistance

and susceptibility is integral to providing strain-specific information that is incorporated in the Grapevine Rootstock Selector tool (Wine Australia 2019). Ten commercial rootstocks have so far been evaluated for resistance/tolerance against six phylloxera strains: G1, G4, G7, G19, G20 and G30 (Powell and Krstic 2015). Here, the resistance/susceptibility of 5C Teleki was tested against six genetically diverse phylloxera that included the G38 strain using potted vines and excised root assays.

Methods

Evaluating resistance

Callused cuttings of 5C Teleki were purchased from Yalumba Nursery in 2018 and planted in 80% potting mix (purchased from Yarra Glen) and 20% Perlite (purchased from Peards Nursery in Albury, NSW). The plantings were thereafter maintained in a shade house for at least four months until fibrous roots were sufficiently developed to infest with phylloxera. *Vitis vinifera* vines were used as control vines (susceptible) and grown under similar conditions. Populations of six phylloxera genetic strains, G1, G4, G19, G20, G30 and G38 were cultured on excised roots of *V. vinifera* cv. Chardonnay under controlled conditions (25±3°C; 12 hours light: dark). The G7 stock was found to be contaminated with G4, hence excluded from all data (see Section 8).

Potted vine assays

Vines were infested as per methods described in Appendix 6. Eight weeks after infestation, the vines were removed from pots and the root pockets examined for the presence of phylloxera life-stages and presence of nodosities on roots. An assessment was made as i) **Resistant** when no phylloxera survival was observed and no eggs were produced after eight weeks; ii) **Tolerant** when phylloxera survived for eight weeks with or without nodosities and non-necrotic pseudo-tuberosities on lignified roots and; iii) **Susceptible** when phylloxera survived for eight weeks and produced necrotic tuberosities on lignified roots.

Excised root assays

Bioassays of excised 5C Teleki and *V. vinifera* roots were pre-prepared according to methods by (Kingston 2007). Ten eggs of a particular phylloxera strain were placed on a 5cm root piece held in a Petri dish. Five root pieces were used per replicate for each of the six phylloxera strains and vine variety. Number of eggs that hatched were recorded at day 8. First instars that attached to feeding sites and subsequently developed to intermediates and adults were recorded at days 18, 25 and 32. Any eggs that were laid on days 18 and 25 were counted and removed from the root assays. Proportion of eggs reaching adulthood, total number of eggs laid per root piece and mean numbers of eggs per surviving female was assessed using a one-way-ANOVA. Performance rating on excised roots was assessed as i) **Resistant** when phylloxera neither attached to feeding sites nor survived after day 18 ii) **Tolerant** when phylloxera survived to adulthood and produced eggs in lower abundance than on *V. vinifera* iii) **Susceptible** when phylloxera survived for 32 days and adults produced eggs in higher abundance than *V. vinifera*.

Results and discussion

Table 1 summarises the result of the 5C Teleki screening trial.
Overall, this rootstock was resistant to G1 and tolerant to G4, G19, G20, G30 and G38.

Table 1 Summary on resistance and susceptibility of rootstock 5C Teleki to feeding by different phylloxera strains, as evaluated using excised roots (ER) and potted vines (PV) assays.

Phylloxera type	Assay type	Rootstock response
G1	ER	Resistant
	PV	Resistant
G4	ER	Resistant
	PV	Tolerant
G19	ER	Tolerant
	PV	Tolerant
G20	ER	Tolerant
	PV	Tolerant
G30	ER	Tolerant
	PV	Tolerant
G38	ER	Tolerant
	PV	Tolerant

Using potted vine assays, phylloxera of G4, G19, G20, G30, G38 phylloxera initiated nodosities on fibrous roots of 5C Teleki (Figure 1 a and b). Though nodosities were found on roots infested with G4, G19, G20, G30, G38 phylloxera, life stages of phylloxera, i.e. eggs, intermediates and adults, were only found on vines infested with G19 phylloxera. 5C Teleki vines infested with the G1 strain neither had nodosities nor phylloxera life stages. Compared to *V. vinifera,* G19, G20, G30 and G38 phylloxera initiated root damage on infested vines and developed to reproductive adults. Necrotic pseudo- tuberosities were observed on the 5C Teleki vines infested with G20, G30 (Figure 1 a and b) and G19 phylloxera.

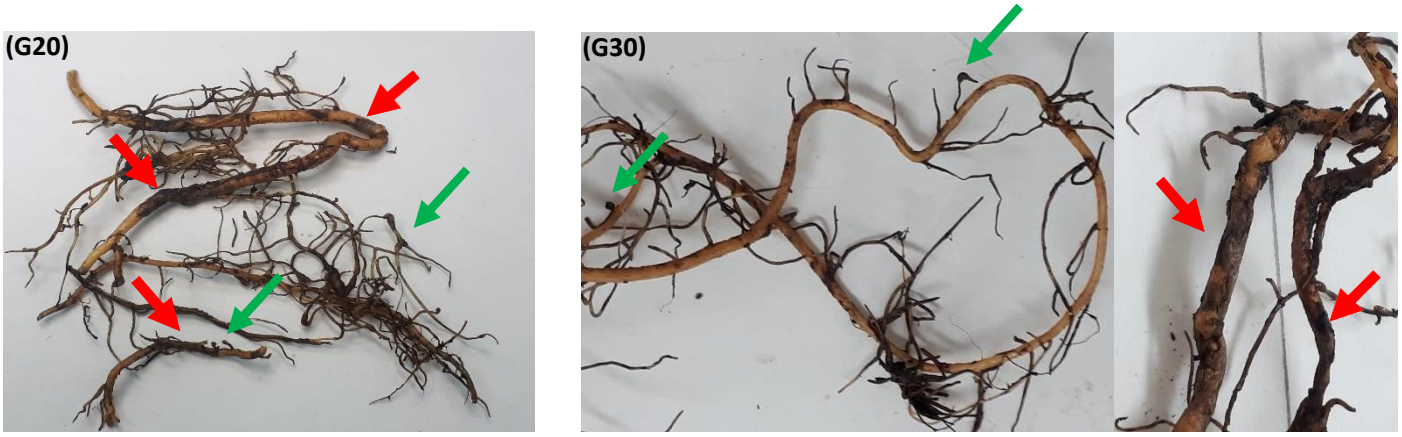


Figure 1 Nodosities initiated on roots of 5C Teleki (green arrows) by (a) G20 and (b) G30 phylloxera and necrotic response on roots (red arrows) eight weeks after inoculation with 20 eggs per replicate vine (eight replicate vines were used for each genetic strain).

On excised roots of 5C Teleki, first instars that emerged from inoculated eggs of G19, G20, G30 and G38 phylloxera survived to reproductive adults, but no first instars developed to adulthood for G1 and G4 (P=0.009; Figure 2a). Compared to the susceptible *V. vinifera* roots, a high proportion of eggs that hatched to first instars that developed to adults was observed for G1 and G4 phylloxera, but the opposite was true for G19, G20 and G30. Though a high number of adults was observed on roots of 5C Teleki inoculated with G19, G20 and G30 phylloxera, the adults laid fewer eggs than on *V. vinifera* (p=0.017; Figure 2b). Across the six phylloxera strains, the numbers of eggs laid per female were lower on 5C Teleki compared to the susceptible *V. vinifera* roots (Figure 2b). These results suggest tolerance characteristics on 5C Teleki by phylloxera using the excised root assays.

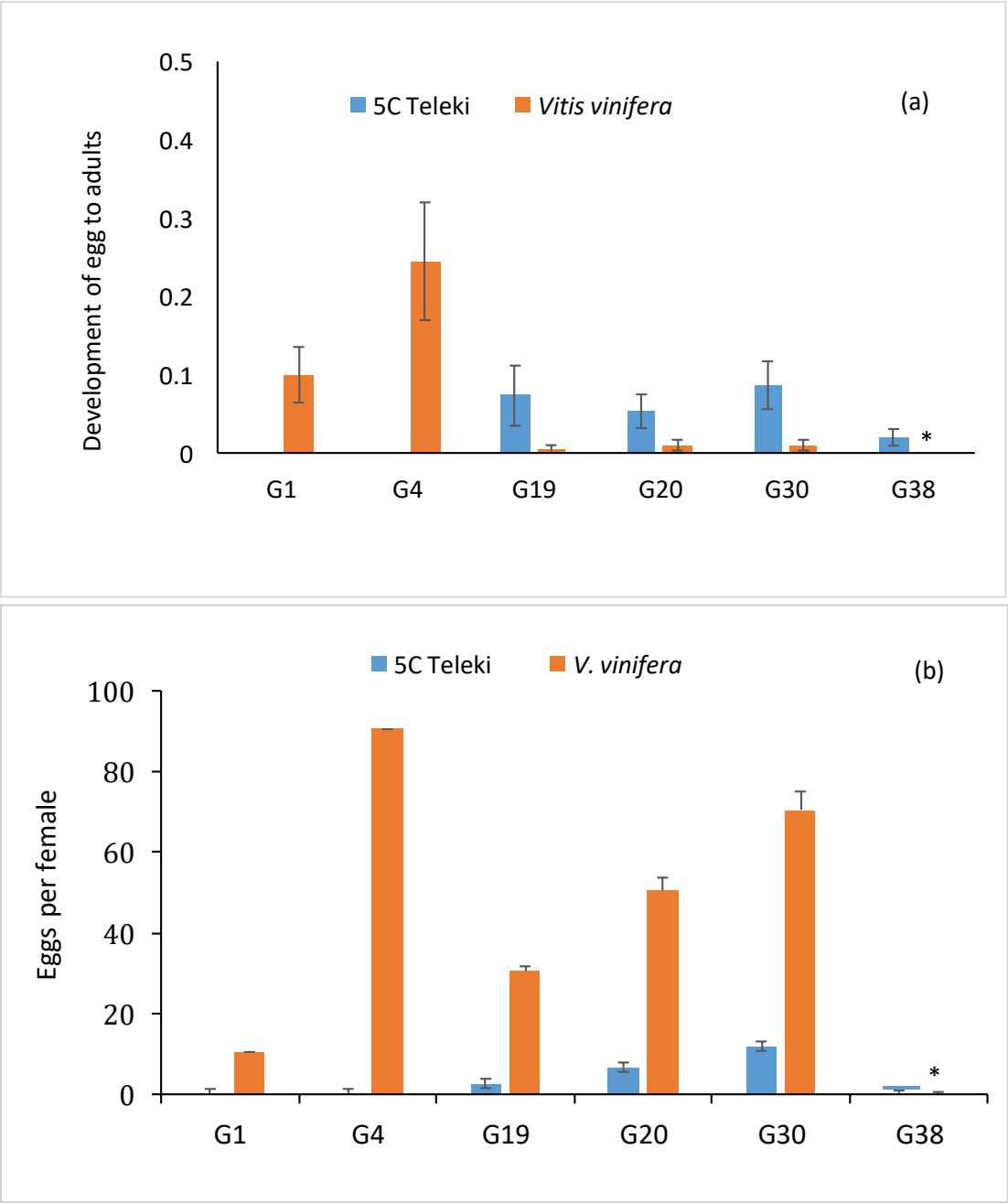


Figure 2 Proportion of adults surviving per root on *V. vinifera* and 5C Teleki rootstock (a) and numbers of eggs per female per root (b) at Day 32. Excised roots were inoculated with 10 eggs of a particular phylloxera genetic strain (five replicates per treatment vine and phylloxera strain). First instars that emerged were monitored until adulthood for 32 days. Error bars in figure 2(a) represent the standard error of the proportion and in 2(b) the mean. Data were analysed by ANOVA ($P < 0.05$). * G38 genetic strain was not tested with *V. vinifera*.

Root damage from particular phylloxera biotypes (i.e. phylloxera strains that are equal in their ability to

establish and develop on a particular host plant (Forneck et al. 2016)), has been reported on 5C Teleki rootstocks under field conditions in Europe (Walker, et al. 1998). Research has further shown that phylloxera can adapt to feeding on rootstock vines (Granett et al., 1987). Recent genome sequencing demonstrated that the specificity of phylloxera genetic strains to rootstocks is an underlying factor influencing adaptation on rootstocks (Rispe, et al. 2020). Findings from this research suggest that vines of rootstocks such as 5C Teleki are likely to become infested with particular phylloxera strains that induce a high level of damage, resulting in deterioration and rapid vine death. In Australia, this scenario could likely happen in regions where strains G19, G20, G30 and G38 phylloxera predominate. High pest populations of these strains in the summer months, when the reproductive capacity is at peak, could lead to substantial root damage. Furthermore, first instars living above-ground can be transferred to clean blocks and vineyards, thus maintaining strict quarantine to stop phylloxera spread is required to minimise dispersal of these phylloxera strains that can populate on 5C Teleki.

Conclusions and recommendations

5C Teleki became one of the most widely used rootstocks after the failure of AXR #1 to phylloxera. This rootstock is widely planted in Victoria and South Australia due to its tolerance to lime and moderate vigour, making it well suited to well-drained and fertile soils (Hadju 2015, Howell 1987). It is also a preferred rootstock where early grape maturity is desired for a particular wine style. The results from this study have implications for regions such as North East Victoria, where a wide range of genetically diverse strains exist across several rootstocks. In PIZs, the knowledge of existing genetic strains is integral to ensure matching of resistant rootstocks. Growers in PIZs should seek to know the phylloxera strains they have and incorporate strict farmgate hygiene protocols to stop the spread of phylloxera between blocks.

We recommend that this information be incorporated into the **Grapevine Rootstock Selector tool** for access by growers who would like to identify key factors that are important to their specific planting site, before planning or replanting on rootstock 5C Teleki.

10.2. IN-FIELD ANALYSIS OF G38 PHYLLOXERA DISTRIBUTION ACROSS DIFFERENT ROOTSTOCKS AND SCREENING OF ROOTSTOCKS FOR RESISTANCE AGAINST G38 STRAIN UNDER CONTROLLED CONDITIONS.

Summary

Field trials in a mixed-rootstock block infested by the virulent "superclone" G38 showed rootstocks 101-14 and Schwarzmann were susceptible to this strain. Standardised laboratory and glasshouse screening assays performed using the same rootstocks also indicated these rootstocks were susceptible to this strain. The study was important in that it validated current screening assays in terms of predicting resistance / susceptibility of rootstocks in vineyards. That rootstocks may be becoming susceptible to G38 is particularly worrying given additional findings in this project that indicate that this genotype may be able to reproduce sexually.

Background

Rootstocks that convey resistance to phylloxera have been selected over generations from *Vitis* species that occur in the native range of this pest (USA), and have evolved mechanisms to prevent (resistant) or withstand (tolerant) attack (Granett et al., 2001). Rootstocks from these vine cultivars can be grafted onto more susceptible vine species to create hybrid varieties that tolerate phylloxera. On tolerant rootstocks, phylloxera can survive and reproduce for many years without killing the vine (Granett et al. 2001). Vine varieties such as *V. vinifera* can be grown on their own roots, but many hybrid varieties are grafted to a rootstock that provides resistance to phylloxera. Rootstock resistance is achieved by stacking different phylloxera resistant genes or loci into a single rootstock via DNA marker-assisted selection (Granett et al., 1987). Aside from phylloxera resistance, rootstocks are used to combat soil-borne pests, such as nematodes and are bred to suit vineyard conditions such as drought, soil pH and porosity (Walker et al. 2014). Planting on hybrids or resistant varieties is considered a long-term management approach for phylloxera.

Agriculture Victoria uses a suite of protocols that integrate lab (excised roots), glasshouse (potted plants) trials, and small-scale field trials to screen commercial and newly developed rootstocks for resistance against phylloxera—each method having different advantages (control, replication, variability, and external validity). Research has so far identified that phylloxera resistance or tolerance to rootstocks is dependent on insect genotype-plant genotype interactions. Recommendations for 10 resistant rootstocks (tested against six genetic strains) were released to the industry in 2015. The knowledge of rootstock and phylloxera interactions is critical for decision making on plantings in greenfields or brownfields (after a phylloxera infestation), and this information is incorporated into the Grapevine Rootstock Selector tool that allows growers to assess other key factors that are critical to a planting site (Wine Australia 2019).

Since the last recommendations of rootstocks to the industry (in relation to tolerance and resistance to phylloxera), the G38 strain was identified in the North East PIZ in the summer of 2015. Field observations showed yellowing leaf symptoms particularly on vines in four rows of a mixed rootstock block in a vineyard located in North East Victoria PIZ. Upon inspection, the vines were found to harbour high populations on grafted rootstocks, particularly on 101-14 in comparison to own roots (*Vitis vinifera*). The G38 strain was first characterised from a leaf galling female sample that was collected from a vineyard in Glenrowan in 2000 (Umina et al., 2007; Final Report LTU 01/01, Ary Hoffmann, September 2003). The study inferred that G38 could be the outcome of sexual reproduction between individuals of G2 and G3 (G2 and G3 were sampled from both leaf and

root (Corrie et al. 2000, 2003). The identity of the rootstock from which the G38 sample was collected is not identified and referred to as the "*Vitis* genotype" in the reports.

The evaluation of resistance and susceptibility under field conditions across multiple rootstocks in a block is exceptional, as it is not always possible to find blocks of vineyards planted with diverse rootstocks. The identification of G38 in a mixed rootstock block with vines grown in a randomised block design offered the perfect opportunity to include a case study on vine decline and population dynamics of a known strain following a phylloxera detection. Here, we conducted a three year in-field rootstock study of G38 across different rootstock cultivars and further, screened the rootstocks for resistance against G38 phylloxera under controlled conditions.

Methods

In-field screening – Emergence traps

A total of 106 emergence traps were installed at the base of 10 rootstock vines in a vineyard in North East Victoria when phylloxera activity was high (November to April) in 2016, 2017 and 2018. Vine selection was randomised across four rows within a block (Appendix 5). Emergence traps consisted of translucent plastic 5 l containers (22 wide by 18 cm in deep) that were wetted with tap water on the internal surface and placed upside down to create an airtight seal that encouraged a build-up of humidity (See detailed methods in Appendix 3 PART A). Phylloxera caught in the condensation within the traps were collected at monthly intervals by washing the sides of the traps with 80% ethanol, which was then poured into sample containers. Emergence traps were then rinsed with tap water and re-installed at the base of the vines until the next collection, and the process repeated for four months each year. Samples were transported to the laboratory at AVR Rutherglen and identified under a low power binocular microscope. Total phylloxera catches in each trap sample were counted and mean numbers of insects per rootstock vine calculated.

Root inspection – confirming presence/absence of phylloxera stages

The dispersal stages of phylloxera; crawlers and alates (winged forms); could have come into traps from neighbouring vines. To determine whether insects were associated with vines under which traps were directly placed, we dug up roots from eight replicate rootstock vines and inspected them for infestation by phylloxera, using a hand lens (See detailed methods in Appendix 3 PART B). Rootstock vines with nodosities that were also found with high populations of phylloxera at all life stages (i.e. eggs, first instars, intermediates, adults) were scored "susceptible". If a rootstock neither had nodosities nor adults with eggs, and yet first instars were found in traps, first instars were assumed to have moved from neighbouring vines.

Glasshouse screening trials using potted vines

Screening of 10 rootstock varieties for resistance against G38 phylloxera was studied using potted vines under controlled conditions in glasshouses. The vines were; *Vitis vinifera*, Ramsey, Schwarzmann, 101-14, 3309C, 5BB Kober, 110 Richter, 1103 Paulsen, 140 Ruggeri and Borner. The rootstock varieties were purchased from Yalumba Nursery, stored at 4°C for no more than four days when they were potted in 80% horticultural soil and 20% Perlite. 20 eggs of G38 phylloxera were placed on roots in a pocket sleeve as per methods described in Appendix 6. The experimental design employed a completely randomised block design with eight replicate vines per rootstock. Experiments were conducted under controlled conditions in the glasshouses (25±3°C; 12 hours L:D).

Eight weeks after infestation, the vines were removed from pots and the root pockets examined for the presence of phylloxera life-stages and presence of nodosities on roots. An assessment was made as i) Resistant, when no phylloxera survival was observed and no eggs were produced after eight weeks; ii) Tolerant, when phylloxera survived for eight weeks with or without nodosities and non-necrotic pseudo- tuberosities on lignified roots and; iii) Susceptible, when phylloxera survived for eight weeks and produced necrotic tuberosities on lignified roots.

Excised root assays

Roots of 10 rootstock vines were cut into 4-5 cm pieces and placed in Petri-dishes according to methods by (Kingston 2007). Ten G38 phylloxera eggs were collected and placed on each root piece, with five replicates of each of the 10 rootstock varieties. Number of eggs that hatched were recorded at day 8. First instars that attached to feeding sites and subsequently developed to intermediates and adults were recorded at days 18, 25 and 32. Any eggs that were laid on days 18 and 25 were counted and removed from the roots. Proportion of eggs reaching adulthood, total number of eggs laid per root piece and mean numbers of eggs per surviving females was assessed using a one-way-ANOVA. Performance rating on the excised roots was assessed as i) Resistant when phylloxera neither attached to feeding sites nor survived after day 18 ii) Tolerant when phylloxera survived to adulthood and produced eggs in lower abundance than *V. vinifera* iii) Susceptible when phylloxera survived for 32 days and adults produced eggs in higher abundance than *V. vinifera*.

Statistical analysis

All sequential and non-sequential variables were initially plotted using boxplots with groups (*Rootstocks*; *Infection status of phylloxera;* Yes/No) to illustrate any potential treatment differences, complementing the formal analyses to follow (boxplots were plotted using R).

Insect numbers caught in traps from each rootstock were fitted in a logistic regression and odds ratios calculated. From the odds ratio, the probability of phylloxera reaching adulthood for each rootstock were calculated using the odds ratio via the formula $p = (\frac{e^z}{1+e^z})$ where *p* = probability and *z* is the relevant odd

ratios for each treatment combination.
Data from potted vines and excised roots assays were analysed using ANOVA with ten levels of rootstocks (*Vitis vinifera*, Ramsey, Schwarzmann, 101-14, 3309C, 5BB Kober, 110 Richter, 1103 Paulsen, 140 Ruggeri and Borner) and *Infestation status of phylloxera* (with two levels: Yes/No) was modelled as the Treatment structure (rootstock*Infestation) with block structure included. All residual values were examined graphically to ensure normality and homogeneity of variances. Observations with standardised residuals greater than 3.0 were excluded from analyses. Fishers protected LSD test ($p<0.05$) were used to separate means where F-tests were significant. For Kruskal-Wallis ANOVA analyses, *Rootstock*Infestation* was modelled as the Group factor and ranks were calculated. The differences between the ranks for the ten rootstocks were then compared using the test statistic H.
All analyses were performed in GenStat 18 th Edition.

Results and discussion

Molecular analysis for in-field G38 biotype distribution study at Whorouly

Two phylloxera genetic strains were identified at the Whorouly study site. Rows 95-98 consisting of mixed rootstock vines had G38 phylloxera while row 38 that was planted on own roots was found to have G20.

Emergence traps

High numbers of phylloxera (crawlers and alates) were recorded from traps placed at 101-14 and Schwarzmann rootstock vines compared to the susceptible own roots (*Vitis vinifera*) in years 1, 2, 3 (Figure 1). Phylloxera mean counts on rootstock 101-14 was 1633 (± 191) compared to the susceptible own root vines with a mean of 161 (± 46) insects over three years. Rootstocks 1103P and R99 had the lowest mean trap count with less than 100 phylloxera caught in traps. Insects caught in traps were highest in the third year of infestation implying a gradual increase of below-ground phylloxera populations across all rootstocks (Figure 1).

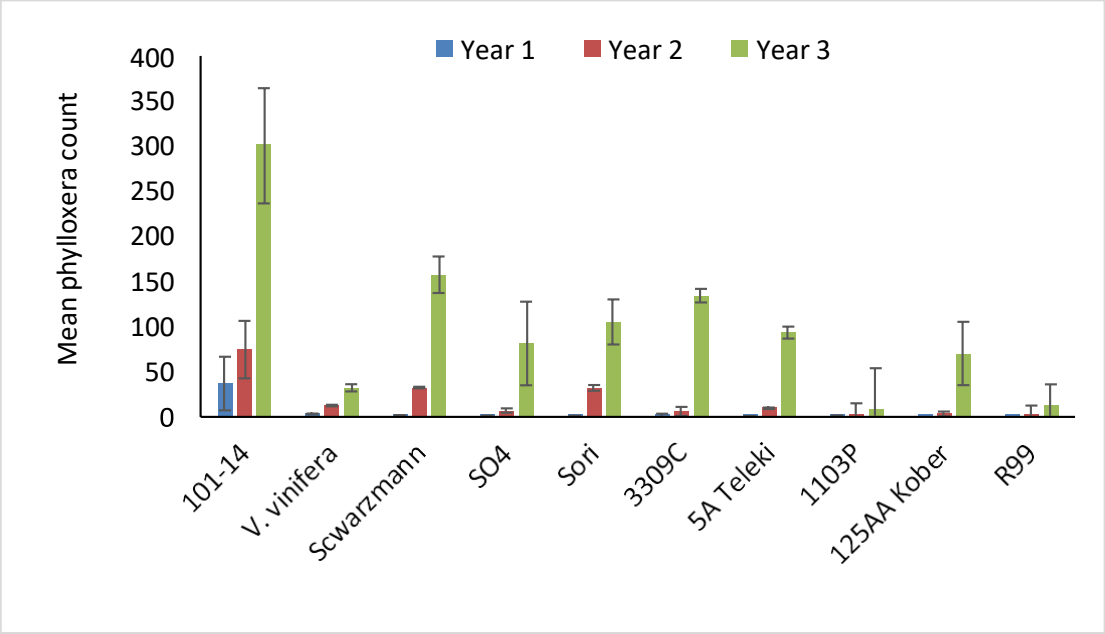


Figure 1 Mean phylloxera (first instars and alates) caught per emergence trap placed on own roots (V. *vinifera*) and different rootstock varieties in three consecutive seasons (2015, 2016 and 2017).

To confirm that insects caught in traps were emerging from the rootstock vine on which trap was placed, (i.e. did not crawl in from neighbouring vines), roots were visually inspected for presence of nodosities and all life stages. Probability of feeding damage as evidenced by presence of nodosities was above 80% for own roots,

Schwarzmann, 101-14 and Sori and between 50 and 60% for 3309C, 125AA Kober and SO4 (Table 1). Probability of finding adults associated with eggs was above 90% for 101-14, own roots and Schwarzmann indicating that the vines are susceptible to G38 phylloxera (Table 1). Neither nodosities nor adults were found on roots of R99 (Table 1) implying that this rootstock is resistant to G38 phylloxera. Only one adult was found on rootstock 1103Paulsen implying a level of tolerance to G38 phylloxera on this rootstock.

Table 1 Probability of feeding damage (presence of nodosities) and successful development (egg to adult cycle) on rootstock vine roots where bucket traps were installed. A logistic regression was fitted, and odds ratio estimates from the regression parameters used to calculate the probabilities that are reported on the table.

Rootstocks	Nodosities	Adults	Eggs	Intermediates
OWN ROOTS	1.000	1.000	1.000	0.874
SCHWARZMANN	1.000	1.000	1.000	0.799
101-14	1.000	0.857	0.857	0.857
SORI	0.802	0.799	0.799	0.599
3309C	0.668	0.666	0.666	0.666
125AA KOBER	0.599	0.599	0.599	0.599
SO4	0.500	0.666	0.666	0.500
5A TELEKI	0.332	0.334	0.334	0.334
1103P	0.198	0.199	0.199	0.199
R99	0.000	0.000	0.000	0.000

First instars and alates of G38 were highest in December signifying a high below ground activity at start of summer (Figure 2) and the optimal time to check for phylloxera infestations. This information is relevant to the industry as it coincides with the best time to monitor symptoms on foliage (Figure 3) and activate disinfestation protocols to apply measures that will minimise further spread between blocks and vineyards.

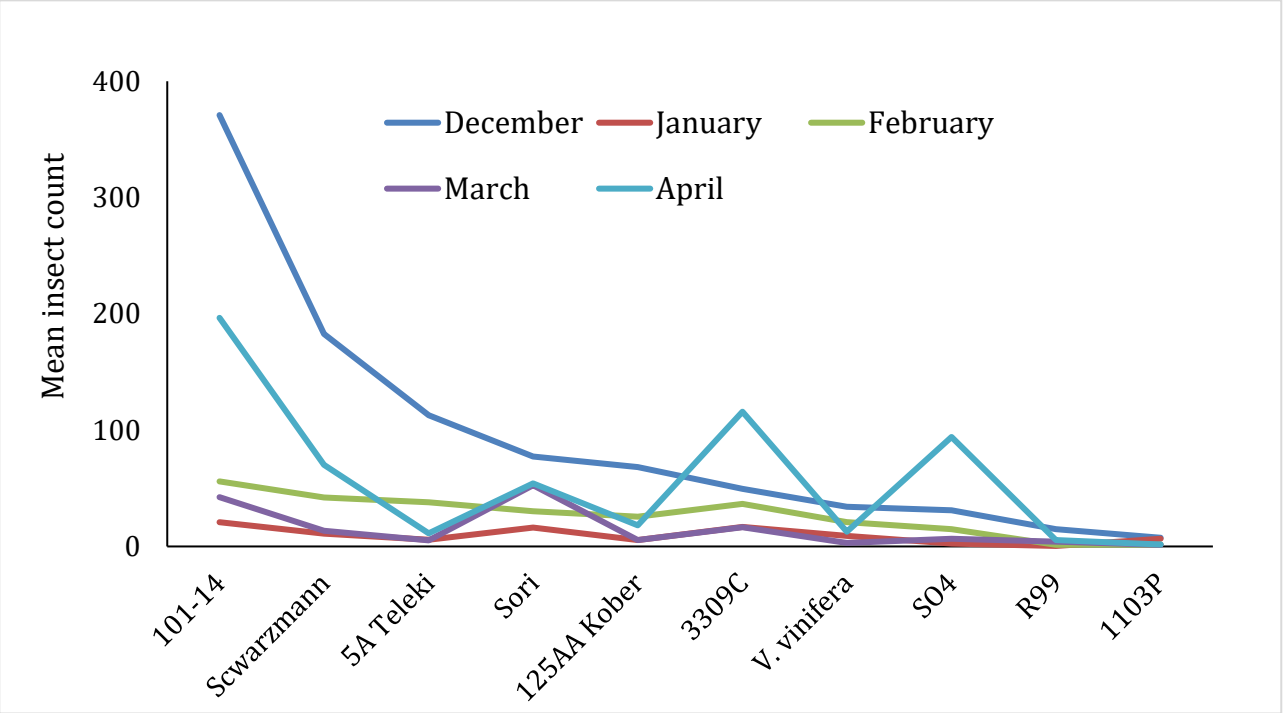


Figure 2 Mean counts of phylloxera (first instars and alates) that were found per emergence trap from December to April over three years (2016, 2017 and 2018) across nine rootstocks and own roots (*V. vinifera*).

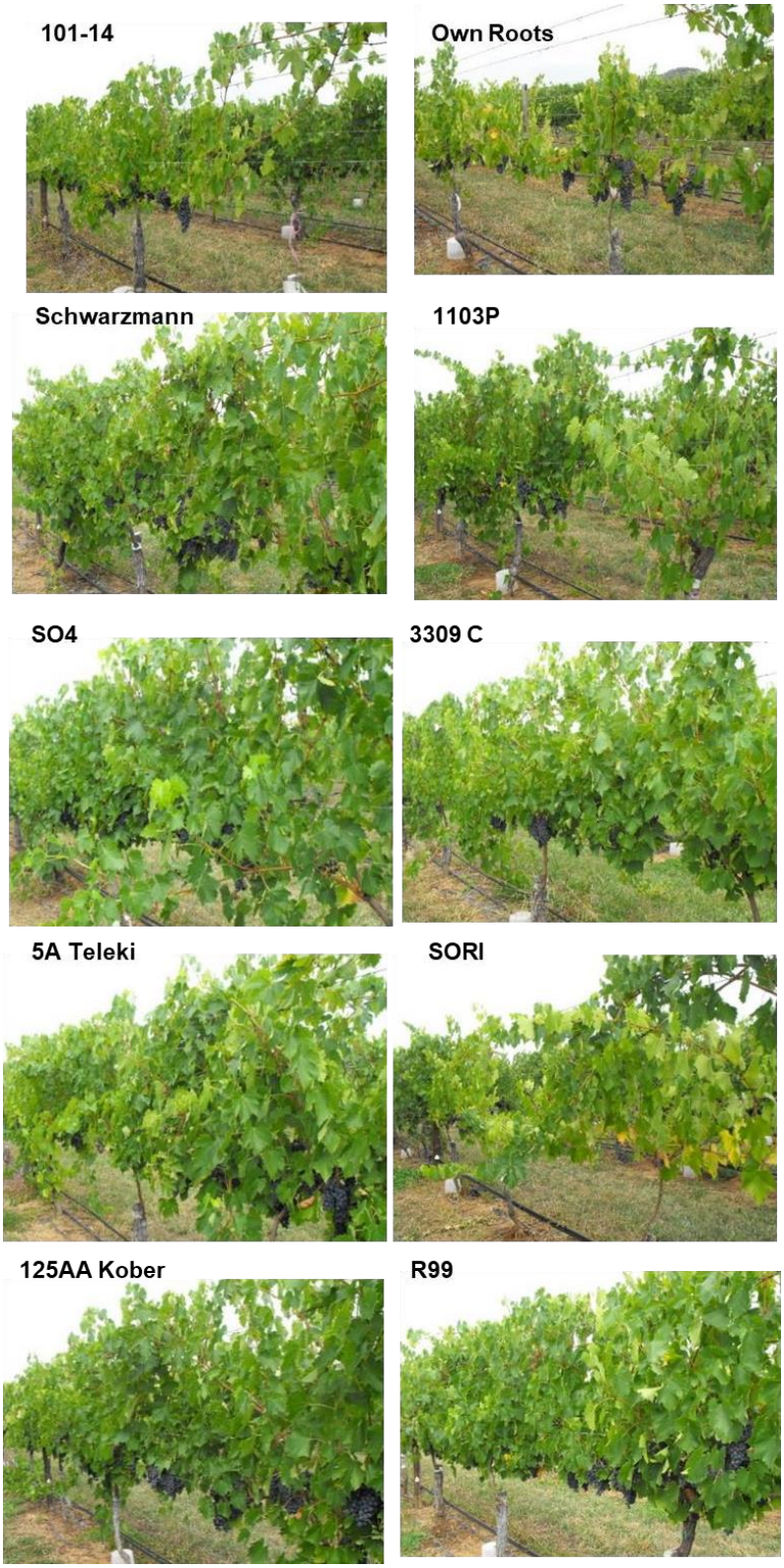


Figure 3 Visual symptoms on vines at the NE Victoria rootstock study site. Vines were monitored for three years for phylloxera infestation (first instars and alates) using bucket traps.

Excised roots

Survival of G38 on excised roots of ten rootstock varieties was highest on 101-14 and Schwarzmann which corroborate findings from emergence trap numbers from field vines (Table 2). Total fecundity as expressed by number of eggs laid per individual that developed to a reproductive adult differed significantly between rootstocks ($p < 0.001$). Mean total eggs laid on excised roots was highest on 101-14 (104 eggs) over 32 days (Table 2). None of the first instars that hatched (out of 50 eggs) developed to reproductive adults on roots of 140 Ruggeri and only one individual reached adulthood that reproduced a single egg on 1103P, Borner, 3309C, 110 Richter and 1103Paulsen (Table 2).

Table 2 Percentage survival of G38 and mean number of eggs laid per adult on excised roots of ten rootstock varieties at day 32. A total of 50 eggs were inoculated per rootstock vine. Within a column, means with different letters are significantly different ($P \leq 0.05$ Fisher's protected least significant difference test).

Rootstock	% survival	Eggs per female
101-14	64^{d}	104 ± 18^{c}
Schwarzmann	23^{c}	25 ± 11^{b}
Vitis vinifera	13^{bc}	10 ± 4^{ab}
Ramsey	10^{ab}	6 ± 2^{a}
5BB Kober	9^{ab}	5 ± 2^{a}
Borner	1^{a}	1 ± 1^{a}
3309C	9^{ab}	1 ± 1^{a}
110 Richter	1^{a}	1 ± 1^{a}
1103 Paulsen	0^{a}	0^{a}
140 Ruggeri	0^{a}	0^{a}

Potted vines assays

Feeding damage due to G38 phylloxera infestations as shown by nodosity formation on rootstocks was apparent on all rootstocks except 110 Richter, 420A and Borner (Table 3). The probability of eggs that hatched to reach reproductive adults was highest on Ramsey (79%) implying that G38 phylloxera adapted better on roots restricted into pockets on this rootstock compared to the other nine rootstocks. The low number of insects counted on roots in the potted vines and the inability of insects to develop in pockets was surprising despite apparent nodosity formation. Most of vine roots that had high nodosities, however, were necrotic and this could be attributed to restricted flow of nutrients to the developing insects that resulted in hatched to lees adults developing to reproductive adults. Trials that use the entire vine root system in a pot would be recommended.

Table 3 Total nodosities (counts) and the probability of successful development (egg to adult cycle) on roots (enclosed in pockets as per methods described in Appendix 6). A logistic regression was fitted, and odds ratio estimates from the regression parameters used to calculate the probabilities that are reported on the table.

Rootstock	Total Nodosities	Probability of reproductive adults
Schwarzmann	71	0.02
V. vinifera	61	0.07
3309C	56	0.16
101-14	29	0.06
1103P	23	0.01
5BB Kober	8	0.00
140 Ruggeri	3	0.00
Ramsey	1	0.79
110 Richter	0	0.00
420A	0	0.00
Borner	0	0.00

Resistance screening trials of rootstocks to phylloxera form an essential component of rootstock selection and evaluation, as well as providing data-driven recommendations to the grape and wine industry. The current study provides robust confirmation for the validity of current laboratory and greenhouse trials, in predicting rootstock performance in the field (Table 4). The study was conducted at a unique site with a block of mixed rootstock vines planted in a randomised block design. The vines were found to be infested with G38 phylloxera in 2015 and the site offered a perfect opportunity for a case study on vine decline and population dynamics within the first years of infestation. Corrie et al. (2003) initially characterised G38 phylloxera as a leaf galling strain. This study clearly showed that the strain exists as a root form as well, causing significant damage on rootstock vines. An important outcome from this work was that two popular commercial rootstocks, 101-14 and Schwarzmann, hosted higher populations of G38 phylloxera than *V. vinifera* over a three year period, indicating that new strains could spread quickly across certain “susceptible” rootstocks if these are widely planted. Five rootstock varieties that were planted in proximity to 101-14 and Schwarzmann were tolerant to G38. Though phylloxera infesting tolerant rootstocks do not kill the vines, these plants can house large populations of the pest. This creates a significant ‘hidden’ risk where crawlers can move to adjacent vineyards with plantings of rootstocks such as 101-14 and Schwarzmann. This outcome has serious implications for management guidelines and attention to include rootstock tolerance with respect to genetic strains in the NPMP should be considered. A root galling phylloxera (G20) was identified on *V. vinifera* vines planted in the same block of the mixed rootstock vineyard with the G38 strain, and an excellent opportunity exists to investigate competition for rootstocks by phylloxera under natural conditions.

Table 4 Resistance ratings of rootstocks to phylloxera strain G38. The table includes the *Vitis* parentage of rootstocks used in three-phase trials (in-field, potted vine and excised root assays). Blank spaces indicate unknown resistance status.

Rootstock	*Vitis* parentage	Screening assay		
		In-field	Potted vines	Excised roots
Vitis vinifera	*vinifera* (own roots)			
101-14	riparia *x rupestris*			
Scwarzmann	riparia x rupestris			
3309C	riparia x rupestris			
1103 Paulsen	berlandieri x rupestris			
420A	berlandieri x riparia	×		
140 Ruggeri	berlandieri x rupestris	×		
110 Richter	berlandieri x rupestris	×		
5BB Kober	berlandieri x riparia	×		
Ramsey	candicans x rupestris	×		
Borner	riparia x cinerea	×		
Sori	solonis x riparia		×	×
5A Teleki	berlandieri × riparia		×	×
SO4	berlandieri x riparia		×	×
125AA Kober	berlandieri x riparia		×	×
R99	berlandieri x rupestris		×	×

Susceptible
Tolerant
Resistant

X indicates that data on screening of a particular rootstock against G38 is unavailable.

The reliance on a few select rootstock varieties is of concern to this management approach. For instance, growers favouring 101-14 or Schwarzmann risk having the rootstocks succumb to infestations by some virulent phylloxera forms. Rootstocks that develop resistance to biotypes of phylloxera have caused unprecedented failure and huge economic losses. For example, the rootstock AXR#1, a hybrid of *V. vinifera* × *V. rupestris,* became susceptible to virulent biotypes of phylloxera in California causing loss in production of between US$ 1 and 6 billion (Granett et al. 2001; Gale, 2011). The failure of AXR#1 led to caution in the use of rootstocks with *V. vinifera* parentage.

The occurrence of high numbers of G38 phylloxera on rootstocks, particularly 101-14 and Schwarzmann, was unexpected and could be attributed to a breakdown in resistance (Grannet et al. 2001) and/or adaptation by G38 phylloxera to the rootstocks (Rispe et al. 2020) (*Due to this unexpected result, the identity of 101-14 and V. vinifera was confirmed using DNA methods (CAGRF on 29/06/2017)).* These findings corroborate those in California where phylloxera biotypes have become highly adapted to feeding on rootstocks with *V. riparia* parentage such as 101- 14, Schwarzmann and some Teleki hybrids (Kocsis et al. 1999, Kocsis et al. 2002, Riaz et al. 2019).

That G38 may be showing signs of virulence on some common rootstocks is particularly worrying given our findings on the increased genetic diversity in the King Valley and that phylloxera may indeed be able to reproduce sexually in Australia (See further details in Section 2.3). The implications are of real concern as strains such as G38 could disperse widely and the ability to reproduce and sustain populations in high numbers suggest a breakdown in resistance by some rootstocks. This finding, in combination with other results discussed below, warrant a major study on the G38 strain and a survey of leaf galling forms to investigate the prevalence of the leaf galling form of G38 between seasons and temperature variations.

Conclusions

This work evaluated G38 phylloxera performance across different rootstock varieties. The study also provided a unique opportunity to validate current laboratory-/glasshouse- based assay in terms of how well they predict resistance, tolerance and susceptibility in the field. The results demonstrated that two of ten rootstocks studied— that is 101-14 and Schwarzmann—were susceptible to G38 phylloxera in all three assays (lab, glasshouse, field), which provides important information to industry concerning plantings of these rootstocks in regions where G38 is prevalent. More generally, the study highlights why knowledge of local phylloxera strains is important for selecting optimal rootstocks at a local and regional level.

Recommendations

- Growers in the region determined in this study where G38 is prevalent should consider that rootstocks 101-14 and Schwarzmann do not appear to be resistant to this strain.
- Current lab / glasshouse rootstock screening assays should be viewed as valid methods for predicting resistance under field conditions.
- Over the last four years the mixed vineyard at NE Victoria has provided invaluable data and should continue as a study site to show longitudinal changes in vine performance and interactions among phylloxera genotypes.

10.3. PHYLLOXERA GENETIC DIVERSITY IN THE KING VALLEY, NORTH EASTERN VICTORIA

Summary

Phylloxera has been known in the King Valley since the early 1990's, where previous studies found only a single strain (G4) present on vine roots. An updated and more detailed survey was conducted to better understand the genetic variation present. A total of 1400 phylloxera were collected from roots of vines in 18 blocks and 764 genotyped. The study revealed that the amount of genetic diversity present in the King Valley is considerably higher than previously thought, with many novel genotypes present on roots. A total of 36 genotypes were identified, with 4 strains known from previous work and 32 new strains. A potential explanation for the high diversity is that sexual reproduction is occurring. If this is the case it is of considerable importance, as sexual reproduction of phylloxera could significantly increase the risk of adaptation to resistant rootstocks and long-range dispersal (reproductive winged forms) occurring in the field. Strong associations of strains with specific rootstocks were not found, and vines were frequently found to have more than one strain of phylloxera on their roots. Currently, there is a single PIZ in northeastern Victoria. Our new data has revealed several groups of genetically related phylloxera strains which appear geographically localized to regions within this PIZ. This evidence, together with the cluster of new (possibly sexually reproducing) variants appearing in the King Valley, might warrant a re-evaluation of the single northeastern PIZ to reduce the risk of novel strains being introduced into new areas. These results show that a larger state-wide survey is needed to update strain-specific distribution maps for other PIZs across Victoria, particularly where the genotypes are still unknown.

Background

The last detailed survey of PIZs in Australia was conducted almost twenty years ago (Corrie *et al.* 2002), with 83 distinct strains being identified. However, the King Valley, in the Victorian northeastern (NE) phylloxera infested zone (PIZ), was only very lightly surveyed for genetic variation (n=12 phylloxera), with G04 being the only genotype found to be present on grapevine roots (Corrie *et al.* 2002, Umina *et al.* 2007).

The aim of the current study was to examine genetic diversity in this region through targeted collection of a large number of phylloxera specimens from grapevine roots in multiple King Valley vineyards and use these results to update the distribution maps of phylloxera genetic variation in the NE PIZ. This data will be used to monitor the spread of phylloxera strains within the NE Victoria PIZ and develop new distribution maps of phylloxera genetic diversity in the region. These will better inform targeted surveillance approaches (based on strain presence), and post-incursion management decisions — such as the selection of strain-specific phylloxera-resistant rootstocks across this and other PIZs.

Phylloxera strains are believed to reproduce asexually (clonally) in Australia (Corrie *et al.* 2002). Strains in the current study were identified using DNA microsatellites, which are currently the only method available for identification of phylloxera strains in Australia (Corrie *et al.* 2002, Corrie *et al.* 2003, Corrie *et al.* 2004, Hoffmann & Corrie 2003, Umina *et al.* 2007).

Materials and Methods

Vineyard selection

Eight vineyards located in the King Valley in the North East (NE) Victoria PIZ were selected based on recent infestations, previous reports (Corrie *et al.* 2002, Corrie *et al.* 2003) and grower contacts sourced from networks in the wine industry. Previous phylloxera genotyping studies differentiated Milawa from the King Valley (e.g. Corrie *et al.* 2002) despite the regions being geographically very close, with Milawa being within the northernmost edge of the King Valley. For ease of comparison with previous work we have continued to utilise the previous naming convention below when discussing Milawa and the King Valley.

Prior to sampling, growers were contacted by phone for a brief overview of the vineyard operations and rootstock plantings. A vineyard was defined as a single viticultural operation, owned and operated by an individual company or person. Samples were collected in 2018 and 2019 between January and March when phylloxera populations are relatively high.

Sampling Protocol

Root and soil containing or suspected to have grape phylloxera were collected from roots of a variety of rootstocks (Appendix 7; Table 1). Vines in a block were initially dug at random in a suspect area to phylloxera infestation. When insects were absent on roots, efforts were aborted, and the search continued in a different block. Where at least one vine (called reference vine) was infested with phylloxera, two and three rows on either side of the reference vine row were selected for sampling, such that six rows in total were surveyed in each block. In each of the six rows selected, a total of up to five vines per row were sampled starting from the first full panel. A maximum of up to 30 root and soil samples were collected from each block (Appendix 7, Table 1).

Sample collection

Phylloxera samples were collected by digging a 15 x 50 cm hole in the soil near the vine trunk to access vine roots. Roots were inspected for the presence of insects and care was taken to ensure that roots sampled originated from below the graft union and insects sampled were feeding on the rootstock type. We aimed to collected adults, nymphs and/or eggs from four different areas around the same vine, if possible, to maximize the chance of detecting a variety of phylloxera. Young and mature-lignified roots were examined for the presence of galls and phylloxera life stages using a hand lens. Where phylloxera was detected or suspected, samples containing galls and roots were excised and placed in a zip-lock plastic bag along with moist soil to prevent the roots from drying. The plastic bags were placed in a cold storage container and transported to the laboratory at Agriculture Victoria - Rutherglen.

Soil, galls and root samples were carefully examined under a 10 x dissecting microscope. Phylloxera adults, intermediates, first instars and eggs were collected into a 1.5 mL Eppendorf tube with 100% ethanol and stored at 4°C for later DNA extraction and genotyping. Many individual phylloxera samples (i.e. eggs, nymphs, adults) were collected for testing per vine (up to four were used for microsatellite genotyping).

Microsatellite Genotyping and Analysis

Microsatellite genotyping was performed following a laboratory protocol (Blacket *et al.* unpublished) that screens the six phylloxera microsatellite loci used to define Australian genotypes (Corrie *et al.* 2002, Umina *et al.* 2007), modified to utilise fluorescently labelled primer tails (Blacket *et al.* 2012) and a multiplex PCR kit (Qiagen, Australia). Capillary separation of fluorescently labelled microsatellites was performed commercially by AGRF (Melbourne), using LIZ-500 size standards. The number of individual phylloxera tested ranged from 1 to 4 per vine. Genotyping of fsa files was performed using the microsatellite plugin in Geneious R11 (https://www.geneious.com). Microsatellite variation was analysed for population structure (AMOVA) and spatial geographic relationships (Spatial Autocorrelation) conducted in GenAlEx 6.502 (Peakall and Smouse 2006, 2012). Relationships between genotypes were estimated based on codominant genotypic distances calculated in GenAlEx and analysed through the construction of a Neighbor-joining tree in MEGA version 7 (Kumar *et al.* 2016).

Strain (Genotype) Mapping

Spatial analysis and maps were produced using ArcGIS® software (Arcmap™ 10.5.1) by Esri. ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. Spatial layers were obtained from the Victorian Government's corporate GIS database and Victorian Phylloxera zones were correct as of 29th July 2020.

Results

Sampling

More than 1400 specimens of phylloxera from multiple rootstocks were collected from eight localities (Appendix 7, Table 1), over two seasons in 2018 and 2019, during January to March, when phylloxera populations are most abundant. One vineyard, Milawa, was located at the northern edge of the King Valley, while the other seven sites were located within the King Valley at Cheshunt, Edi, Whitfield and Whitlands.

A total of 764 of these phylloxera were genotyped for six microsatellites. Of the 30 vines sampled per block, between 4 to 30 positive vines were found to be positive for phylloxera. For each vine, up to 3 phylloxera samples were genotyped. The total number of phylloxera samples tested per vineyard (block) ranged from 9 to 63 (Appendix 7; Table 1). Of the 764 samples tested, 39 did not yield a result. We excluded these samples from the analyses. Thus, the final data used for analysis was 725.

We detected a total of 36 genotypes, with 4 genotypes known from previous work (G04, G20, G35 and G41) and 32 new genotypes (G84 to G115) (Table 1). Overall, the most abundant genotype found in this study was G20 (26% of all samples), followed by G92 (12%), G104 (9%), G94 (8%) and G35 (7%). The remaining genotypes represented less than 5% of the samples (Table 2).

The distribution of genotypes was not uniform. For instance, three blocks from MIL-1 with vines on Schwarzmann rootstock had G20 or G35, whereas a fourth block on 1202 rootstock had a mix of three genotypes (G04, G20 and G109) (Table 1). Genetic diversity (as determined by the number of genotypes) was highest in a CHE-2 block with 14 genotypes, followed by three blocks with eight genotypes each (two WHF-1 blocks on Paulsen 10114 and Schwarzmann rootstocks, and a CHE-2 block also on Schwarzmann) (Table 1).

Genotype analyses by rootstock revealed a high genetic diversity of phylloxera (as determined by the number of genotypes) on Schwarzmann rootstock with 25 genotypes found, followed by 14 genotypes

found in an unknown rootstock in the CHE-2 vineyard and nine found on Paulsen 10114. Only three genotypes were detected in 1202 rootstock (Appendix 7; Table 2).

Genotype Results

Table 1 Genotyping results for each vineyard and block sampled (new genotypes indicated in grey)

Vineyard name	Location	Rootstock	Genotype	Count of Genotype	Overall frequency of genotype (%)	Block frequency of genotype (%)
MIL-1	Milawa	1202	G04	24	3.31	51.06
			G20	22	3.03	46.81
			G109	1	0.14	2.13
	Milawa	Schwarzmann	G20	19	2.62	100.00
	Milawa	Schwarzmann	G35	49	6.76	100.00
	Milawa	Schwarzmann	G20	33	4.55	100.00
CHE-2	Cheshunt	Schwarzmann	G94	16	2.21	55.17
			G95	12	1.66	41.38
			G96	1	0.14	3.45
		Unknown1	G94	13	1.79	48.15
			G95	7	0.97	25.93
			G96	1	0.14	3.70
			G97	2	0.28	7.41
			G98	1	0.14	3.70
			G99	2	0.28	7.41
			G114	1	0.14	3.70
	Cheshunt	Schwarzmann	G04	2	0.28	3.77
			G92	12	1.66	22.64
			G99	39	5.38	73.58
WHL-1	Whitland	Schwarzmann	G20	59	8.14	100.00
	Whitland	Schwarzmann/Riparia Grigio	G20	48	6.62	100.00
CHE-1	Cheshunt	Schwarzmann	G88	2	0.28	6.06

			G91	16	2.21	48.48
			G92	2	0.28	6.06
			G93	3	0.41	9.09
			G94	10	1.38	30.30
WHF-2	Whitfield	Schwarzmann	G04	4	0.55	8.33
			G20	2	0.28	4.17
			G105	33	4.55	68.75
			G106	6	0.83	12.50
			G107	2	0.28	4.17
			G108	1	0.14	2.08
WHF-1	Whitfield	Paulsen 10114	G94	2	0.28	22.22
			G103	3	0.41	33.33
			G104	4	0.55	44.44
	Whitfield	Paulsen 10114	G100	3	0.41	5.77
			G101	1	0.14	1.92
			G102	3	0.41	5.77
			G91	5	0.69	9.62
			G92	27	3.72	51.92
			G94	13	1.79	25.00
	Whitfield	Schwarzmann	G84	9	1.24	90.00
			G85	1	0.14	10.00
	Whitfield	Schwarzmann	G20	3	0.41	12.00
			G41	6	0.83	24.00
			G84	3	0.41	12.00
			G86	8	1.10	32.00
			G87	1	0.14	4.00
			G88	1	0.14	4.00
			G89	2	0.28	8.00
			G90	1	0.14	4.00
CHE-2P	Cheshunt	Schwarzmann	G101	1	0.14	1.67
			G102	1	0.14	1.67
			G88	2	0.28	3.33
			G92	47	6.48	78.33
			G94	2	0.28	3.33
			G95	4	0.55	6.67
			G96	1	0.14	1.67
			G99	2	0.28	3.33

	Cheshunt	Unknown2	G101	7	0.97	11.11
			G104	6	0.83	9.52
			G107	3	0.41	4.76
			G110	1	0.14	1.59
			G111	3	0.41	4.76
			G112	2	0.28	3.17
			G113	1	0.14	1.59
			G115	1	0.14	1.59
			G84	1	0.14	1.59
			G88	15	2.07	23.81
			G90	1	0.14	1.59
			G92	2	0.28	3.17
			G94	3	0.41	4.76
			G99	17	2.34	26.98
EDI-1	Edi	Schwarzmann	G104	57	7.86	93.44
			G105	4	0.55	6.56

Table 2 Genotype Frequency

(new genotypes indicated in grey)

Genotype	Count of genotype	Frequency of genotype (%)
G04	30	4.14
G20	186	25.66
G35	49	6.76
G41	6	0.83
G84	13	1.79
G85	1	0.14
G86	8	1.10
G87	1	0.14
G88	20	2.76
G89	2	0.28
G90	2	0.28
G91	21	2.90
G92	90	12.41
G93	3	0.41
G94	59	8.14
G95	23	3.17
G96	3	0.41
G97	2	0.28
G98	1	0.14
G99	60	8.28
G100	3	0.41
G101	9	1.24
G102	4	0.55
G103	3	0.41
G104	67	9.24
G105	37	5.10
G106	6	0.83
G107	5	0.69
G108	1	0.14
G109	1	0.14
G110	1	0.14
G111	3	0.41
G112	2	0.28
G113	1	0.14
G114	1	0.14
G115	1	0.14
Grand Total	725	

Phylloxera Genotype Relationships

Our large-scale survey of the King Valley region in the current study brings the total number of known Australian phylloxera strains from 83 to 115, a 39% increase in genetic diversity. The new genotypes detected (Figure 1) are closely related to each other and to genotypes previously detected in Milawa in the earlier survey (Corrie *et al.* 2002), which as noted above is located at the northern edge of the King Valley. Most genotypes are localized to single regions, while some - G01, G04, G35, G37, G41 - appear more widespread, having been found at multiple locations.

Interestingly, there is no new allelic variation (i.e. no new microsatellite size fragments) found in the King Valley, only new combinations of previously characterized (Umina *et al.* 2007) alleles. Most of the new genetic variation was detected within the King Valley at two locations – Cheshunt and Whitfield – with many of these new genotypes found only at these locations (Figure 2).

Previous studies from the Milawa region sampled in the year 2000 included phylloxera samples from roots (sites ML1 and ML3, n=16) and from leaf galls (site ML1, n=33) (Corrie *et al.* 2002). In this previous study, the roots at site ML1 all had the G04 strain present, whereas the leaf galls of the same vineyard had predominantly G34 and G41 strains, with G05 being the only genotype found to be shared between roots and the leaves in this region. Likewise, the root system in the ML3 site were all found to be a single genotype, G04.

Unfortunately, it is not possible to directly link the sites sampled previously with our current study (Hoffmann *pers comm*). In our survey we found that there has probably been an increase of the G20 genotype on roots at Milawa, as this was the dominant genotype found across three of the four blocks sampled from this region (Table 1). Interestingly, the oldest vines (circa. 1937) sampled in our study, on rootstock 1202, were found to possess a mixture of G04 and G20 (Table 1). Also, the genotype G05 found in the older studies on roots and leaves was not detected on roots in our study (note, leaf samples were not surveyed in the current study, but would be valuable to include in future studies).

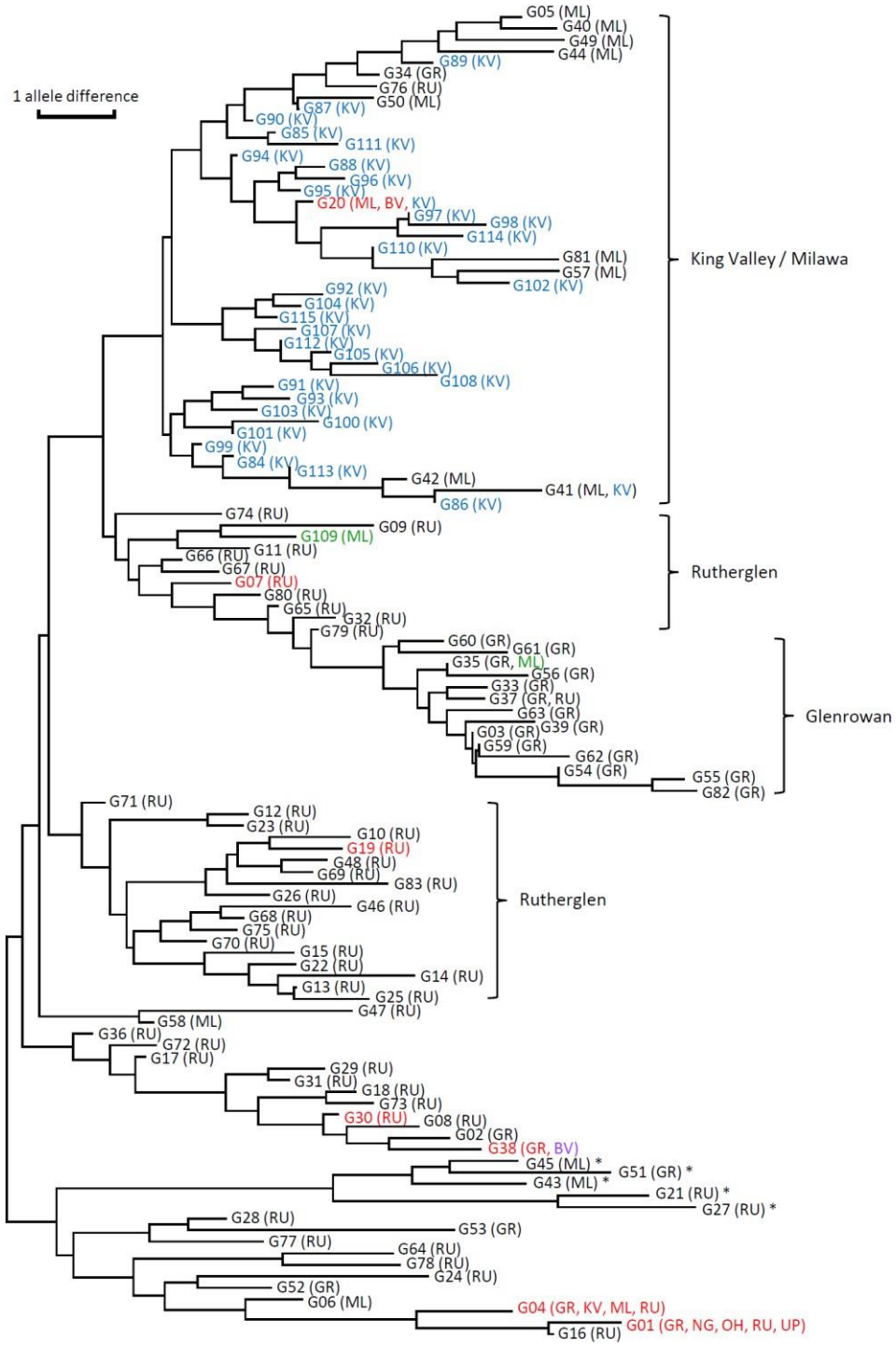


Figure 1 Relationship between 115 Australian phylloxera genotypes (Neighbor-joining tree). Black colour indicates previously recognized (x 83) Australian phylloxera genotypes (Umina *et al.* 2007), red indicates genotypes maintained as laboratory colonies at Rutherglen (AgVic), blue indicates new King Valley (KV) genotypes, green new Milawa (ML) genotypes, purple new Buckland Valley (BV) genotypes. Five existing genotypes previously described from incomplete microsatellite data (Umina *et al.* 2007) are indicated by an asterix. Groups of genotypes primarily localized to a geographic region are indicated by bracket and the region's name. Other location abbreviations follow Umina *et al.* 2007.

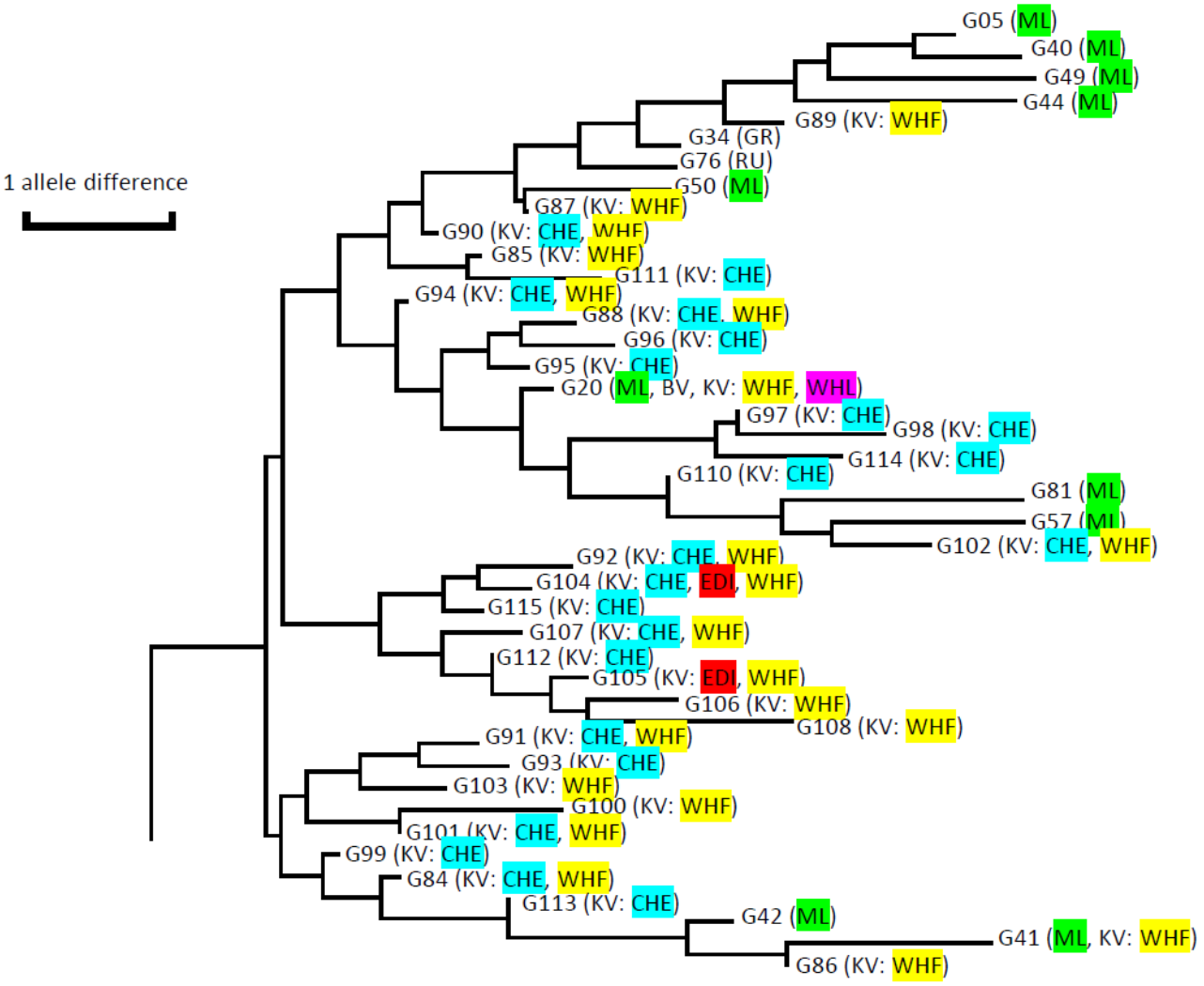


Figure 2 Detail of relationships between Australian phylloxera genotypes in the "Milawa / King Valley" group (Neighbor-joining tree). Colors indicate sampled Milawa / King Valley subregions: ML = Milawa (green), WHF = Whitfield (yellow), CHE = Cheshunt (blue), EDI = Edi (Red), WHL = Whitlands (purple).

Patterns of microsatellite variation

Distribution of genetic variation between sites

Phylloxera genetic variation was found to vary greatly between sampled sites, with some sites possessing a single genotype, while others had up to 14 different genotypes present in a vineyard block (Table 1, Figure 3).

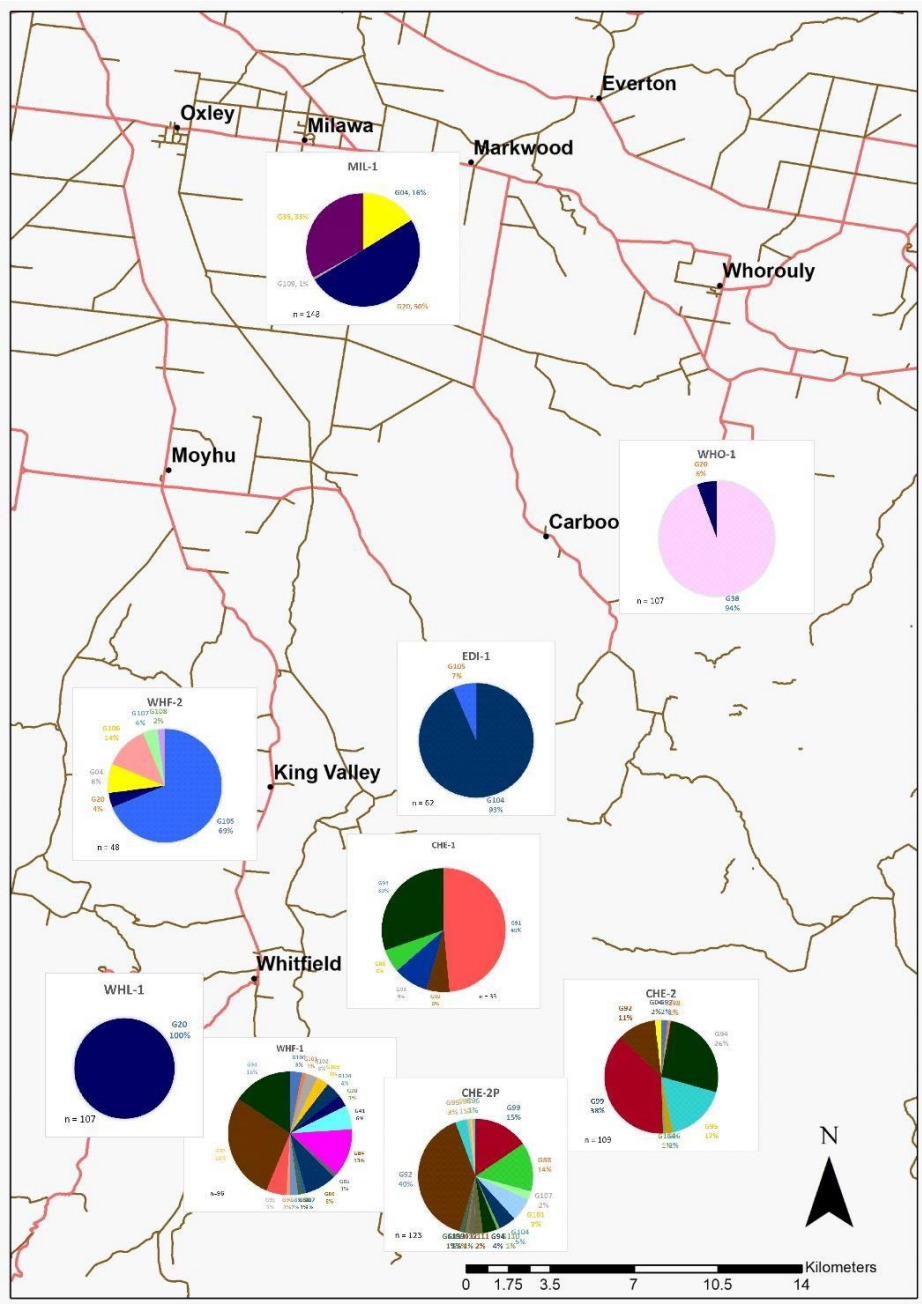


Figure 3 Distribution of phylloxera genotypes across King Valley vineyards. Each colour represents a different genotype.

Geographic partitioning of genetic variation

An analysis of molecular variance (Figure 4) indicated that there was generally a high level of variation found between individuals, with most of the genetic variation found between individual phylloxera (78%), with some variation localized to blocks (15%) and vineyards (7%). This indicates that a reasonably large amount of the genetic variation observed (>20%) was found to be localized to specific geographic areas, i.e. vineyards and blocks.

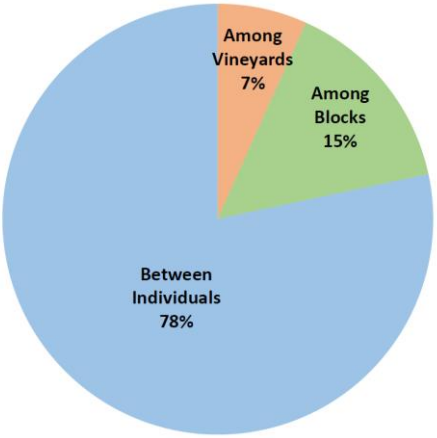


Figure 4 AMOVA analysis (GenAlEx) comparing microsatellite genetic variation among individuals, blocks and vineyards.

Further indications of geographic localization of genotypes was shown by the spatial autocorrelation analysis (Figure 5). Positive correlations were indicated between some adjacent sites, indicating genetic relatedness of these samples, at geographically close sites (2km and 4km) as well as some more distant sites, which were up to 10 km apart. Negative correlations, indicating non-relatedness between sites, were apparent at sites located 8km, 12 km and 40km apart. This indicates a general relationship between the proximity of sites and relatedness of strains, with sites which are geographically close (less than approx. 8-10 km apart) more likely to be similar genetically than they are to more distant sites (greater than approx. 8-10km apart).

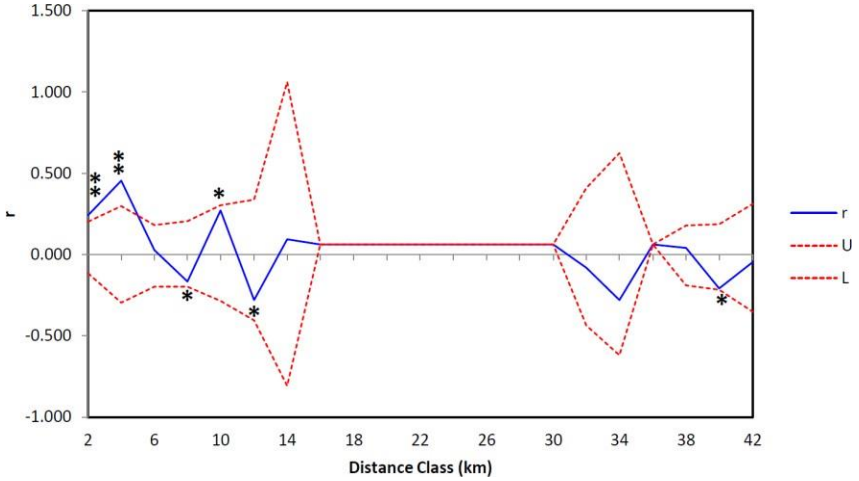


Figure 5 Spatial autocorrelation analysis of microsatellite variation (GenAlEx), with 2km distance classes, including all samples from Milawa and the King Valley. Double asterisk indicates significant correlations P<0.01, single asterisk indicates significant correlations P<0.05.

Regionalization of genetic variation

An examination of the combined microsatellite genotype data from previous genetic surveys (Umina *et al.* 2007) and the current study reveals a general pattern of non-random distribution of phylloxera strains in the north eastern PIZ (Figure 6). The two regions previously extensively sampled, Rutherglen and Glenrowan, both predominantly have unique strains of phylloxera, with a small number of strains that are more widespread. The large-scale survey of the Milawa / King Valley conducted in the current study reveals a very similar pattern to the other two regions, with a large number of strains almost entirely localized to this region, with two widespread genotypes (G04 and G35) also present.

Rutherglen: G01, G02, G04, G08, G09, G10, G11, G12, G13, G14, G15, G16, G17, G18, G19, G21, G22, G23, G24, G25, G26, G27, G28, G29, G31, G32, G36, G37, G46, G47, G48, G64, G65, G66, G67, G68, G69, G70, G71, G72, G73, G74, G75, G76, G77, G78, G79, G80, G83

Glenrowan: G01, G02, G03, G04, G33, G34, G35, G37, G38, G39, G51, G52, G53, G54, G55, G56, G59, G60, G61, G62, G63, G82



Milawa / King Valley: G05, G04, G06, G20, G35, G40, G41, G42, G43, G44, G45, G49, G50, G57, G58, G81, G84, G85, G86, G87, G88, G89, G90, G91, G92, G93, G94, G95, G96, G97, G98, G99, G100, G101, G102, G103, G104, G105, G106, G107, G108, G109, G110, G111, G112, G113, G114, G115

Figure 6 Regionalization of phylloxera genotypes in the north eastern Victoria PIZ. Genotypes shared between multiple areas are shown in red; while black / green / blue genotypes have been found only in a single one of these regions to-date (combined data from Umina *et al.* 2007 and the current study).

Discussion and recommendations

Novel genetic variation in the King Valley

Most of the NE PIZ is believed to have had phylloxera present for more than a century (Corrie *et al.* 2002, Powel *et al.* 2013), while phylloxera are believed to have been introduced to the King Valley relatively recently (early 1990's, Corrie *et al.* 2002). Previously only a very limited genetic survey had been conducted in the King Valley, with only G04 known to be present predominantly in the roots of grapevines (Corrie *et al.* 2002). The current survey has revealed that the amount of genetic diversity present in the King Valley is actually very high, with many novel genotypes present. Contrastingly, only a single new genotype (i.e. one individual of G109) was detected from Milawa, despite the Milawa samples representing one fifth of all the phylloxera examined.

Corrie *et al.* 2002 regarded the high level of genetic diversity present in northeastern Victoria (as indicated by the presence of 83 strains in the region) to be predominantly due to asexual (parthenogenetic) reproduction, based on the widespread presence of some heterozygous genotypes, such as G01 and G04. They note that if sexual reproduction was occurring "the majority of samples of any one genotypic class should be found within the same location in the presence of related recombinant genotypes", a pattern that exactly matches what we observed in the current study, with localization of related genotypes to regions. A possible explanation for the high diversity in the recently colonized King Valley may be that sexual reproduction has occurred, mixing up existing alleles into new genotype combinations, which are probably now reproducing asexually. If this is the case then it is the first evidence of sexual reproduction occurring in phylloxera in Australia to give rise to new strains. This finding is highly significant, as increasing the genetic diversity of phylloxera will increase the risk of resistance adaptation occurring in the field (Corrie *et al.* 2002).

Regionalization of Phylloxera Genetic Variation

Currently, there is a single PIZ in northeastern Victoria. The King Valley was previously recognized as a separate PIZ (e.g. Corrie *et al.* 2002) and the genetic data from the current study supports the genetic distinctiveness of the Milawa / King Valley region. Likewise, the Rutherglen and Glenrowan regions appear to predominantly consist of unique strains of phylloxera, while additional adjacent regions, such as the Buckland Valley, do not appear to be very genetically diverse. A re-evaluation of the current NE PIZ would be worthwhile in light of the findings of the current study. Rezoning the NE PIZ would obviously have major implications for the movement of equipment and produce between zones, but once done it would better reflect the underlying genetic structure of phylloxera populations within the region and would reduce the risk of novel strains being introduced into new areas.

Genotype Rootstock Relationships

Five different rootstocks, including three known and two unknown, were sampled in this study (Appendix 7, Table 2). Genetic diversity appeared highest in Schwarzmann (25 different genotypes), followed by Paulsen 10114 (8 genotypes), 1202 (3 genotypes) and Schwarzmann/Riparia Grigio (1 genotype). The two unknown rootstocks had considerable genetic diversity: rootstock "Unknown1" (from CHE-2 vineyard) had 7 genotypes and, rootstock "Unknown2" (from CHE-2P vineyard) had 14 genotypes (Appendix 7, Table 2).

Although not strong, there seems to be some level of genotype / rootstock interaction detected in the current study. Genotype G20 was the most common strain found in high frequency in Schwarzmann,

Schwarzmann / Riparia Grigio and 1202. This genotype however was not found in either of the two unknown rootstocks (despite these being planted next to Schwarzmann; Table 1) or Paulsen 10114. Similarly, G04 was found in high frequency in Schwarzmann and 1202 but not in the remaining rootstocks despite some being planted next to Schwarzmann (Table 1). The genotypes found at high frequency (> 10%) were G92 and G94 (Paulsen 10114), in unknown rootstocks were G94 and G95 (Unknown1), and G88, G99 and G101 (Unknown2) (Appendix 7, Table 2). Some of these genotypes were also found in Schwarzmann although in low frequencies. For instance, G94 was found in high frequency in Paulsen 10114 (24.6%), and in Unknown1 (48.2%) but in low frequency in Schwarzmann (6%).

There seems to be two rootstock groups; one with Schwarzmann, Schwarzmann / Riparia Grigio and 1202 that have 'old' and 'new' genotypes including G20 and G04 and, a second rootstock group with Paulsen 10114, Unknown1 and Unknown2 that exclusively have 'new' genotypes only.
This is the first Australian study to highlight the presence of multiple genetic strains of phylloxera on single vines. In some blocks, we found more than one genotype coexisting (Table 3).

Table 3 Frequency of vines with more than one genotype / vine

Vineyard	Location	Rootstock	Number of vines sampled	Number of vines with more than one genotype	Frequency of vines with more than one genotype (%)	Frequency of vines with one genotype
MIL-1	Milawa	1202	28	4	14.3	85.7
		Schwarzmann	10	0	0.0	100.0
		Schwarzmann	28	0	0.0	100.0
		Schwarzmann	30	0	0.0	100.0
CHE-2	Chesthunt	Schwarzmann	13	4	30.8	69.2
		Unknown1	13	3	23.1	76.9
		Schwarzmann	28	5	17.9	82.1
WHL-1	Whitland	Schwarzmann	30	0	0.0	100.0
		Schwarzmann/Riparia Grigio	26	0	0.0	100.0
CHE-1	Chesthunt	Schwarzmann	17	1	5.9	94.1
WHF-2	Whitfield	Schwarzmann	23	4	17.4	82.6
WHF-1	Whitfield	Paulsen 10114	3	2	40.0	60.0
		Paulsen 10114	27	7	25.9	74.1
		Schwarzmann	13	4	30.8	69.2
		Schwarzmann	6	0	0.0	100.0
CHE-2P	Chesthunt	Schwarzmann	30	5	16.7	83.3
		Unknown2	31	19	61.3	38.7
EDI-1	Edi	Schwarzmann	30	1	3.3	96.7
Overall Average					16.0	84.0

We found that when more than one phylloxera was sampled from single vines, multiple genotypes were often present on the roots, with 16% of the vines examined possessing more than one genotype. The frequency of multiple genotypes on a vine was not found to be correlated with the total number of vines sampled at a site (-0.1), however it was moderately positively correlated (0.6) with the total number of genotypes present at a site. For instance, out of the 13 vines sampled from the WHF-1 block, four vines (31%) had two genotypes e.g., vine B-1-25-11 had G86 and G87 and vine B-1-29-11 had G20 and G84 (data

not shown). This finding has implications for interactions between phylloxera strains and rootstocks, and for the level of sampling required to ascertain which phylloxera strain is present on a grapevine.

Update of state-wide distribution maps with recent detections

The results from this study imply that there could be much more diversity in phylloxera than previously thought, and genotypes could be widely distributed in PIZs. This information would enable region-specific rootstock recommendations to be developed. There is, therefore, need for a larger state-wide survey to update strain-specific distribution maps for other PIZs across Victoria especially where the strain identity has not been confirmed. To monitor the spread of endemic strains within PIZs, the phylloxera distribution map was updated (Figure 7). The distribution maps are integral to informing targeted surveillance approaches (based on endemic strain presence), and post-incursion management decisions—such as the selection of strain- specific phylloxera-resistant rootstocks across a region.

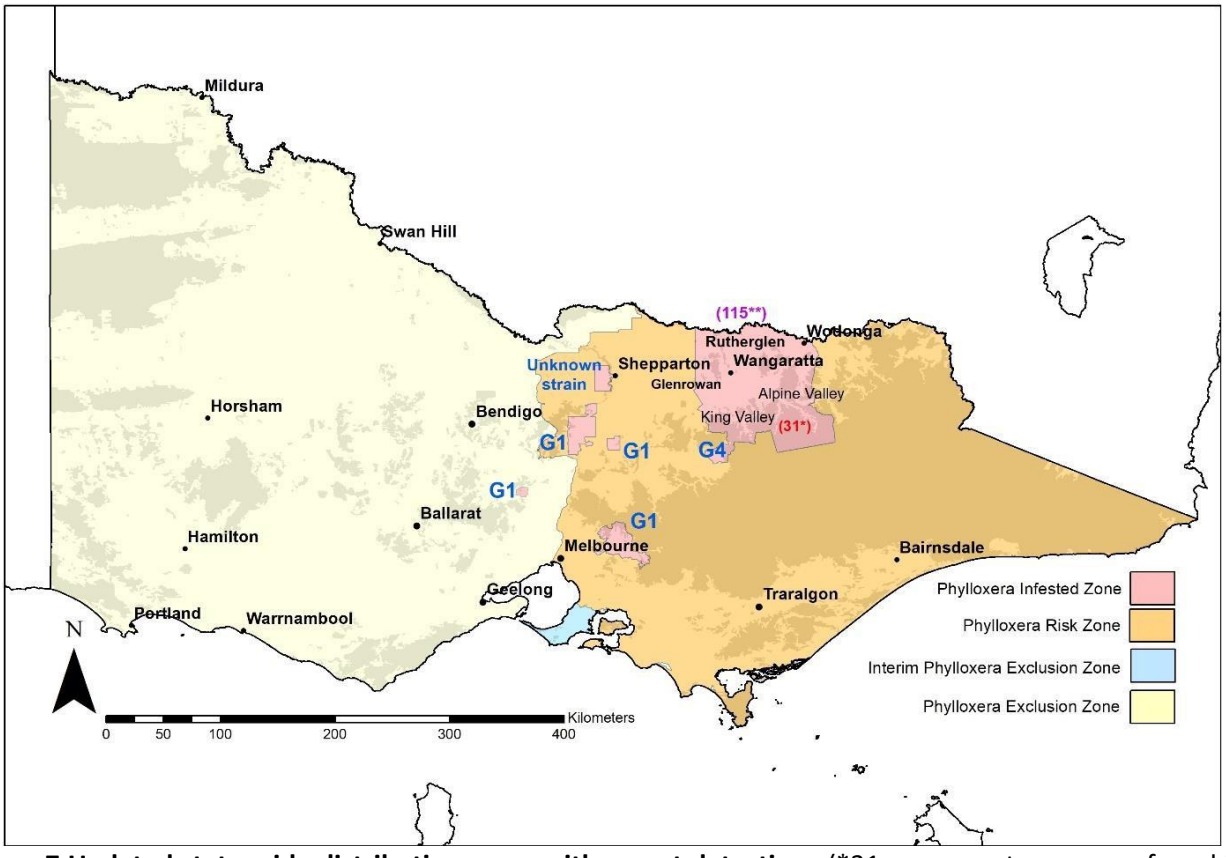


Figure 7 Updated state-wide distribution maps with recent detections (*31 new genotypes were found in the King Valley region; ** current genetic diversity in the North East PIZ is 115 up from previously known 83)

RESEARCH THEME 3

IMPROVED QUARANTINE PROTOCOLS

11.0 IMPROVED QUARANTINE PROTOCOLS

11.1. EVALUATION OF NEW TREATMENTS FOR DISINFESTATION OF FOOTWEAR AND HAND-HELD TOOLS.

Summary

Household bleach is currently recommended for disinfecting footwear and small hand-held tools against phylloxera, but this product is not favoured by growers. Off-the-shelf products were reviewed and scored using a matrix that looked at availability, safety and practicality. Twenty-two products were then tested in laboratory assays using first instar G4 phylloxera in 30 or 60 second immersions, with or without a follow-on water rinse. Products that were effective were further screened across genetically diverse strains (G1, G19, G20, G30 and G38). Only Dettol (5% active ingredient Chloroxylenol) achieved 100% mortality against the six phylloxera strains tested and was deemed suitable for use in a practical setting. We can therefore recommend Dettol as a suitable substitute to bleach for disinfestation of footwear and handheld tools.

Background

People movement, vineyard tools and clothing have been a source of concern with regulators and vignerons due to the risk they pose in dispersing phylloxera. People encounter first instars on shoes and clothing during the peak reproductive period, when this “crawler” life stage moves above ground and onto the soil surface, and the fruit, foliage and stem of the vine. Above-ground phylloxera populations are particularly prevalent during vintage, a time when risk of dispersal via people and machinery is highest (Herbert at al., 2006).

Quarantine protocols have played a major role in containing phylloxera infestations within infested zones (PIZ) in central and North East Victoria and South East New South Wales. Specific procedures for movement of people, tools, vine material, machinery and equipment are recommended and specified in the National Phylloxera Management Protocol document (National Vine Health Steering Committee 2009). These procedures have recently been validated (Powell 2017; Clarke et al. 2017, Clarke et al. 2018a, b) with recommendations to re-examine durations of exposure, temperature thresholds, rates of chemicals and the consideration of diverse phylloxera strains in order to ensure effectiveness. The research indicated that virulence differences between phylloxera strains are likely to increase the risk of spread in some regions, due to high population build -up of the pest under some environmental conditions, especially if a disinfestation procedure is ineffective.

Farm gate practices that involve disinfesting tools (such pruners, trowels, shovels, shears, fertilizer dispersers, and tagging machines) and managing people movement prior to moving from one vineyard to another are a high priority. This is currently achieved by immersing footwear and tools into a bath of 2% sodium hypochlorite (NaOCl) for 60s without a water rinse afterwards (Clarke et al. 2017). Omitting a water rinse after treatment, however, damages footwear and corrodes handheld tools, further making bleach unfavourable for uptake by growers. Furthermore, chlorine residues and odours have been highlighted as an occupational health and safety hazard. The recommended one-minute immersion in a bleach bath is regarded as being too long in terms of practicality. A shorter immersion time and the option to rinse off harmful residues using recommended disinfestation products would be beneficial from a practical perspective provided that these are effective against all phylloxera strains.

Thus, an evaluation of alternative disinfestation treatments was conducted on products that are commercially available and/or recommended by the wine industry.

Methods

Insects

Eggs of G1, G4, G19, G20, G30 and G38 phylloxera were collected from excised roots using an artist's paint brush and placed on moistened filter paper in a 90 x 25mm Petri dish. The Petri dishes were sealed with cling film to create a hatching chamber and incubated in the dark at a constant temperature of 22°C. Eggs were monitored daily and newly emerged active first instars were collected and used for all experiments.

Initial product screening and evaluation

The initial list of products was based on industry suggestions and a desktop review of pest control products in orchards, with consideration for those which are acceptable for use in vineyards. Seventeen products were evaluated through an assessment of availability, safety, practicality (ease of use), and cost (calculated as 5 ml active ingredient per footbath as per the manufacturers recommendations) (see Figure 1 and Appendix 8). Products that were deemed suitable against these selection criteria were then screened for effectiveness against G4 phylloxera for 30 and 60 s, with and without a water rinse afterwards. The G4 phylloxera was used in the preliminary screening because it is one of the most virulent strains and it populates well in laboratory cultures, hence sufficient numbers were available when needed for experiments. For products that achieved 100% mortality for a given treatment combination (immersion time and rate of active ingredient (a.i)), screening was then performed against five other phylloxera strains.

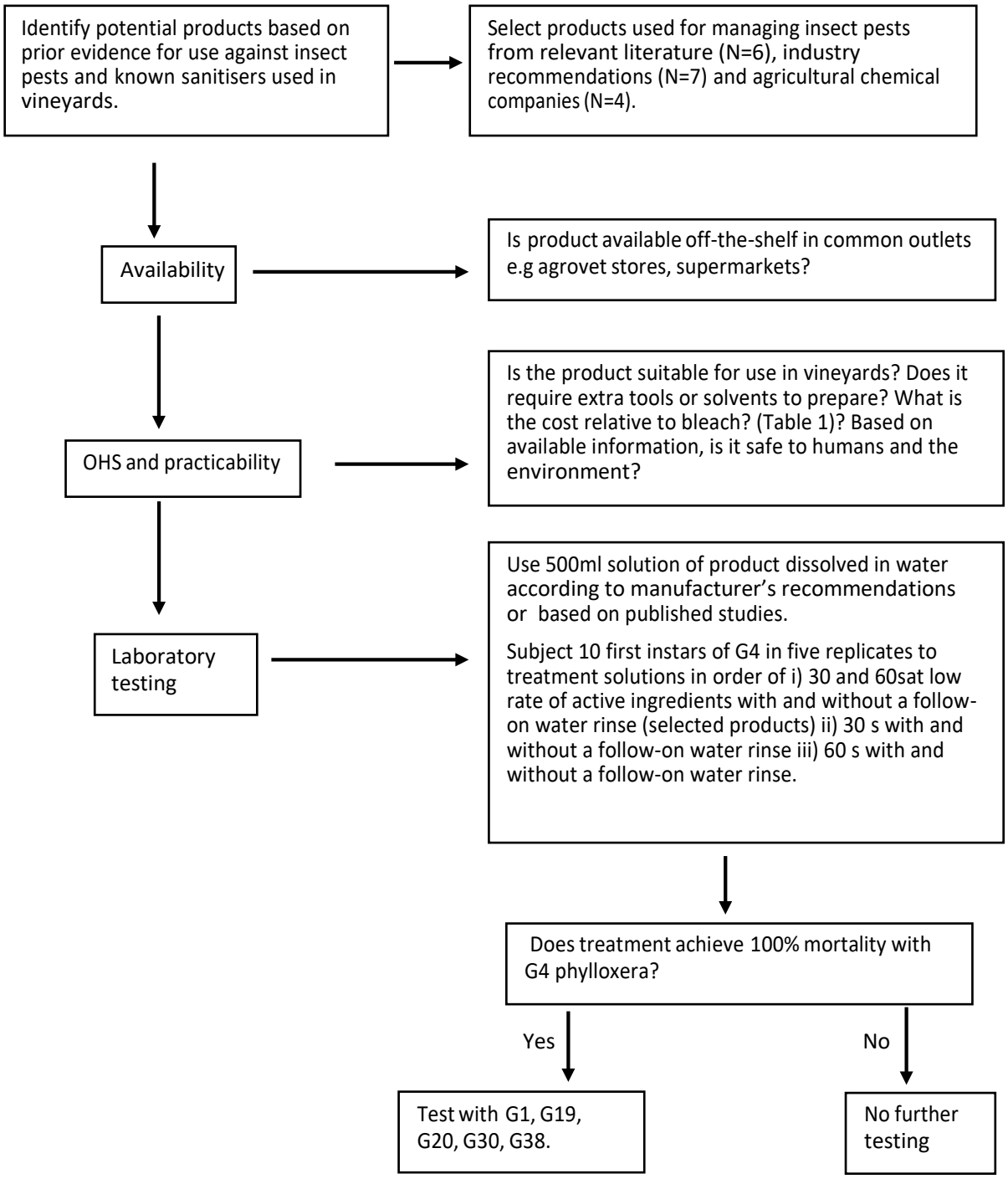


Figure 1 Process flow and decision-making matrix used in selecting and testing effectiveness of products against grapevine phylloxera.

Laboratory screening trials

Ten newly emerged first instars were placed in a plastic vial (5.5 m high x 2.5 m diameter) and subjected to treatment solutions in products (listed in Appendix 8) for 30 s, followed by a 30 s water rinse. This represented the "most preferable" treatment (short immersion time, water rinse). Where survival of G4 phylloxera was recorded (i.e. an ineffective treatment), treatments were then conducted without the water rinse (to see if rinsing, though preferable, was contributing to survival). In all trials where 100% mortality was achieved against G4, the sequence of trials (30s / 60s, with / without rinse) was repeated. Where a product was found to be effective against G4 (100% mortality), the experiment was repeated using G1, G19, G20, G30 and G38 phylloxera, to establish if the treatment product was effective across diverse strains.

Experiments were conducted in the laboratory at 22°C. A water control was conducted for the six phylloxera strains to compare the reliability and effectiveness of treatments. Each disinfestant and water treatment used five replications. 500 ml of test solutions was prepared in a beaker, using the dose specified for insect pests according to the manufacturers rates (Appendix 8), except for white vinegar and coca cola that were used undiluted.

Evaluating survival

Following each treatment, first instars were removed from vials using a sable-haired paintbrush moistened in water, placed onto clean filter paper in a Petri dish and examined under a low power microscope immediately and two hours after treatment. Where movement was observed, including antennal or tarsal movement when stimulated with the tip of the paintbrush, insects were classified as having survived (or dead with no apparent movement). The total number of first instars that were alive were recorded.

Statistical Analysis

Treatments that were tested against G4 were fully validated scientifically to select suitable candidates for footwear and tools by comparing effectiveness to the recommended bleach treatment (2% NaoCl for 60s without a follow- on water rinse). Data of seven products; bleach, ethanol, methylated spirit, Virkon, pulse penetrant, phytoclean and biopest were sourced from experiments conducted under the project DED1301 (Risks and Management of Endemic and Exotic Phylloxera; Powell, 2017; Clarke et al., 2017). These data were collated from testing conducted with the same phylloxera cultures used in this study and similar methods and conditions.

Counts of insects that survived in treatments was converted to percentage data (live insects/10 × 100) for each replicate. The percentage data were subjected to the non-parametric Kruskal-Wallis ANOVA. Disinfestant product was modelled as the group factor across the 23 products. The differences between the ranks for the 22 groups were then compared against a standard, using the test statistic H. The test statistic H was formed by ranking the combined data set, then considering the sum of these ranks within each sample. When there were at least five cases in each of the samples, H has approximately a Chi-square distribution on K-1 degrees of freedom, where K was the sample size.

For first instars that survived in each disinfestant product type, a logistic regression was fitted, and odds ratios calculated from the regression parameters. The probability of first instars that survived in each disinfectant product type for each of the treatment combination (time/concentration/rinse) were

calculated using the odds ratio via the formula p=(ez/(1+ez)) where p = probability and z is the relevant odd ratios for each treatment combination.

All residual values were examined graphically to ensure normality and homogeneity of variances. Observations with standardised residuals greater than 3.0 were excluded from the analyses. This impacted <1% of datapoints evaluated. There were no cases where excluded datapoints indicated survival for time–disinfectant combinations. A permutation test with 5000 iterations were also performed to check the F-probabilities due to a relatively small number of residual degrees of freedom in the ANOVA). All analyses were performed in GenStat 18th Edition (VSN International 2016).

Results and discussion

The initial matrix selected 26 potential products for testing against phylloxera. Four of the 26 products, Teracep, Byotrol, Kiwi Lustre and Citrox, did not pass the criteria for safety or practicality and were not selected for laboratory testing (Figure 1; Appendix 8). Teracep (a.i. Hydrogen peroxide) and Byotrol (a.i. dodecyl dimethylammonium chloride and benzalkonium chloride) were deemed toxic following a review of the potential hazards of the respective active ingredients. Kiwi Lustre and Citrox required a minimum immersion time of 120s and were regarded counterproductive as their use would require people to stand on a foot bath for longer than the current recommendation.

Twenty-two of the 26 products that passed the initial matrix screening were evaluated through laboratory bioassays that tested their effectiveness at killing G4 phylloxera (0% survival), using two immersion times (30/60 seconds) and follow-on treatments (with / without water rinse). Though farm-fluid and mineral turpentine passed the initial time test of 30 seconds with a follow-up water rinse, trials were terminated due to safety concerns. Testing with farm-fluid necessitated the use of a fume cabinet due to irritant odours while mineral turpentine was discontinued when test containers were corroded during the first replicate trial.

Lower concentrations of the active ingredient (than recommended by manufacturers) were trialled to evaluate if products were effective (0% survival) against G4 phylloxera and, therefore, would be favoured for reduced impact on people and the environment. Testing was performed using Dettol (2.5% *Chloroxylenol*), environsan (0.4% *Dodecylbenzenesulfonic and lactic acids*) and common salt (0.5% *sodium chloride*) because information was available based on the manufacturer's recommendations and a literature review showed that they were effective against other insect pests at an alternative low rate (Appendix 8). First instars of G4 phylloxera survived when subjected to solutions of the three products at a low rate of the respective active ingredient for 30 seconds, with and without a follow-on water rinse. Survival was also observed in Envirosan and common salt for 60 seconds, with and without a follow-on water rinse, and subsequent test solutions used the high rate of active ingredient (1% Environsan and 3.5% common salt). Dettol was, however, effective at 60 seconds at the lower rate against G4 phylloxera without a follow-on rinse, and hence further trials were conducted for G1, G19 and G20 phylloxera. Survival of 4%, 50% and 16% was recorded for G1, G19 and G20 phylloxera, respectively, and further trials with Dettol used the higher rate (5% *Chloroxylenol)*. Based on the finding that a low product rate was ineffective, further trials with G4 used the manufacturers recommendations.

A follow-on water-rinse when applied after a 30 second treatment was tested for 19 products to evaluate short duration disinfestations that would be effective across diverse phylloxera, an option that would offer a more practical solution to the grape and wine industry. None of the products were effective with G4 phylloxera, therefore, 60s seconds follow-on water treatment was further conducted, nonetheless with similar results. The 19 products were further tested for 30 seconds without a follow-on water rinse, where Dettol, ethanol and methylated spirit were effective (0% survival) against G4 phylloxera (Figure 2). The latter three products were then further tested with five other genetic strains. Results showed that the 30 seconds treatment without a follow-on water rinse was effective for G1, G19, G20, G30 and G38 phylloxera with methylated spirit (Figure 2). The 30 seconds non-water rinse treatment was, however, ineffective for G19 phylloxera with Dettol and ethanol where, 4% and 9% first instars survived, respectively. This finding corroborates with recent studies by Clarke et al., (2017) where bleach treatments that omitted a follow-on water rinse at 30s were found to be ineffective across diverse phylloxera strains. Omitting a water-rinse step allows the active ingredient more contact time to penetrate the cuticle allowing oxidation or protein degradation to take place (Bloomfield 1996), which leads to mortality.

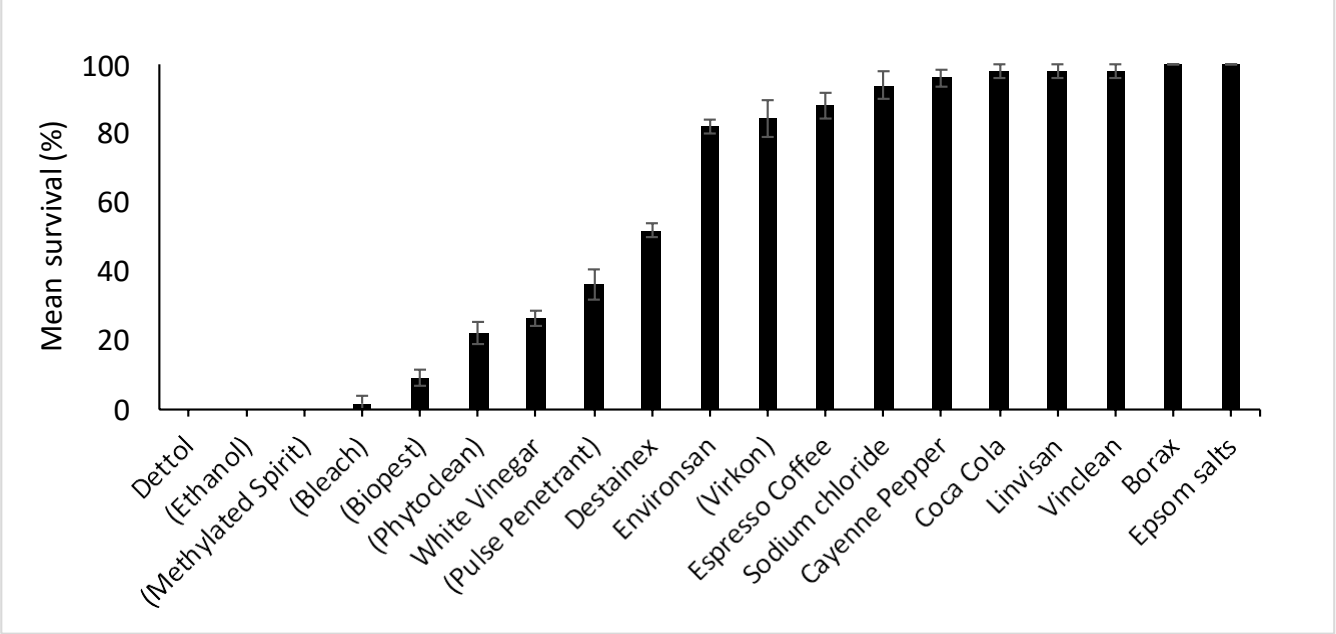


Figure 2 Mean percentage survival of first instar G4 phylloxera when subjected to test solutions of selected products for 30 seconds without a follow-on water rinse.

Further testing of 22 off-the-shelf products was conducted to evaluate effectiveness at killing G4 phylloxera (0% survival) after 60 seconds using the rate of active ingredient recommended by manufacturers and without a follow-on water rinse. Cayenne pepper, destainex, klorkleen, borax, epsom salt, common salt and coffee were considered safe for testing against G4 phylloxera but none of the products were effective (Figure 3). Practicality issues also arose with cayenne pepper, which required hot water and five drops of soap as a surfactant to dissolve and the experiments were conducted using a stirrer in solution. Destainex, klorkleen, borax, coffee, epsom and common salts were dissolved in water heated to 40-60°C and cooled to

room temperature. Three products, Dettol, ethanol and methylated spirit achieved 100% mortality of G4 first instars (Figure 3) and were fully evaluated for effectiveness against five other phylloxera strains.

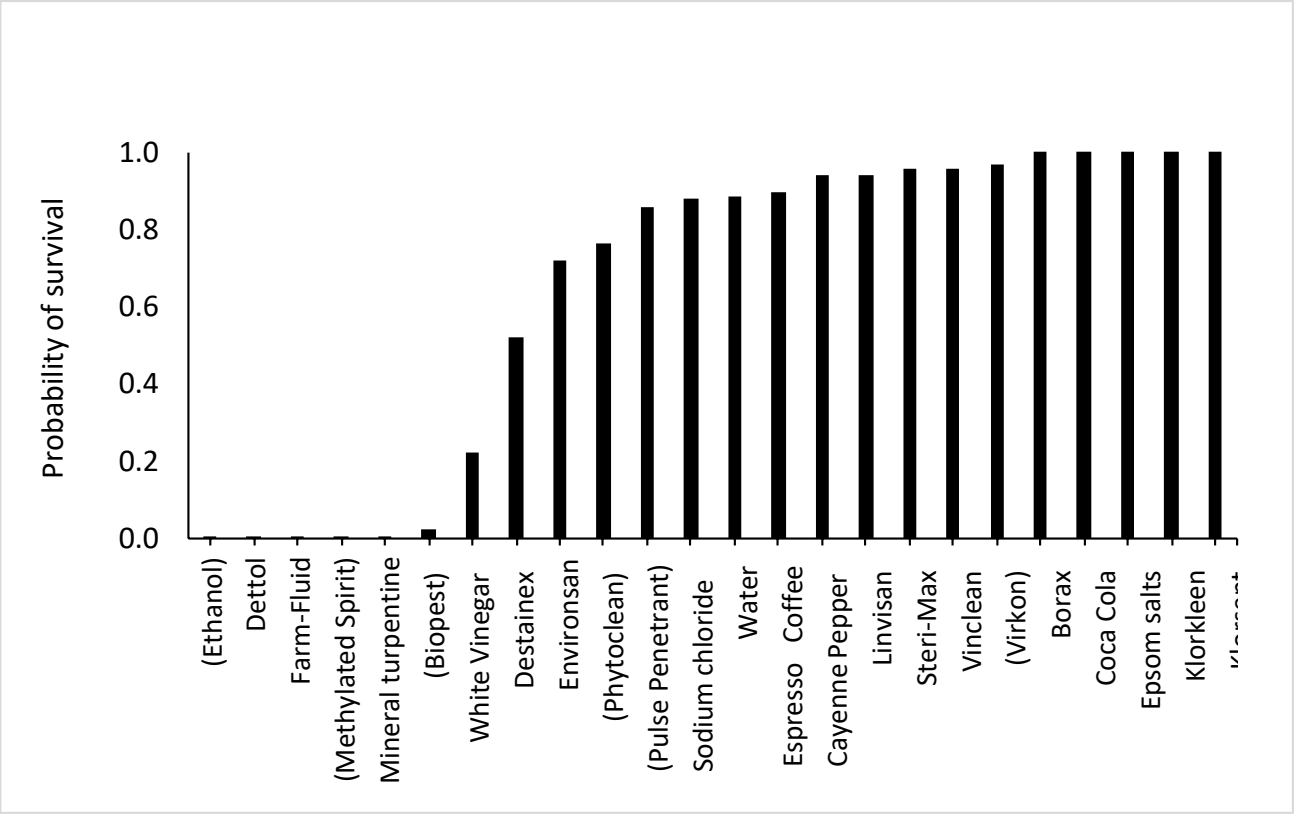


Figure 3 Probability of survival in a range of products when first instars of G4 phylloxera were immersed in solutions for 60 seconds without a follow-on water rinse. A logistic regression was fitted, and odds ratio estimates from the regression parameters used to calculate the probabilities that are reported on the graph. In brackets, data used to compare product effectiveness for G4 phylloxera was obtained from project DED 1301 (Powell 2017).

Dettol and methylated spirit were the only two products found to be effective (0% survival) across all six strains at 60 seconds without a follow-on water rinse treatment (Table 1) and their efficacy compares with the current footwear recommendation of bleach (2% NaOCl). Significant differences between genetic strains ($p>0.001$) were observed with ethanol, which was effective for G19, G30 and G38 phylloxera, but not G1 and G20 (Table 1). A study by Grzegorczyk and Walker (1997) found that phylloxera eggs survived in 50% ethanol after a seven-minute treatment. Ethanol at 70% ethanol is commonly used in tissue cultures and is a widely used fixative in research (Stanta et al., 2006). The mode of action for ethanol (active ingredient ethyl alcohol) is tissue shrinkage and hardening (Moelans et al., 2011) and more contact time (>60 seconds) and a high concentration of the active ingredient (>80%) could be required to disinfect phylloxera contaminated items. However, the time threshold required for ethanol effectiveness has not been validated scientifically and its use should not be recommended for phylloxera disinfestation at this stage.

Table 1 Mean percentage first-instar survival (± standard error) of six phylloxera strains (G1, G4, G19, G20, G30 and G38) in Dettol, methylated spirit and ethanol compared to bleach (recommended for phylloxera disinfestation) after a 60 seconds disinfestation treatment without a follow-on water rinse.

Product name	G1	G4	G19	G20	G30	G38
(Bleach)	**0**	**0**	**0**	**0**	**0**	**0**
Dettol	0	0	0	0	0	0
(Methylated Spirit)	0	0	0	0	0	0
(Ethanol)	0	0	5 ± 3	0	12.22 ±4	2.5 ± 3

Kruskal-Wallis one-way ANOVA was calculated for first instar survival in solutions of the four products across six genetic strains. In brackets, data used to compare product effectiveness across the six phylloxera was obtained from project DED 1301 (Powell, 2017). In blue font, bleach (2% NaOCl) is currently recommended for footwear and small tools disinfestation.

An effective disinfestant should offer rapid mortality across phylloxera strains, be non-phytotoxic and non-corrosive to tools and footwear. Efficacy of a disinfestation treatment is particularly significant because phylloxera reproduces asexually and it only takes one individual that survives treatment to start the spread. The risk of transfer in shoes or small tools is especially high during vintage when workers move between zones and the season corresponds to peak phylloxera activity when the most mobile and dispersive life stages are above ground.

Of the products tested in this study, Dettol appears to be most suitable due to its ease in preparation under field conditions, relative safety, and its accessibility from outlets, such as supermarkets. Though results showed that methylated spirit is effective as a disinfestant product at 30s across six phylloxera strains tested, flammability concerns negate its recommendation for field use. Active ingredients of common disinfestation products used in agricultural practices include hypochlorite (e.g. bleach), Quartenary Ammonium Compounds, phenols (e.g Dettol) and alcohols (e.g. methylated spirit) (Celar et al., 2007). Of the products used in this study, Dettol and methylated spirit contain these active compounds whose mode of action is the disruption of the cell membrane causing cell death (McDonell et al., 1991). This study demonstrated the importance of considering that pests and pathogens have different sensitivity, tolerance to toxicity and reactivity to disinfestants (Celar et al., 2007), and some products, though effective against pathogens, and easily accessible and practical, are not effective against phylloxera. For instance, products such as Linvisan, Vinclean and Virkon were selected for testing because they are effective disinfectants used in vineyards against pathogens while biopest, pulse penetrant and common salt have been used against aphids and mealy bugs (Mukhopadhyay et al., 2010). However, these products lacked the active ingredients and the probability of phylloxera survival was surprisingly higher (>0.90) than that in water.

Some compounds have been shown to achieve effectiveness if used in combination with other chemicals. For instance, a study by (Khan, 2003) demonstrated that common salt enhances efficacy of some broad- spectrum insecticides against mirids. A combination of a compound that is less toxic (though not investigated in this project), can allow vignerons to reduce the rate of chemicals in the environment while keeping the desired level of efficacy across phylloxera genetic strains. This current study provides a guide that could be used in selecting future products to use or test with potential to killing phylloxera and allows growers

and researchers to eliminate further product testing, making informed choices based on the mode of action that kills cells and specific for insects rather than disease-causing pathogens e.g. bacteria, viruses and fungi.

Phylloxera protocols for shoes and small hand-held tools aim to minimise corrosiveness caused by bleach and hence the recommendation to follow-on with a water rinse or coat items with oil (National Vine Health Steering Committee 2009). This procedure, however, is not effective in killing phylloxera and surviving insects do develop into reproductive adults as shown in a previous study with NaOCl (Clarke et al., 2017). Here, we explored products that are less corrosive alternatives, practical, cost effective and, achieved 100% mortality across a range of phylloxera strains and found that only Dettol satisfied these criteria.

Conclusions and Recommendations

This study provides an alternative option that is safe and effective across multiple phylloxera genetic strains. Growers should avoid the selection of alternative products simply on the basis that they are toxic to other pests. Dettol can be used as an alternative to bleach for the treatment of footwear and hand-held tools, at 5% for 60s duration. Rinsing with water is not recommended. Methylated spirit could be used as an alternative product for disinfecting small handheld tools where fire danger is minimal.

11.2. VALIDATING FERMENTATION TREATMENT AS A DISINFESTATION PROTOCOL FOR GRAPE PRODUCTS

Summary

Currently, the endorsed procedure in the National Phylloxera Management Protocols is for grape products to complete at least three days (72 hours) of fermentation to ensure disinfestation of phylloxera (Procedure D). The efficacy of this procedure was evaluated under laboratory conditions testing five phylloxera strains in red and wine ferments, with and without the addition of yeast, for 6, 12, 24, 48 and 72 hours. 100% mortality was achieved across all phylloxera strains when subjected to yeast fermentation of grape products lasting 48 and 72 hours. The results therefore validated that the current protocol is effective against the strains tested when fermentation takes place in presence of yeast, for both red and white wine ferments. 100% mortality was achieved across all phylloxera strains when subjected to fermentation lasting 48 and 72 hours, when yeast was added to grape products. However, phylloxera survived in ferments without yeast at 48 and 72 hour time points. The current procedure that allows movement of must or juice from a PIZ or PRZ into a PEZ requires completion of at least three days (72 hours) of fermentation; this is only valid when yeast is added to wine ferments. The fermentation protocol in the NPMP requires a review in light of these findings and new protocols should be developed and evaluated to determine mortality in naturally fermented products.

Background

Procedures that govern the movement of vine material, machinery and grape products exist within infested and risk areas (National Vine Health Steering Committee, 2009). The previous research project "DEP1301 Risks and Management of Exotic and Endemic Phylloxera" started the validation of the recommended protocols, with results revealing varied responses to both heat and chemical disinfestation treatments across diverse phylloxera strains (Powell, 2017; Clarke et al., 2017, 2018b). Consequently, recommendations have been made through respective State Biosecurity Committees and Vinehealth Australia to modify procedures for disinfestation of planting material, vineyard equipment, machinery, clothing and footwear (Agriculture Victoria, 2019; Vine Health Australia 2020).

Winemaking takes place through fermentation of grape must (red wine) and juice (white wine) and is driven by either added or naturally occurring yeasts, which metabolises and convert sugars into alcohol and carbon dioxide (Crabtree, 1929). Fermentation, therefore, is recommended for disinfestation of grape products such as must and juice before movement of such products from an infested zone ((National Vine Health Steering Committee, 2009; National Phylloxera Management Protocol; Procedure D). Fermentation is typically driven by commercial yeast, *Saccharomyces cerevisiae*, which can withstand high levels of carbon dioxide (Diaz, 2013), is cost-effective and easy to grow after storage for long periods in suspension (Romano, 2003). Adding *S. cerevisiae* to wine ferments is also favoured because the beginning of fermentation is controlled and not influenced by exogenous factors such as grape variety, microbial environment on skins and weather conditions (Marais, 2003).

However, some winemakers choose to use naturally occurring yeasts on skins of grapes for particular wine styles (Provenzano et al., 2010), in which fermentation is spontaneous (herein referred to as *wild* fermentation). In white winemaking, the grapes are pressed before fermentation, skins and seeds are discarded (through a strainer) and, juice is fermented at low temperatures (7-18°C). With red wine making, grapes are

crushed and the must fermented at 20-30°C, with the skins and seeds present to add the colour compounds and tannins (Berry 1998).

Grape harvesting takes place in summer (vintage) which coincides with peak phylloxera populations above and below ground (Herbert, et al. 2006). High numbers of crawlers above-ground can be present on vine stems, leaves and grapes and are a potential risk for transfer from the canopy via fruit, vine leaves and canes to the must and the marc (the solid remains of grapes after pressing).. The fermentation process potentially kills any insects that survive initial processing at the winery (e.g. washing and filtering), and current recommendations necessitate that a 72 h fermentation period has passed before moving grape products. However, this has not yet been scientifically validated. This study aimed to evaluate the effectiveness of fermenting wine for 72 hours against diverse genetic strains and wine types (red and white) and establish the minimum time required for fermentation to ensure phylloxera mortality.

Methods

Insects / fermentation products. Eggs of G1, G4, G19, G20, G30 and G38 phylloxera were collected from excised roots as described previously, placed in a Petri dishes (90 x 25 mm) sealed with cling film, and incubated in the dark at 25°C. Eggs were monitored daily and newly emerged active first instars were collected and used in the experiments. Cabernet sauvignon grapes were provided by Pffeifers winery (Rutherglen) and Phaedrus Estate (Mornington Peninsula) within 12-15 h of crushing. To replicate the red wine making process, must (skins and juice) of Cabernet sauvignon grapes (250ml w/v of skins and seeds and 250 ml juice) were aliquoted into 1 litre containers (130 x 90 mm). For white wine, grapes were pressed, skins and seeds discarded and 500 ml aliquots of juice placed in 1 litre containers. Both the red must and white juice were then stored at -20^{o}C until insects were available for experiments. Containers were defrosted overnight at 22°C prior to the start of each experiment.

Fermentation methods. Most winemakers use the yeast *Saccharomyces cerevisiae* in the winemaking process. Wild fermentations (in which the indigenous yeast on grapes drive the fermentation process), is however, gaining popularity. We therefore tested the effectiveness of moving grape products with and without yeast added, during the fermentation process as used in both red and white winemaking processes. The yeast *Saccharomyces cerevisiae* (>10^{10} cells/g dry vinification yeast) that was used in the experiments was supplied by Pfeiffer Wines (Rutherglen Victoria).

Validating the effectiveness of fermentation as a disinfestation procedure of diverse phylloxera strains as applied in the red winemaking process with added yeast.

Red wine ferments (RWFs) were prepared as follows; one teaspoon (2g) of dried *S. cerevisiae* was rehydrated with 15 ml warm ultra-pure water (40°C) in a 500 ml glass beaker; the water / yeast mixture was allowed to stand at room temperature (22°C) for 15 min; water /yeast mixture was added to the red grape must contained in a 1 litre cylindrical container (described in methods above); grape and yeast mixture was stood on the bench (stirring twice) for a further 30 min.

To assess phylloxera survival, 10 first instars of either G1, G4, G19, G20, G30 or G38 were collected into a single vial. Three replicate vials containing the first instars of a particular strain (Figure 1a) were then

submerged into the prepared grape and yeast solution (Figure 1b) and incubated at 22°C. Red wine fermentation causes skins and seeds to rise to the top of the liquid (called the *cap*) within the first 6-12 hrs of fermentation. Therefore, we used a plunge to push down the "*cap*" into the liquid every 24 hrs to keep the skins and seeds moist and to release ensuing carbon dioxide (as applied by winemakers to enhance colour and tannin development in red wines), while checking that the vials were fully immersed in the test liquids.

Survival of the first instars in the RWFs was evaluated after 24, 48, 72 h. To establish a lower time threshold for phylloxera survival in the RWF, treatments of 6 and 12 h were conducted for G4 and G38 (the strains were available in adequate numbers to conduct the experiments). A water control (ultra-pure water) treatment using three replicate vials of a particular phylloxera strains was set up at the same temperature. The experiment used a randomised complete block design with full factorial combination for each genetic strain and time combination (i.e. 3 replicates x 3 times x 6 genetic strains).

(a) (b)



Figure 1 Disinfestation vials (a) containing 10 first instars of a known phylloxera strain and red wine ferments in 1 litre plastic containers with a screw top (b) placed loosely to allow carbon dioxide to escape as used in the experiments

Validating the effectiveness of fermentation as a disinfestation procedure in white wine ferments with added yeast using G4 phylloxera

This experiment aimed at verifying that fermentation as used in white winemaking is consistent with the red winemaking process and used G4 phylloxera. G4 phylloxera were used due to ease of laboratory culturing and it being widespread within PIZs (Umina et al. 2007). First instars of G4 phylloxera were subjected to treatments in white juice inoculated with *S. cerevisiae* for 6, 12, 24, 48 and 72 h using the same procedure as described in (i) above. To mirror the white wine fermentation process as applied by winemakers, lower temperatures of 18°C (compared to 22°Cfor the red wines), were used.

Survival of G4 phylloxera in red and white wine ferments without added yeast -wild fermentations

Survival of phylloxera was assessed by subjecting first instars in RWFs and WWFs *without adding the commercial yeast S. cerevisiae,* at 6, 12, 24, 48 and 72 hours. The RWFs were incubated at 22°C and the WWFs incubated at 18°C. First instars were processed using the same procedures described for red and

white wine ferments in (i) and (ii) above and immersed in the non-yeast must and juice. Each fermentation treatment was replicated three times for each genetic strain and time combination (i.e. 3 replicates x 3 times x 6 genetic strains).

Assessing survival

A single vial of each genetic strain was removed at 24, 48 and 72 h time points and drained, and at 6 and 12h for G4 and G38 for the RWFs (with added yeast). A vial was also removed at 6, 12, 24, 48 and 72 h time points and drained for G4 phylloxera in WWF (with added yeast) and in WWF and RWF (without added yeast). First instars were carefully transferred from the vial using a sable-haired paintbrush and placed onto a filter paper where they were examined under a low-power microscope. Survival was determined if antennae and / or tarsi showed movement when touched with the tip of a paintbrush immediately after treatment, and 2h thereafter. Alcohol content was subsequently measured using a hydrometer based at Pfeiffer Wines, using standard wine practices (Berry, 1998).

Statistical analysis. Percentage first instar survival per replicate was determine for the six phylloxera genetic strains and analysed using ANOVA in GenStat (GenStat Release 2016). G4 survival at specific time points in WWFs and red wine wild ferments was explored using linear regression (R, Kruskal-Wallis with Dwass post hoc test, significance at $p<0.05$). The blocking factor was replication.

Results and discussion

Validating effectiveness of red wine fermentation with added yeast across six phylloxera strains

No first instar phylloxera survived in RWF with yeast added at 24, 48 and 72h (Table 1). These findings validate the effectiveness of the current recommendation for moving red wine that has been fermented for 72 hours, with consistent results across the six phylloxera strains trialled. The results also suggest that a 48 hour fermentation for red wine with yeast might also provide effective control.

A 24h fermentation was ineffective for G4 phylloxera where 16% first instars survived in treatments of RWF with added yeast. When tested at 6 and 12 hour, however, at least 60% first instars of G4 and G38 first instars survived in RWFs (Table 1). The proportions of G4 and G38 that survived in RWF at 12 hours were significantly different ($P=0.001$), at 62% and 10%, respectively (Table 1), indicating differences in survival between genetic strains.

Over 70% of first instars across the six genetic strains survived in the water treatments up to the 72 hour duration (Table 1), demonstrating that fermentation is a valid protocol for phylloxera disinfestation.

Table 1 Percentage mean (±) survival of first instars of six diverse phylloxera genetic strains in red wine ferments with the yeast, *S. cerevisiae* added. Data were analysed using a general ANOVA (LSD (5%)) in Genstat.

Treatments		Mean survival (%) Phylloxera genetic strains					
Time (h)	**Ferment**	**G1**	**G4**	**G19**	**G20**	**G30**	**G38**
6	RWF	*	78 ± 9	-	-	-	60 ± 10
12	RWF	-	62 ± 10	-	-	-	10 ± 6
24	RWF	0	16 ± 8	0	0	0	0
48	RWF	0	0	0	0	0	0
72	RWF	0	0	0	0	0	0
6	Water	-	100	-	-	-	98 ± 3
12	Water	-	97 ± 3	-	-	-	98 ± 3
24	Water	93 ± 3	97 ± 3	100	100	100	97 ± 3
48	Water	83 ± 3	80 ± 1	100	97 ± 3	97 ± 3	97 ± 3
72	Water	73 ± 2	67 ± 9	93 ± 3	97 ± 3	73 ± 3	90 ± 6

The alcohol content in the yeast-driven RWFs increased proportionately over the fermentation time, with 12.7% alcohol produced at the 72 h time point (Figure 2). Primary fermentation has been shown to start within 12hrs (Chambers 2010) and an estimated 70% of alcohol can be produced in some cases. A measure of alcohol content in wine ferments, in addition to the 72h time recommendation, can be used to determine the effectiveness of the fermentation process in killing phylloxera. A measure of alcohol content in wine ferments before moving grape products from an infested zone would be a complementary procedure that growers and biosecurity personnel could use at different time points to enable transport of grape products under different conditions such as when bottling needs to take place at different locations.

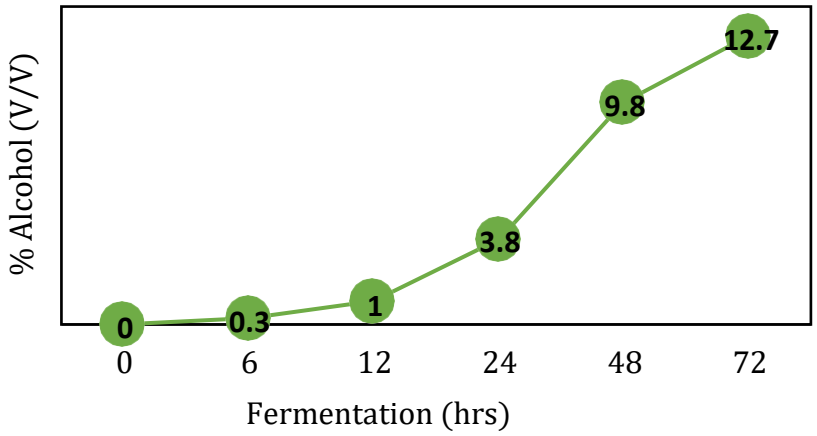


Figure 2 Changes in alcohol content as measured in red wine ferments with added yeast, *S. cerevisiae*

Validating the effectiveness of white wine fermentation (with added yeast) as a disinfestation procedure. Using G4 phylloxera, our results validated the current recommendation for moving white grape products for wine making after 72 hours of yeast-driven fermentation (Figure 3). The findings were also consistent with the RWFs in that first instars did not survive fermentation treatments at 48 hours.

A 24hr fermentation is not recommended as a disinfestation procedure. As for RWF treatments, first instar survival was observed for G4 in the WWF at the 24 hour time point (Figure 3).

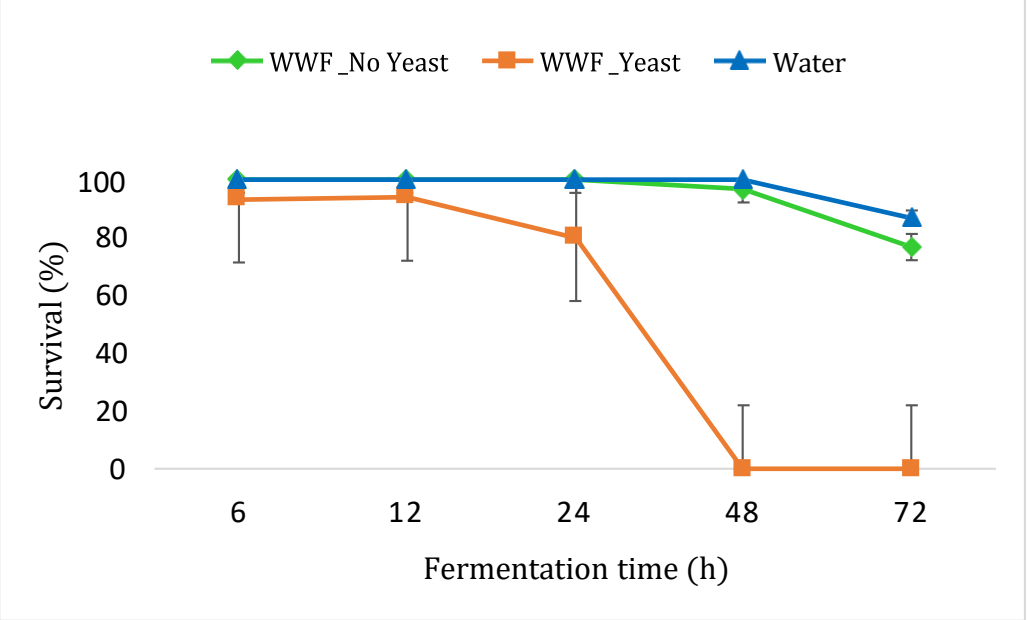


Figure 3 Mean survival of first instar phylloxera (G4) in white wine ferments with added yeast (WWF_Yeast) and without added yeast (WWF_No Yeast) compared to the water control treatment

Survival of G4 phylloxera in red and white ferments without yeast added

Statistical analysis (t-test) showed a significant difference in first instar survival between wild and yeast -driven RWF and WWF ($p=0.001$). Fermentation in the absence of yeast improved phylloxera survival (G4 only) compared to yeast ferments (Figure 3 and 4). When subjected to WWF without yeast, G4 first instar survival was similar to that of the water control for up to 72 hours (Figure 3). This is in contrast to yeast- driven WWFs where no first instars survived at the 48 and 72 hour time points (Figure 3).

The findings in RWFs were consistent with that of the WWF, where survival of G4 was observed at the 6,12, 24 hour time points in ferments without added yeast (Figure 4). However, first instar survival was reduced in RWFs without yeast compared to WWFs at the 24hr time point (Figure 3 and 4) and none of the G4 phylloxera first instars survived in the non-yeast RWFs at the 48 and 72 hour time points (Figure 4).

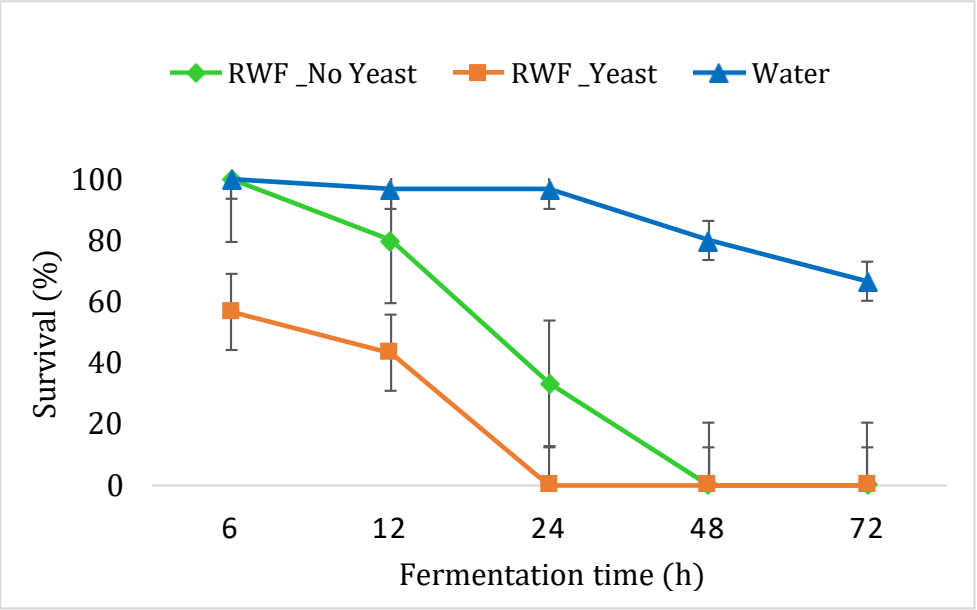


Figure 4 Mean survival of first instar phylloxera (G4) in red wine ferments with added yeast (WWF_Yeast) and without added yeast (WWF_No Yeast) compared to the water control treatment

Specific procedures are in place for movement of grape must and fresh juice from an infested or risk zone for the purpose of winemaking in an exclusion zone. Fermenting grape must and juice for 72 hours as specified in the NPMP (National Vine Health Steering Committee, 2009) is effective across the six phylloxera strains tested but only when yeast is added to wine ferments. The procedure in the NPMP does not specify if ferments are yeast driven or whether wines are fortified. In fortified winemaking, ferments are stopped with a high 'residual sugar content' usually after 2-3 days and a spirit added which kills the yeast (Chorniak, 2020). It is likely that spirits would kill any phylloxera present if the alcohol content is more that 80%. Our results, however, did show differences in phylloxera survival in the red and white wine ferments without yeast added, with increased survival that impacts on the effectiveness of the disinfestation procedure. If fermentation is halted after 24 hours, for instance because of a desired winemaking style, there would need to be guidelines for moving those grape products from a PIZ, as this time point, fermentation was ineffective against G4 phylloxera whether or not yeast was added to wine ferments.

Differences were observed between G4 and G38 at the 12 hour fermentation time point with added yeast in red wine ferments. This finding corroborates previous studies that have highlighted differences in survival of genetic strains at different disinfestation treatments, especially those based on temperature (Clarke et al., 2017; 2018b). In this study, there was no obvious indication of a rise in temperature in the ferments and the levels of alcohol would be the primary effect on phylloxera mortality. The strains used in this study are a representative of key clades that include both root and leaf galling. Result showed that the G38 phylloxera was more sensitive to the red wine ferments (with added yeast) compared to the G4 phylloxera. Studies to compare effectiveness of fermentation using more leaf galling phylloxera would be important as this form is highly likely to be in grape products especially during vintage.

Conclusions and recommendations

The results demonstrated that yeast and time of fermentation are critical factors to consider before allowing grape products to move from a PIZ. This study highlights the need to include a condition in the NPMP that the 72 hour recommendation is only effective when commercial yeast is added to grape ferments. Wild fermented grape products should not be moved from an infested zone. An alternative method to disinfest grape products where commercial yeast is not used in the winemaking process needs to be determined.

RESEARCH THEME 4

PHYLLOXERA BIOLOGY – KNOWLEDGE GAPS

12.0 PHYLLOXERA BIOLOGY – KNOWLEDGE GAPS

12.1 THE EFFECT OF TEMPERATURE ON PHYLLOXERA SURVIVAL, DEVELOPMENT AND REPRODUCTION UNDER CONTROLLED ENVIRONMENTS.

Summary

A better understanding of phylloxera strain-specific temperature tolerance could help with predictions for phylloxera distribution under climate change. The effect of temperature on phylloxera survival, development and reproduction was studied in laboratory assays, using temperatures of 18, 22, 26 and 30°C. Survival was evaluated on excised roots (i.e. with a feeding site), and also in the absence of roots (i.e. without a food source) under dry and wet conditions. On excised roots, only G1 phylloxera developed to adulthood at 18°C. Temperatures of 22 and 26°C were optimal for survival and reproduction across the five phylloxera strains tested (G1, G4, G19, G20 and G30). At 30°C and above, G19, G20 and G30 did not develop beyond egg hatch. These results suggest that the threshold temperature for establishment of a feeding site (for assimilation of nutrients) by phylloxera strains in Australia is <30°C, and that the optimal temperature to maintain a feeding site and develop to adulthood is >18°C. Survival at lower temperatures while phylloxera is not attached to a vine root was found to be longer than previously thought, with G20 strain surviving for up to 29 days at 18°C. This finding has implications for managing movement of contractor workers or vineyard machinery from PIZs to exclusion zones and highlights the importance of strict adherence to protocols for disinfesting clothing, footwear and machinery.

Background

The current geographic distribution of phylloxera strains in Australia appears to be relatively localised, with 119 strains identified in the North East (NE) region (Corrie et al. 2002; section 8.3 of this study). Of the 119 strains, the G1 strain appears to have spread widely outside the NE Phylloxera Infested Zone (PIZ). The reason for the localisation of the majority of strains has been attributed to favourable environmental conditions, particularly temperature, which could impact on strain distribution under climate change (McLeod 1990, Yin et al. 2019). In the cool climates, such as the Yarra Valley, phylloxera detections have increased gradually in the last two years (Agriculture Victoria 2019, Vinehealth Australia 2019), with now over 40 vineyards known to be infested. Leaf galling strains have recently been observed in NE Victoria, and a G38 strain having first been recorded on leaves in the Glenrowan region, has been found to adapt well on roots of commercial 'resistant' rootstocks (Further details in section 8.1).

Overwintering early stages (1st and 2nd instars) become active and start feeding on roots as soon as vine growth commences in spring (Herbert et al. 2006) while attached to feeding sites (nodosities and tuberosities). The insects reproduce asexually throughout summer. Eggs hatch to first instars that undergo four nymphal stages, developing to adults that produce an average of 100 to 400 eggs per adult (Buchanan and Whiting 1984). Studies overseas have shown that phylloxera survival is dependent on temperature, humidity and daylength (Turley et al. 1996, Omer et al. 1997). In the USA, the optimal temperature for phylloxera survival, development and reproduction ranges between 21 and 36°C (Granett and Timper 1987, Fisher and Albrecht 2003), with differences between individual phylloxera populations. This study explored temperature effects on the performance of Australian phylloxera populations.

In Australia, recent validation on the effectiveness of heat as a disinfestation treatment for phylloxera revealed differences in mortality when first instars of genetically diverse strains were subjected to temperature ranges of 40 – 45°C for short duration exposures (i.e. 2 – 2.15 hours) (Korosi et al. 2012, Clarke et al. 2018). Data provided evidence for how temperatures influence the rate of survival in exposed insects, but less is known about development and survival of Australian strains at lower ambient temperatures, and particularly in the context of an insect feeding on a root. We examined this here, investigating survival, fecundity and development of phylloxera strains under laboratory controlled conditions, at different temperatures on vine root material and in the absence of roots.

Methods

Vitis vinifera roots were collected from a vineyard in the NE Victoria during the phylloxera active period (February 2020). This ensured that root physiological characteristics equated with optimal nutrition required for insects throughout the experiment. Excised roots (5-7cm in length) for bioassays were prepared as for culturing of insects. Experiments used five phylloxera strains, G1, G4, G19, G20 and G30 cultured on bioassay plates. When required for experiments, 1-3 day old eggs of each phylloxera strain were separately removed from stock cultures using a dampened fine sable-hair paintbrush. To determine the temperature range to use in experiments, 18, 32 and 36°C were first trialled in preliminary trials with G4 phylloxera, by inoculating ten eggs on excised roots (five replicates for each temperature) in a Petri dish (Figure 1a). No eggs hatched at 36°C, and no first instars that hatched at 32°C developed to reproductive adults, only reaching the 4th instar stage. At 18°C, eggs developed to reproductive adults within 60 days, and roots were often contaminated with fungus. Based on these preliminary findings, 18 and 30°C were used as the lower and upper temperatures for subsequent experiments. All experiments were ended at day 32 as per Kocsis et al. (1999, 2002).

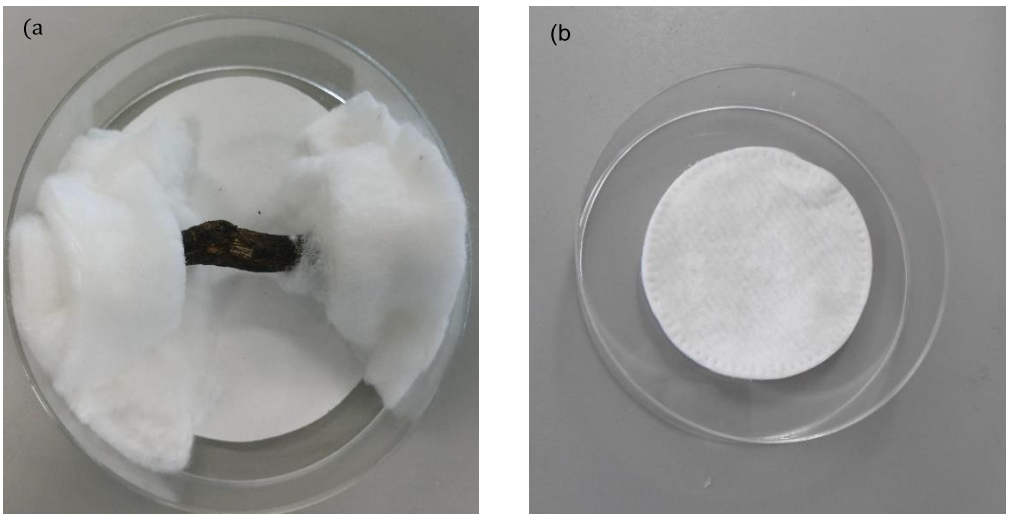


Figure 1 Bioassays of 90 x 25 mm Petri dishes used for (a) excised roots experiments to determine the influence of temperature on survival, development and reproduction with a food source (b) cotton eye make-up pad used to evaluate temperature effects in wet and dry conditions.

Phylloxera survival on excised roots (Vitis vinifera) at 18, 22, 26 and 30°C

A single excised root of *V. vinifera* (5-7mm) in a 90 x 25 mm Petri dish (Figure 1a) was separately inoculated with ten 1-3 day- old eggs of G1, G4, G19, G20 or G30. Petri dishes were each labelled accordingly (replicate, phylloxera strain and temperature treatment), sealed with a cling film, covered with aluminium foil and incubated at 18, 22, 26 and 30 ˚C. Temperatures in the incubator were monitored hourly using a Gemini data logger over the experiment duration. Five replicates were used for each phylloxera strain and temperature combination (i.e. 5 phylloxera strains x 4 temperatures x 5 replicates treatment combinations). The roots were kept alive by adding four drops of sterile water to cotton wool wrapped at each excised end, once a week. On Day 8 after inoculation, roots were examined for the presence of eggs that had hatched (Day 8 is the optimal time when eggs are expected to hatch at 25°C) and first instars were recorded. Numbers of insect at the different developmental stages (first instars, intermediates and adults) that were attached to feeding sites on Days 18, 25, and 32, were recorded. Adults were always associated with adjacent eggs. Eggs found on any roots on Day 18 and 25 were removed to ensure data collected was from the 1st generation cohort.

Percentage survival of insects at 18, 22, 26 and 30 ˚C was determined for each of the five strains (total phylloxera first instars, intermediates and adults attached to feeding sites/original eggs placed on each root x 100) on Days 18, 25, and 32. The *index of population development* was determined per root as the proportion of adults / total adults and intermediates at Day 32, for each of the five phylloxera strains at the four temperatures. Total eggs produced per excised root from the initial 10 eggs was calculated for the entire 32 day bioassay duration for each of the five strains at the four temperatures. *Fecundity* was determined as total eggs by all adults /number of adults per root, for each phylloxera strain at the four temperatures. Eggs laid prior to Day 18 were not used to determine egg production and fecundity. Survival, development, total egg production and fecundity were compared across temperatures and genetic strains using an ANOVA in Genstat ((GenStat Release 18) (VSN International, Hemel Hempstead, England)).

Phylloxera survival without a vine root material as a food source at 18, 22, 26 and 30°C

Survival of phylloxera when not feeding on vine roots was determined under wet and dry conditions. Ten eggs of strains G1, G4, G19, G20 and G30 were placed on separate cotton eye make-up pads in a 90 x 25 mm Petri dish (Figure 1b). To create wet conditions, 60 drops of purified water were added to pads, whilst no water was added to the dry treatments. All treatments were replicated five times for each of the five phylloxera strains and four temperatures (5 replicates x 5 strains x 4 temperatures). Each Petri dish was thereafter labelled accordingly and separately sealed with a cling film, covered with aluminium foil to exclude light and create an egg hatching chamber before incubating at 18˚C, 22 ˚C ,26 ˚C and 30 ˚C.

Evaluating survival

Phylloxera egg survival and hatching were evaluated under a low-powered binocular microscope at x40 magnification. Eggs that hatched were counted and longevity of first instars was recorded. Percentage of eggs that hatched per bioassay and numbers of first instars that survived at 18, 22, 26 and 30 ˚C were calculated as number of eggs that hatched/10 X 100. Data on percentage of eggs that hatched per excised root and longevity of first instars were compared across phylloxera strains, treatment conditions and temperatures using a two-way ANOVA, with temperature and phylloxera strains as main effects and egg hatch and longevity as response variables. A Kaplan–Meier test was used to analyse first instar survival in

the wet and dry experiments and survival curves were drawn to visualize the data (Kaplan and Meier, 1958). A log-rank test with all variables included was used to significance of the model. Comparisons between treatments were tested and the log-rank test was used to correct for multiple comparisons. Data were analysed in Genstat (GenStat Release 18) (VSN International, Hemel Hempstead, England)

Results and discussion

Survival, development and reproduction of phylloxera on excised roots at 18, 22, 26 and 30°C

Survival on excised roots was measured as the numbers of insects (first instars, intermediates and adults) that were attached to feeding sites on Days 18, 25 and 32. Optimal survival was recorded at 18, 22 and 26°C and dropped significantly at 30°C, a trend that was consistent across the five phylloxera strains. At 30°C, no phylloxera of G20 and G30 were found on excised roots on Day 32 (Table 1). Overall and across the five genetic strains, survival at 18 and 22°C was higher (overall mean of 70% at both the temperatures) and lower at 26°C (overall mean of 55%).

Table 1 Percentage survival (mean ± standard error) of phylloxera first instars, intermediates and adults that were attached to feeding sites on a given day (18, 25 and 32) on excised *V. vinifera* roots at 18, 22, 26 and 30°C.

Phylloxera	Day 18	Day 25	Day 32
18°C			
G1	82 ± 13	78 ± 12	76 ± 13
G4	84 ± 7	84 ± 6	84 ± 7
G19	30 ± 12	26 ± 12	26 ± 12
G20	78 ± 10	72 ± 14	72 ± 14
G30	68 ± 11	70 ± 11	70 ± 11
22°C			
G1	82 ± 8	80 ± 8	78 ± 8
G4	74 ± 10	72 ± 9	72 ± 9
G19	50 ± 12	44 ± 11	42 ± 11
G20	64 ± 16	64 ± 16	64 ± 16
G30	64 ± 17	68 ± 17	68 ± 17
26°C			
G1	60 ± 10	60 ± 10	58 ± 10
G4	92 ± 5	84 ± 11	82 ± 11
G19	54 ± 14	46 ± 15	42 ± 13
G20	64 ± 11	48 ± 19	48 ± 19
G30	42 ± 9	30 ± 11	22 ± 11
30°C			
G1	20 ± 3	18 ± 3	8 ± 2
G4	12 ± 7	8 ± 8	8 ± 8
G19	32 ± 7	30 ± 6	24 ± 5
G20	16 ± 7	4 ± 4	0
G30	8 ± 5	2 ± 2	0

Development to adulthood was determined per root as adults/(intermediates+adults) at Day 32 for each of the four temperatures. The developmental index differed significantly across the four temperatures ($P = 0.008$). Only G1 phylloxera developed into adults that produced eggs at 18 °C (Figure 2). Optimal temperature for development was generally 22°C for the phylloxera strains except for G19 which was 26°C (Figure 2). The indices of development on the excised roots were low relative to survival across the genetic strains and temperatures. This inconsistency, that, first instars established feeding sites and survived on roots but did not complete development to adulthood could be attributed to the fact that excised roots under controlled laboratory conditions do not exhibit plant responses such as the galls and tuberosities that phylloxera feed on (Forneck 2011, Griesser et al. 2015, Eitle and Forneck 2017). This implies that some phylloxera strains, such as G19, are sensitive to physiological changes in the vine root (galls and tuberosities) that could be induced by temperature variation, and may explain some of the differences found in phylloxera strain and rootstocks interactions.

Future work could focus on the physiological mechanisms involved in the interaction between environmental factors (e.g. soil type and temperature) and rootstocks, which could help future breeding and selection of resistant rootstocks.

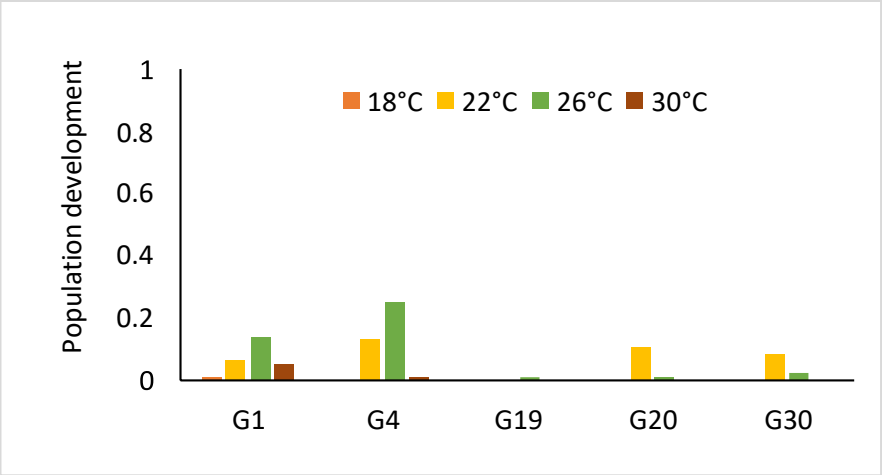


Figure 2 The index of population development on excised *V. vinifera* roots at 18, 22, 26 and 30°C for G1, G4, G19, G20 and G30 phylloxera. The index of development was determined per root as the proportion of adults / total adults and intermediates at Day 32 and compared across the five phylloxera strains and four temperatures (ANOVA ($P<0.05$)).

Total eggs produced per excised root from first instars that hatched from 10 initial eggs was calculated for the entire 32-day bioassay duration for the five strains at the four temperatures as a measure of population growth. Total eggs produced differed significantly across the four temperatures ($P < 0.001$). G4 phylloxera produced the greatest number of eggs (mean = 16 eggs per roots combined for the four temperatures), compared to G1 (6), G19 (1), G20 (2) and G30 (2). No first instars and intermediates of G4, G19, G20 and G30 phylloxera reached the adult stage, and therefore no eggs were found on roots of these strains at 18° C (Table 2). The trend was similar at 30°C bar one individual of G4, which was laying eggs between Day 25- 32. Peak egg production was on Day 32 and was highest for G1 and G4 phylloxera at 26°C, and at 22°C for phylloxera of G20 and G30. Optimal temperature for egg production was 22 and 26°C, for all strains except for G19 phylloxera that did not produce eggs at any given day at 22°C (Table 2). At 26°C, G1, G4 and G30 phylloxera produced enough eggs (Table 2) by Day 18 to potentially increase the population through a second generation; eggs that were laid on bioassay plates on Day 18 and 25 were removed to discount the second generation in the data.

This finding is significant because early egg production (as demonstrated by G1, G4 and G30) indicates a quicker generation turnaround and higher risk of below-ground populations moving above-ground and spreading quickly among vines at the optimal temperatures due in high numbers. Secondary peaks in phylloxera populations have been observed on vine roots in Australian (Powell et al. 2000) and Californian (Omer et al. 1997) vineyards. For regions where vines are predominantly *V. vinifera* (own roots) such as South Australia, this study highlights the risk of rapid spread of strains such as G1, G4 and G30 due to their potential to reproduce quicker and in significantly high numbers compared to G19, G20 and G30 at mid-temperature ranges. At these temperatures, several generations would increase the number of nodosities and tuberosities proportionately, causing wounding and rapid vine death (Granett and Kocsis 2000).

Fecundity was estimated as the number of eggs per adult per root at any given day (18, 25 and 32). At 18 and 30°C, only G1 and G4 had laid any eggs at the lower and higher threshold temperatures where one adult of G1 phylloxera laid a total of 120 eggs between days 18 and 32 (Table 2). No eggs were laid at 18°C by G4, G19, G20 and G30 phylloxera and, at 30°C for G19, G20 and G30 (Table 2). Fecundity was highest at 22 and 26°C at 8 and 11 for G1 and G4 phylloxera, respectively, between Days 25-32, and lowest (1-4) for G19, G20 and G30 phylloxera. Fecundity of G19 phylloxera appears to be most impacted at the four temperatures compared to the other four genetic strains, given that only one individual laid eggs at 26°C. Phylloxera of G1 and G4 appear to be well adapted to temperatures of 22 and 26°C whilst G1 and G30 have potential to produce eggs in large numbers at lower temperatures (Table 2).

Table 2 Total egg production per excised root from the initial 10 eggs for the entire 32 day bioassay and fecundity (total eggs by all adults /number of adults per root in brackets) on excised-root *V. vinifera* roots by G1, G4, G19, G20 and G30 recorded at 18, 22, 26 and 30°C.

Phylloxera	Day 18	Day 25	Day 32
		18°C	
G1	40 (1)	40 (1)	40 (1)
G4	0	0	0
G19	0	0	0
G20	0	0	0
G30	0	0	0
		22°C	
G1	0	170 (2)	323 (2)
G4	0	448 (5)	600 (6)
G19	0	0	0
G20	0	173 (2)	308 (4)
G30	0	57 (1)	184 (2)
		26°C	
G1	40 (1)	792 (8)	745 (8)
G4	20 (1)	706 (8)	1077 (11)
G19	0	40 (1)	40 (1)
G20	0	0	40 (1)
G30	40 (1)	0	120 (2)
		30°C	
G1	10 (1)	10 (1)	30 (1)
G4	0	0	80 (1)
G19	0	0	0
G20	0	0	0
G30	0	0	0

Phylloxera survival without root material at 18, 22, 26 and 30°C

Egg hatching into first instars was impacted by temperature and whether bioassay conditions were wet or dry (P>0.001). Under dry conditions, none of the G4 phylloxera eggs hatched at the four temperatures,

implying high sensitivity to lack of moisture resulting in dessication; the eggs appeared shrivelled when observed under the microscope. Temperatures of 18 and 22°C were favourable for egg hatching under the dry conditions for G1, G19 and G20 phylloxera eggs, implying that these strains could adapt to cooler climates more readily than other strains. G30 phylloxera eggs appear to be highly susceptible to cool and dry conditions as no eggs hatched at 18°C (Figure 3). By contrast, eggs of G20 phylloxera appear comparably better than other strains in dry conditions at 18°C, with 72% (N=50) of eggs hatching into first instars (Figure 3). Results showed that temperatures reaching 30°C impede phylloxera egg hatching in dry conditions, with only 2% of eggs hatching for G19 and G30 phylloxera, and none from G1, G4 and G20 (Figure 3). Under wet conditions, 95% of eggs hatched, a result that was consistent across the four temperatures and phylloxera strains (P=0.748).

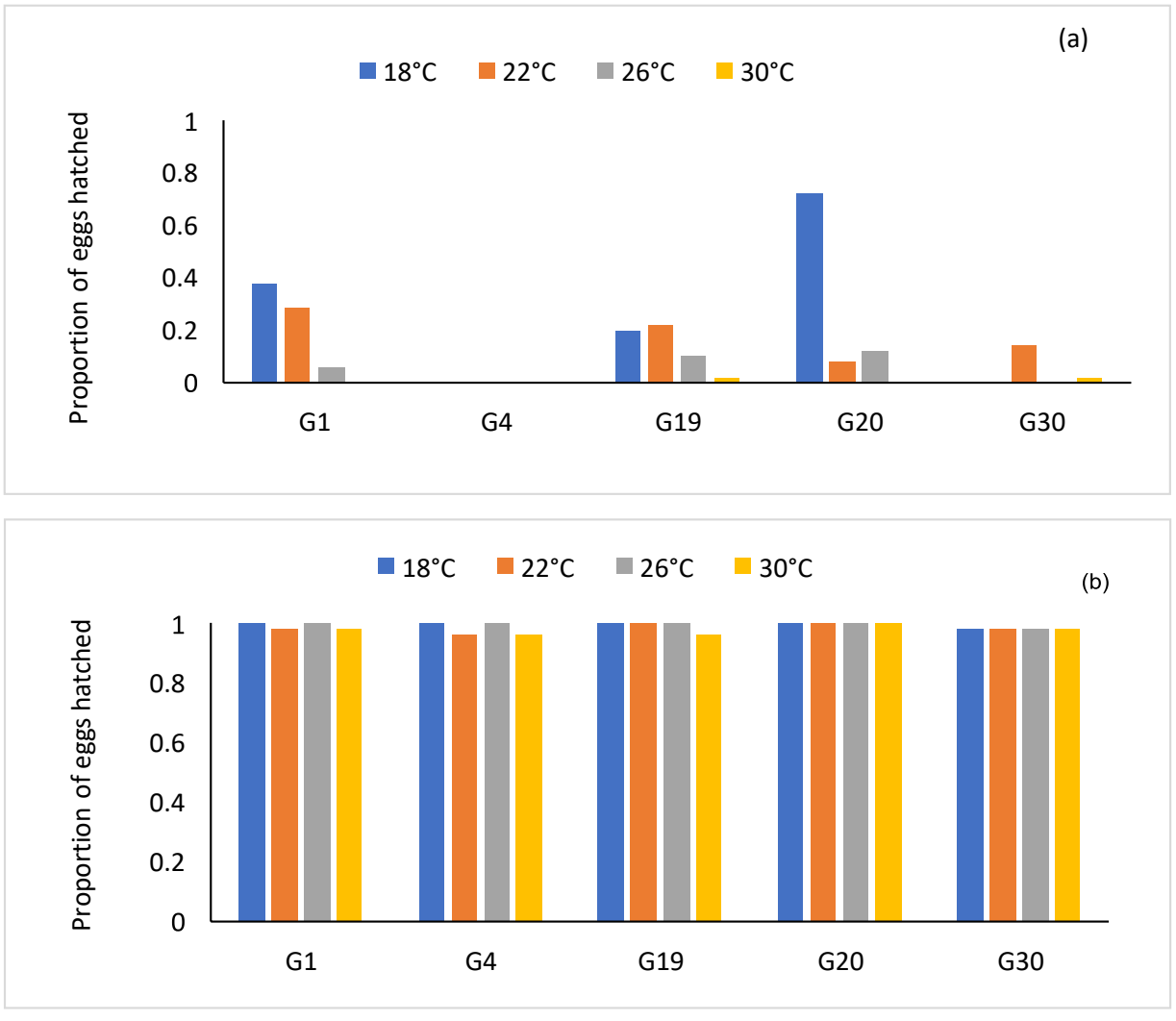


Figure 3 Proportion of *eggs that hatched* under (a) dry and (b) wet conditions at 18, 22, 26 and 30°C for G1, G4, G19, G20 and G30 phylloxera (N=50).

First instar survival under dry and wet conditions was significantly impacted by wet or dry conditions (Log-rank = 174, P < 0.001; Figure 3) and temperatures (Log-rank = 66, P < 0.001; Figure 4).

Under dry conditions, survival probability of first instars across all the five phylloxera strains was longer (5- 10 days) at 18°C and shorter (2-5 days) at 30°C (Figure 4a). First instars of G1, G4, G19 and G30 lived for 5 -6 days at 18°C, while G20 first instars survival time was highest at 10 days at this lower temperature (Figure 4a). Under wet conditions, survival probability followed a similar trend of first instars living longer at 18°C (17-29 days) and short-lived at 30°C (10-12 days) (Figure 4b). Survival time of G19, G20 and G30 phylloxera was the highest – up to 25 days at 18°C. At 22°C, survival time of first instars across the five strains ranged from 15-20 days. These findings imply that phylloxera have the propensity to withstand wet and cool conditions at the first instar stage, when they are active and at greater risk of being spread. Findings from this work suggest susceptibility of G1 phylloxera eggs to cool temperatures and corroborate a study by Korosi et al. (2012) that showed that increasing humidity increased the survival of phylloxera first instars that were subjected to heat treatments of 40-45°C for 75-90 min.

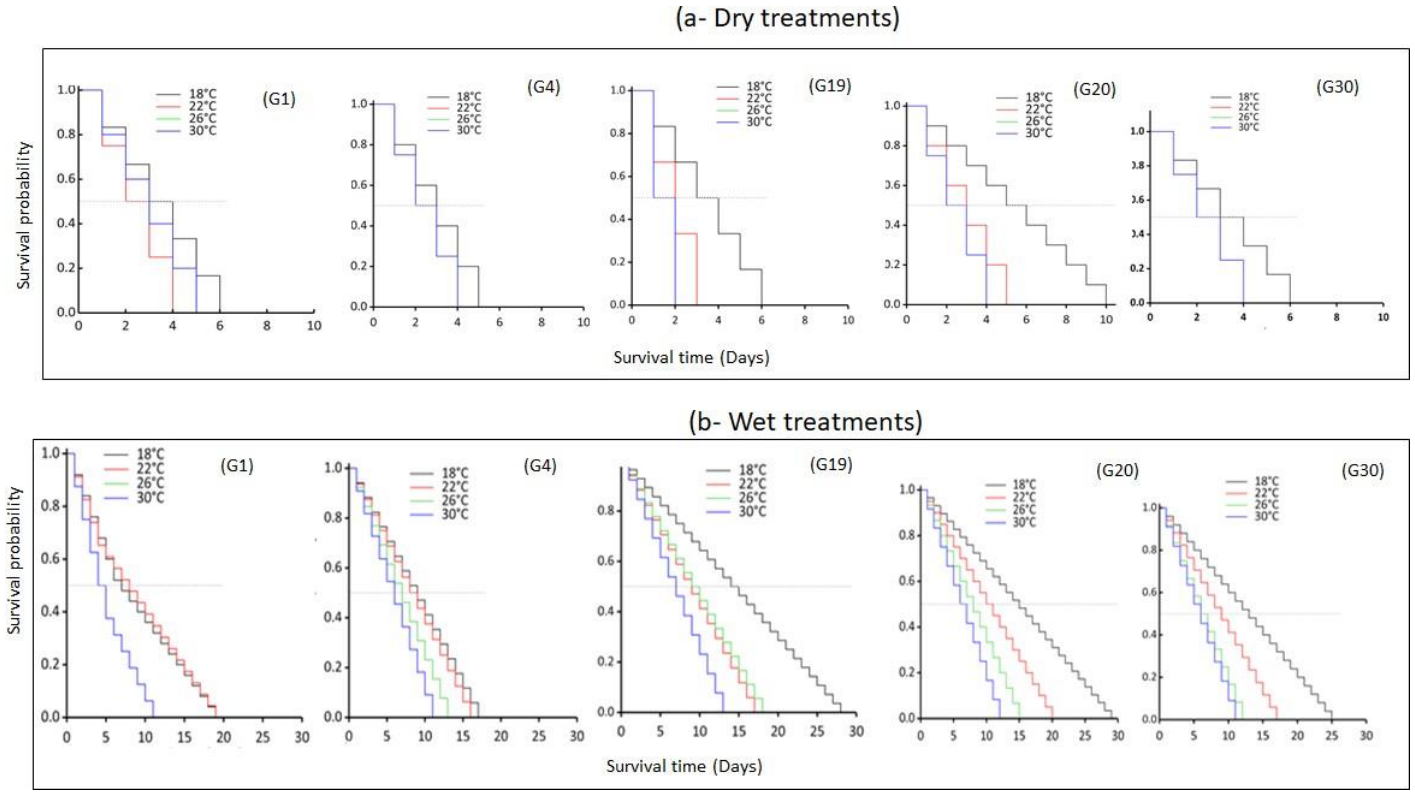


Figure 4 Kaplan–Meier curves showing the proportion of surviving first instars over time under (a) dry and (b) **wet conditions.** All data were taken until the last instar on each Petri dish was dead.

Survival of eggs and first instars at low temperatures (18°) and conditions (wet and dry) influences how early in spring phylloxera can begin to feed. The findings that eggs do hatch and first instars do survive under dry conditions for most of the phylloxera strains are notable because dry vineyard conditions are associated with phylloxera outbreaks, whilst wet conditions are associated with lower root-galling phylloxera populations (Helm et al. 1991). However, formation of galls on leaves is believed to occur in conditions of high humidity and cool temperatures (Benheim et al. 2012, Powell et al. 2013). For phylloxera that exists as both root and leaf forms, there is potential that these strains could adapt to wet and cold

conditions, and switch forms depending on prevailing temperatures. Research overseas has shown that environmental conditions with high moisture content and low temperatures could give rise to the winged form of phylloxera (alate) (Granett et al. 2001, Forneck and Huber 2009). Alates produce sexual offspring and genetic recombination that occurs could give rise to diverse genetic strains and new virulent biotypes (Granett et al. 2005, Skinkis et al. 2009). Further studies would shed light as to whether temperature and water availability conditions are influencing factors in the generation of new strains in the King Valley (see section 2.3).

Conclusions and recommendations

Survival, developmental and reproduction rates as used to describe fitness in insects were observed among the five phylloxera strains on excised *V. vinifera* roots at different temperatures with and without feeding on grapevine root material. Fitness response in survival and reproduction in this assay was between 22°C and 26°C, with upper and lower temperature thresholds recorded at 18°C and 30°C. The upper temperature threshold at 30°C survival and developmental time of phylloxera was significantly lower than that at the other tested temperatures (>32°) overseas (Granett and Timper 1987, Turley et al. 1996, Omer et al. 1997). In preliminary lab trials at a 32 and 36°C we found no survival beyond the intermediate stage. These findings imply that phylloxera in Australia appears to be sensitive to temperatures above 30°C and that mid-twenty temperature ranges are optimal for development and reproduction. Individual strains such as G1 laid eggs at 18°C, and this could partly explain the predominance of this strain in the Yarra Valley, compared to some areas in the NE Victoria where strains such as G4 (Umina et al., 2000) and G20 have been recorded (see section 8.3). These findings have implications for quarantine management. Producers should be mindful of changing conditions and that presence of water (humidity) could prolong first instar survival on equipment and machinery and other known transfer risks. Furthermore, relaxing vigilance, especially in cooler months of the year (when peak phylloxera activity is expected to be low) is a risk because movement of items could aid in transfer of first instars to areas where the insects could start reproducing if they find a suitable host.

Notably, this study was carried out under controlled conditions rather than in the field. The effect of temperature on development and reproduction of both the root and leaf galling phylloxera under different soil conditions (temperature, soil type and moisture content) and as related to vine phenology (bud burst, bloom, fruit set, cluster closure, véraison and fruit maturity) are not yet known. Particularly, these interactive effects are expected to impact on the ability of phylloxera 1st and 2nd instars to overwinter and break the dormancy state. This knowledge is integral to developing an understanding of phylloxera spread and in developing protocols to govern the movement of people and vineyard items in different climatic conditions. Comparing the biological characteristics of phylloxera under different temperature ranges will enable development of risk maps of phylloxera distribution under future climate change scenarios.

RESEARCH THEME 5

RE-EVALUATION OF BIOCONTROL OPTIONS

13.0 RE-EVALUATING BIOCONTROL OPTIONS

Summary

Drawing on expert advice from North America, Europe and Australia, an extensive review (Appendix 8) on phylloxera biocontrol evaluated approaches including the potential release of exotic natural enemies from the pest's native range, commercially-reared predatory insects, the application of insect killing microorganisms such as nematodes and fungi, and practices to enhance native predators in vineyards. The review found 16 predatory species, of which only two attack root- feeding phylloxera. A predatory syrphid fly is particularly promising and warrants further investigation. We also suggest targeted field surveys in eastern USA to search for root predators and insect pathogens (entomopathogens). Similarly, in Europe most predators were of the leaf-galling form. In Australia, similar predatory guilds occur in vineyards and could be encouraged through habitat manipulation for conservation biocontrol. There is enormous scope to further investigate the use of entomopathogenic nematodes and fungi as biological pesticides with the aim of combining multiple biocontrol approaches for an integrated phylloxera management.

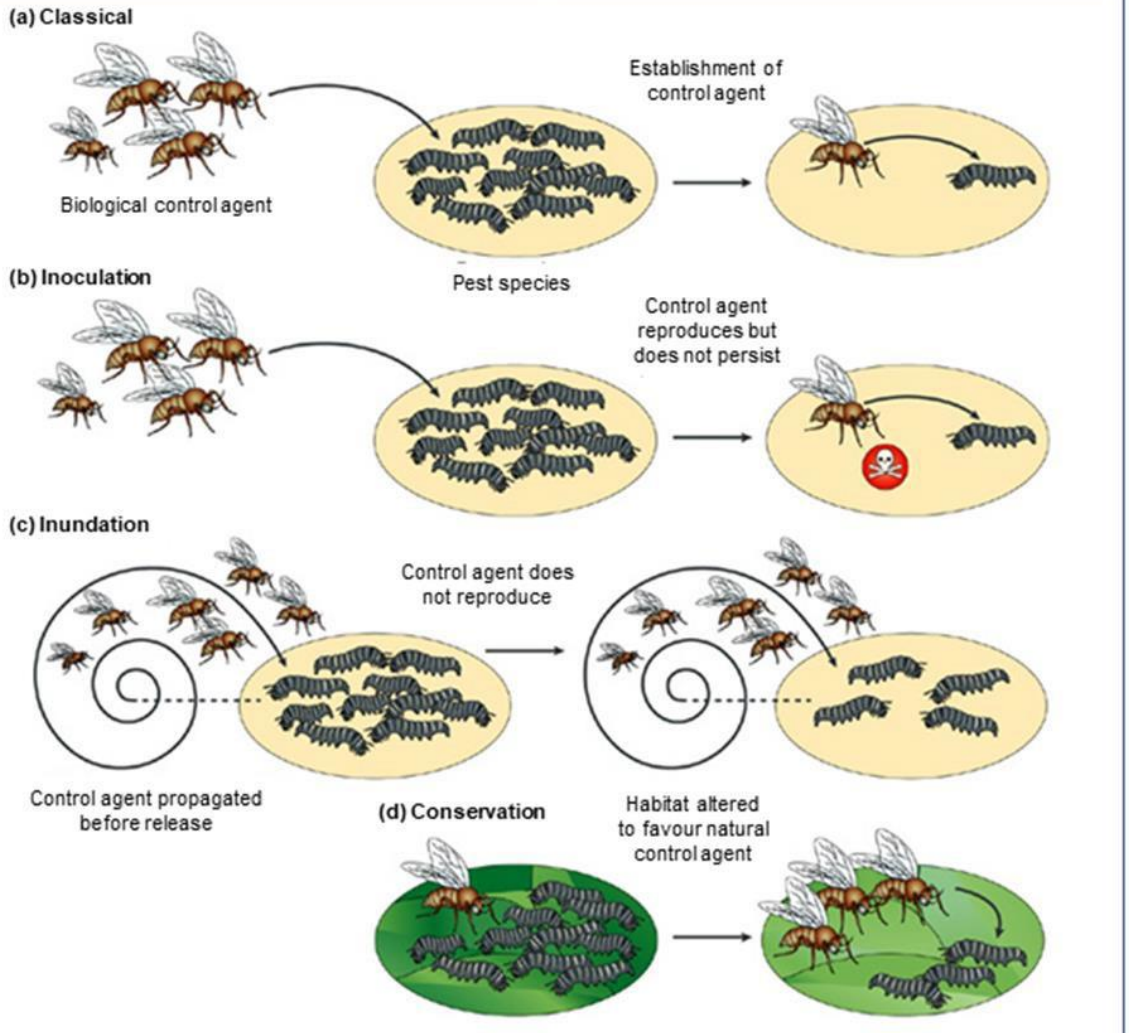
Background

There is currently no effective treatment for phylloxera, with no chemicals registered for the control of phylloxera in Australia. Grape phylloxera management around the world has predominantly relied on the use of resistant rootstocks. However, there is evidence that this resistance is being overcome by new strains of the pest (Bao et al., 2015; Forneck et al., 2016; Umina et al., 2007). In Australia, 83 strains have so far been identified (additional 32 new strains identified on roots in this study see section 10.3), of which around 49 are confined to roots only, 23 are confined to leaves only, and 11 strains attack both leaf and root material (Umina et al., 2007). The evolution of new strains necessitates both the development of new rootstocks and research on integrated control methods, including biological control. However, research into the latter, such as the use of natural enemies of grape phylloxera from the native range, the manipulation of the vineyard habitat to encourage native predators, and the mass release of insect pathogens such as nematodes, bacteria and fungi, has been limited in comparison to research into chemical control and rootstock breeding (Benheim et al., 2012).

Biological control, or biocontrol, can simply be explained as the use of natural enemies (biological control agents) to reduce the impacts of a pest. In practical terms, biocontrol consists of four main types: (1) Classical Biological Control, (2) Inoculation Biological Control, (3) Inundation Biological Control, and (4) Conservation Biological Control (Eilenberg et al., 2001), which can be used either alone, or in combination (see Figure 1). The purpose of the study was to conduct a thorough review to identify prospects for the biological control of grape phylloxera in Australia.

(a) Classical Biological Control involves the intentional introduction of an exotic natural enemy for permanent establishment and long-term pest control. The natural enemy (also known as 'biocontrol agent', or 'agent') can be a parasite, pathogen or predator and is typically collected from the native range of the pest. The agent enemy must be sufficiently specific to the target pest to be approved for release by regulatory authorities.

(b) Inoculation Biological Control involves the intentional release of biocontrol agents with the expectation that they will multiply and control the pest for an extended period, but not permanently. This approach is often used in glasshouses or periodic crops such as the annual release of egg parasitoid wasps (*Trichogramma* spp.) for Lepidopteran pests of corn, cotton and other broadacre crops.



(c) Inundation Biological Control involves the mass release of natural enemies that are typically produced on a commercial scale. Control is achieved exclusively by the released organisms themselves, hence reproduction of the organism is not expected after release. Certain entomopathogenic fungi, bacteria, viruses and nematodes can be readily cultured *in vitro* and applied using conventional pesticide techniques. In essence, these pathogenic organisms are applied as a microbial insecticide.

(d) Conservation Biological Control involves the alteration of the habitat or cultural practices to increase the numbers or efficiency of resident populations of predators, parasites or pathogens. Such alterations might include providing refuges within or adjacent to crops to provide food and shelter for natural enemies; and changing the timing of cultivation and pesticide applications.

Figure 1 Types of biological control used against arthropod pests (adapted from Roderick and Navajas (2003).

Methods

A list of predators and pathogens associated with grape phylloxera in the native range (North America) and introduced ranges (predominantly Europe) was built through intensive internet and literature searches. In addition, data were gathered from the grey literature and through direct communication with phylloxera experts via face-to-face meetings or email correspondence (refer to the Acknowledgements section in Appendix 8).

References were examined for their relevance to this study and organisms found to be associated with grape phylloxera were further checked for information on their current taxonomy and nomenclature, host-range, mode of action and location and summarised in easy-to-read tables. For each group of natural enemies found to be associated with grape phylloxera, such as predatory Coccinellidae (lady beetles), a brief overview was provided on the importance of the organism in the biological control of aphids in general, and the prospects of the group for the biological control of grape phylloxera through classical, inoculation, inundative and conservation approaches.

Results and discussion

Classical Biological Control using specialised arthropod predators

The exotic origin of grape phylloxera makes it particularly amenable to Classical Biological Control through the importation and establishment of specific natural enemies from its native range in North America. While these natural enemies are poorly documented and even more poorly understood, we know of two insect species that warrant further investigation as candidates for biological control. The syphid fly, *Heringia salax* is of greatest interest because it is known to attack both leaf and root-galling phylloxera and is likely to be host-specific. Other members of this genus have been utilised in classical biological control programs against aphids that have both arboreal (leaf-galling) and edaphic (root- galling) forms, such as *H. calcarata* against woolly apple aphid in the USA (Bergh and Short, 2008). A second fly species, *Leucopis simplex*, which is reportedly the most common predator of grape phylloxera in North America, is considered of lower priority because it is likely to be a predator of only the leaf-galling form. Targeted natural enemy surveys in the eastern USA may reveal the identity of other arthropod predators of root-galling phylloxera with potential as candidate biological control agents for importation and release into Australia.

Theoretically, the probability of finding a biocontrol agent that is deemed “safe for release” into Australia is likely to be higher if there are few or no native species that are closely-related to the target pest. In the case of phylloxera, there are no native species within the Phylloxeridae family, making classical biological control a highly feasible option (Table 1).

Recommendations

- Undertake natural enemy surveys in the native range concentrating on soil-dwelling arthropod predators in collaboration with USA entomologists.
- Through laboratory and field studies in the USA, determine which predators have potential as classical biocontrol agents based on specificity, effectiveness and likelihood of establishment under Australian climate and soil conditions.
- Pending results of native range studies, initiate a classical biological control program against grape phylloxera in Australia.

Inoculation Biological Control using commercially available biocontrol agents

There are several commercially available biological control agents that are utilised in Australia against soft-bodied pests such as aphids, scale insects and thrips. While it is not known if these species will attack grape phylloxera, similar predatory guilds are key predators of grape phylloxera in other countries and may play a similar role here. Hence the prospects of inoculation biological control of grape phylloxera using commercially available agents warrants further investigation. Of interest are the predatory mites, *Gaeolaelaps aculeifer* and *Stratiolaelaps scimitus*, as these are already sold in Australia for the control of soil-dwelling soft-bodied insects including fungus gnats and western flower thrips. Arboreal and ground - dwelling predators such as ladybirds, lacewings and predatory bugs may be useful in reducing the spread of grape phylloxera within and between vineyards by attacking the vulnerable dispersing juveniles and alate (winged) adults (Table 1).

Recommendations

- Conduct laboratory bioassays to determine if commercially available biological control agents, particularly soil-dwelling predatory mites, predate upon root-galling and dispersal stages of grape phylloxera.
- If so, determine effective agent release strategies and methods to conserve populations for longer-term benefits.

Inundation biological control using entomopathogenic fungi and nematodes

Entomopathogenic fungi (EPF) and nematodes (EPN) are among the most promising biocontrol agents of root pests because they are naturally soil-dwelling organisms, they are easy to isolate from the field, and they can be readily mass-produced on artificial media and applied using conventional insecticide application techniques. In comparison to chemical pesticides, the multiple mode of action of EPF and EPNs lessens the possibility of resistance development in insects. Plus, they are often highly host- specific, avoiding unexpected deleterious effects on non-target beneficial organisms (Kergunteuil et al., 2016). However, the use of EPF and EPN against grape phylloxera has to date been surprisingly limited, despite the increasing development of commercial products for other pests, resulting from significant advances in the isolation of effective strains, formulation, shelf-life and application techniques in recent years (van Lenteren et al., 2018).

Entomopathogenic fungi and nematodes have considerable potential for use against grape phylloxera in Australia, and this area of research warrants further investigation. As classical biological control agents, phylloxera-specific species and strains would need to be identified from the native range and subjected to prey-range studies under quarantine conditions to assess their safety for release into the Australian environment. If approved for release, studies would need to be conducted to determine if these entomopathogenic organisms can maintain self-sustaining populations or if they need to be developed into commercial products for inundation biological control. Various commercial species and strains of EPF and EPN are already available in Australia against other soil-dwelling pests, and these could be easily screened for their effectiveness against grape phylloxera. Finally, further research should seek to identify endemic entomopathogenic organisms present within Australian vineyards and their potential for development as commercial biopesticides (Table 1).

Recommendations

Native range studies:

- Undertake field surveys to identify the EPF and EPN associated with grape phylloxera in the eastern USA in collaboration with key universities and other research agencies.
- Conduct laboratory and field studies to better understand the role of entomopathogens in grape phylloxera population dynamics.
- For entomopathogenic nematodes, investigate the interactions between these nematodes and their symbiotic bacteria.
- Pending results of native range studies, initiate a classical biological control program against grape phylloxera in Australia.

Australian studies:

- Undertake laboratory bioassays to screen the effectiveness of commercially available strains of EPF (*Beauvaria bassiana* and *Metarhizium*) and EPN *(Steinernema* and *Heterorhabditis*)
- Determine if endemic fungi and nematodes present in Australian vineyards have potential for commercial development as biopesticides for grape phylloxera.

Conservation Biological Control

Australian vineyards are host to a diverse range of predatory arthropods whose populations can be enhanced through the provision of insectary plants that provide 'SNAP', an acronym that refers to shelter, nectar, alternative prey and pollen (Retallack et al., 2019). While we do not know if these predators attack grape phylloxera in Australia, it is highly likely that some of them do because similar functional groups are known to attack grape phylloxera in other countries. For instance, two species of *Scymnus* lady beetles in North America (native range) plus a further six species in Europe (invaded range) are known to predate upon grape phylloxera. Therefore, it is likely that the native *Scymnus* identified in Australian vineyards are also predators of grape phylloxera (Table 1).

Recommendations

- Conduct local ground surveys within Australian phylloxera infested zones (PIZs) to elucidate endemic predators using next generation sequencing of gut contents for the presence of grape phylloxera.
- Identify suitable native plant species that provide 'SNAP" resources (i.e. shelter, nectar, alternative prey and pollen) to encourage predators of phylloxera.

Table 1 Prospects for Classical, Inoculation, Inundation and Conservation Biological Control of grape phylloxera (GP) in Australia.

Type	Habitat	Phylloxera target	Potential biological control organisms	Priority for biocontrol
Classical	Soil and canopy	Root-feeding, leaf-galling and dispersal stages	Syrphid fly, *Heringia salax* (Diptera).	**High priority**. *H. salax* is known to attack both root and leaf-galling GP in the native range. Research is required to understand the biology and prey-range of *H. salax* before its suitability as a biocontrol agent for GP can be assessed.
	Canopy	Leaf -galling	Silver fly, *Leucopis simplex* (Diptera).	**Low priority**. A common predator of leaf-galling GP in the native range. Research is required to understand the biology and prey-range of *H. salax* before its suitability as a biocontrol agent for GP can be assessed.
	Soil	Root-feeding	Entomopathogenic nematodes.	**High priority**. Nothing is known about the EPN associated with GP in the native range. Native range surveys are required to identify EPNs of root-galling GP and their prospects for classical biological control.
Inoculation	Canopy and ground	Leaf-galling and dispersal stages	Green lacewing, *Mallada signatus.* Brown lacewing, *Micromus tasmaniae.* Australian spotted ladybird *(Harmonia conformis).* Damsel bug, *Nabis kinbergii.* Orius - minute pirate bug, *Orius armatus.*	**Medium priority.** Adult lacewings will persist in the crop if nectar and pollen are present. Practices such as strip intercropping and encouraging flowering plants will give best results. Ladybird beetles are very effective predators of aphids, but they may be harmed by pesticides. The damsel bug is an aphid predator currently under development for commercial use. Minute pirate bug is predominantly a thrips predator but will also feed on aphids.
	Soil	Root-feeding	Predatory mites, *Gaeolaelaps aculeifer*, and *Stratiolaelaps scimitus.*	**High priority**. Commercially sold for soil-dwelling pests such as fungus gnats and western flower thrips, but overseas, similar species attack soil-dwelling aphid pests. Studies are required to determine if species available in Australia will attack grape phylloxera.

Type	Habitat	Phylloxera target	Potential biological control organisms	Priority for biocontrol
Inundation	Soil	Root-feeding	Entomopathogenic fungi, *Beauvaria, Metarhizium, Isaria* spp..	**Medium priority**. Identify EPF species and isolates attacking root-galling GP in the native range and assess their potential for commercial development. **Medium priority**. Identify EPF species and isolates present in Australian vineyards and assess their efficacy against GP and potential for commercial development. **High priority**. Investigate the potential for off-label use or registration of BIPESCO 5/F52/Met 52 through the APVMA in Australia.
			Entomopathogenic nematodes, *Steinernema* spp, and *Heterorhabditis.*	**High priority**. Conduct screening trials to determine if commercially available EPN are effective against GP.
Conservation	Canopy	Leaf-galling and dispersal stages	Native ladybirds, hoverflies (Syrphids) .	**Medium priority.** Identify which native ladybirds will attack GP and develop strategies to encourage their presence in vineyards.
	Ground	Dispersal stages	Native predatory bugs and rove beetles.	**Medium priority** Predatory bugs and rove beetles occur in Australian vineyards, many of which predate of ground-dwelling pests. However, their role as predators of GP is unknown.
	Soil	Root-feeding	Native entomopathogenic nematodes, fungi, predatory arthropods.	**High priority** Little is known of the native soil-dwelling predators or pathogens in Australian vineyards and the role they may play, if any, on phylloxera control.

Conclusions

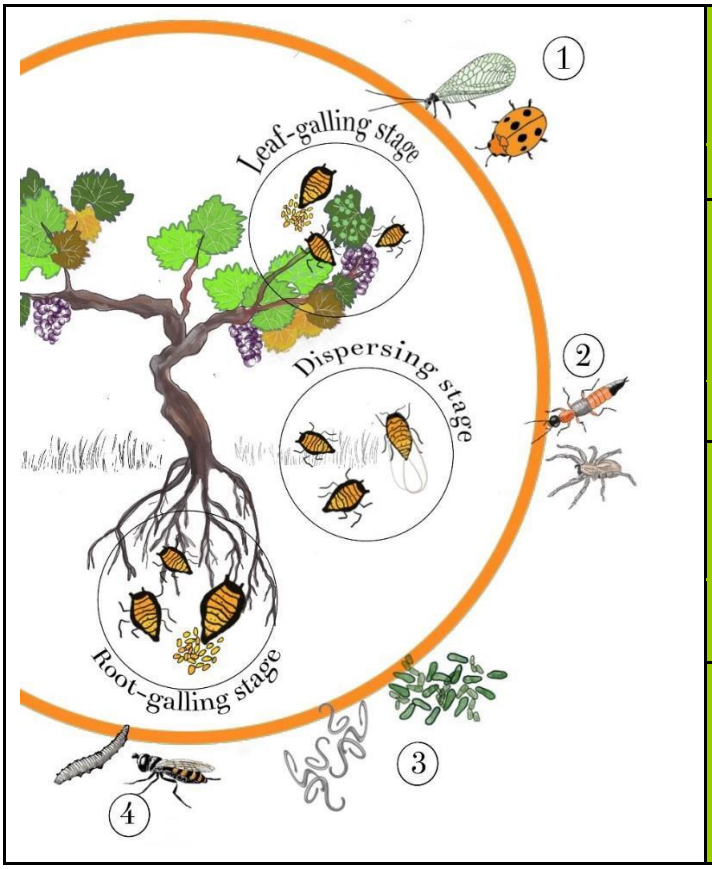
Classical Biological Control using a predatory syrphid fly (*Heringia salax*) has considerable potential because it is one of the few documented predators that attacks the adult and juvenile stages of root-galling grape phylloxera in its native range in the USA. In addition to conducting prey-range studies to determine if *H. salax* would be suitable for release into Australia, native range field surveys should be undertaken in North America to identify other predators and pathogens that may make effective biocontrol agents.

Biopesticides. Around the world, considerable scientific advances have been made in the development of biopesticides providing Inundation Biological Control against a range of soil- dwelling pests using entomopathogenic fungi and nematodes. Surprisingly, relatively little research has been undertaken on the development of biopesticides for grape phylloxera, apart from the studies conducted in Europe that showed that the fungal pathogen, *Metahrizium* sp. provided control of root-galling grape phylloxera for up to three years.

Supporting and enhancing natural enemy communities. In Europe and the USA, over 30 different predatory insects and mites have been recorded attacking grape phylloxera, yet nothing is known about the predation of grape phylloxera in Australia, despite the presence of similar predatory guilds occurring within and around vineyards. Recent research on Conservation Biological Control in Australian vineyards has shown that native plant species can attract and support insect predators, providing food (pollen, nectar, alternative prey) and shelter (Retallack 2011). Yet the impact of native predators on phylloxera infestations in Australian vineyards has not yet been validated through field studies.

Recommendations

Future phases of research should aim to develop and adopt a multi-faceted approach that combines different forms of biological control targeting all stages and forms of the pest. This concept is outlined in Figure 2 below.



(1) Inoculation Biological Control providing early-season control through the mass release of commercially available biological control agents.

(2) Conservation Biological Control using resident populations of insect predators to attack phylloxera root and leaf-galling dispersing stages as they emerge from the soil.

(3) Inundation Biological Control using entomopathogenic nematodes and fungi (to provide "knock-down" control of priority infestations.

(4) Classical Biological Control using exotic natural enemies to provide long-term suppression of root-galling populations.

Figure 2 A multi-layered or truly integrated approach to the biological control of grape phylloxera in Australia that combines various approaches to target specific pest stages such as root -galling, leaf-galling and the dispersing stages of both.

To achieve a multi-faceted approach to biological control, research should focus on the following priorities as outlined in Table 2:

1. **Inoculation Biological Control**

Inoculation Biological Control should be trialled as an early-season strategy using a range of commercial- reared aphid predators such as predatory mites, ladybeetles, lacewings and predatory bugs. Initial research should determine if these predators do feed on phylloxera and if so, what release strategies should be adopted to maximise their impact.

2. **Conservation Biological Control**

A holistic Conservation Biological Control strategy should be developed that targets all key pests, including root and leaf-galling forms of phylloxera. This could be achieved by conducing local ground surveys in Australia phylloxera-infested vineyards to collect predatory insects. Next generation sequencing of gut contents could be used to elucidate which endemic predators feed on phylloxera. This will allow native plant species that are known to provide suitable habitat for these predators to be trialled for use in vineyards. For instance, the planting of wallaby grasses, *Rytidosperma* spp. between rows could provide habitat for ground-dwelling spiders, earwigs, shield bugs, assassin bugs, lacewings and ground beetles to predate upon dispersing crawlers and winged adults as they emerge from the soil. Similarly, native shrubs that attract aphid-specific predators such as

hunter/ambush spiders (Salticidae, Thomisidae), hoverflies, lacewings, earwigs, shield bugs, and ladybeetles could assist in the predation of leaf-galling phylloxera or dispersing stages.

3. Inundation Biological Control

A recent study revealed that a diverse community of entomopathogenic fungi (EPF) are present in vineyard soils of southern Australia (Korosi, et al. 2019). These fungi, consisting of three *Beauveria* and six *Metarhizium* species, should be examined further for their efficacy against phylloxera. In addition, screening trials should also be conducted to assess the effectiveness of currently available commercial products against Australian strains of grape phylloxera. Finally, techniques for the effective application of EPF into vineyards should be trialled, such as dissemination though existing irrigation systems.

4. Classical Biological Control

Natural enemy surveys in the USA, the native range of phylloxera, should be conducted concentrating on soil-dwelling arthropod predators in collaboration with USA entomologists, Dr Mark Hoffman and Dr Hannah Burrack from North Carolina State University. Through laboratory and field studies in the USA, natural enemies with potential as classical biocontrol agents would then be prioritised based on specificity, effectiveness and likelihood of establishment under Australian climate and soil conditions. In particular, the predatory syrphid fly (*Heringia salax*) should be studied and preliminary host specificity testing conducted in the USA. Pending positive results of the native range studies, a formal application to nominate Phylloxera (*Daktulosphaira vitifoliae*) a Target for Biological Control would be made to the Plant Health Committee (PHC) prior to the initiation of a classical biological control program against grape phylloxera in Australia. Further information on the regulatory steps required to enable a biological control agent to be released into the Australian environment can be found on the Department of Agriculture, Water and the Environment website (https://www.agriculture.gov.au/biosecurity/risk-analysis/biological-control-agents).

14.0 COMMUNICATION AND INDUSTRY ENGAGEMENT

Industry Reference Group

A Terms of Reference document was established by Vinehealth Australia, and key industry, grower and biosecurity representatives were invited to be members of the Industry Reference Group (IRG) (Appendix 10). The first IRG meeting for the project was held on 8th November 2019, at Agriculture Victoria (Attwood). The meeting was organised and chaired by Vinehealth Australia (Inca Lee) and attended by 13 participants, including representatives from the project team, Wine Australia, Vinehealth Australia, AWRI, CSIRO, Agriculture Victoria Biosecurity (CPHO unit), and representative growers and consultants. The project team leads (Paul Cunningham and Catherine Clarke) gave a detailed presentation on progress, tracking, results, and research outcomes achievements over the 2 year project. The meeting included a discussion about the scope and priorities for the next phase of phylloxera research (2020-2023). To evaluate outputs from this project and inform extension of results, a second IRG meeting was held on 17th June 2020. The meeting was held online (due to travel restrictions during the COVID-19 pandemic). The project team leads (P. Cunningham and C. Clarke) were joined by research team members (Mark Blacket, Rae Kwong and John Weiss). Team leads (PC & CC) gave updates from the current project and highlights on the way forward by discussing the next phase of research (2020-2023). A third and final IRG meeting for this project is scheduled for the 4th November 2020.

Recommendations provided to key biosecurity stakeholders and committees on NPMP procedures.

Recommendations for disinfestation of footwear, machinery and vineyard equipment were prepared and presented to Agriculture Victoria (Chief Plant Health Office Unit) and Vine Health Australia (via email, on 17th September 2018) (Appendix 11). Subsequently, a face to face meeting was held (8th October 2018, Agriculture Victoria Attwood) to discuss the latest scientific findings with respect to disinfestation protocols and the implications for respective Plant Quarantine Standards and legislation. The meeting initiated the development of a new national management strategy for phylloxera to reflect the latest research outcomes with regard to disinfestation procedures and update the standard operating procedures.

Conference attendance and, presentations to stakeholders and industry groups

Conference presentations

i. **Clarke CW.** Grape phylloxera quarantine management and importance of diverse genetic strains. Front-line pest and disease management for healthy vineyards. ASVO seminar, Mildura 25-26 July 2018. https://www.wineaustralia.com/news/articles/frontline-pest-and-disease-management

Stakeholders and industry groups engagements

i. **Clarke CW** and **Carmody BM**. Phylloxera biology and finding phylloxera on vines. Training of contract workers for rezoning of Mornington Peninsula (December 2017, 1018 and 2019)
ii. **Clarke CW.** Disinfestation protocols of diagnostic samples; Smoke Taint Briefing, Brown Brothers, Milawa; 15th January 2020; https://www.bordermail.com.au/story/6582547/worried-winemakers-gather-to-understand-the-science-of-smoke-taint/

iii. **Clarke CW.** Phylloxera tour shows the impact of outbreak; Phylloxera immersion tour at the Yarra Valley; 26-27th November 2019; https://www.youtube.com/watch?v=e80PHTKqAEE; https://winetitles.com.au/phylloxera-tour-shows-the-impact-of-outbreak/; https://vinehealth.com.au/2019/12/phylloxera-tour-shows-the-impact-of-outbreak/;
iv. **Clarke CW.** Vive la difference – Tasmania's cool challenge, Wine Tasmania field day; 11th November 2018; https://www.wineaustralia.com/news/articles/vive-la-difference-%e2%80%93-tasmania%e2%80%99s-cool-challenge.
v. **Clarke CW.** Mornington Peninsula Regional Phylloxera Forum, Elgee Park, Dromana; Victorian Viticulture Biosecurity Committee; 8th May 2018; https://www.youtube.com/channel/UCPXHj0lSjRXKJUCybU8Ia8Q/videos?disable_polymer=1;
vi. **Clarke CW** Risks and management of grape phylloxera - research updates; Heathcoate Phylloxera Forum; https://www.awri.com.au/events/heathcote-phylloxera-forum/; 17th July 2018.
vii. **Clarke CW.** Grape Phylloxera- Biology, Risks and Management; 15th June 2018; Gippsland viticulture growers meeting, Lardener Park Warragul;
viii. **Cunningham JP; Clarke CW.** Risks and Management of Endemic and Exotic Grape Phylloxera; Yarra Valley Workshop; 30th January 2018. https://www.awri.com.au/events/yarra-valley-pre-vintage-workshop-shiraz-winemaking-trials-tasting/.

Publications

Publications in peer reviewed Journals

The following articles on improved disinfestation techniques for endemic grape phylloxera were published.

i. Clarke, C.W., Norng, S., Yuanpeng, D., Carmody, B., and Powell, K.S. (2018). Dry heat as a disinfestation treatment against genetically diverse strains of grape phylloxera. Australian Journal of Grape and Wine Research. https://doi.org/10.1111/ajgw.12340. Published 27th February 2018.
ii. Clarke, C.W., Norng, S., Yuanpeng, D., Carmody, B., and Powell, K.S. (2019). Hot water immersion as a disinfestation treatment for grapevine root cuttings against genetically diverse grape phylloxera *Daktulosphaira vitifoliae* Fitch. Australian Journal of Grape and Wine Research. https://doi.org/10.1111/ajgw.12407. Published 5th August 2019.

Publications in preparation

- Farnier K, Tobin S, Clarke CC, Carmody BC, Cunningham JP. Enhanced early detection (odour sensing technologies): Electronic noses and GC-MS analysis (Journal to be determined).
- Clarke CW, Carmody BC, Cunningham JP. Sniffer dogs and phylloxera – views of the industry (To Wine and Viticulture Journal).
- Miyazaki J, Agarwal A, Clarke CW, Carmody BM, Weiss JW, Cunningham JP, Blacket MJ. Shining a light on surveillance: extending LAMP for in-field detection of grape phylloxera infestation in vineyards (Journal of Pest Science).
- Clarke CW, Carmody BC, Cunningham JP. Alternative disinfestation for footwear and small hand- held tools against grapevine phylloxera (Australian Journal of Grape and Wine Research).
- Clarke CW, Carmody BC, Cunningham JP. Performance of the G38 phylloxera strain on varieties of *Vitis* species in South east Australia – a case study (Journal to be determined).

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i. Mark Blacket; New portable genetic test for phylloxera; https://agriculture.vic.gov.au/about/media-centre/media-releases/new-portable-genetic-test-for-phylloxera; 13 February 2020.
ii. Mark Blacket; Agriculture Victoria develops pest test to help fight vineyard threat; https://www.bendigoadvertiser.com.au/story/6632089/agriculture-victoria-develops-pest-test-to-help-fight-vineyard-threat/; 15 February 2020.
iii. Paul Cunningham; Wine grape growers hope a new test for phylloxera is good news; Victorian Country Hour - Victorian Country Hour - ABC Radiohttps://www.abc.net.au/radio/programs/vic-country-hour/victorian-country-hour/11952352; 17 February 2020

An award for excellence

Catherine Clarke; The ASVO viticulture paper of the year; Paper title "Effectiveness of sodium hypochlorite as a disinfestation treatment against genetically diverse strains of grape phylloxera *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae)" Published in Australian Journal of Grape and Wine Research; Authors Clarke, C.W., Wigg, F., Norng, S. and Powell, K.S. (2018). ASVO 2018 Awards for Excellence; https://onlinelibrary.wiley.com/doi/10.1111/ajgw.12380; https://agriculture.vic.gov.au/about/media-centre/media-releases/agriculture-victoria-scientists-win-distinguished-award; https://www.wineaustralia.com/news/articles/congratulations-to-australias-rd-e-community.

15.0 LIST OF APPENDICES

Appendix 1. Project Outputs aligned against five key themes

Appendix 2. (Part B) looking for key identifying compounds in the odour profiles of infested and uninfested plants using gas chromatography-mass spectrometry (GC-MS).

Appendix 3. Phylloxera surveillance methods

Appendix 4. New Detection Tools: LAMP

Appendix 5. In-field screening of G38 across multiple rootstocks. Randomised rootstock vines across four blocks in the North East Victoria study site.

Appendix 6. Methods – Screening of rootstocks in potted vines

Appendix 7. Phylloxera genetic diversity in the King Valley

Appendix 8. Products screened against phylloxera

Appendix 9. Feasibility of biological control of grape phylloxera (see separate document)

Appendix 10. IRG Terms of Reference phylloxera project (see separate document)

Appendix 11. Recommendations for the NPMP review (see separate document)

16.0 ABBREVIATIONS

ABBREVIATION	FULL TITLE
Ag Vic	Agriculture Victoria
ANOVA	Analysis of variance
ASVO	Australian Society of Viticulture and Oenology
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DJPR	Department of Jobs, Precincts and Regions
Enose	Electronic nose
GC-MS	Gas chromatography–mass spectrometry
IPM	Integrated Pest Management
IRG	Industry Reference Group
LAMP	Loop-mediated isothermal amplification
NPMP	National Phylloxera Management Protocol
PEZ	Phylloxera Exclusion Zone
PHA	Plant Health Australia
PIZ	Phylloxera Infested Zone
PRZ	Phylloxera Risk Zone
SIMPER	Similarity Percentage analysis
SPME	Solid phase microextraction
VHA	Vinehealth Australia

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