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Abstract

This project aimed to improve the management of grapevine phylloxera in Australia and prevent its spread into regions that are currently free of this pest. Specifically this project i) compared in-field detection methods for phylloxera and found that LAMP tests of root samples could improve the probability of detecting phylloxera, ii) developed new simulation models for biosecurity agencies to improve confidence in declarations of phylloxera absence, iii) screened novel rootstocks for resistance against prevalent phylloxera genotypes, allowing growers to make informed replanting choices, iv) evaluated disinfestation protocols under different hygiene conditions, developing an improved dry-heat disinfestation protocol for the movement of machinery between quarantine zones, v) assessed overwintering phylloxera populations to determine the risk of phylloxera dispersal during winter operations, vi) characterised new leaf galling genotypes while investigating the increased prevalence of leaf galling phylloxera in King Valley and vii) explored potential biocontrol options for phylloxera to support growers managing this pest.

Executive Summary

Grapevine phylloxera is Australia's most damaging viticulture pest. Management practices aimed at preventing the spread of phylloxera outside defined quarantine boundaries incorporate a suite of management tools and strategies including i) effective surveillance and diagnostics, ii) availability of resistant rootstocks, iii) validated disinfestation protocols, iv) comprehensive understanding of the insect's ecology, and v) sustainable management options for growers. This project aimed to improve the industry's ability to sustainably manage grapevine phylloxera by building on the previous research under these five broad themes.

Effective surveillance and diagnostics: Current surveillance practices for phylloxera rely on a time- and resource-limited "grid approach" that involves visually inspecting the roots of vines. Using this method it is, however, difficult to provide assuredness of the insect's absence when no individuals are detected following a surveillance event.

Field surveys were conducted in an infested vineyard to develop simulation models that could improve confidence in declarations of phylloxera absence. These models have been provided to Biosecurity, Agriculture Victoria, as an interactive spreadsheet where users can enter surveillance details (e.g. vineyard size, survey method, season) and be provided with an estimate of the probability of detecting phylloxera and the time needed to survey the vineyard.

Data from the field surveys were also used in a side-by-side comparison of three detection methods; visual inspection of roots, LAMP tests on emergence trap samples, and LAMP assays on root samples. Regardless of season, LAMP tests on root samples was the most effective surveillance method, followed by visual inspections and then LAMP tests on emergence trap samples. A combination of both LAMP tests on root samples and visual inspections captured over 90% of all positive detections. Incorporating LAMP tests on root samples is thus likely to benefit surveillance efforts for phylloxera by improving the probability of detecting the pest, improving confidence in declarations of absence (i.e. when LAMP tests are negative, and no physical specimen is detected), and providing overlapping layers of protection against one detection method failing.

Additionally, data collected from the field surveys was used to determine if the season (summer or autumn) or the experience of the surveyor (expert or beginner) had an impact on the probability of detecting phylloxera and the time needed to survey the vineyard. All three detection methods were significantly more effective in autumn compared to summer and experienced surveyors took significantly less time to detect phylloxera on vine roots compared to beginners. Biosecurity agencies could improve the probability of detecting phylloxera and reduce the time needed to conduct vineyard surveys by prioritising vineyard surveys in autumn and ensuring at least some of the surveyors have more than one year of experience.

The relative effectiveness of the different methods (visual inspections, LAMP tests on root samples, and LAMP tests on emergence trap samples) in winter (outside of the peak phylloxera activity) was compared. All three detection methods were significantly less effective in winter but were still able to detect the pest. The possibility of off-season surveys could be beneficial for biosecurity efforts by increasing the surveillance window and allowing surveys to be conducted at the first sign of infestation regardless of season.

Improved selection of resilient rootstocks: Grafting European vine varieties onto American rootstock is recognized as the best viticultural practice to safeguard vineyards from phylloxera-induced damage. Ongoing rootstock screening trials are essential in managing grapevine phylloxera, allowing growers to make informed replanting decisions based on the prevalent phylloxera genotype/s on or surrounding their vineyard.

This project assessed the susceptibility of two new rootstocks, M5512 and M5489, to phylloxera genotypes G4 and G38. Each vine was infested with eggs belonging to either the G4 or G38 phylloxera. Additionally, *Vitis vinifera* vines (own rooted vines) were similarly infested with phylloxera and used as a positive control.

Both M5512 and M5489 rootstocks inoculated with G4 or G38 had significantly fewer nodosities, adults and total insects compared to their positive control counterparts. M5512 roots infested with G38 phylloxera eggs showed signs of phylloxera feeding (nodosities), but no individuals of any life stage were observed. This suggests that phylloxera were able to feed and induce damage on this rootstock, but were not able to develop into adults. Nodosities were also present on M5489 roots infested with G38 phylloxera eggs, and a single individual phylloxera was also recorded. Nodosities and individuals of all life stages were observed on both M5512 and M5489 rootstocks infested with G4 phylloxera eggs. Taken together, these results suggest that both M5512 and M5489 rootstocks should be rated tolerant for both the G4 and G38 genotypes. Although no individuals were observed on M5512 rootstocks inoculated with G38 phylloxera eggs, the presence of nodosities suggests that this genotype can feed and induce damage on this rootstock. This information should be provided to growers by updating the Rootstock Selector Tool.

Improved disinfestation procedures: In order to prevent the spread of phylloxera outside of quarantine boundaries, vineyard machinery needs to be disinfested before it can be transported outside of infested or risk zones. The current disinfestation protocol requires machinery to be thoroughly cleaned (including the removal and cleaning of parts that can hide or trap soil), and then placed in a dry heat shed for either 40°C for 2 hours and 15 minutes, 40°C for 3 hours, or 45°C for 2 hours to kill any phylloxera that may have been transferred from the soil or grapevines onto the equipment. These temperatures and time requirements were determined in the laboratory under sterile conditions.

The effectiveness of dry heat disinfestation was assessed under different hygiene conditions, specifically while phylloxera were attached to a piece of root and protected by soil or grapevine material. The current dry heat disinfestation protocols were not 100% effective at killing phylloxera, regardless of the hygiene condition tested.

An improved disinfestation procedure was subsequently tested, exposing phylloxera that were attached to a piece of root to 50°C for 2 hours. This resulted in 100% mortality. The new protocol was then tested in a commercial dry heat shed using phylloxera that were either: i) attached to a root, ii) attached to a root and protected by grapevine material, iii) attached to a root and protected by soil or iv) attached to a root and protected by soil and grapevine material across 6 phylloxera genotypes (G1, G4, G19, G20. G30 and G38). 100% mortality was observed in all genotypes tested when phylloxera were attached to a root in the absence of soil and plant material, except for G30. Four percent of G30 phylloxera initially survived the dry heat exposure however, these insects died before they were able to complete development, suggesting a delayed effect of the treatment. When phylloxera were attached to the root and protected by just grapevine material, 100% mortality was achieved across all genotypes. Unfortunately, 100% mortality was not achieved when phylloxera were attached to a root and protected by soil or by both soil and grapevine material. Forty-two percent of phylloxera protected by soil survived the initial dry heat exposure, with 30% subsequently developing into adults. Similarly, when phylloxera were protected by both soil and grapevine material 50% survived both the initial exposure and the following 30 days, developing into adults. This suggests that soil but not grapevine material offers additional protection from the dry heat treatment.

These findings suggest that changing the disinfestation protocol to 50°C for 2 hours may increase the effectiveness of the dry heat disinfestation treatment. These results also highlight the importance of cleaning machinery and removing parts that can hide or trap dirt before placing them in the dry heat shed to remove any soil that may protect phylloxera from the heat.

Overwintering population dynamics: Phylloxera are slow-moving insects and dispersal is predominantly through human activity. This project investigated the risk of phylloxera dispersal by human activity during winter (outside of peak phylloxera activity), in the North-East Phylloxera Infested Zone (PIZ) by studying phylloxera development in the field during spring, winter and autumn, and assessing phylloxera development and survival at winter temperatures for three phylloxera genotypes (G1, G4, and G19) in the laboratory.

Field surveys found that the number of crawlers on vine roots was highest in winter. However, no nodosities were observed on roots in winter suggesting that phylloxera may not be feeding and may have reduced activity during this season. Laboratory studies suggest that at 4 or 9°C, phylloxera are unlikely to survive or develop into adults, but at 14°C (which is within the maximum winter soil temperatures recorded for the North-East PIZ) a small number of individuals survive and can develop from eggs into crawlers. Taken together this suggests that at least at mild winter temperatures, human-assisted dispersal of phylloxera is possible and effective disinfestation and quarantine protocols should continue to be implemented in winter.

Additionally, laboratory studies demonstrated genotypic variation in phylloxera sensitivity to winter temperatures. G1 and G4 phylloxera eggs maintained at 14°C were able to develop into crawlers and continued developing into adults once returned to control temperatures (25°C). A small number of G19 eggs maintained at 14°C were able to develop into crawlers, but these individuals died following their transfer to control temperatures (25°C). Understanding how different phylloxera genotypes develop and survive in different climates is critical in predicting how the changing climate will influence the prevalence and distribution of different genotypes in Australia. This may have significant implications for growers replanting on resistant rootstocks, as their choice of rootstock is highly dependent on the risk of infestation from specific phylloxera genotypes.

The project also investigated soil characteristics that may contribute to variation in the population dynamics of phylloxera in the field. Field surveys revealed significant negative relationships between soil moisture and the number of root galls and adults observed on roots, and soil pH and the total number of insects observed on roots. Findings from previous research on the relationships between phylloxera populations and soil characteristics have been largely inconsistent. Empirical laboratory studies are likely needed to determine which soil characteristics influence phylloxera population dynamics and whether they can be manipulated to limit their ability to establish, reproduce, or disperse.

Prevalence of leaf galling phylloxera in the King Valley: Recently, occurrences of leaf galling phylloxera in commercial vineyards have increased in many parts of the world including the North-East Phylloxera Infested Zone (PIZ) in Victoria, Australia. The project investigated whether the abundance of leaf galling phylloxera in infested vineyards is influenced by vineyard practices (such as row spacing), grape variety, or rootstock.

Field studies found no significant difference in the abundance of galls between grape varieties or rootstocks, however, several unaccounted-for variables may be masking differences among grape varieties or rootstocks (such as the number of founding phylloxera, time since initial infestation, and the age of the vines) and controlled studies in a greenhouse setting may provide a more definitive answer. There was insufficient variation in vineyard practices to statistically determine if these contribute to the abundance of leaf galling phylloxera. Similarly, controlled laboratory studies where vineyard practices can be manipulated may be beneficial.

Following field surveys, leaf galls were dissected in the laboratory to determine if there was any relationship between the number of galls on leaves and the level of infestation within the gall. There was a significant correlation between the number of phylloxera per gall and the number of galls per leaf. Whether this is due to the length of time the leaves had been infested, or whether specific leaf characteristics are better able to support or attract leaf galling phylloxera requires further study. Interestingly, approximately 17% of the leaf galls dissected did not contain any individuals. Future research could be directed at determining what is driving the absence of individuals inside some galls as this information could be used to develop management strategies that help growers reduce populations of leaf galling phylloxera.

Genetic diversity of leaf galling phylloxera in the King Valley: This study aimed at characterising the genetic diversity of leaf gall phylloxera from the King Valley. The genetic diversity of root galling phylloxera have previously be characterised in this region, this is the first study to characterise leaf galling phylloxera in the North-East Phylloxera Infested Zone.

A total of 513 samples were examined for microsatellite variation from leaf galls (497) and roots (16) collected from six vineyards, including some ornamental vines. A total of 76 genotypes were characterised. Twenty-seven of these genotypes had previously been characterised, but 42 were new. Most genotypes detected were unique to a single vineyard, whilst 12 genotypes were found at multiple sites and in leaves and roots. Almost 50% of leaves had more than one genotype (between 1 and 12), whilst galls contained up to three genotypes.

These results indicate a higher-than-expected genetic diversity of phylloxera in Victoria and future research should consider if sexual reproduction is occurring. Furthermore, this work shows the potential for genotypes to move between vineyards, regardless of the type of vines i.e., ornamental or commercial or, the part of the plant (same genotypes were found in the roots and the leaves). In-depth studies of the genetic diversity of phylloxera such as this enable appropriate selections of rootstock, which should be based on the most abundant local genotype present.

A conservation biological control approach: This study aimed to conduct field surveys to identify potential phylloxera predators in Victorian vineyards and assess the efficacy of commercial aphid predators in laboratory settings for potential use in “inundation biological control”. Field surveys were conducted in Victorian vineyards, focusing on phylloxera-infested roots and leaves to identify resident predatory arthropods. Laboratory trials tested the predation capabilities of ladybirds (*Harmonia octomaculata*) and green lacewing larvae (*Mallada signatus*) on phylloxera eggs and crawlers.

Field surveys identified 56 arthropod specimens from 24 families, with predators comprising 38% of the total taxa. Predators included spiders, ladybeetles, lacewings, ants, and predatory mites, primarily found on grapevine leaves. Laboratory feeding trials showed ladybird larvae as effective predators, consuming all phylloxera crawlers within an hour. Ladybird adults and lacewing larvae also predated phylloxera eggs, with ladybirds showing higher predation rates.

The study highlighted the potential of both conservation biological control and inundation biological control strategies. Field surveys cataloged a modest diversity of phylloxera predators, suggesting opportunities for enhancing these populations. Laboratory trials confirmed the predation capacity of commercially available aphid predators, though their practical deployment requires further investigation. Future research should focus on validating predator-prey associations through direct observation or molecular analysis and exploring large-scale application methods for these biocontrol agents.

Biological control presents a promising alternative for sustainable phylloxera management. Enhancing resident predator populations and utilizing commercial aphid predators could assist in reducing the prevalence of dispersing stages of phylloxera. Further research is essential to optimize these strategies and ensure their effectiveness in diverse vineyard conditions.

Introduction

Grapevine phylloxera (*Daktulosphaira vitifoliae*) is considered one of Australia's most damaging viticulture pests. This small aphid-like insect feeds on either the roots or the leaves of the vine. Root feeding phylloxera typically lead to vine death. There is currently no treatment that can remove phylloxera from the soil once it has been established in a vineyard. As such, delivering sustainable improvements to the management of grapevine phylloxera is a key priority of the wine industry (Strategy 3: Enhance Grape and Wine Excellence - Wine Industry Development Strategy (2020-2025)).

Managing phylloxera within quarantine boundaries requires effective surveillance and diagnostics, availability of resistant rootstocks, validated disinfestation protocols, knowledge of the insect's ecology, as well as the development of new management strategies for growers. This project aimed to improve the industry's ability to sustainably manage grapevine phylloxera by building on previous research in these areas, specifically findings from Wine Australia's Phylloxera Integrated Pest Management (IPM) Project DED1701.

Effective surveillance and diagnostics: Compared to most of the world, Australia has been incredibly successful at reducing and preventing the spread of phylloxera outside of defined quarantine boundaries. Effective surveillance of phylloxera plays a critical role in defining these boundaries. Current surveillance practices for phylloxera rely on a time- and resource-limited "grid approach" which involves visually inspecting the roots of vines. Importantly, due to the cryptic nature of phylloxera, it is difficult to provide assuredness of the insect's absence when no individuals are detected following a surveillance event. New diagnostic tools (such as the new LAMP tests developed by Agriculture Victoria as part of Wine Australia's project DED1701) may improve the probability of detecting phylloxera when it is present. Additionally, statistical models could be developed to help estimate the probability of detecting phylloxera when it is present, allowing biosecurity agencies to determine the time and resources needed to effectively survey a vineyard, increasing confidence in declaring phylloxera absences.

In this project field surveys were conducted in an infested vineyard to develop simulation models that can be used to estimate both the probability of detecting phylloxera if it is present and the time needed to survey the vineyard. Data from the field surveys was also used to compare three detection methods specifically, visual inspection of roots, LAMP tests on emergence trap samples, and LAMP tests on root samples. Additionally, data collected from the field surveys was used to determine if the season (summer or autumn) or the experience of the surveyor (expert or beginner) had an impact on the probability of detecting phylloxera and the time needed to survey the vineyard respectively. Lastly, the relative effectiveness of different surveillance methods were compared in winter (outside of the peak phylloxera activity),

Improved selection of resilient rootstocks: Currently no treatment can remove phylloxera from the soil and grafting European vine varieties onto American rootstock is recognized as the best viticultural practice to safeguard vineyards from phylloxera-induced damage. Ongoing rootstock screening trials are essential in managing grapevine phylloxera. These trials determine the susceptibility of novel rootstocks to specific phylloxera genotypes, by comparing the ability of phylloxera to feed (induce nodosities), survive, and reproduce on novel rootstocks to a positive control (i.e. a rootstock highly susceptible to grapevine phylloxera). As phylloxera genotypes vary in their ability to feed and survive on different rootstocks, novel rootstocks need to be screened against specific genotypes. This allows growers to make informed decisions when replanting with resistant rootstocks based on the presence of specific phylloxera genotypes on or surrounding their vineyard. Merbein 5512 (M5512) and Merbein 5489 (M5489) are two novel rootstocks bred from complex hybrids of *V. berlandieri.* These rootstocks have been selected for their low uptake of potassium in their berries, and their ability to use water efficiently. Although the rootstocks have good nematode tolerance, their tolerance to phylloxera is unknown.

In this project rootstock screening trials were conducted to determine the susceptibility ratings for M5512 and M5489 against tow prevalent phylloxera genotypes: G4 and G38.

Improved disinfestation procedures: Due to their small size, phylloxera's ability to disperse naturally is limited (although wind dispersal has been documented), as such new phylloxera infestations are predominately the result of human activity. Phylloxera crawlers (first instars) can travel above the ground, where they can be collected by vineyard machinery, people's clothing or footwear, or plant material. These crawlers can be transferred to new vineyards if the contaminated material is not effectively disinfested before

leaving the vineyard or quarantine boundary. Most disinfestation protocols require exposing phylloxera-contaminated material to hot or cold temperatures that are outside the limits of the insect's physiological tolerance. The current disinfestation protocols for machinery require removing any parts of the machinery that may hide or contain dirt and thoroughly cleaning the machinery, before placing the machinery in a dry heat shed under specific temperatures and time-periods. The specific temperatures and time-periods for dry heat disinfestation were previously validated in the laboratory and found to result in 100% mortality across a diverse range of phylloxera genotypes. In a laboratory setting phylloxera are typically exposed to the disinfestation temperature under sterile conditions, while in practice, phylloxera may be exposed to the disinfestation temperatures under different hygiene conditions. For example, phylloxera may still be attached to a small piece of root or may be covered by soil or grapevine foliage when exposed to the heat treatment. Protocols need to be validated in the field to ensure they are effective under different hygiene conditions.

This project evaluated the current dry-heat disinfestation protocols in the presence of soil and grapevine material in both the laboratory and the field against a diverse panel of phylloxera genotypes

Overwintering population dynamics: The risk of phylloxera dispersal from human activity may vary between seasons. Seasonal differences in the abundance of different phylloxera life-stages have been observed including phylloxera crawlers (the mobile life-stage of phylloxera). Improved knowledge of the seasonal variation in phylloxera population dynamics and survival overwinter could inform polices that help prevent or minimize human-assisted dispersal of phylloxera during winter operations.

This project investigated the risk of phylloxera dispersal by human activity during winter in the North-East (PIZ) by assessing phylloxera development under field conditions and assessing phylloxera development and survival at winter temperatures in the laboratory. Additionally, field surveys investigated soil characteristics that may contribute to variation in the population dynamics of phylloxera in the field.

Prevalence of leaf galling phylloxera in the King Valley: There has been a recent rise in the prevalence of leaf galling phylloxera in vineyards in many parts of the world including the North-East Phylloxera Infested Zone (PIZ) in Victoria. Unlike root galling phylloxera and despite inducing striking galls on the surface of the leaf, leaf galling phylloxera are thought to have little impact on the health of grape vines. As such, few studies have investigated the factors that impact their abundance.

This project investigated whether the abundance of leaf galling phylloxera in infested vineyards is influenced by vineyard practices (such as vine spacing, irrigation, row spacing and the cardinal direction of the vines), grape variety, or rootstock by counting the number of galls on leaves collected from different vineyards in the North-East PIZ. Galls were then dissected to determine whether there was any relationship between the number of galls on leaves and the level of phylloxera infestation within each gall.

Genetic diversity of leaf galling phylloxera 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. A recent study also from roots, revealed a considerably higher than previously thought number of genotypes, with a total of 36 genotypes characterised. Despite reports of leaf-galls from this area, no leaf-gall study had ever been taken place. Yet, understanding the genetic relationships between leaf-gall and root-gall phylloxera is important for its management as well as understanding if these could be involved in a sexual life cycle. 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.

This project investigated the genetic diversity of more than 500 leaf-gall phylloxera from the King Valley and adjacent areas, from leaf-galls and from a subset of root samples using microsatellite markers.

A conservation biological control approach: Biological control is a fundamental component of many Integrated Pest Management (IPM) systems. However, grape phylloxera has traditionally been managed with a focus on chemical treatments and phylloxera-resistant rootstocks, leaving biological control relatively underexplored. Although there have been some successes, particularly with entomopathogenic fungi in laboratory and field trials abroad, other biological control methods have not been thoroughly investigated.

A literature review conducted under the Wine Australia project DED 1701 evaluated various biological control strategies for grape phylloxera in Australia. These strategies included Classical Biological Control (releasing exotic natural enemies from the pest's native habitat), Inoculation Biological Control (introducing commercially reared predatory insects, mites, or parasitoids), Inundation Biological Control (utilizing

entomopathogenic organisms like nematodes and fungi), and Conservation Biological Control (promoting resident native predators through habitat enhancement).

The review indicated significant potential for these approaches. Classical Biological Control showed promise, especially with the predatory syrphid fly (*Heringia salax*), which effectively targets both adult and juvenile stages of root galling grape phylloxera. Inundation Biological Control has made global strides using entomopathogenic fungi and nematodes, though research on biopesticides for grape phylloxera remains limited. Conservation Biological Control could benefit from the promotion of native predators in Australian vineyards, with recent studies highlighting the potential of native plants to attract diverse insect predators.

This study aimed to explore the potential of both Inundation and Conservation Biological Control for managing grape phylloxera. Field surveys in Victorian vineyards identified resident predatory arthropods on phylloxera-infested roots and leaves, assessing their suitability for Conservation Biological Control. Laboratory trials tested commercially available aphid predators (lacewings and ladybirds) for their efficacy in preying on phylloxera eggs, evaluating their potential for Inundation Biological Control. This approach aims to foster biological control approaches and promote sustainable pest management practices in vineyards

Project Objectives

This project aimed to improve the industry's ability to sustainably manage grapevine phylloxera across five key areas. Specifically:

1. More effective surveillance and diagnostics
2. Improved selection of resilient rootstocks
3. Improved disinfestation procedures
4. Improved knowledge of Australian phylloxera ecology
5. A conservation biological control approach

Detailed project objectives for each key area are provided in the corresponding technical reports in this document

Project Outputs

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Theme and short title	Output	Milestones	Page number
MORE EFFECTIVE SURVEILLANCE AND DIAGNOSTICS			
Statistical models to increase confidence in declarations of phylloxera absence	Interactive excel spreadsheet which allows users to estimate the probability of finding phylloxera if it is present across seasons and using different detection methods	3a, 3d	Appendix 5 (provided as a separate document)
Large-scale comparison of different detection methods for phylloxera across seasons	Technical report on field survey techniques for effectiveness at detecting phylloxera	4c	Page 20
Surveillance and phylloxera detection training module	Draft slide deck outlining the key findings from this research along with instructions on how to use the interactive spreadsheet to be finalised with Biosecurity and Agricultural Services	4c	Page 115
Extension	Training of new Biosecurity and Agriculture Services (BAS), and Agriculture Victoria Research (AVR) staff on phylloxera detection and surveillance in the field during data collection for this activity	4h	Page 115
Publications	Publication on the use of LAMP for phylloxera detection	4h	Page 115
IMPROVED SELECTION OF RESILIENT ROOTSTOCKS			
Screening M-series rootstocks across phylloxera strains	Technical report on screening rootstock M5512 and M5489 across G4 and G38 phylloxera strains with detailed updated screening methods and recommended susceptibility ratings.	3b, 4g	Page 42

Extension	Stakeholder engagement at Mornington Peninsula Growers meeting November 2021; AWRI Seminar series 2021; AWITC conference 2022	4h	Page 115
Publications	Publications on interactions of phylloxera and rootstocks	4h	Page 115
IMPROVED KNOWLEDGE OF AUSTRALIAN PHYLLOXERA ECOLOGY			
Environmental- and rootstock-related factors that may account for increased prevalence of leaf galling strains in vineyards, and associated risks to industry.	Technical report on study on ecology and prevalence of leaf galls in the North-East Victoria Phylloxera Infested Zone (PIZ), including recommendations to state biosecurity and industry	2c, 3e	Page 80
Genotype mapping of leaf galls in North-East Victoria phylloxera infested zone	Technical report on genotyping study of leaf galling phylloxera including recommendations to state biosecurity and industry	4d	Page 91
Extension	State biosecurity meeting organised by CPHOU and AVR March 2021, HPIS conference	4h	Page 115
IMPROVED DISINFESTATION PROCEDURES			
Assessment of phylloxera life-stage development over wintering *under field conditions*	Technical report on phylloxera populations on vine roots over winter in a focal study site in North-East Victoria	2a, 4b	Page 64
Assessment of phylloxera survival through "overwintering" *on excised roots under laboratory conditions*	Technical report on the impact of overwintering temperatures on phylloxera survival	4a, 4e	Page 64
Evaluating the effectiveness of the dry heat disinfestation procedures under soil and grapevine material contamination	Technical report on improved validity of disinfestation procedures for machinery and vineyard equipment through assessment in laboratory and industry aligned protocols	1b, 2b, 3c	Page 52

Extension	State biosecurity meeting organised by CPHOU and AVR March 2021, Seminar to diploma of Viticulture students October 2023	4h	Page 115
A conservation biological control approach			
Assessment of potential for conservation biological control of phylloxera in Victorian vineyards	Technical report on possible phylloxera predators on vine leaves and roots in King Valley vineyards	2d, 4f	Page 106
A conservation biocontrol approach for phylloxera	Technical report on laboratory feeding trials using commercially available aphid predators	4f	Page 106
Extension	State biosecurity meeting organised by CPHOU and AVR March 2021	4h	Page 115
Communication outputs			
At least one industry publication / website contribution / conference attendance / Industry stakeholder engagement / media release on other outputs of the project	Appendix 1: complete list of all publications, industry articles, conference presentations, and other communication outputs	4h	Page 115
FINAL REPORT SUBMITTED	Full final report	4i	This document

Research Theme 1: Effective Surveillance and Diagnostics

Improved Surveillance and Detectability of Grapevine Phylloxera

Executive Summary

Effective vineyard surveillance and accurate identification of phylloxera are crucial in defining and updating the quarantine boundaries that have been instrumental in preventing and slowing the spread of grapevine phylloxera. Current surveillance practices for phylloxera rely on visual inspection of vine roots (often referred to as the dig method) by trained biosecurity officers during summer and autumn at the peak of phylloxera activity. As it is not feasible to inspect every vine, vineyards are surveyed using a time- and labour-intensive grid approach. Surveyors dig to expose the root system of the target vine and inspect roots for insects using a hand lens. Other in-field detection methods for phylloxera have been developed but are not widely used during field surveys; these include emergence traps and LAMP. Emergence traps (bucket traps) can be deployed throughout a vineyard to capture phylloxera crawlers (or crawler DNA) for morphological or molecular testing. The LAMP assay is a portable device that enables rapid and accurate molecular ID of phylloxera in-field, and can be used on insects, root samples and emergence trap samples.

Phylloxera are small insects and difficult to detect even with a hand lens, as such if phylloxera are not detected during vineyard surveillance, it is difficult for biosecurity agencies to be confident that phylloxera was not present. Here, we used data collected from our own field surveys, along with historical data to develop simulation models that can be used to improve confidence in declarations of phylloxera absence. These models were then used to create an interactive Excel spreadsheet that will allow users to estimate the probability of detecting phylloxera if it is present in a vineyard, as well as the time required to survey a vineyard. Both visual inspections and LAMP tests of root samples were included in the model. LAMP test on emergence trap samples were not included due to the low number of positive detections. The interactive Excel spreadsheet (Appendix 5) has been provided to Agriculture Victoria's Biosecurity and Agriculture Services Branch for feedback.

Field surveys were also used to conduct a side-by-side comparison of the three detection methods (visual inspections of roots, LAMP tests on emergence trap samples and LAMP tests on root samples). **LAMP tests on root samples was the most effective detection method**. 48.33% of root samples tested with LAMP were positive for phylloxera. 22.08% of roots tested positive for LAMP with no corresponding positive sample for the other detection methods, while the remaining 26.25% of root samples tested positive for LAMP with a corresponding positive detection for a least one other method. Visual inspection of roots had the next highest proportion of positive detections (34.58% of the visual inspections resulted in a positive detection) and LAMP assays on emergence trap samples was the least effective detection method (19.16% of LAMP tests on emergence trap samples were positive). A combination of both LAMP tests on root samples and visual inspections captured over 90% of all positive detections. **Incorporating LAMP tests on root samples is likely to benefit surveillance efforts for phylloxera** by i) improving the probability of detecting the pest, ii) improving confidence in declarations of absence (i.e. when LAMP tests are negative, and no physical specimen is detected) and iii) providing overlapping layers of protection against one detection method failing.

Further analysis of our field data revealed that all three detection methods were significantly more effective in autumn compared to summer. Biosecurity agencies could consider prioritising vineyard surveillance in autumn to improve the probability of detecting phylloxera. Additionally, we found that surveyors with more than one year of experience conducing vineyard surveys (experts) took significantly less time to find phylloxera during visual inspections of roots compared to beginners (surveyors with less than one year of experience). The time needed to detect phylloxera during vineyard surveys may be significantly less if at least some of the surveyors are experienced.

Lastly, we conducted a field survey during winter (outside of the peak phylloxera activity) to compare the relative effectiveness of the three detection methods. All three detection methods were significantly less effective in winter (compared to surveys conducted during peak phylloxera activity). Nonetheless, all three detection methods were able to detect phylloxera during winter and the off-season surveys could be beneficial by increasing the surveillance window and allowing surveys to be conducted at the first sign of infestation regardless of season.

Key findings and recommendations

Simulation models to improve confidence in declarations of absence

- Data from field surveys, along with historical data were used to build simulation models to improve confidence in declarations of absence
- These models have been built into an interactive Excel spreadsheet, allowing users to estimate the probability of detecting phylloxera and the time needed to survey a vineyard
- To provide estimates on the probability of detecting phylloxera and the time needed to conduct the survey, the interactive Excel spreadsheet asks users to enter information on the surveillance approach, detection method, vineyard size, prevalence of phylloxera and the percentage of symptomatic vines.
- The interactive Excel spreadsheet has been provided to Agriculture Victoria's Biosecurity and Agriculture Services Branch for feedback

Comparisons of detection methods

- LAMP analysis of root samples was the most effective surveillance method, followed by visual examination of roots.
- Our results suggest that incorporating LAMP tests of root samples (or similar molecular techniques) into the current surveillance practices would improve phylloxera detectability.

Effect of season

- All three detection methods were more effective in autumn compared to summer
- Biosecurity agencies may consider prioritizing vineyard surveillance in autumn

Effect of experience

- Surveyors with more than one year of experience conducting vineyard surveys for phylloxera took significantly less time to find phylloxera compared to surveyors with less than one year of experience
- Biosecurity agencies should consider how the experience of the surveyors will impact the time needed to conduct vineyard surveys

Surveillance outside of peak phylloxera activity

- All three detection methods were significantly less effective in winter
- Biosecurity agencies could consider prioritizing surveys in autumn or summer. Noting that off-season surveys could be beneficial by increasing the surveillance window

Introduction

Grapevine phylloxera (*Daktulosphaira vitifoliae)* is considered one of the most damaging viticulture pests worldwide. Phylloxera feed on either the roots or leaves of the vine, but root galling phylloxera are typically more damaging to the plant (Benheim et al., 2012). Root-feeding phylloxera induce nodosities or tuberosities on the roots of the vine, which inhibit water uptake (Clarke et al., 2023) and create rips and tears in the root system which exposes the vine to soil-borne pathogens (Edwards et al., 2006; Granett et al., 1998). Vines infested with root-feeding phylloxera typically die within three to six years of the initial infestation (Buchanan, 1990). Australia has been incredibly successful at slowing, if not preventing, the spread of phylloxera outside of defined quarantine boundaries. Phylloxera Infested Zones (PIZ) are quarantine boundaries that contain at least one vineyard currently or previously infested with phylloxera and extend a minimum of five kilometres from the closest infested vineyard (National Vine Health Steering Committee, 2009). Enforceable regulations restrict the movement of vineyard equipment or material originating from within PIZs. As dispersal of phylloxera is predominately driven by human activity (Deretic et al., 2003; Powell et al., 2008), this severely limits the dispersal of this pest. Effective vineyard surveillance

and accurate identification of phylloxera are crucial in defining and updating these quarantine boundaries and therefore decreasing the risk of new infestations.

Current surveillance practices for phylloxera rely on visual inspection of vine roots (often referred to as the dig method) by trained biosecurity officers during summer and autumn at the peak of phylloxera activity. Vineyards are surveyed using a time- and labour-intensive grid approach where a single vine in every 5th panel of every 2nd or 3rd row is inspected. Inspection of vines requires surveyors to first dig at the base of the vine (Figure 1.1) and collect small pieces of root which are then inspected in the field for phylloxera and root galls using a hand lens (Buchanan, 1987; National Vine Health Steering Committee, 2009). Any suspect samples are then sent to Agriculture Victoria Research (AVR) diagnostic scientists, who use morphological and molecular tools to determine whether the sample is phylloxera.



Figure 1.1: AVR researcher digging at the base of a vine to expose the root system to conduct a visual inspection of the root system for grapevine phylloxera

In addition to the grid approach, biosecurity officers will also inspect any vines showing symptoms which may indicate phylloxera infestation, such as stunted growth (Powell et al., 2009), yellow leaves, and a decline in canopy vigour (Benheim et al., 2012). Unfortunately, these symptoms are also common to other pests and diseases as well as vines experiencing stress due to high temperatures or dehydration (Benheim et al., 2012). Additionally, phylloxera's small size (approx. 1mm) and cryptic nature, make it difficult for biosecurity officers to be confident in declarations of phylloxera absence if no individuals or root galls are found during a survey. Importantly the probability of detecting phylloxera if it is present is unknown but is likely to vary with the pest's prevalence and may vary with the experience of the surveyors (i.e. biosecurity officers) (Hauser et al., 2021) and season (Powell et al., 2009). Biosecurity officers require tools that allow them to make reliable decisions on the amount of time they should spend surveying individual vineyards and the probability that they will find phylloxera if it is present.

Although the dig method is the most common surveillance method used in Victoria, other methods have been developed. Emergence traps (sometimes referred to as bucket traps) capture the mobile life stages of phylloxera (crawlers and alates) which sometimes travel above the ground to infest new vines (Powell et al., 2007). Emergence traps are typically made of translucent plastic containers (between 4 and 5 L). A fine spray of water is applied to the internal surface of the trap, which is then placed upside down next to its target vine (Figure 1.2). The condensation that forms inside the trap, intercepts some of the mobile individuals traveling from the roots to above the soil. This condensation is collected by biosecurity officers periodically and, as with visual surveillance, suspect samples are sent to AVR diagnostic scientists for morphological and/or molecular confirmation. In-field molecular detection tests for grapevine phylloxera are also available. LAMP (loop-mediated isothermal amplification, (Notomi et al., 2000) is a relatively new molecular technology that provides rapid and accurate diagnostics of target species (e.g. Blacket et al., 2020). LAMP tests can be conducted on portable devices using species-specific kits and are relatively easy to perform and provide results that are easy to interrupt (Figure 1.3). Agriculture Victora have previously developed a LAMP assay for grapevine phylloxera (Agarwal et al. 2020). More recently, this LAMP assay was demonstrated to detect phylloxera in condensation wash samples collected from emergence traps and from pieces of root collected in the field (Clarke et al., 2020). Whether the use of LAMP assays on emergence trap samples or root samples can improve the probability of detecting phylloxera has not previously been tested and is unknown.



Figure 1,2: Emergence traps (bucket traps) deployed next to target vines and surveyors inspecting vine roots with a hand lens

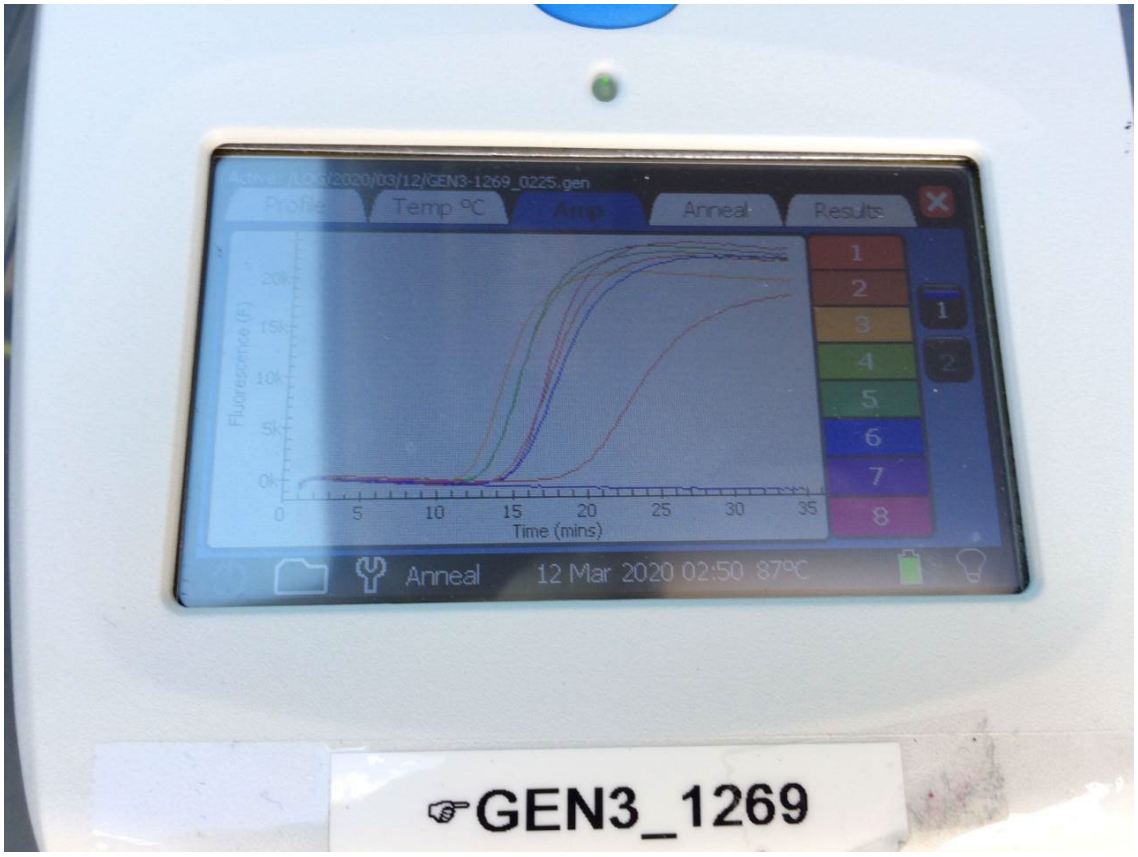


Figure 1.3: Portable LAMP machine displaying positive detections for grapevine phylloxera in the field during validation of LAMP assays (Clarke et al., 2020). The presence of coloured annealing curve indicates that phylloxera DNA was detected in the corresponding-coloured sample (numbers 1 to 8 on the right hand side of the machine)

Here we conducted field surveys in an infested vineyard to develop simulation models that can be used to estimate both the probability of detecting phylloxera if it is present using different detection methods, namely visual inspections (dig method) and LAMP assays of root samples. The simulation models also provide users with an estimate of the time needed to survey the vineyard. Data from the field surveys was also used to compare three detection methods specifically: i) visual inspection of roots, ii) LAMP assays on emergence trap samples, and iii) LAMP assays on root samples. Additionally, we used the data collected from our field surveys to determine if the season (summer or autumn) or the experience of the surveyor (expert or beginner) had an impact on the probability of detecting phylloxera and the time needed to survey the vineyard respectively. Lastly, we compared the relative effectiveness of different surveillance methods in winter (outside of the peak phylloxera activity), when surveys are not normally conducted.

Project Objectives

The aims of this project were to:

i) Develop simulation models to establish a level of confidence in detecting a phylloxera infestation at different levels of prevalence.

ii) Conduct a large-scale side-by-side comparison of the different detection methods specifically: visual inspection, LAMP analysis of emergence traps, and LAMP analysis of root samples.

iii) Review prior and within-project data for any relationship between rootstock, genotype, and environmental factors on phylloxera detection.

iv) Assess the relative effectiveness of using different surveillance methods outside of peak phylloxera activity.

Methodology

Field survey

Monthly field surveys were conducted at an infested vineyard in the Yarra Valley that between December 2021 and April 2022, January – April 2023, and June – August 2023 (outside of the peak phylloxera activity: Powell, 2009). Three detection methods were used to examine vines for the presence of phylloxera during each survey: i) visual inspection of roots, ii) LAMP assays of emergence trap samples, and iii) LAMP assays of root samples. All vines examined belonged to the same two rows of own-rooted vines infested with the G1 phylloxera genotype. This vineyard had previously participated in phylloxera surveillance and diagnostics research developing methods for LAMP assays (Clarke et al., 2020), noting that the rows of vines sampled in this research belonged to a different block.

Visual inspections

During each survey, surveyors were instructed to dig at the base of each vine to expose the root system. Roots were then cut from the vine and examined for phylloxera using a hand lens (Figure 1.2), in line with current vineyard surveillance practices. Surveyors were given ten minutes to find phylloxera and the time taken to find phylloxera for each vine inspection was recorded, along with the vine identification (vine ID) and the previous experience of the surveyor. Surveyors were sourced from Biosecurity Agricultural Services (BAS), Agriculture Victoria Research (AVR) scientists, PhD students, and AVR interns. The prior experience of each surveyor was classified as either *beginner* (defined as less than one year of experience conducting field surveys for phylloxera) or *expert* (with more than one year of experience conducting field surveys for phylloxera).

All cut roots inspected, regardless of whether phylloxera was detected were placed in sterile vials (250 ml) containing 100% ethanol for LAMP analysis (described below: *LAMP analysis of root samples*). All vials were carefully labelled with the vine ID and stored at 4°C upon returning to the laboratory until they could be prepared for LAMP assays.

LAMP analysis of emergence traps

Emergence traps were deployed in two rows of vines, one month prior to the beginning of each field surveying period and were reset each month during the survey (See Table 1.1 for details on the number of emergence traps deployed each year). Each trap consisted of a translucent round plastic container (21cm width and 18cm depth). A fine spray of water was applied to the internal surface of each trap, which was then placed upside down next to its target vine (Figure 3). Metal tent pegs were used to secure the trap in place, ensuring that a strong seal was formed between the trap and the ground to allow condensation to collect. This condensation trapped phylloxera crawlers and alates when travelling from the roots of the vine to the surface inside the trap.

Table 1.1: Number of visual inspections of roots, LAMP assays on emergence trap samples, and LAMP assays on root samples for each month across all sampling periods. The number of visual inspections performed each month varied with the number of surveyors available during each field survey.

Sampling period	Month	Number of visual inspections	Number of LAMP assays on emergence trap samples	Number of LAMP assays on root samples
Year 1	**December**	**31**	**86**	**20**
	January	**28**	**86**	**20**
	February	**71**	**0***	**20**
	March	**41**	**86**	**20**
	April	**32**	**86**	**20**
Year 2	**January**	**63**	**80**	**20**
	February	**30**	**80**	**20**
	March	**67**	**80**	**20**
	April	**36**	**80**	**20**
Outside of peak phylloxera activity	**June**	**30**	**80**	**20**
	July	**24**	**80**	**20**
	August	**30**	**80**	**20**
Total across all sampling periods and months		**483**	**904**	**240**

*Emergence trap samples were not collected in February in Year 1 as transportation difficulties limited the time available to conduct the survey. We prioritised the visual inspection and collection of root samples over the collection of emergence trap samples during this surveillance event.

During each monthly survey (except for February 2022 – see Table 1.1 for details), emergence trap samples were collected by rinsing the inside of the trap with 100% ethanol to collect any insects captured by the condensation on the internal surface of the trap. The ethanol was then carefully collected into individual sterile plastic vials (250 ml) labelled with the vine ID. Each trap was then reset or replaced (if damaged). All samples were then stored at 4°C upon returning to the laboratory until LAMP tests could be performed.

Emergence trap samples were prepared for LAMP assays as described by Clarke et al., 2020. In brief, each sample was filtered using coffee filter paper placed in a funnel, by carefully washing the vial and its lid with 100% ethanol. The filter paper and the remaining residue (which contains any trapped insects) were then removed from the funnel and allowed to air dry. Once all the ethanol had evaporated, the filter paper was trimmed down to the section containing only the residue and the paper was placed in a 2 ml microtube along

with between 500 – 600 µl of HotShot buffer (consisting of 25 mM NaOH and T10E1 buffer: Truett et al., 2000), ensuring the filter paper was completely submerged in the buffer. The samples were then incubated at 95°C for 10 minutes to extract any DNA from the filter paper. The samples (DNA extracts) were subsequently chilled on ice for 1 minute before being stored at 20°C until the LAMP assays could be performed.

Due to the large number of samples collected each month (see Table 1.1 for details), LAMP assays were performed on a qPCR machine (Thermo Fisher Quant Studio 3), as the portable GenieIII LAMP machine can only analyse eight LAMP reactions at a time, including at least one positive and negative control. qPCR machines, however, can simultaneously analyse up to 96 samples, using standard LAMP reagents. As such, the qPCR machine can run the same test as the portable LAMP machine at a much higher scale.

All LAMP assays were conducted following Agarwal et al., (2020). For each test 25µl of reaction mix (consisting of 14µl of "Isothermal Master Mix DR001" (made following the manufacturer's instructions) and 10µl of primer master mix (See Table 1.2 for details) was added to each well of a 96-well qPCR plate. 1µl of DNA extract was then added to 90 of the wells. Four of the remaining wells were assigned positive controls by adding 1µl of phylloxera synthetic phylloxera DNA. No DNA was added to the two remaining wells (negative control). LAMP amplification involved 120 cycles of amplification at 65°C for 30 minutes (with fluorescence measured every 15 seconds), followed by an annealing curve analysis from 98°C to 73°C with ramping at 1.6°C per second.

Table 1.2: Primer concentrations and volumes used in the 100 µl Primer Master Mix for LAMP assays on emergence trap and root samples

Reagent	Concentration (µM)	Recipe (µL)
Phylloxera_F3	10	10
Phylloxera_B3	10	10
Phylloxera_FIP	100	6
Phylloxera_BIP	100	6
Phylloxera_FL	100	3
Phylloxera_BL	100	3
H_2O	N/A	62

LAMP analysis of root samples

For each monthly survey, 20 randomly selected root samples collected during the visual inspection of vine roots (described previously) were analysed for the presence of phylloxera using LAMP assays following Clarke et al., 2020. As the root samples were too large to fit inside the 2ml microtube, three segments of each root were removed using sterile razor tips. Segments of roots bearing signs of phylloxera infestation such as nodosities were prioritized but otherwise, the segments were selected at random. The root segments were placed in 2ml microtubes along with between 500 – 600 µl of HotShot buffer (described above), ensuring the root segment was fully submerged in the buffer. The samples were then incubated at 95°C for 10 minutes to extract any DNA from the root segment. The samples (DNA extracts) were chilled on ice for 1 minute and then stored at 20°C until the LAMP assays could be performed. Prior to conducting the LAMP assays all DNA extracts were diluted to 25x using ultrapure water as previous research found this improved the performance of these assays (Clarke et al., 2020). LAMP assays were then performed on a qPCR machine as described above.

Development of simulation models

Data collected from the above surveys during summer and autumn (but not winter) along with historical data were used to build simulation models in Excel. These models were then used to create an interactive Excel spreadsheet (**Appendix 5**) that will allow users to estimate the probability of detecting phylloxera if it is present in a vineyard, as well as the time required to survey a vineyard. Both visual inspections and LAMP

assays of root samples were included in the model. LAMP assays on emergence trap samples were not included due to the low number of positive detections (see side-by-side comparison results below).

To build the models we assumed that **the probability of detecting phylloxera in 1 hectare of vineyard** is equal to the probability of detection for a specific visual inspection or LAMP assay x the number of vines examined x the prevalence of the pest in 1 hectare. The formula can be expressed as

$$Pr\ Pr\ (detecting\ phylloxera\ in\ 1\ ha)\ = Pr\ Pr\ (detect_t)\ \times\ n\ \times\ prop(invested\ vines)$$

To calculate the probability of detecting phylloxera on a specific vine by visually inspecting the root we used the formula:

$$Pr\ Pr\ (detect_t)\ = 1 -\ e^{(-\lambda t)}$$

Where t equals the search effort (i.e. the time spent examining a specific vine) and λ equals the detection rate.

The detection rate (λ) for visual inspections of vine roots was estimated by dividing the total number of vine examinations by the sum of all detection times. The detection rate was calculated separately for beginner and experienced surveyors and summer and autumn surveys. Where phylloxera was not detected, the detection time was substituted for ten minutes. The formulae can be expressed as:

$$\lambda = \frac{n}{\sum t}$$

To estimate the time needed to survey a vineyard we made the following assumptions:

i) Rows of vines are planted between 2.2 to 3 meters apart, in line with standard vineyard practices

ii) Vines are planted evenly along each row, approximately 1.5 meters apart, in line with standard vineyard practices

iii) Surveyors are using the 'grid' approach (i.e. surveyors are examining a single vine in every 5th panel of every 2nd or 3rd row)

iv) Surveyors are also examining any symptomatic vines encountered during the survey

v) The time to walk between rows is between 6 to 10 seconds (unpublished data John Weiss)

vi) The time to walk between panels is between 15 to 20 seconds (unpublished data John Weiss)

vii) It takes approximately 30 seconds to place roots in sterile vials with ethanol for future LAMP analysis

Statistical analysis

All statistical analyses were performed in R studio (version 4.1.1). Survival analyses were used to compare the time taken for surveyors to find phylloxera in autumn and summer, and to compare the time taken for expert and beginner surveyors to find phylloxera. Survival analyses were conducted with the R package "survival".

Chi-square tests were then used to compare the number of positive detections for each detection method between summer and autumn, within the peak of phylloxera activity, and outside of the peak of phylloxera activity (winter). To meet the assumptions of chi-square tests positive detections were only included when all three detection methods were conducted on the same vine during the same sampling event.

Additionally, to ensure equal sampling effort when comparing the relative effectiveness of the different detection methods outside of the peak of phylloxera activity, we analysed a subset of positive detections in summer and autumn comprising only the positive detections in the three months immediately prior-to the winter surveys.

Key Research Findings

Across all field surveys, we assessed a total of 1627 samples for grapevine phylloxera, comprising 483 visual inspections, 904 LAMP assays on emergence trap samples, and 240 LAMP assays on root samples. Positive phylloxera results were obtained in 17.64% of visual examinations, 8.97% of emergence trap sample LAMP assays, and 42.08% of root sample LAMP assays. **These findings suggest that LAMP assays on root samples is the most effective detection method.**

Simulation models

An interactive Excel spreadsheet (Figure 1.4) has been provided (**Appendix 5**) that will allow users to estimate the probability of detecting phylloxera at different levels of prevalence using visual inspections or LAMP assays of root samples. LAMP analysis of emergence trap samples was not included due to the low rate of detection (8.97%). The spreadsheet asks users to enter information on i) the number of hectares being surveyed, ii) the season (summer or autumn), iii) the surveillance method (LAMP or visual), iv) the experience of the surveyors (expert, beginner or mixed), v) the time spent examining each vine, vi) the survey strategy (every 2nd row or every 3rd row), vii) the prevalence of phylloxera (i.e the proportion of vines expected to be infested), viii) the number of vines per hectare, ix) the walking speed between rows, x) the walking speed between panels, xi) the percentage of vines showing symptoms, and xii) the probability of surveyors encountering symptomatic vines.

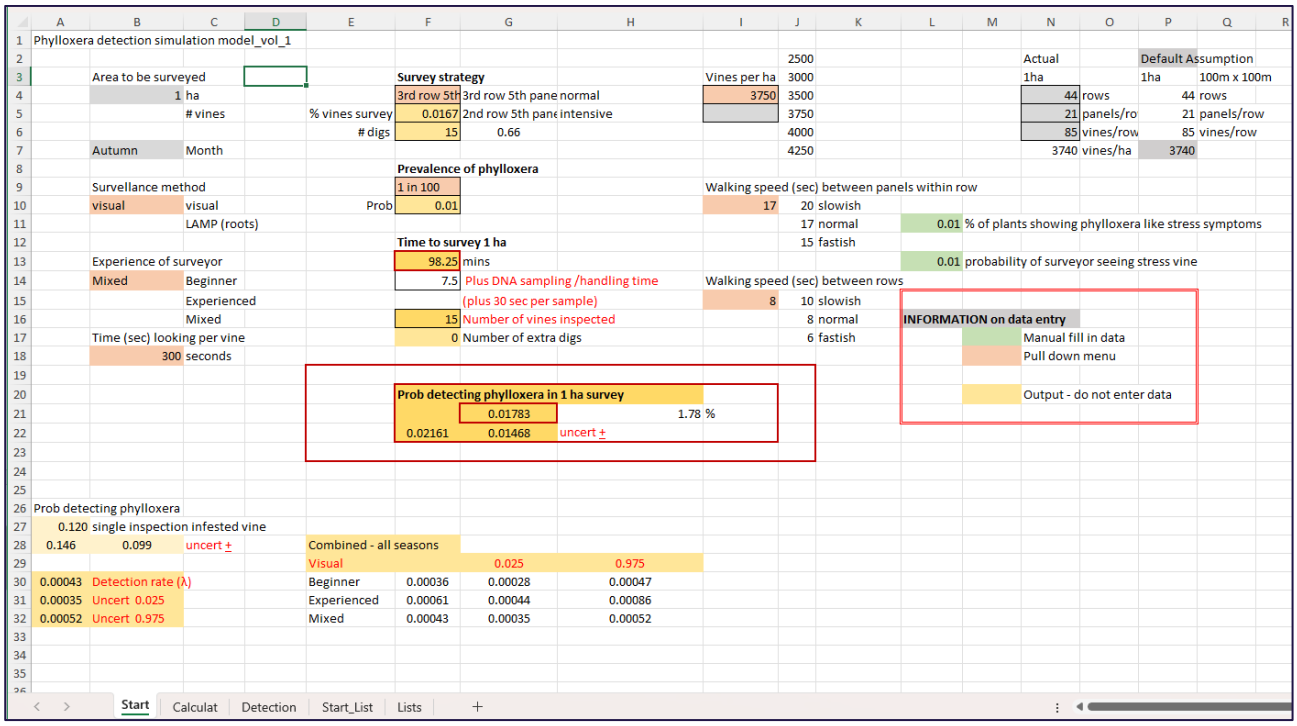


Figure 1.4: Simulation models were developed into an interactive spreadsheet, allowing users to estimate the probability of detecting phylloxera if it is present and the time needed to survey the vineyard. The spreadsheet has been provided to Agriculture Victoria's Biosecurity and Agriculture Services branch for feedback

This information is then used to calculate the number of vines to be examined and to estimate the time required to survey 1 hectare. The formulas described above are then used to estimate the probability of detecting phylloxera if it is present.

For example, assuming: i) the survey area is one hectare, ii) the season is autumn, iii) the surveillance method is visual inspections (I.e. the dig method), iv) the surveyors have mixed experience, v) the surveyors

spend five minutes examining each vine, vi) every third row is surveyed, vii) one in every 100 vines are infested, viii) the number of vines per hectare is 3740 (standard vineyard practice) ix) the walking speed between rows is normal (8 seconds), x) the walking speed between panels is normal (17 seconds), xi) 0.1% of vines are showing symptoms, and xii) the probability of surveyors encountering symptomatic vines is also 0.1% it would take approximately 223 minutes (3.7 hours) to conduct the survey and the probability of detecting phylloxera would be approximately 7.5%.

If we conserve the above assumptions but substitute LAMP assays of root samples for visual inspections, we not only decrease the time it would take to survey the area to approximately 43 minutes (<1 hour) but also increase the probability of detecting phylloxera to 47.97%.

Side-by-side comparisons

Our side-by-side comparison only included vines that were tested across all three detection methods (n = 240 vines across all three sampling periods, treating the three segments of root belonging to the same vine as a single sample, totalling 720 samples). Of the 240 vines tested, 159 had a positive detection for at least one of the detection methods used. **Consistent with the findings above, LAMP assays of roots had the highest proportion of positive detections** (Figure 1.5). 48.33% of the 240 root samples tested with LAMP were positive for phylloxera. 53 (22.08%) of roots tested positive for LAMP with no corresponding positive sample for the other detection methods. The remaining 63 (26.25%) root samples tested positive for LAMP with a corresponding positive detection for a least one other method. **Visual inspection of roots had the next highest proportion of positive detections**. 34.58% of the visual inspections resulted in a positive detection. 27 (11.25%) visual inspections had no corresponding positive sample. The remaining 56 (23.33%) positive visual inspections had corresponding positive samples for at least one other detection method. Lastly, **LAMP assays on emergence trap samples had the lowest proportion of positive detections**. Only 19.17% of the emergence trap samples tested with LAMP were positive for phylloxera: 15 had no corresponding positive samples for the other detection method and the remaining 31 had positive corresponding samples for at least one other detection method.

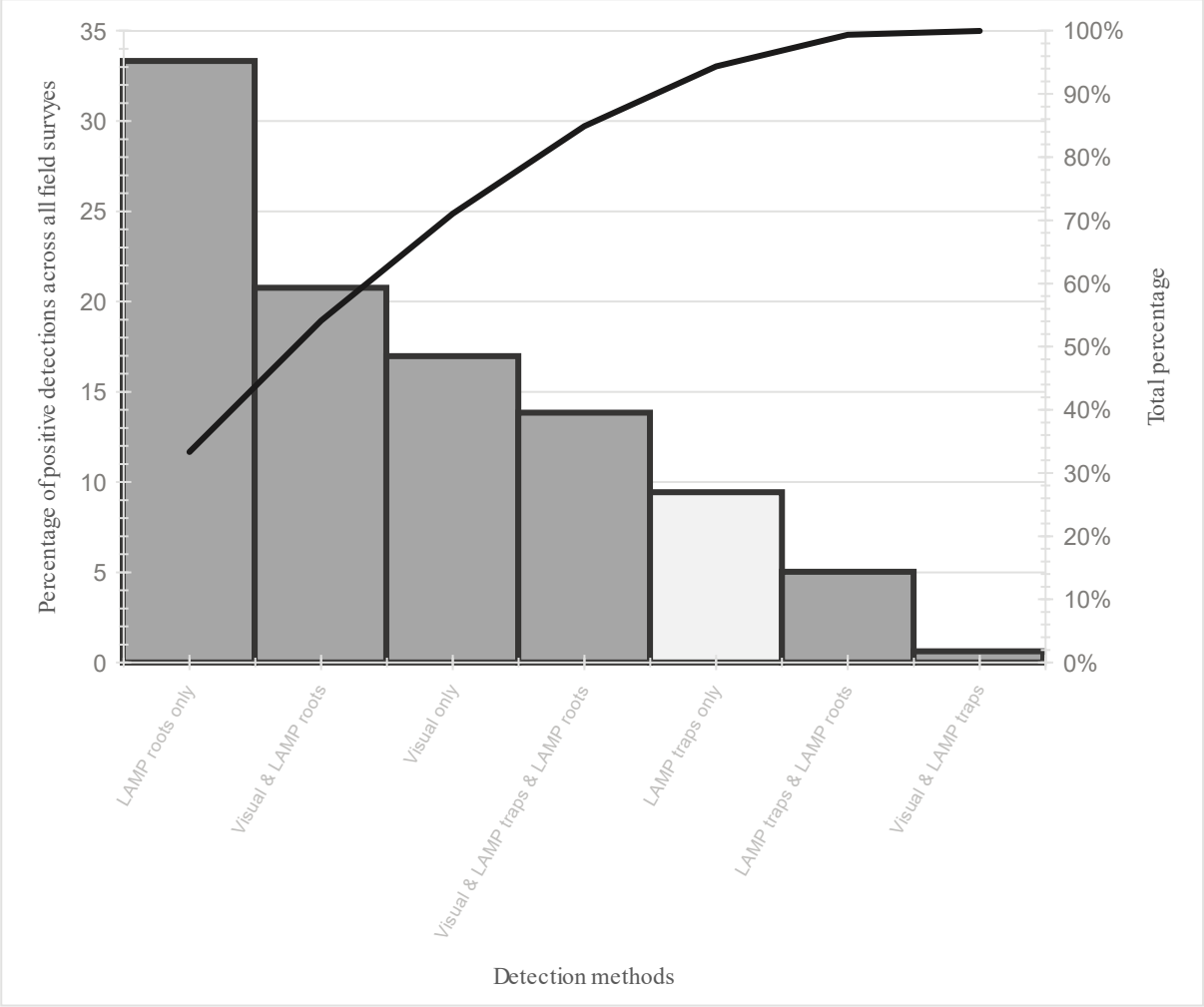


Figure 1.5: The percentage of vines positive for one or a combination of the different detection methods tested: LAMP roots (LAMP assays on root samples), Visual (visual inspections of roots), and LAMP traps (LAMP assays on emergence trap samples) across all field surveys. LAMP assays on root samples alone had the highest proportion of positive detection. 90.76% of positive detections were captured by a combination of LAMP assays on root samples and visual inspections of roots (dark grey)

As 59.73% of the 159 positive detections were samples that tested positive for a single detection method (i.e. had no corresponding positive sample for the other detection methods), **using a combination of detection methods would improve detectability. For example, a combination of visual examinations and LAMP assays on root samples would have captured 90.57% of the 159 positive detections**.

Relationships between rootstock, genotype and environmental factors that contribute to phylloxera detection.

There was insufficient variation in rootstock and phylloxera genotype in the historical and within-project data to determine whether these factors contribute to phylloxera detectability (Table 1.3). Historical data from visual surveys at 11 vineyard blocks was sourced from previous projects. The rootstocks at these vineyards were either not recorded (n = 5) or were own-rooted (n = 6). Similarly, the phylloxera genotype was either not recorded (n = 8) or was G1 (n = 3). Within-project data was collected at a vineyard with own-rooted vines infested with the G1 phylloxera genotype. As the experience of the surveyor and season are factors that may

influence detection we used within-project data to determine if these factors contribute to phylloxera detection.

Table 1.3 Details on the rootstock and genotype for historical and within-project field surveys. There was insufficient variation in the rootstock and genotype to determine if there is any relationship between these factors and phylloxera detection.

Data	Year	Vineyard/Block	Rootstock	Genotype	Number of vine roots inspected
Historical	2015	Vineyard 1 block A	Not recorded	Not recorded	46
		Vineyard 1 block B	Not recorded	Not recorded	47
		Vineyard 1 block C	Own-rooted	Not recorded	54
		Vineyard 2 block A	Own-rooted	G1	41
		Vineyard 2 block B	Own-rooted	G1	49
		Vineyard 3	Own-rooted	G1	43
		Vineyard 4	Not recorded	Not recorded	34
		Vineyard 5	Own-rooted	Not recorded	32
		Vineyard 6 block A	Not recorded	Not recorded	42
		Vineyard 6 block B	Not recorded	Not recorded	42
	2016	Vineyard 7	Own-rooted	Not recorded	32
Within-project	2021/2022	Vineyard 8	Own-rooted	G1	203
	2023	Vineyard 8	Own-rooted	G1	280

Our survival analysis found no significant difference in the time it takes to find phylloxera in autumn or summer ($\chi2 = 1.9$, df = 1, $p = 0.4$). Our chi-squared tests however revealed that the effectiveness of all three detection methods was significantly higher in autumn compared to summer (visual examination: $\chi2 = 11.07$, df = 1, $p = 0.00088$, LAMP analysis of emergence trap samples: $\chi2 = 8.89$, df = 1, $p = 0.0029$, LAMP analysis of root samples: $\chi2 = 13.36$, df = 1, $p = 0.00026$; Figure 1.6).

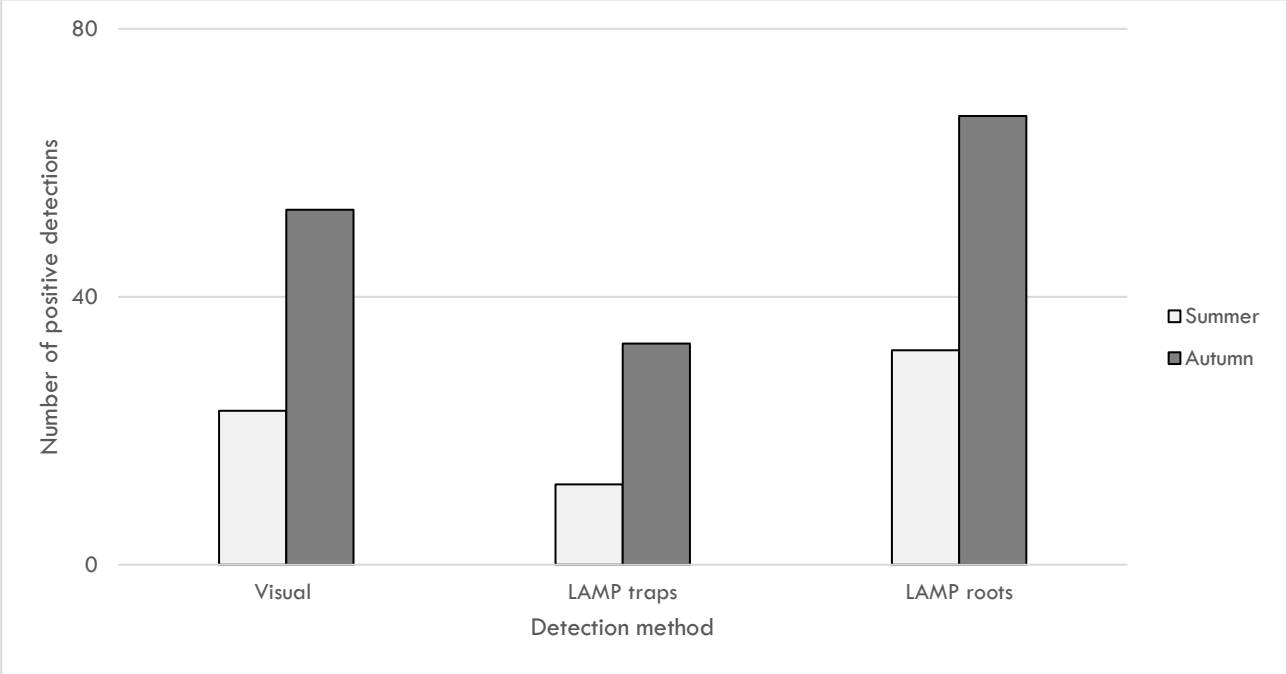


Figure 1.6: There were significantly more positive detections in autumn compared to summer across all three detections methods (visual examination: $\chi^2 = 11.07$, df = 1, p = 0.00088, LAMP analysis of emergence trap samples: $\chi^2 = 8.89$, df = 1, p = 0.0029, LAMP analysis of root samples: $\chi^2 = 13.36$, df = 1, p = 0.00026.

Our survival analysis suggests that experienced surveyors will take significantly less time to find phylloxera if it is present during visual inspections of roots compared to inexperienced (beginner) surveyors ($\chi 2 = 15.60$, df = 1, $p < 0.0001$; Figure 1.7).

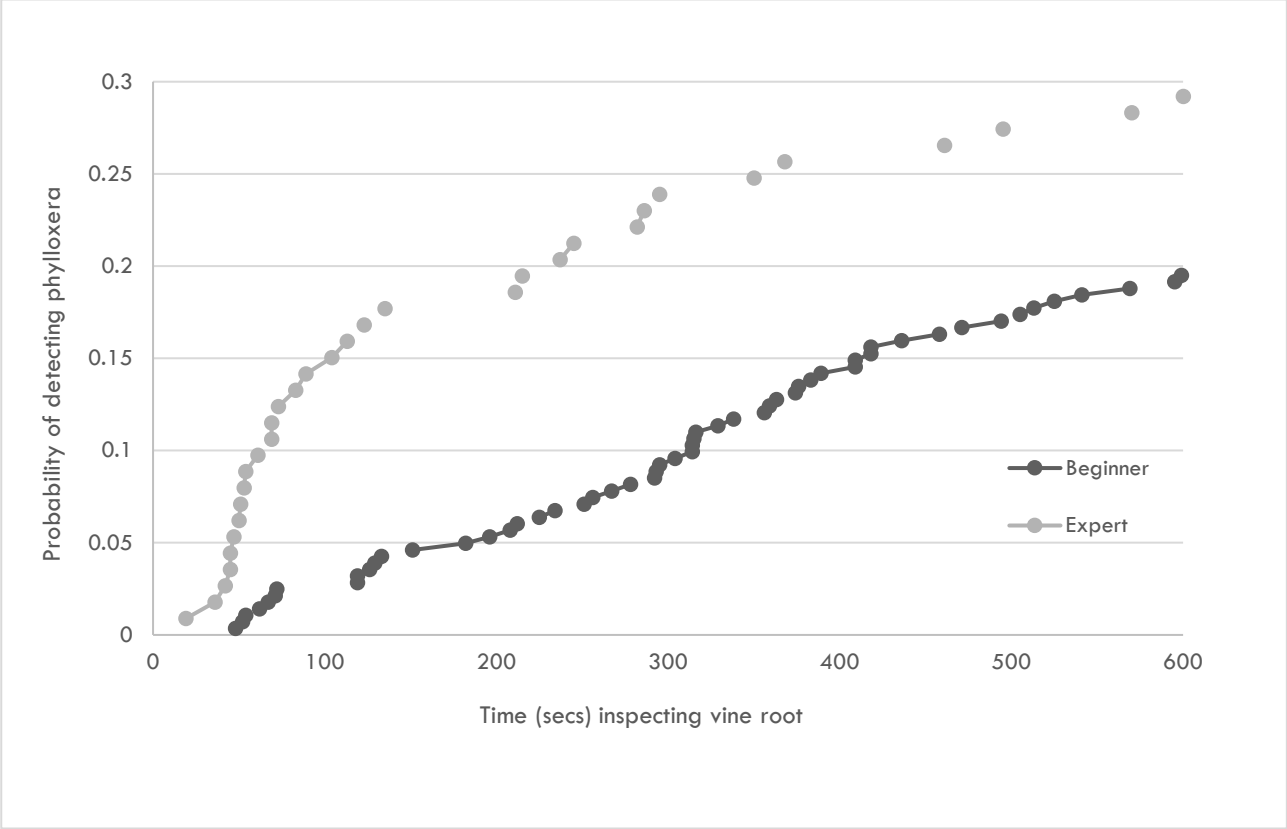


Figure 1.7: Surveyors with more than one year of experience conducting field surveys (expert) were able to find phylloxera significantly faster compared to surveyors with less than one year of experience (beginner) ($p < 0.0001$)

Relative effectiveness of different surveillance methods outside of peak phylloxera activity

The relative effectiveness of the different surveillance methods outside of the peak phylloxera activity was assessed between June and August 2023. Consistent with surveys within the peak of phylloxera activity, LAMP assays of root samples had the highest proportion of positive detections at 26.67% (n = 60), followed by visual inspection at 8.33% (n = 84) and LAMP assays of emergence trap samples at 4.56% (n = 241) across all three months.

This pattern holds when we consider only vines that were tested across all three detection methods (n= 60 vines; Figure 1.8). 23 (38.33%) of the 60 root samples tested with LAMP were positive for phylloxera. 15 (25%) roots tested positive for LAMP with no corresponding positive sample for the other detection methods and an additional 2 roots tested positive for LAMP with a corresponding positive visual inspection. Visual inspection had the next highest proportion of positive detections, with 7 of the visual inspections resulting in positive detections (5 of which had no corresponding positive sample for any other detection method). Lastly, a single emergence trap sample tested with LAMP was positive for phylloxera.

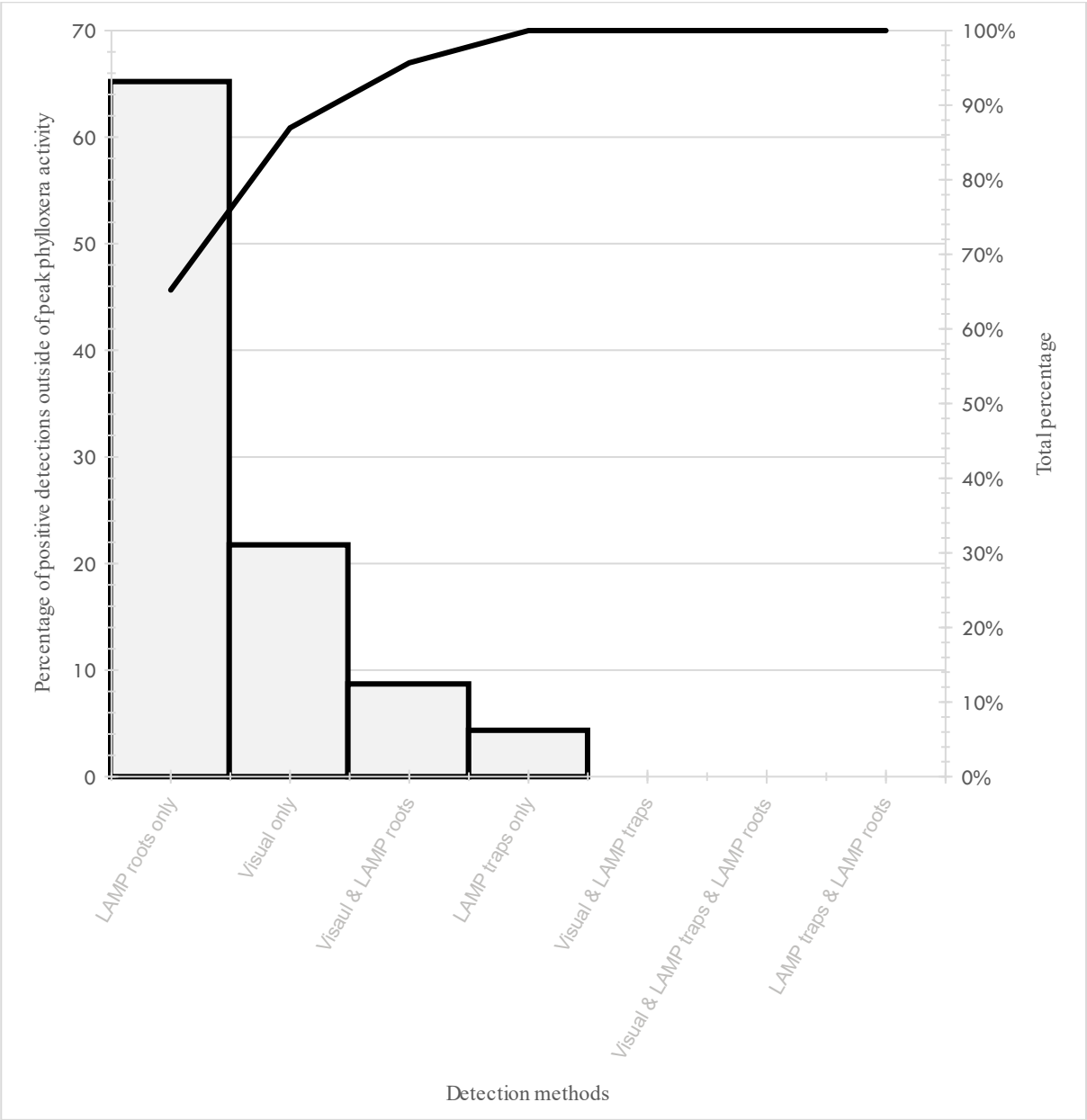


Figure 1.8: The percentage of vines positive for one or a combination of the different detection methods tested: LAMP roots (LAMP assays on root samples), Visual (visual inspections of roots), and LAMP traps (LAMP assays on emergence trap samples) outside of peak phylloxera activity. LAMP assays on root samples alone had the highest proportion of positive detection.

Our chi-squared test revealed that all three detection methods were significantly less effective during winter compared to the previous three months of sampling where phylloxera activity is expected to peak (visual inspection: $\chi^2 = 7.50$, df = 1, p = 0.0062, LAMP analysis of emergence trap samples: $\chi^2 = 21.33$, df = 1, p < 0.0001, LAMP analysis of root samples: $\chi^2 = 11.76$, df = 1, p = 0.00061; Figure 1.9)

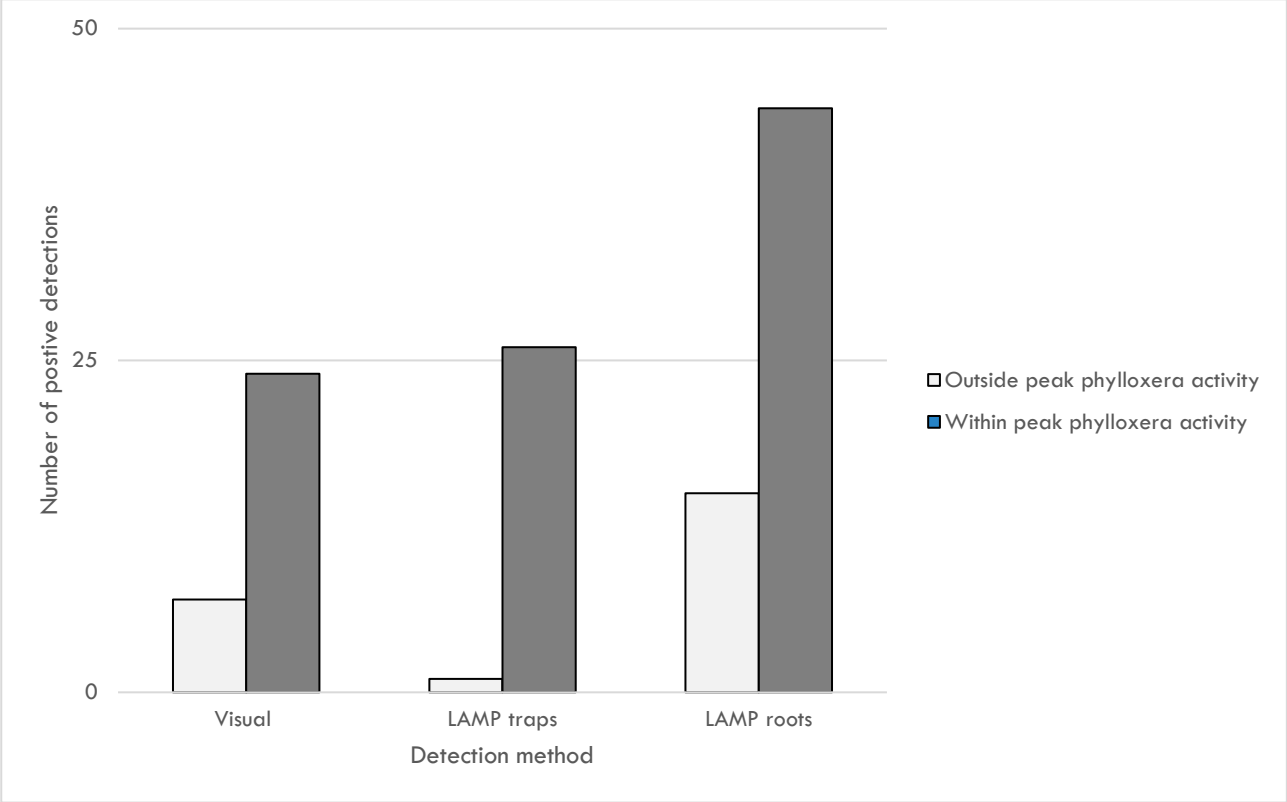


Figure 1.9: There were significantly more positive detections within peak phylloxera activity (February – April 2023) compared to outside of peak phylloxera activity (June – August 2023) across all three detections methods: visual inspection: $\chi^2 = 7.50$, df = 1, p = 0.0062, LAMP analysis of emergence trap samples: $\chi^2 = 21.33$, df = 1, p < 0.0001, LAMP analysis of root samples: $\chi^2 = 11.76$, df = 1, p = 0.00061.

Discussion and Recommendations

Our simulation models have been used to build an interactive spreadsheet that could assist users in determining the probability of detecting phylloxera if it is present and estimating the time needed to survey a vineyard. This spreadsheet has been made available to Biosecurity and Agricultural Services for their feedback. An improved version incorporating their feedback could be made available more widely.

Our simulation models and side-by-side comparisons suggest that biosecurity agencies could improve the probability of detecting phylloxera by incorporating LAMP assays of root samples into their surveillance practices. However, at present the detection of target DNA (i.e. a positive LAMP detection from either emergence trap samples or root samples) without a physical specimen is not considered sufficient evidence to declare a vineyard infested with phylloxera. Rather, the integration of LAMP assays of root samples into the current surveillance practise could improve surveillance efforts in two key ways i) negative LAMP tests may improve confidence in declaring the absence of grapevine phylloxera in vineyards when no insects are found during visual inspections; and ii) LAMP assays on root samples could assist biosecurity agencies as a triaging tool by determining which sites should be resurveyed if no insects are observed on roots but one or more LAMP samples are positive for phylloxera.

Our side-by-side comparisons also demonstrate the benefit of incorporating different detection methods during surveys. Although LAMP assays of root samples was the most effective detection method, a combination of visual examinations and LAMP assays of root samples captured more than 90% of the positive detections during our surveys. As such the integration of LAMP assays could improve surveillance in a third way: iii) by providing overlapping and independent layers of protection, such that if one method fails to detect phylloxera, there is an opportunity for the second independent method to detect the pest.

In contrast to previous research, we found that LAMP assays on emergence trap samples was the least effective detection method. Herbert et al., (2008) found that emergence traps were more effective than visual inspections of roots. Emergence traps only capture the mobile life stages of phylloxera, alates (winged adults), and crawlers (first-instar), and only when they disperse above ground. Above-ground dispersal is expected to occur when the roots become overcrowded and/or the vine experiences nutritional stress (Granett et al., 2001; Herbert et al., 2006). Thus, the effectiveness of emergence traps likely varies between vineyards depending on the abundance of the phylloxera population and the health of the vines. Additionally, the condensation that capture phylloxera inside these traps would degrade any DNA belonging to insects if they die within the trap (Martoni et al., 2021) reducing the effectiveness of this trap over time.

In the same study, Herbert and colleagues also validated a DNA probe (i.e., qPCR test) for phylloxera surveillance, finding that the probe was also more effective than visual inspections of root samples (Herbert et al., 2008). This probe was not included in this study and is not currently used by biosecurity agencies for phylloxera surveillance in Victoria. Our results suggest that using a combination of detection methods would likely improve detectability. Our findings suggest that incorporation of molecular tests (LAMP or otherwise) during phylloxera surveillance would be beneficial to surveillance efforts, by improving the probability of detecting phylloxera and providing overlapping layers of protection against false negatives.

Although there was insufficient variation in historical data to determine whether genotype or rootstock contributes to phylloxera detection, our within-project data suggests that the probability of detecting phylloxera across all three detection methods is significantly higher in autumn compared to summer. Biosecurity agencies could consider prioritizing vineyard surveys in autumn although this may vary with genotype, rootstock, and/or environmental conditions.

For visual inspections of roots (the dig method) our findings suggest that experienced surveyors will take significantly less time to find phylloxera than inexperienced surveyors. Our findings suggest that at least some of the surveyors present during field surveys should have more than one year of experience conducting field surveys to reduce the time needed to survey a vineyard.

We found that all three detection methods were less effective in winter compared to the peak of phylloxera activity and biosecurity agencies. Nonetheless, phylloxera was detected across all three methods. Positive detections from any of the emergence trap samples in the current winter study were especially unexpected. The activity of phylloxera mobile life stages (alates and crawlers) above ground is expected to peak in summer and autumn (Powell et al., 2009). Additionally, previous research suggests that phylloxera over-

winter as crawlers and are thus not expected to disperse during this season (Hoffman et al., 2016), noting that this research was conducted in Europe where the climate, rootstock, and genotypes differ. Our within-project research suggests that genotypes may vary in their sensitivity to winter temperatures (page xxxx of this report) which may explain differences between studies.

Our results suggest that biosecurity agencies should consider prioritizing surveys in autumn or summer where the probability of detecting phylloxera is twice as high but the possibility of off-season surveys could be beneficial by increasing the surveillance window and allowing surveys to be conducted at the first sign of infestation regardless of season.

Conclusion

Our simulation models have been used to build an interactive spreadsheet that could assist users in determining the probability of detecting phylloxera if it is present, and in estimating the time needed to survey a vineyard. This spreadsheet has been made available to biosecurity agricultural services for their feedback and an improved version incorporating their feedback could be made available more widely.

Taken together, our results strongly suggest that of the three detection methods assessed during this study, **LAMP assays of root samples is the most effective**. Although the detection of phylloxera DNA at present is not sufficient to declare a vineyard infested, **the incorporation of these LAMP tests (or similar molecular techniques) during field surveys has several potential benefits specifically:**

i) **Improving confidence in declarations of absence**

ii) **Providing a triage tool to identify suspect vineyards to resurvey**

iii) **Providing overlapping independent layers of protection, ensuring against false negatives**

Our results suggest that **all three detection methods may be more effective in autumn compared to summer**, although this may vary with genotype, rootstocks, and/or environmental conditions. We also found that the experience of the surveyor had a significant effect on the time needed to detect phylloxera during visual surveys. Surveyors with more than one year of experience took significantly less time to find phylloxera compared to surveyors with less than one year of experience. **The time needed to find phylloxera is likely to be less if at least some of the surveyors have more than one year of experience conducting field surveys.**

Lastly, all three detection methods were significantly less effective outside of phylloxera peak activity (winter), Biosecurity agencies could consider prioritizing surveys in autumn or summer where the probability of detecting phylloxera is twice as high**. Nonetheless, the possibility of off-season surveys could be beneficial by increasing the surveillance window and allowing surveys to be conducted at the first sign of infestation without needing to wait for warmer weather.**

Research Theme 2: Improved Selection of Resilient Rootstock

Improved Selection of Resilient Rootstocks M5512 and M5489

Executive Summary

Phylloxera is considered one of the most damaging viticulture pests worldwide. Phylloxera damage vines by inducing swellings (nodosities and tuberosities) on the roots during feeding. These swellings inhibit water uptake by the vine and cause the roots to split, exposing them to pathogens in the soil. *Vitis vinifera* vines infested with phylloxera typically die within three to five years following infestation. Although there is no treatment that can remove phylloxera from the soil, grafting European vine varieties onto American rootstock is currently recognised as the best viticultural practice to safeguard vineyards from phylloxera induced damage. Phylloxera is native to North America, and American rootstocks have evolved resistance or tolerance to phylloxera infestations and/or damage.

Managing phylloxera with resistant rootstocks involves breeding novel rootstocks that are not only resistant or tolerant to phylloxera but meet the individual needs of growers and their land. Importantly, rootstocks vary in their resistance to specific phylloxera genotypes (genetic makeup, sometimes referred to as biotypes or strains) and in Victoria, the prevalence of different phylloxera genotypes vary across phylloxera infestation zones. It is therefore crucial to assess the interaction between phylloxera genotypes and rootstock before widespread adoption of new rootstocks.

This project assessed the susceptibility of two novel rootstocks, M5512 and M5489, to phylloxera genotypes G4 and G38. We infested each vine with 20 eggs belonging to either the G4 or G38 phylloxera genotype and maintained them under controlled conditions in a greenhouse for approximately eight weeks. Additionally, *Vitis vinifera* vines (own rooted vines) were similarly infested with phylloxera and used as a positive control. The entire root system of each vine was then harvested, and the number of nodosities, tuberosities, eggs, intermediate life stages and both winged and wingless adults were recorded. Performance ratings were then calculated by comparing the number of nodosities, the number of adults, and the total number of insects recorded for each genotype x novel rootstock interaction to their positive control counterpart (*Vitis vinifera*).

Nodosities and all phylloxera life stages were recorded on positive control (*V. vinifera*) vines infested with G4 or G38 phylloxera genotypes. Both M5512 and M5489 rootstocks inoculated with G4 or G38 had significantly fewer nodosities, adults and total insects compared to their positive control counterparts. M5512 roots infested with G38 phylloxera eggs showed signs of phylloxera feeding (nodosities) but no individuals of any life stage were observed, suggesting that phylloxera were able to feed and induce damage on this rootstock, but were not able to develop into adults. Nodosities were also present on M5489 roots infested with G38 phylloxera eggs, and a single individual phylloxera was also recorded. M5512 roots infested with G38 phylloxera eggs had significantly more nodosities compared to their M5489 counterparts, but there was no significant difference in the number of adults or insects observed between the two rootstocks. Nodosities and individuals of all life stages were observed on both M5512 and M5489 rootstocks infested with G4 phylloxera eggs. M5512 roots infested with G4 phylloxera eggs had a significantly higher number of nodosities, adults and insects compared to their M5489 counterparts.

Our results suggest that both M5512 and M5489 rootstocks be rated tolerant for both the G4 and G38 phylloxera genotypes. Although no individuals were observed on M5512 rootstocks inoculated with G38 phylloxera eggs, the presence of nodosities suggest that this genotype can feed and induce damage on this rootstock. **This information could be provided to growers.** For example, by updating the Rootstock Selector Tool. **These rootstocks could also be screened against additional phylloxera genotypes** so that growers exposed to or at risk of exposure to alternative phylloxera genotypes can make reliable decisions on these novel rootstocks.

Key findings and recommendations

G4 phylloxera

- M5512 and M5489 roots infested with G4 phylloxera eggs had significantly fewer nodosities, insects and adults compared to our positive control.
- Nodosities and insects at all life stages were observed on both M5512 and M5489 roots infested with G4 phylloxera eggs.
- M5512 roots infested with G4 phylloxera eggs had significantly more nodosities, adults and insects compared to M5489 roots.
- Our results suggest that M5512 and M5489 should be rated tolerant against the G4 phylloxera genotype.

G38 phylloxera

- M5512 and M5489 roots infested with G38 phylloxera eggs had significantly fewer nodosities, insects and adults compared to our positive control.
- M5512 roots infested with G38 phylloxera eggs showed signs of feeding (nodosities) but no individuals of any life stage were observed.
- Nodosities and a single individual was recorded on M5489 roots infested with G38 phylloxera eggs.
- M5512 roots infested with G38 phylloxera eggs had significantly more nodosities compared to M5489 roots, but there was no significant difference in the number of adults or insects recorded between the two rootstocks.
- Our results suggest that M5512 and M5489 should be rated tolerant against the G38 phylloxera genotype but the absence of insects on M5512 and low number (single individual) on M5489 suggest these rootstocks may be especially beneficial for growers managing vineyards where the G38 phylloxera genotype is prevalent.

Introduction

Grapevine phylloxera (Daktulosphaira vitifoliae) is considered one of the most damaging viticulture pests worldwide. This small aphid-like insect led to the collapse of the wine industry in Geelong in the mid-1800s and has been established in Northeast Victoria since the early 1900s (Clarke et al., 2023). Phylloxera damages grapevine (Vitis spp.) by feeding on either the roots or the leaves of the vines. Root-feeding phylloxera (called radicicoles) induce nodosities on young fibrous roots, and tuberosities on older lignified roots during feeding (Clarke et al., 2023a). The nodosities and tuberosities act as food storage organs for the insect (Forneck 2011) and inhibit water uptake by the vines resulting in reduced leaf surface area for photosynthesis, loss of vigour, and production of small berries (Forneck 2011, Granett et al. 2003, Granett and Kocsis 2000). These swellings subsequently split and expose roots to soil pathogens and infections, typically resulting in vine death (Edwards, et al. 2006, Granett, et al. 1998).

Phylloxera can reproduce both sexually and asexually. Asexual females can produce several hundred eggs during the growth season (Forneck and Huber, 2009). First-instars are mobile and can establish new feeding sites. Root-feeding phylloxera develop into either wingless adults or winged adults (alates). Alates can emerge from the soil in search of new vines (Clarke et al., 2023). In Australia, there is no evidence of sexual reproduction among phylloxera populations (Corrie and Hoffman, 2004). There is however considerable genetic diversity, and to date over 100 phylloxera genotypes have been characterised (Clarke et al., 2020). Characterising these genotypes is critical to the management of phylloxera in Australia due to plant x genotype interactions (Powell and Krstic, 2015) and the potential for genotypic variation in response to management practices (e.g., hygiene treatments).

Although there is no treatment that can eliminate phylloxera from the soil, grafting European vine varieties onto American rootstock is currently recognised as the best viticultural practice to safeguard vineyards from phylloxera induced damage (Hadju, 2015). North American Vitis species have co-evolved with phylloxera and can host populations of phylloxera for several years without experiencing yield loss or death. Managing

phylloxera with rootstocks requires breeding novel rootstocks that are not only resistant or tolerant to phylloxera but meet the individual needs of growers and their land, such as tolerance to lime, acceptability of scions, and desired vine and fruit size (Hadju 2015, Hajdu and Bakonyi 2006). Importantly, rootstocks vary in their resistance to specific phylloxera genotypes. It is crucial to assess the interaction between phylloxera genotype and rootstock before widespread adoption of any novel rootstocks (Clarke et al., in press, Lund., et al. 2017, Powell and Krstic 2015). Information on the performance of specific phylloxera genotypes on rootstock varieties is used to develop tools that allow growers to make reliable decisions on rootstock adoption, such as the Grapevine Rootstock Selector (Wine Australia 2019).

Merbein 5512 (M5512) and Merbein 5489 (M5489) are two novel rootstocks bred from complex hybrids of V. berlandieri. These rootstocks have been selected for their low uptake of potassium in their berries, and their ability to use water efficiently. Although the rootstocks have good nematode tolerance, their tolerance to phylloxera is unknown. Here we assessed the susceptibility of these two rootstocks to two phylloxera genotypes, G4 and G38, by comparing their ability to survive and reproduce on these rootstocks in potted vines to a positive control (own-rooted Vitis vinifera).

Project Objectives

The aim of this project was to:

i) Screen two novel rootstocks M5512 and M5489 for their susceptivity to phylloxera genotypes G4 and G38 using updated screening methodology.

ii) Determine susceptibility ratings for M5512 and M5489 rootstocks against G4 and G38 phylloxera genotypes.

Methodology

Phylloxera cultures

The G4 and G38 phylloxera cultures were established from eggs laid by a single individual placed on excised roots (*V. vinifera* Chardonnay) at 25 ± 2°C, following Kingston 2007 and maintained under quarantine at Agriculture Victoria, Rutherglen. The identity of each phylloxera genotype was confirmed prior to the potted vine assays (described below) using six nuclear microsatellite markers (Umina et al., 2007).

Rootstocks

Dormant vines of M5512, M5489 and *V. vinifera* were provided by Glenavon nursery in July 2022. All vines were planted in 20 cm diameter pots in 80% general purpose potting mix composed of composted pine bark, peat moss and humic peat (Spotswood potting mixes and fertilisers, Yarra Glen, Victoria) mixed with 20% Perlite and maintained in a shade house. All vines were drip irrigated for 2 minutes daily, and fertilised with 3.5 g Osmocote™ (Scotts Osmocote®, Scotts Australia Pty Ltd) and 500 ml Thrive™ (Yates ©, DuluxGroup Australia) as required.

Potted vine assays

Each potted vine was infested with 20 eggs belonging to either the G4 or G38 phylloxera genotype (n = 9 vines per rootstock and phylloxera genotype). We collected eggs from each culture using a moistened fine artist's paintbrush and then placed them on a piece of filter paper inside a small Petri dish (3.5 cm diameter). We then exposed the root system of each vine by digging a hole into the soil until a combination of fibrous and lignified roots were visible. The filter paper was then carefully placed face down on the roots and immediately covered with soil. The Petri dish was subsequently buried in the same pot, as insurance against any eggs that may have come free from the filter paper. To prevent cross contamination, we wrapped a mesh netting (50 µm, Bugdorm Nylon Netting, Megaview Science CO, Ltd, Taiwan) around each infested potted vine and applied a thin layer of sticky insect trap coating around the base of each vine stem and the rim of each pot (TanglefootTM, The tanglefoot company, Grand Rapids, MI, USA). The vines were then maintained in a glasshouse at 22 ± 2°C and a relative humidity of 60 – 70%, and drip irrigated for 2 min daily for eight weeks. Eight weeks is sufficient time for the eggs to hatch, reach their adult stage and produce a second generation of phylloxera. Following the eight weeks, each vine was removed from its pot and the entire root system was harvested. To remove soil from the roots, we rinsed each root system in water over a series of fine mesh sieves, collecting the water in sterile vials (300 ml). The entire root system was then removed from the vine using secateurs and placed divided into 2 - 3 sterile vials (300 ml) depending on the size of the root system. All vials were then frozen at -20 °C until inspected for the presence of nodosities and phylloxera. We inspected both the entire root system, and a 100 ml sub-sample of the soil water from each vine by counting the number of eggs. Intermediates, adults, alates (winged adults), nodosities and tuberosities under a dissection microscope. All water samples were briefly vortexed prior to sub-sampling and the workbench and dissecting microscope were cleaned with 80% ethanol between sample inspections to prevent contamination.

Performance rating

Performance ratings were calculated following Clarke et al. (2023b). Rootstocks are rated resistant when neither nodosities nor phylloxera of any life-stage are found living on the roots, tolerant when nodosities or phylloxera are found on roots at a level below their positive control counterpart (*V. vinifera*), and susceptible when the number of nodosities and phylloxera are at the same level or higher than their positive control counterpart (*V. vinifera*).

Statistical analysis

All statistical analyses were performed in R-studio (R version 4.1.1). We performed a series of generalized linear models (GLMs) with poisson distributions to determine if variation in i) *the number of nodosities*, ii) *the total number of adults* (including alates) and iii) *the total number of insects* observed could be explained by rootstock, phylloxera genotype or their interaction. Models were assessed using likelihood ratio tests. Post-

hoc comparisons were performed using the least square means tests for the number of nodosities recorded and pairwise Wilcoxon tests for the total number of insects and the total number of adults recorded. Least square means tests could not be performed for the total number of adults or the total number of insects as these variables had zero counts recoded for all replicates in some treatments (specifically total adults recorded for M5512 and M5489 rootstocks infested with G38 phylloxera eggs and total insects recorded for M5512 rootstocks infested with G38 phylloxera eggs). All GLMs were performed using the "MASS" package and least square means tests were performed using the "emmeans" package. All post-hoc tests were adjusted for multiple comparisons using the Bonferroni method.

Key Research Findings

Nodosities and all phylloxera life stages were recorded on the positive control (*V. vinifera*) vines infested with either the G4 or G38 phylloxera genotype. Nodosities were also observed on both the M5512 and M5489 rootstocks regardless of the phylloxera genotype. Both the M5512 and the M5489 were able to support all phylloxera life stages when inoculated with the G4 phylloxera genotype, however no individuals of any life stage were recorded on M5512 roots infested with the G38 phylloxera genotype and only a single crawler (intermediate life stage) was recorded on M5489 roots infested with the G38 phylloxera genotype. No tuberosities were recorded on any roots during this study.

Nodosities

Our GLM revealed a significant interaction between rootstocks and phylloxera genotype on the number of nodosities recorded ($df = 2$, $\chi^2 = 214.88$, $p < 0.0001$). Post-hoc analysis revealed *V. vinifera* roots inoculated with either the G4 or G38 phylloxera genotypes had significantly more nodosities than M5512 or M5489 rootstocks (all $p < 0.0001$: Figure 1). Additionally, M5512 roots infested with either the G4 or G38 phylloxera strains had significantly more nodosities than M5489 (all $p < 0.0001$: Figure 2.1).

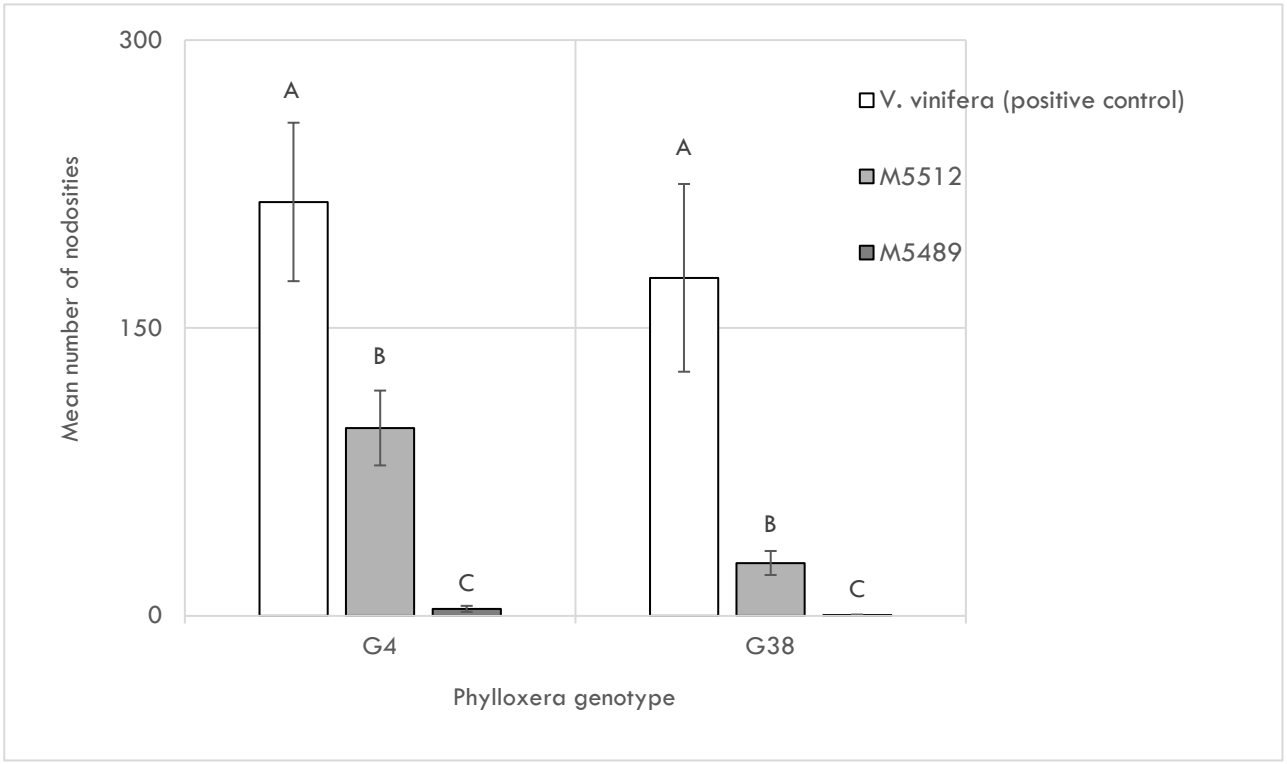


Figure 2.1: Mean number of nodositis on roots of potted vines infested with eggs of either the G4 or G38 phylloxera genotype. Treatments separated by letters are statistically different. Nodosities were recorded on all rootstock and genotype combinations.

Total adults

Our GLM revealed a significant interaction between rootstocks and phylloxera genotype on the number of adults (including alates) recorded (df = 2, $\chi^2 = 200.86$, $p < 0.0001$). Post-hoc analysis revealed that significantly more adults were recorded from *V. vinifera* roots inoculated with either the G4 and G38 phylloxera genotypes than M5512 or M5489 rootstocks (all $p < 0.021$: Figure 2). Additionally, M5512 roots infested with the G4 had significantly more adults than M5489 (all $p = 0.00076$: Figure 2.2). There was no significant difference in the number of adults recorded on M5512 and M5489 roots inoculated with the G38 phylloxera genotype.

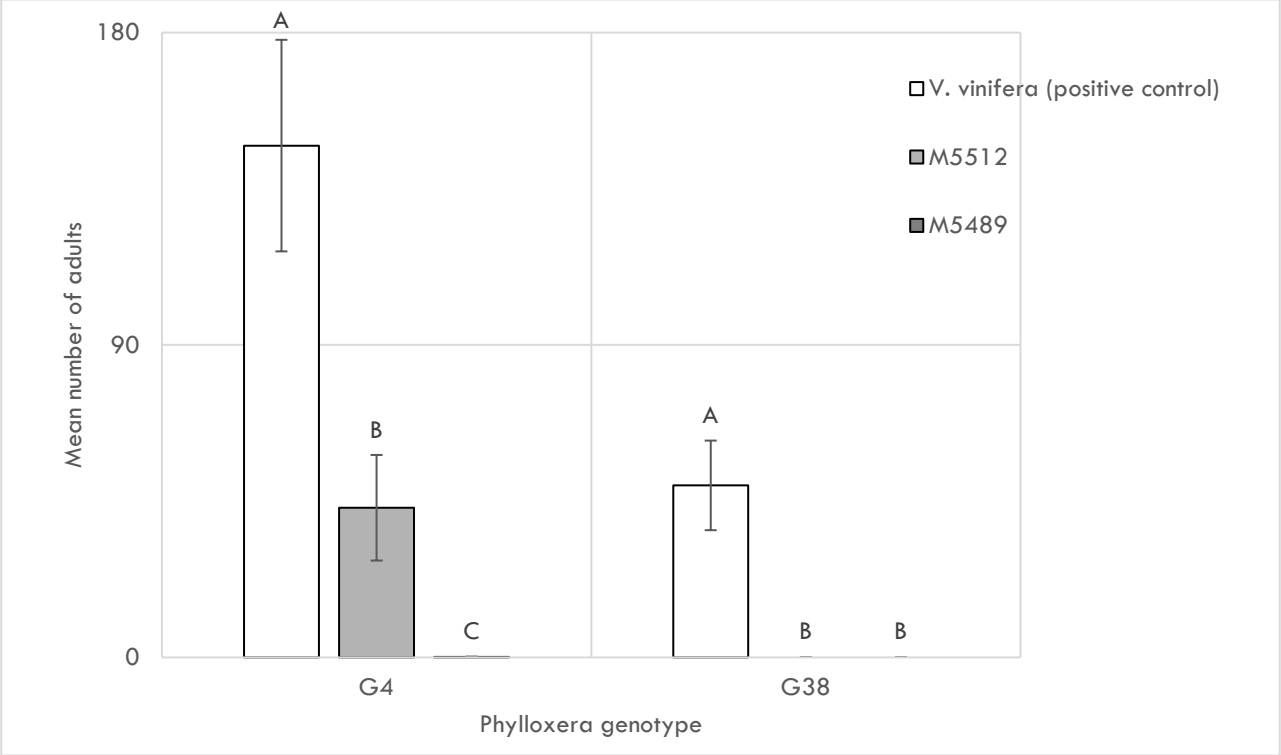


Figure 2.2: Mean number of adults including alates on roots of potted vines infested with eggs of either the G4 or G38 phylloxera genotype. Treatments separated by letters are statistically different. No adults were recorded on M5512 or M5489 roots infested with the G38 phylloxera genotype.

Total insects

Our GLM revealed a significant interaction between rootstocks and phylloxera genotype on the total number of insects recorded (df = 2, $\chi^2 = 200.86$, $p < 0.0001$). Post-hoc analysis revealed that significantly more insects were recorded from V. vinifera roots inoculated with either the G4 and G38 phylloxera genotypes than M5512 or M5489 rootstocks (all $p < 0.012$: Figure 3). Additionally, M5512 roots infested with the G4 had significantly more insects than M5489 (all $p = 0.00094$: Figure 2.3). There was no significant difference in the number of adults recorded on M5512 and M5489 roots inoculated with the G38 phylloxera genotype.

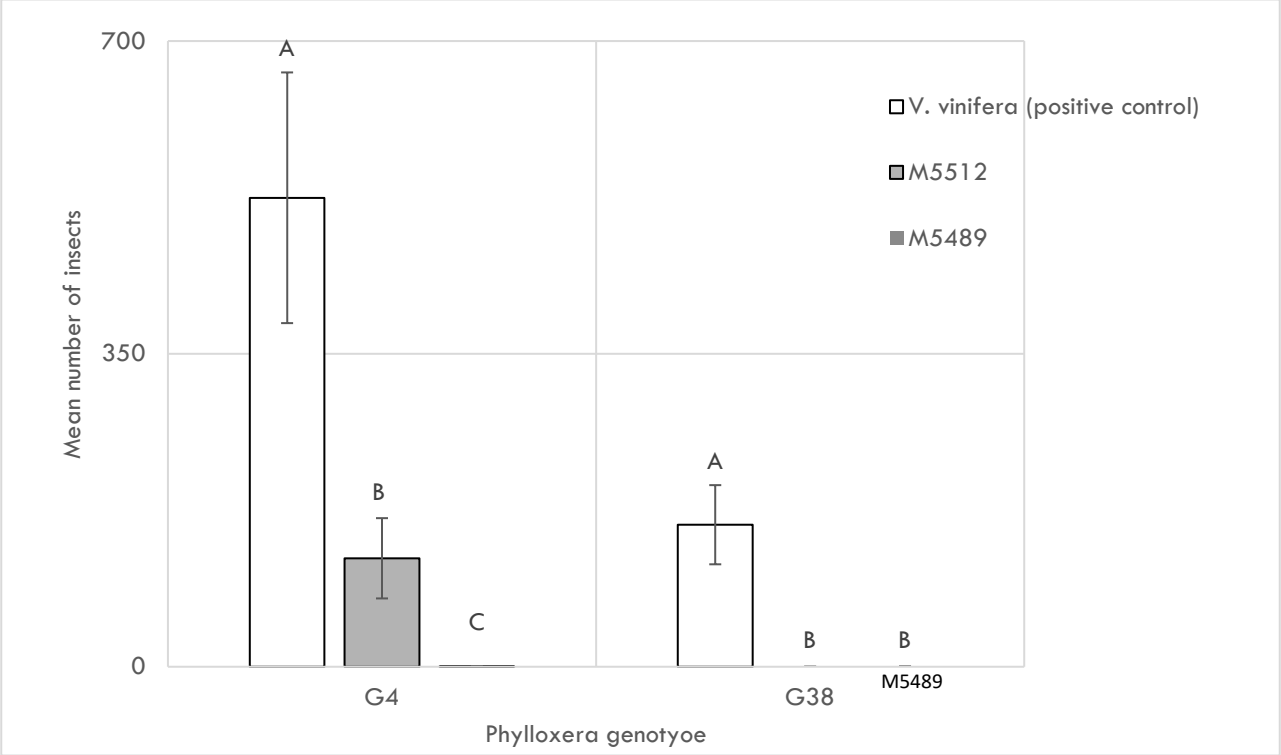


Figure 2.3: Mean number of insects on roots of potted vines infested with eggs of either the G4 or G38 phylloxera genotype. Treatments separated by letters are statistically different. No insects were recorded on M5512 roots infested with the G38 phylloxera genotype.

Performance ratings

Despite few if any individuals being observed for some of our rootstock x phylloxera genotype treatments (Table 2.1), both the G4 and G38 phylloxera genotypes were able to induce nodosities on both novel rootstocks screened in this study. In all cases the number of nodosities induced was significantly lower compared to their positive control counterparts. **Our results suggest that the M5512 and M5489 rootstocks are rated tolerant against both the G4 and G38 phylloxera genotypes.**

Table 2.1: Mean number of nodosities, eggs, intermediates, adults (including alates), and total insects recorded on rootstocks inoculated with G4 or G38 phylloxera eggs.

Phylloxera genotype	Rootstock	Mean number of nodosities	Mean number of eggs	Mean number of intermediate life-stages	Mean number of adults (including alates)	Mean number of insects (all life-stages)
G4	Positive control (*V. vinifera*)	215.67	44.56	332.67	147.44	524.67
	M5512	97.89	32.78	45.44	43.11	121.33
	M5489	3.67	3.56	0.11	0.11	0.33
G38	Positive control (*V. vinifera*)	176.11	34.44	74.89	49.56	158.89
	M5512	27.56	0	0	0	0
	M5489	0.44	0	0.11	0	0.11

Discussion and Recommendations

We found that M5512 and M5489 roots infested with G4 phylloxera eggs had significantly fewer nodosities, insects and adults compared to our positive control counterparts. Nodosities and insects of all life stages were observed on both roots infested with G4 phylloxera eggs, but M5512 roots had significantly more nodosities, adults and insects compared to M5489 roots infested with this genotype.

Our results suggest that **M5512 and M5489 are rated tolerant against the G4 phylloxera genotype.**

We found that M5512 and M5489 roots infested with G38 phylloxera eggs had significantly fewer nodosities, insects and adults compared to our positive control counterparts. Nodosities were observed on both rootstocks infested with G38 phylloxera eggs, but no insects were observed on M5512 roots and only a single insect was recorded on M5489 roots infested with this genotype.

Our results suggest that **M5512 and M5489 are rated tolerant against the G38 phylloxera genotype,** but due to the low number (if any) insects recorded, these rootstocks may be especially beneficial to growers managing vineyards in areas where G38 in highly prevalent.

We recommend that **information on the tolerance ratings of M5512 and M5489 against G4 and G38 is made available to growers**. For example, by updating the Grapevine Rootstock Selector Tool.

We also recommend that **additional genotypes are screened to determine their susceptibility ratings against the M5512 and M5489 rootstocks.**

Conclusion

Grafting European vine varieties onto American rootstock is currently recognised as the best viticultural practice to safeguard vineyards from phylloxera induced damage. Rootstocks vary in their resistance to specific phylloxera genotypes. It is therefore crucial to screen novel rootstocks against different phylloxera genotypes before widespread adoption. This information can then be provided to growers to ensure that they are able to make reliable decisions on rootstock adoption based not only on their land and viticultural practices, but also their risk of exposure to specific phylloxera genotypes

In this study we screened two novel rootstocks, M5512 and M5489, against two phylloxera genotypes, G4 and G38. Both M5512 and M5489 rootstocks infested with G4 or G38 phylloxera eggs had significantly fewer nodosities, adults and total insects compared to their positive control counterparts. Nodosities and insects of all life stages were observed on M5512 and M5489 roots infested with G4 phylloxera eggs. But M5512 had significantly more nodosities, adults and insects compared to M5489. k. Although no insects were observed on M5512 and only a single individual was recorded on M5489 rootstocks infested with G38 phylloxera eggs, the presence of nodosities indicates that this genotype can feed and induce damage on this rootstock

In summary our results suggest that **both rootstocks should be rated tolerant against the G4 and G38 phylloxera genotypes**. Our findings also suggest that growers looking to adopt a low potassium rootstock in regions where the G4 phylloxera genotype dominates, should consider the M5489 over the M5512 rootstock and the low number (if any) insects recorded on M5512 and M5489 rootstocks suggests that these may be especially beneficial for growers in regions where the G38 phylloxera genotype is prevalent.

Our results also provide further evidence of genotype X rootstock interactions, and **we recommend that these rootstocks are screened against additional phylloxera genotypes** so that growers exposed to or at risk of exposure to alternative phylloxera genotypes can make reliable decisions on the adoption of these rootstocks.

Research Theme 3: Improved disinfestation procedures

Improved Disinfestation Procedures Under Different Hygiene Conditions

Executive Summary

A key component of Australia's quarantine strategy is the implementation of three quarantine boundaries: Phylloxera Infested Zones (PIZs), Phylloxera Risk Zones (PRZs) and Phylloxera Exclusion Zones (PEZs). Movement of plant material and machinery among zones is under enforceable regulations, which often require the material or machinery to be disinfested prior to leaving the zone. As first-instar phylloxera (crawlers) are typically transported to new vineyards on contaminated machinery, clothing or plant material, effective disinfestation protocols play a critical role in preventing the spread of phylloxera.

Most disinfestation protocols require exposing phylloxera contaminated material to hot or cold temperatures that are outside the limits of the insect's physiological tolerance. The current disinfestation protocols for machinery requires removing any parts of the machinery that may hide or contain dirt and thoroughly cleaning the machinery, before placing in a dry heat shed at designated temperatures for set periods of time: either 40°C for 2 hours and 15 minutes, 40°C for 3 hours, or 45°C for 2 hours, depending on the state's requirements. These protocols were previously validated in the laboratory and found to result in 100% mortality across a diverse range of phylloxera genotypes. In a laboratory setting phylloxera are typically exposed to the disinfestation temperature under sterile conditions, while in practice, phylloxera may be exposed to the disinfestation temperatures under different hygiene conditions. For example, phylloxera may be still attached to a small piece of root or may be covered by soil or grapevine foliage. Crevasses on the surface of root pieces, soil or grapevine foliage may provide phylloxera with a microclimate or a source of moisture, reducing desiccation and/or reducing the maximum temperature experienced by the insect during the dry heat treatment. As such, insects attached to roots or protected by soil or plant material during dry heat disinfestation may experience reduced mortality, which could result in the transport of live phylloxera between quarantine boundaries.

In this study, we first assessed in the laboratory whether the current dry heat disinfestation protocols are effective against phylloxera when they are protected by soil and plant material by exposing phylloxera crawlers under three different hygiene conditions i) clean (not attached to a feeding site), ii) clean root (phylloxera are attached to a piece of root) and iii) root protected by soil and grapevine material (phylloxera are attached to a piece of root and protected by soil and grapevine material) to the current recommended disinfestation procedures: 40°C for 2 hours and 15 minutes, 40°C for 3 hours, and 45°C for 2 hours. In a second laboratory experiment we trialled an improved disinfestation protocol by increasing temperature to 50°C across diverse phylloxera genotypes using crawlers attached to a piece of root. Lastly, we assessed the improved disinfestation protocol (50°C for 2 hours) in a commercial dry heat shed under different hygiene conditions i) clean root, ii) root protected by grapevine material, iii) root protected by soil and iv) root protected by grapevine material and soil across, diverse phylloxera genotypes.

The current dry heat disinfestation protocols were found to be ineffective in this study, regardless of the hygiene condition. Across all disinfestation treatments and hygiene conditions tested, a minimum of 48% of phylloxera survived and developed into adults. We found that increasing the temperature improved mortality: 100% mortality was observed on phylloxera exposed to 50°C for 2 hours in the laboratory. Similarly, 100% mortality was observed in all genotypes tested in the field using a commercial dry heat shed when insects were attached to a clean root, except for G30. Four percent of G30 phylloxera initially survived the dry heat exposure however, these insects died before they were able to complete development, suggesting a delayed effect of the treatment. When phylloxera were attached to the root and protected by just grapevine material, 100% mortality was achieved across all genotypes. Unfortunately, 100% mortality was not achieved when phylloxera were attached to a root and protected by soil or by both soil and grapevine material. Forty-two percent of phylloxera protected by soil survived the initial dry heat exposure, with 30% subsequently developing into adults. Similarly, when phylloxera were protected by both soil and grapevine material 50% survived both the initial exposure and the following 30 days, developing into adults. This suggests that soil but not grapevine material offers additional protection from the dry heat treatment.

Our findings suggest that the current dry heat disinfestation protocols are not fully effective and might be **improved by changing the dry heat disinfestation protocol to 50°C for 2 hours**. We also suggest that growers continue to remove any machinery parts that hide dirt and thoroughly clean equipment before disinfestation. Further research on disinfestation protocols that are effective in the presence of soil are needed. Additionally, industry might benefit from disinfestation protocols that do not require a heat shed, as they are not accessible to all growers.

Key findings and recommendations

Laboratory assays

- Between 48 and 100% of phylloxera exposed to the current dry heat treatment protocols under different hygiene conditions survived
- Increasing the temperature of dry heat disinfestation protocol to 50°C and holding phylloxera at this temperature for 2 hours resulted in 100% mortality when phylloxera were attached to a clean feeding site.
- Dry heat disinfestation protocol could be improved by changing the protocol to 50°C for 2 hours.

In-field assays using a commercial dry heat shed

- 100% mortality was achieved when phylloxera attached to a clean root or attached to a root protected by grapevine material were placed in a commercial dry heat shed, maintained at 50°C for 2 hours.
- When phylloxera were placed in a commercial dry heat shed maintained at 50°C for 2 hours and were attached to root protected by either soil alone or by both soil and grapevine material, 100% mortality was not achieved.
- These findings provide additional support for changing the dry heat disinfestation protocol to 50°C for 2 hours but highlight the importance of removing parts of vineyard equipment that can hide dirt and thoroughly cleaning the equipment prior to commencing the dry heat treatment.

Introduction

Grapevine phylloxera (*Daktulosphaira vitifoliae*) is considered one of the most damaging viticulture pests world-wide. Phylloxera feed on either the roots or leaves of grapevines. Root-feeding phylloxera (called radicicoles) attach to feeding sites on the roots and damage vines by inducing swelling on the roots during feeding. These swellings inhibit water uptake by the vine and cause the roots to split, exposing them to pathogens in the soil. *Vitis vinifera* vines infested with phylloxera typically die within four to five years following infestation (Powell, 2012). In Australia, quarantine efforts have significantly reduced and slowed the spread of phylloxera (Powell, 2008). A key component of Australia's quarantine strategy is the implantation of three quarantine boundaries: Phylloxera Infested Zones (PIZs), Phylloxera Risk Zones (PRZ), and Phylloxera Exclusion Zones (PEZ). Each zone has specific and enforceable quarantine regulations with the aim of preventing the spread of phylloxera from PIZs into PEZs (NVHSC, 2009). Disinfestation protocols are critical in ensuring phylloxera infestations are contained within Phylloxera Infestation Zones by preventing movement of phylloxera out of infested vineyards. Phylloxera are small, slow-moving insects, and as such are thought to rarely infest new vineyards independently. First-instar phylloxera (crawlers) are mobile and infest new vines by walking above the ground or by wind dispersal (King and Buchanan, 1986). Importantly, within vineyard studies suggest phylloxera dispersal is limited to just 100m a year (King and Buchanan, 1986). Between vineyards, phylloxera are most likely transported by contaminated equipment, machinery, plant material, clothing, or footwear (Powell, 2008). In order to prevent new infestations, these transportation sources need to be disinfested before leaving the vineyard using validated disinfection protocols.

Most disinfestation protocols require exposing phylloxera contaminated material to hot or cold temperatures that are outside the limits of their physiological tolerance (e.g., Clarke et al., 2018; Clarke et al., 2019; Powell, 2017). Dry heat disinfestation in Victoria, which is typically used for vineyard machinery, requires placing the machinery in a dry heat shed or similar at either 40°C for 2 hours and 15 minutes or 45°C for 2 hours. These temperatures were validated in the laboratory by holding crawlers (the life-stage that exists above the ground and are therefore more likely to be picked up on machinery) at the desired temperature for

the specified amount of time and then recording mortality (Clarke et al., 2018). A protocol is considered effective if it results in 100% mortality across a diverse range of phylloxera genotypes, as genotypes vary in their susceptibility to disinfestation protocols (Clarke et al., 2018). These protocols are typically validated in a laboratory under sterile conditions. Importantly, laboratory conditions do not completely mimic field conditions. In practice, phylloxera may be exposed to the disinfestation temperatures while still attached to a feeding site on a piece of root, or while covered by soil or plant material. Although rarely studied in the context of disinfestation (Schortemeyer et al., 2011), such hygiene conditions may decrease the effectiveness of the disinfestation treatment by either i) limiting desiccation by providing the insect with a source of moisture from the soil or plant material, or ii) by providing the insect with a microhabitat (e.g., crevasses on the surface of lignified roots) that does not reach the disinfestation protocol's designated temperature (Prinzing, 2001; Teriau et al. 2023). These hygiene conditions may result in decreased mortality, potentially resulting in the transport of live phylloxera to new vineyards. This may be especially true for vineyard machinery, which contain parts that can hide dirt or plant material. Although the current dry heat protocols include the removal of any parts of the machinery that can hide dirt and thoroughly cleaning the machinery before treating (NVHSC, 2009), soil and plant material can be missed due to the size and complexity of vineyard machinery.

Here, we evaluated the effectiveness of the dry heat disinfestation protocols under different hygiene conditions in the laboratory. First, we assessed the current recommended disinfestation procedures for different states by exposing phylloxera under different hygiene conditions to either 40°C for 2 hours and 15 minutes (Victoria and New South Wales), 40°C for 3 hours (South Australia), or 45°C for 2 hours (Victoria and New South Wales). We then trialed a new disinfestation protocol across diverse phylloxera genotypes in the laboratory, by increasing the temperature to 50°C. Lastly, we assessed a disinfestation protocol of 50°C for 2 hours in a field setting, using a commercial dry heat shed and diverse phylloxera genotypes maintained under different hygiene conditions.

Project objectives

The aim of this project was to:

i) Evaluate the effectiveness of the current dry heat disinfestation protocols under different hygiene conditions.

ii) Trial a new disinfestation protocol involving higher temperatures in the laboratory.

iii) Evaluate the new dry heat disinfestation protocol in a commercial dry heat shed under different hygiene conditions across diverse phylloxera genotypes.

Methodology

Phylloxera cultures

All phylloxera cultures included in this study (G1, G4, G19, G20, G30 and G38) were established from eggs laid by a single individual placed on excised roots (*V. vinifera* Chardonnay) held at 25 ± 2°C, following Kingston 2007, and maintained under quarantine at Agriculture Victoria, Rutherglen. The identity of each phylloxera genotype was confirmed using six nuclear microsatellite markers (Umina et al., 2007). For each experiment, phylloxera eggs or crawlers were carefully collected using a fine artists paintbrush. We ensured that all eggs and crawlers used in each experiment were from the same cohort (i.e., all individuals used in a single experiment were laid within 48 hours of one another).

Laboratory assays

Recommended dry heat treatments under different hygiene conditions

We evaluated the current dry heat disinfestation treatment for different Australian states using one-day old G4 phylloxera crawlers under different hygiene conditions. The dry heat disinfestation treatments were: i) 40 °C for 2 hours and 15 minutes (Victoria and New South Wales), ii) 45 °C for 2 hours (Victoria and New South Wales), and iii) 40 °C for 3 hours (South Australia). Phylloxera under different hygiene conditions were held at the desired temperature using a fan-forced oven (Qualtex Model Number OM24SE3D. FSE Scientific Marrickville, NSW Australia) for the duration of the treatment. Three hygiene conditions were tested: i) *clean* (not attached to a root and no soil or plant material), ii) *clean root* (phylloxera are attached to a piece of root with no soil or plant material) and iii) *root protected by soil and grapevine material* (phylloxera are attached to a root and protected by soil and grapevine material). For the *clean* treatment, 10 crawlers were held in a vial (5.5 cm high and 2.5 cm in diameter) for the duration of dry heat treatments. To ensure crawlers were attached to the roots for both the *clean root* and *root protected by soil and grapevine material* treatments, phylloxera were first collected as eggs (n = 25) and placed on a 5cm piece of excised root (*Vitis vinifera*), the eggs were allowed to hatch and the newly emerged crawlers were given time to establish feeding sites on the roots before exposure to the dry heat treatment. The number of crawlers to hatch and establish feeding sites on each root was recorded prior to the dry heat treatment. For the *clean root* treatment, the root was held in a Petri dish (90 mm in diameter) for the duration of the dry heat treatments. For the *root protected by soil and grapevine material* treatment, the root was placed in a mesh pouch (Figure 3.1a). The mesh pouch was then placed on a Petri dish (90 mm in diameter) and covered with 1g of grapevine foliate, 0.4g of vine bark, and 150g of clay (Unimin Clay Grp 1) moistened with 50ml of rainwater (Figure 3.1b). Five replicates per hygiene condition x dry heat disinfestation treatment were tested, except for *clean root* x 45 °C for 2 hours where n = 10 and noting that a *root protected by soil and grapevine material* was not tested at 40 °C for 2 hours and 15 minutes). See Table 3.1 for a description of all treatments.

Table 3.1: Treatment descriptions across all three experiments.

	Hygiene conditions tested	**Dry heat treatments tested**	**Genotypes tested**
Recommended dry heat treatments under different hygiene conditions.	*Clean*: phylloxera were not attached to a root and no soil or plant material was present	40°C for 2 hours and 15 minutes	G4
		45°C for 2 hours	G4
		40°C for 3 hours	G4
	Clean root: phylloxera were attached to a root and no soil or plant material was present	40°C for 2 hours and 15 minutes	G4
		45°C for 2 hours	G4
		40°C for 3 hours	G4
	Root protected by soil and grapevine material: phylloxera were attached to a feeding site and protected by both soil and grapevine material	40°C for 2 hours and 15 minutes	Not tested
		45°C for 2 hours	G4
		40°C for 3 hours	G4
Improved dry heat treatment	*Clean root*: phylloxera were attached to a root and no soil or plant material was present	50°C for 1 hour	G4
		50°C for 2 hours	G1
			G4
			G19
			G20
			G30
			G38
In-field assays	*Clean root*: phylloxera were attached to a root and no soil or plant material was present	Commercial heat shed (50°C) for 2 hours	G4
			G1
			G4
			G19
			G20
			G30
			G38
	Root protected by grapevine material: phylloxera were attached to a root and protected by grapevine material	Commercial heat shed (50°C) for 2 hours	G1
			G4
			G19
			G20
			G30
			G38
		Commercial heat shed (50°C) 1 hour and 45 minutes	G4
		Commercial heat shed (50°C) 1 hour and 30 minutes	G4
	Root protected by soil: phylloxera were attached to a root and protected by soil	Commercial heat shed (50°C) for 2 hours	G4
	Root protected by soil and grapevine material: phylloxera were attached to a root and protected by both soil and grapevine material	Commercial heat shed (50°C) for 2 hours	G4

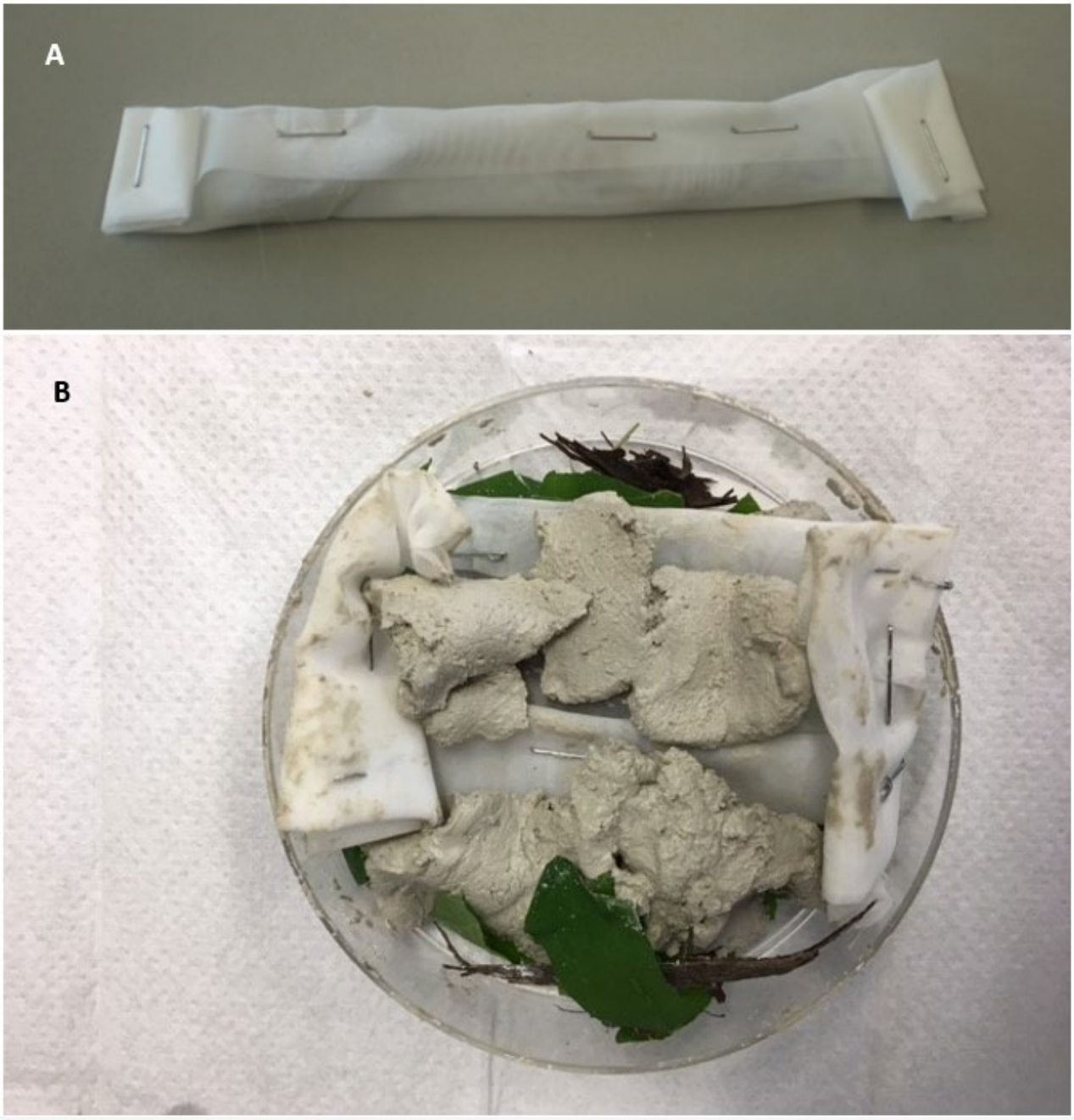


Figure 3.1A: Mesh pouch containing an excised root (Vitis vinifera) with phylloxera crawlers attached to the feeding site.

Figure 3.1B: Mesh pouch containing an excised root with phylloxera crawlers on a petri dish protected by grapevine foliage, vine bark and clay moistened with rainwater to mimic hygiene conditions where phylloxera are protected from dry heat disinfestation by soil and plant material.

Following the dry heat treatment, each root was placed on a clean Petri dish and maintained in a growth chamber as per the phylloxera cultures described previously. Phylloxera in vials (*clean* hygiene condition) were first transferred onto clean pieces of roots (5cm in length) using a fine artist's paintbrush, which was then placed on a clean Petri dish. Roots placed in pouches (*root protected by soil and grapevine material* hygiene condition) were first removed from the mesh and all soil and grapevine material was inspected for phylloxera under a microscope to ensure no insects had escaped the mesh pouch during treatment. Roots from all treatments were then inspected weekly and the number of living crawlers, dead crawlers, and the number of individuals to complete development into adults were recorded for four weeks.

Improved dry heat treatment.

We investigated whether an increased temperature of 50 °C could improve phylloxera mortality. Phylloxera crawlers were prepared as the *clean root* treatment described previously. In brief: 25 eggs of either G1, G4, G19, G20, G30, or G38 phylloxera were placed on excised *V. vinifera* roots (approximately 5cm in length) and the number of crawlers to hatch and establish feeding sites was recorded. The roots were then placed on Petri dishes (90 mm in diameter) and held at 50°C for either 1 (G4 phylloxera only: n = 5) or 2 hours (all phylloxera genotypes listed above: n = 5 per genotype) in a fan forced oven as previously described. See **Table 1** for a description of all treatments. Following the dry heat treatment, each root was placed on a clean Petri dish and maintained in a growth chamber. Roots were inspected weekly and the number of living crawlers, dead crawlers, and the number to phylloxera to develop into adults were recorded for four weeks.

In-field assays using a commercial dry heat shed

The dry heat treatment of 50 °C was tested in field using a commercial dry heat shed in the Yarra Valley. Heat sheds vary in their heating methods. The dry heat shed used in our experiment had two diesel burners and one gas burner for heating the shed, and had both ceiling and blast fans to circulate the heat. Phylloxera crawlers attached to feeding sites were prepared as described previously. Crawlers of G1, G4, G19, G20, G30 or G38 phylloxera genotypes were allowed to establish feeding sites on excised *V. vinifera* roots (5cm in length). The roots were then sealed in a fine mesh pouch to prevent escape (Figure 3.1a). The pouch was then placed on a Petri dish (90mm in diameter) and prepared under different hygiene conditions:: i) *clean root* (all genotypes), ii) *root protected by grapevine material* (1g of grapevine foliage: all genotypes), iii) *root protected by soil* (150g of Unimin Clay Grp 1 clay moistened with 50ml of rainwater: G4 only), or iv) *root protected by soil and grapevine material* (1g of grapevine foliage with 150g of Unimin Clay Grp 1 clay moistened with 50ml of rainwater: G4 only; Figure 3.1b). All treatments were placed on a wire rack within the heat shed (Figure 3.2), approximately 50cm above the ground. Four temperature probes were placed in the shed during the experiment as part of a concurrent study (The Dirt Dude, 2022). Probes placed at 0.45 and 0.6 m confirm that the temperature inside the shed did reach 50°C during the experiment although there was some variation (The Dirt Dude, 2022). The Petri dishes were held in the heat shed for either 2 hours (n = 5 per genotype x hygiene condition), 1 hour and 45 minutes (n = 5: G4 *root protected by grapevine material*), or 1 hour and 30 minutes (n = 5: G4 *root protected by grapevine material*). See **T**able 3.1 for a description of all treatments.



Figure 3.2: Plates containing diverse phylloxera genotypes under different hygiene conditions were placed on wire racks in a commercial dry heat shed.

All Petri dishes containing the pouches were then removed from the heat shed and returned to the laboratory. Each root was then carefully removed from the pouch and inspected under a microscope. The number of dead and living phylloxera was calculated. Each root was then transferred to a clean Petri dish and maintained in the growth chamber as previously described for 4 weeks, the roots were then inspected under a microscope and the number of individuals to survive and complete development was recorded.

Effectiveness ratings

Each disinfestation protocol was given an effectiveness rating of either i) not effective (did not result in 100% mortality regardless of the hygiene condition), ii) effective following cleaning (resulted in 100% mortality under specific hygiene conditions), and iii) effective (resulted in 100% mortality under all hygiene conditions tested).

Key Research Findings

Laboratory assays

Recommended dry heat treatments under different hygiene conditions.

100% mortality was not achieved for any of the current recommended dry heat treatments tested, regardless of the hygiene condition (Table 3.2). Crawlers across all dry heat treatments and hygiene conditions were also able to complete development to the adult stage (Table 3.2)

Table 3.2: The proportion of G4 crawlers to survive and complete development following the recommended dry heat disinfestation treatments for different states.

Dry heat disinfestation treatment	Hygiene condition	Percentage to survive (two weeks post treatment)	Percentage to develop into adults (approximately 30 days post treatment)
40 °C for 2 hours & 15 minutes	Clean (no root)	100%	95%
	Clean root	96%	92%
45 °C for 2 hours	Clean (no root)	86%	81%
	Clean root	94%	78%
	Root protected by soil and grapevine material	48%	48%
40 °C for 3 hours	Clean (no root)	59%	51%
	Clean root	98%	91%
	Root protected by soil and grapevine material	90%	90%

Improved dry heat treatment.

100% mortality was achieved for all genotypes tested namely G1, G4, G19. G20. G30 and G38 at 50°C for 2 hours (Table 3.3), noting that only a single hygiene condition was tested (*clean root*). 100% mortality was not achieved when this time was reduced to 1 hour (G4 phylloxera tested only) with some individuals completing development post treatment (Table 3.3)

Table 3.3: The proportion of phylloxera crawlers to survive and complete development after either 1 or 2 hours at 50°C in the laboratory.

Phylloxera strain	Time spent at 50 °C	Percentage of crawlers to survive (two weeks post treatment)	Percentage of crawlers to develop into adults (30 days following treatment)
G1	2 hours	0	0
G4	1 hour	74%	39%
	2 hours	0	0
G19	2 hours	0	0
G20	2 hours	0	0
G30	2 hours	0	0
G38	2 hours	0	0

In-field assays

When placed in a heat shed for 2 hours, 100% mortality was achieved across all genotypes except G30 when attached to a clean root or attached to a root protected by grapevine material. (Table 4). Four percent of G30 phylloxera crawlers attached to a clean root, initially survived the treatment, but died before they were able to complete development, suggesting a delayed effect of the disinfestation treatment. G4 crawlers attached to a root and protected by soil alone or by soil and grapevine material were able to survive and complete development (Table 4). When the time of exposure was decreased to 1 hour and 45 minutes, four percent of G4 crawlers attached to a root and protected by grapevine material survived. However, the surviving crawlers were unable to complete development. When the time of exposure was decreased further to 1 hour and 30 minutes, all G4 crawlers attached to a feeding site and protected by grapevine material died after the initial treatment (Table 3.4).

Table 3.4: The proportion of phylloxera crawlers to survive and complete development in a dry heat shed maintained at 50°C under different hygiene conditions.

Phylloxera strain	Time in heat shed	Hygiene condition	Percentage of crawlers to survive post treatment	Percentage to develop into adults (30 days following treatment)
G1	2 hours	Clean root	0	0
		Root site protected by grapevine material	0	0
G4	2 hours	Clean root	0	0
		Root protected by grapevine material	0	0
		Root protected by soil	42%	30%
		Root protected by soil and grapevine material	50%	50%
	1 hour and 30 minutes	Root protected by grapevine material	0	0
	1 hour and 45 minutes	Root protected by grapevine material	4%	0
G19	2 hours	Clean root	0	0
		Root protected by grapevine material	0	0
G20	2 hours	Clean root	0	0
		Root protected by grapevine material	0	0
G30	2 hours	Clean root	4%	0
		Root protected by grapevine material	0	0
G38	2 hours	Clean root	0	0
		Root protected by grapevine material	0	0

Effectiveness ratings

All current recommend dry heat disinfestation treatments are rated *not effective* as they did not result in 100% mortality regardless of the hygiene condition tested. The disinfestation treatment of 2 hours at 50°C is rated *effective following cleaning* as it resulted in 100% mortality under some, but not all of the hygiene conditions tested.

Discussion and Recommendations

We found that the currently recommended dry-heat disinfestation protocols were not fully effective against phylloxera regardless of the hygiene condition. Survival and subsequent completion of development was considerably higher than expected, as these protocols had already been validated against G4 (Clarke et al., 2018). Clarke and colleagues placed diverse phylloxera (not attached to a root and in the absence of soil and plant material) in an oven at both 40°C for 2 hours and 15 minutes, and 45°C for 75 minutes and achieved 100% mortality (Clake et al., 2018). We were unable to replicate these results. Instead, **we found that 100% mortality was only achieved when individuals were held at 50°C for 2 hours and we recommend the National Phylloxera Management Protocols are updated to reflect these findings.**

Our field assessment in a commercial dry heat shed resulted in 100% mortality across all genotypes when phylloxera were either attached to a root, or were attached to a root and protected by grapevine foliage, and held at 50°C for 2 hours. Although 4% of G30 phylloxera attached to a root survived the initial treatment, they

died before completing development, suggesting a delayed response to the treatment. Areas within commercial dry heat sheds can reach temperatures of greater than 70°C. but this varies between sheds and temperatures are typically lower closer to the ground (The Dirt Dude, 2022). **Elevating equipment using blocks of wood could help ensure all parts of the equipment reach the minimum temperature and is already practiced by some dry heat shed operators** (The Dirt Dude, 2022).

Unfortunately, even when the temperature was increased to 50°C, phylloxera that were protected by soil alone or by both soil and grapevine material were able to survive and develop into adults; 30% of phylloxera attached to root and protected by soil alone and 50% of phylloxera attached to a root and protected by both soil and grapevine material developed into adults within 30 days of the dry heat **treatment. This result highlights the importance of thoroughly cleaning machinery before the dry heat disinfestation treatment is applied. Although it may be easier to remove soil from machinery compared to plant material (personal communication – Warren Birchmore, VineHealth Australia), further research could develop additional disinfestation protocols for machinery contaminated with soil.** It is worth nothing that many dry heat shed operators leave machinery in the heated sheds overnight (The Dirt Dude, 2022), and whether this improves phylloxera mortality under different hygiene conditions has not been empirically investigated.

Taken together, out findings demonstrate the need to consider hygiene conditions when developing disinfestation protocols. Few studies (if any) consider the impact that variation in microclimate or moisture may have on the effectiveness of dry heat treatments for pest disinfestation (Schortemeyer et al., 2011). **Where possible, protocols should also be validated in the field, under realistic conditions**. This not only protects against the sterile nature of some laboratory studies but also provides researchers with the opportunity to develop protocols alongside their users (e.g., growers and heat shed operators), ensuring these protocols are can be readily adopted by their users.

Conclusion

Our findings suggest that the current dry heat disinfestation treatment temperature should be increased to 50°C for a minimum of 2 hours following removal of parts that hide dirt and a thorough cleaning of the object.

Additionally, **we recommend further research is conducted to develop a dry heat disinfestation protocol that is effective under hygiene conditions in which phylloxera are protected by soil**. These protocols should be validated using diverse phylloxera genotypes and ideally using commercially available heat sheds.

Phylloxera Population Dynamics and Survival Over Winter

Executive Summary

Phylloxera first-instar crawlers are a mobile life stage, with some individuals traveling above ground in search of new vines. These individuals are sometimes collected by vineyard machinery or equipment or on the clothing or tools of vineyard workers and can subsequently be transferred to new blocks or vineyards if effective disinfestation protocols are not implemented. In Australia, quarantine efforts have significantly slowed the spread of phylloxera by limiting human-assisted dispersal through the development and implementation of effective disinfestation protocols. As phylloxera population dynamics vary between seasons, the risk of crawlers being collected by vineyard machinery or personnel may also vary between the seasons. Previous research in Victoria, Australia suggests that phylloxera overwinter as crawlers, meaning there are high numbers of the mobile life stage in the soil in winter. Although this may suggest an increased risk of human-assisted dispersal, crawlers may be unable to develop or establish new feeding sites at cooler temperatures which may reduce the risk of dispersal.

Here we investigated the risk of phylloxera dispersal by human activity during winter in the North-East Phylloxera Infested Zone (PIZ) by i) assessing phylloxera development under field conditions and ii) assessing phylloxera development and survival at winter temperatures for three phylloxera genotypes (G1, G4, and G19) in the laboratory. Additionally, we investigated soil characteristics that may contribute to variation in the population dynamics of phylloxera in the field.

In line with previous field studies, we found that the number of crawlers on vine roots was highest in winter. We also did not observe any root galls (galls that form on the roots in response to feeding phylloxera) in winter suggesting that phylloxera may not be feeding and may have reduced activity during this season. Our laboratory studies suggest that at 4 or 9°C, phylloxera are unlikely to survive or develop into adults, but at 14°C (which is within the maximum winter soil temperatures recorded for the North-East PIZ) a small number of individuals survive and can develop from eggs into crawlers. Taken together this suggests that at least at mild winter temperatures **human-assisted dispersal of phylloxera is possible and we therefore recommend that effective disinfestation and quarantine protocols continue to be implemented in winter.**

Additionally, our laboratory studies demonstrated genotypic variation in phylloxera sensitivity to winter temperatures. G1 and G4 phylloxera eggs maintained at 14°C were able to develop into crawlers and continued developing into adults once returned to control temperatures (25°C). A small number of G19 eggs maintained at 14°C were able to develop into crawlers, but these individuals died following their transfer to control temperatures (25°C). Few studies have investigated variation in the survival of different phylloxera genotypes at different temperatures outside of the context of disinfestation protocols. Yet, understanding how different phylloxera genotypes develop and survive in different climates is critical in predicting how our changing climate will influence the prevalence and distribution of different genotypes in Australia. This may have significant implications for growers replanting on resistant rootstocks, as their choice of rootstock is highly dependent on the risk of infestation from specific phylloxera genotypes. We recommend future research investigates the temperature thresholds of prevalent phylloxera genotypes and maps their predicted distributions under different climate scenarios to help future-proof the replanting efforts of growers.

Lastly, during field surveys, we found weak but significant negative relationships between soil moisture and the number of root galls and adults observed on roots, and soil pH and the total number of insects observed on roots. Findings across different field surveys on relationships between phylloxera populations and soil characteristics and mostly inconsistent and empirical laboratory studies are likely needed to determine which soil characteristics influence phylloxera population dynamics and whether they can be manipulated to limit their ability to establish, reproduce, or disperse. As many different and interacting soil characteristics may influence phylloxera populations, we recommend a literature review is conducted to collate previous and within-project research in this area which also considers the potential implications of any soil-based management strategy on vine health and productivity.

Key findings and recommendations

Risk ofhuman-assisted dispersal during winter operations

- Our field studies suggest that phylloxera overwinter as the most mobile life stage (crawlers)
- Our laboratory studies suggest that at mild winter temperatures phylloxera can survive and are able to continue to develop and reproduce shortly after exposure to warmer temperatures
- Taken together this suggests that human-assisted dispersal of phylloxera during winter is possible and we recommend effective disinfestation and quarantine protocols continue to be implemented during winter operations.

Genotypic variation in sensitivity to winter temperatures

- Laboratory studies revealed genotypic variation in sensitivity to winter temperatures.
- In a changing climate, we may expect the distribution of phylloxera genotypes to shift which may have consequences for growers replanting on resistant rootstocks based on the specific genotypes on or surrounding their vineyard.
- Future research could investigate the temperature thresholds of prevalent phylloxera genotypes and map their predicted distributions under different climate scenarios

Relationship between phylloxera abundance and soil characteristics

- There were weak but significant negative relationships between the number of root galls and adults and moisture
- There was also a weak but significant negative relationship between the total number of insects and soil pH.
- Empirical laboratory studies are needed to determine which soil characteristics influence phylloxera population dynamics and whether manipulating these characteristics could be used as a management tool for this pest.
- A literature review could be conducted to collate previous and within-project research on potential soil-based management strategies for phylloxera.

Introduction

Grapevine phylloxera (*Daktulosphaira vitifoliae*) is widely considered to be one of the most damaging viticulture pests, feeding on either the roots or the leaves of the vine (Benheim et al., 2012). Root-feeding phylloxera (called radicicoles) are especially damaging to grapevines. Root-feeding phylloxera induce galls on the roots of the vine, which inhibit water uptake and cause tears and rips in the root system exposing the vine to soil-borne pathogens typically resulting in vine death (Powell et al., 2012). In Australia, quarantine efforts have significantly reduced and slowed the spread of phylloxera (Powell, 2008). A key component of Australia's quarantine strategy is the implantation of three quarantine boundaries: Phylloxera Infested Zones (PIZs), Phylloxera Risk Zones (PRZ), and Phylloxera Exclusion Zones (PEZ). Each zone has specific and enforceable quarantine regulations with the aim of preventing the spread of phylloxera from PIZs into PEZs (NVHSC, 2009). Disinfestation protocols are critical in ensuring phylloxera infestations are contained within Phylloxera Infestation Zones by preventing the movement of phylloxera out of infested vineyards. Phylloxera are small, slow-moving insects, and as such disperse predominantly through human activity (Deretic et al., 2003; Powell, 2008). First-instar (crawlers) are mobile, with some individuals traveling above ground in search of new vines (King and Buchanan, 1986). These individuals may be collected by vineyard machinery or equipment or on the clothing or tools of vineyard workers and subsequently transferred to new blocks or vineyards if effective disinfestation protocols are not followed.

The risk of human activity resulting in new phylloxera infestations may vary between seasons. Previous research in Victoria, Australia suggests that in temperate climates there are three distinct seasonal stages of grapevine phylloxera population dynamics i) *reproducing:* occurs between early summer and mid-autumn and consists of insects across all life stages, ii) *overwintering*: occurs between late autumn to early spring, and consists only of first-instars, and lastly iii) *declining*: occurs between early spring and early summer and consists of low numbers of individuals (Hoffman et al., 2016). Although the overwintering life stage is the

most mobile, these individuals may not be active at cooler temperatures. The exact mechanisms triggering the above-ground dispersal of grapevine phylloxera are unknown but previous research suggests it is seasonal (Herbert et al., 2006), however the lower temperature threshold for crawlers to establish a feeding site is 18°C (Turley et al., 1996) which is outside of the maximum winter temperatures recorded for the North-East Phylloxera Infested Zone. At lower temperatures, crawlers may be unable to infest new vines and we might expect them to limit above-ground dispersal. Importantly, the risk of overwintering populations of phylloxera infesting new blocks or vineyards is currently unknown.

In addition to varying with the season, phylloxera population dynamics may also be influenced by soil characteristics such as pH, moisture, or organic matter content. Findings from field studies investigating whether soil characteristics influence grapevine phylloxera population dynamics are inconsistent. For example, Hoffman and colleagues, found no effect of soil characteristics (pH, water content, organic matter content, and basic respiration) on phylloxera density by comparing soil samples taken from vines at a single field site (Hoffman et al., 2016), whereas, a multi-site study conducted by Powell and colleagues, found that vineyards with low soil pH and high aluminium exchange capacity had higher numbers of phylloxera (Powell et al., 2001). Whether manipulating soil characteristics can help reduce populations of grapevine phylloxera requires further study, which must also consider how changing these characteristics impacts the productivity and health of different grapevine varieties. Importantly, soil is a complex environment that interacts with the plants, animals, and microbes within it, and manipulating one aspect of the soil will likely have consequences for other soil characteristics. Nonetheless, an improved understanding of how soil characteristics shape grapevine phylloxera population dynamics has potential applications in the development of new disinfestation protocols and/or management strategies.

Here we investigated the risk of phylloxera dispersal by human activity during winter in the North-East (PIZ) by i) assessing phylloxera development under field conditions and ii) assessing phylloxera development and survival at winter temperatures in the laboratory. Additionally, we investigated soil characteristics that may contribute to variation in the population dynamics of phylloxera in the field.

Project Objectives

The overarching aim of this project was to assess the risk of human-assisted dispersal of phylloxera during winter by:

i) Assessing phylloxera development under field conditions.

ii) Assessing phylloxera survival at overwintering temperatures in the laboratory across at least two diverse strains

Additionally, we investigated soil characteristics that may contribute to variation in the population dynamics of phylloxera in the field.

Methodology Part A: Phylloxera development under field conditions

Field assessment of phylloxera development

All field surveys were conducted in a commercial vineyard in the North-East Phylloxera Infested Zone (PIZ) in Victoria. Australia. All vines at this site were planted in 1988 on uncultivated soil without a previous history of grapevine cultivation. Root and soil samples were collected from four rows of vines planted in a randomised complete block of *Vitis vinifera* either own-rooted or grafted to 101-14 or Schwarzmann rootstocks. Vineyard surveys confirmed that these vines were infested with the G38 phylloxera genotype. Nineteen vines in an additional row within the same vineyard were also sampled. These vines were own-rooted *V. vinifera* and were infested with both the G20 and G38 phylloxera genotypes.

Samples were collected monthly between Spring 2021 and Winter 2022. All sampled vines were sampled only once during the study. Roots samples were collected by digging at the base of the vine and soil samples were taken using a tube soil sampler at a depth of 15 - 20 cm. All samples were placed in 2L plastic bags and were maintained at 4°C upon returning to the laboratory.

Root examination

Each root sample was examined under a dissecting microscope (10X) and the number of root galls, eggs, crawlers, alates (winged adults), and adults were recorded. All root samples were examined within one week of collection.

Soil characteristics

To measure soil pH, 10g of soil was suspended in 25 ml of a 0.01mol/L CaC12 solution. The pH of the solution was then measured with a pH meter after 30 minutes.

The soil water content was measured by first sieving and then weighing the soil, before drying the soil for 24 hours at 100 °C. The soil's moisture content was then calculated using the formula:

$$\frac{wet\ soil\ mass - dry\ soil\ mass}{dry\ soil\ mass}$$

To measure the soil's organic matter content the dried soil (described above) was incubated at approximately 400°C for 24 hours. The ash produced was then weighed and the soil's organic matter content was calculated using the formula:

$$\frac{dry\ soil\ mass - ash\ mass}{dry\ soil\ mass}$$

Statistical analysis

All statistical analyses were performed in JMP (JMP version 17 SAS institute). We performed a series of generalized linear models (GLMs) with Poisson distributions to determine if variation in i) the number of root galls ii) the number of eggs iii) the number of crawlers, iv) the number of adults, and v) the total number of insects could be explained by season. Post-hoc comparisons were then performed using Tukey's t-tests

We then performed a series of linear regression models to determine if there was any relationship between soil pH, soil moisture, and the soil's organic matter content and i) the number of root galls ii) the number of eggs iii) the number of crawlers, iv) the number of adults, and v) the total number of insects.

Methodology Part B: Phylloxera development and survival at overwintering temperatures

Phylloxera cultures

The G1, G4, and G19 phylloxera cultures were established from eggs laid by a single individual placed on excised roots (*V. vinifera* Chardonnay) at 25 ± 2°C, following Kingston 2007 and maintained under quarantine at Agriculture Victoria, Tatura. The identity of each phylloxera genotype was confirmed prior to the overwintering assays (described below) using six nuclear microsatellite markers (Umina et al., 2007).

Overwintering assays

To assess the survival of phylloxera at overwintering temperatures, we placed 5 eggs belonging to the G1. G4 or G19 phylloxera genotypes on excised roots following Kingston, 2007. The excised roots were then maintained at one of four temperatures: 4°C, 9°C, 14°C, or 25°C (control) for 4 weeks (n = 5 roots per temperature x genotype). Winter soil temperatures in the North-East Phylloxera Infested Zone are between approximately -0.94 and 14.25°C (unpublished data Catherine Clarke). After 4 weeks, all excised roots were maintained at 25 °C for an additional 2 weeks. The excised roots were checked weekly and the number of phylloxera eggs, crawlers, adults, and any dead phylloxera were recorded.

Statistical analysis

All statistical analyses were performed in JMP (JMP version 17 SAS institute). We performed a series of generalized linear models (GLMs) with Poisson distributions to determine if variation in i) the number of crawlers and ii) the number of adults at four weeks, and if variation in the iii) number of eggs, iv) the number of crawlers, v) and the number of adults at seven weeks could be explained by temperature, phylloxera genotype or their interaction. The high number of second-generation individuals in the control group masked differences between the overwintering temperature treatments. As such, this treatment was excluded from the analysis. Post-hoc comparisons were then performed using Tukey's t-tests.

Key Research Findings Part A: Phylloxera development under field conditions

Individuals of all life stages were observed on roots across all seasons, except for spring. In spring, no eggs were observed on roots, despite the presence of adults during this season. Root galls were found on roots in spring and autumn but not winter, suggesting that the individuals found in winter may not be feeding. Additionally, no alates were observed in the field during any of these surveys.

Number of root galls

Our GLM revealed a significant effect of season on the number of root galls. (df = 2, $\chi^2 = 371.84$, $p < 0.0001$). Post-hoc analysis revealed that there were significantly less root galls in winter compared to spring ($p = 0.001$) or autumn ($p = 0.011$) (Figure 3.3).

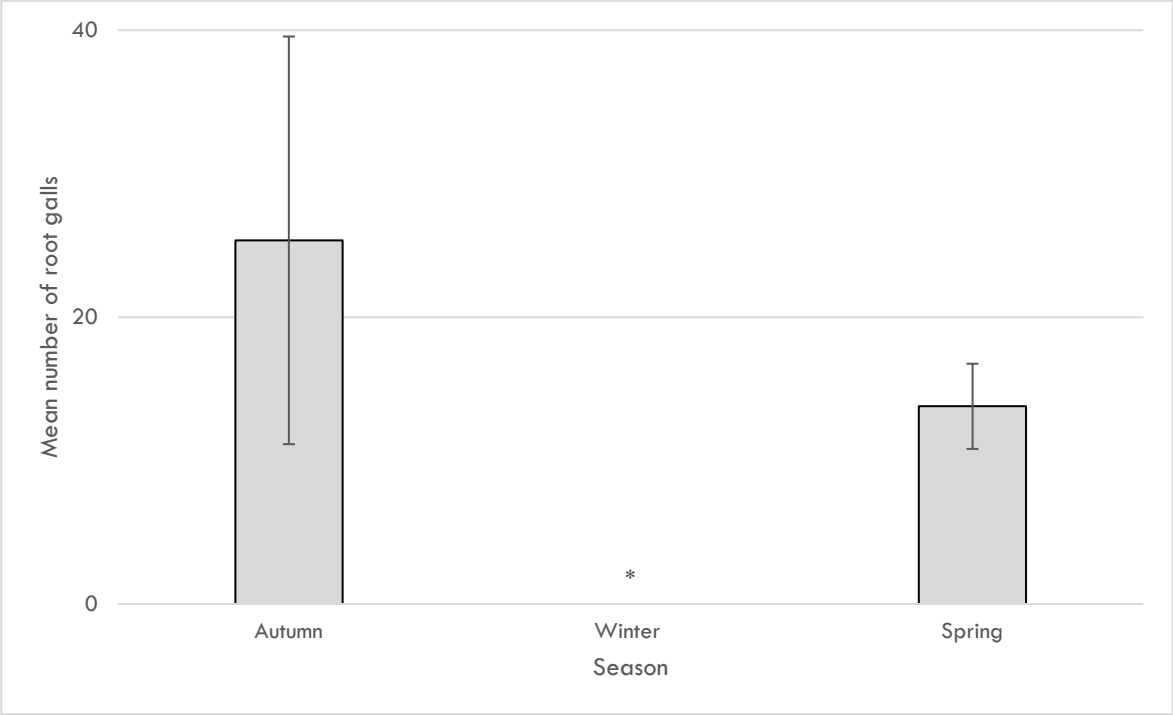


Figure 3.3: The mean number of root galls observed on roots collected from a commercial vineyard in autumn, winter, and spring. No root galls were found on roots collected in winter.

Additionally, our linear regression revealed a weak but significant negative relationship between the number of root galls and soil pH ($r^2 = 0.24$, F = 13.71, p = 0.0006: Figure 3.4) and moisture ($r^2 = 0.15$, F = 7.41, p = 0.0093: Figure 3.5). There was no relationship between the number of root galls and the organic matter content of the soil (p = 0.66).

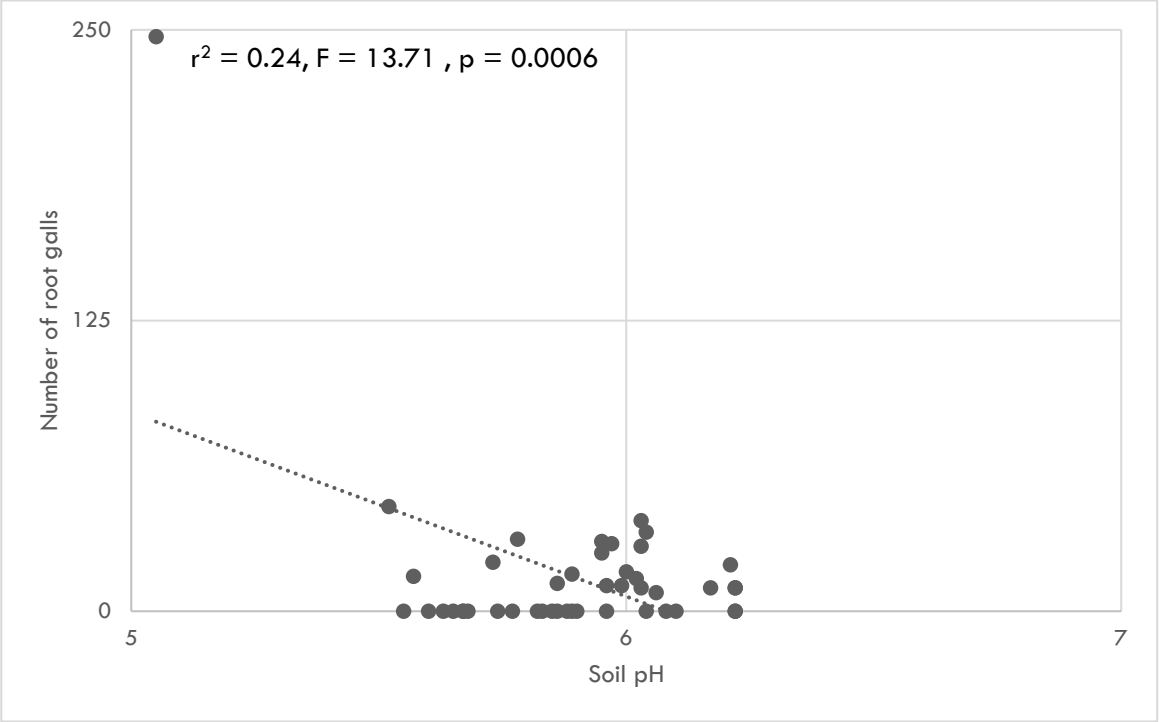


Figure 3.4: There was a weak but significant negative relationship between the number of root galls and soil pH.

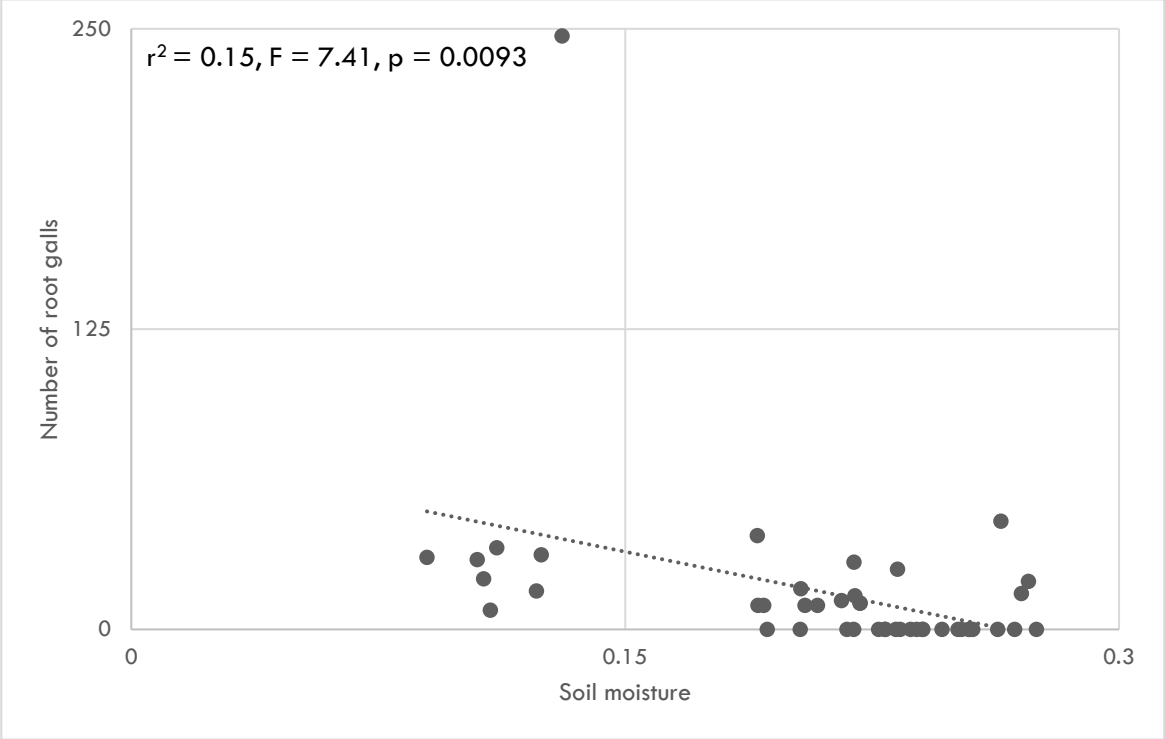


Figure 3.5: There was a weak but significant negative relationship between the number of root galls and soil moisture. This relationship holds when the outlier (root with 247 galls) is removed (r^2 = 0.24, F = 13.47, p = 0.00068)

Number of eggs

Our GLM revealed a significant effect of season on the number of eggs. (df = 2, $\chi^2 = 86.93$. $p < 0.0001$: Figure 3.6). Post-hoc analysis revealed that significantly less eggs were observed in spring compared to autumn ($p < 0.0001$)

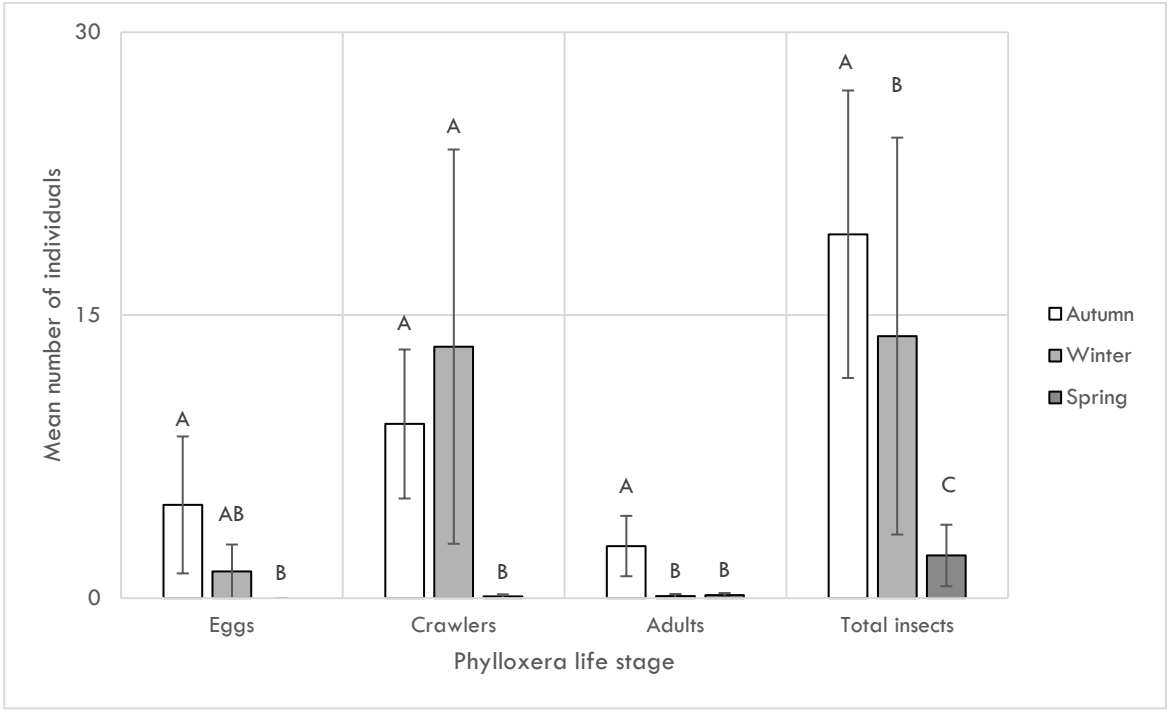


Figure 3.6: The mean number of individuals of different life stages observed on roots collected in a commercial vineyard in autumn, winter, and spring. Significantly fewer crawlers and total insects were observed on roots in spring compared to both autumn and winter.

Additionally, our linear regression revealed a significant negative relationship between the number of eggs and the soil pH ($r^2 = 0.26$, $F = 15.14$, $p = 0.0003$), however when a single outlier is removed from the data (a root with approximately 32 times the mean number of eggs calculated for all root samples) this relationship is lost ($p < 0.7$). There was no relationship between the number of eggs and the soil's moisture or organic matter content (all $p > 0.1$).

Number of crawlers

Our GLM revealed a significant effect of season on the number of crawlers. (df = 2, $\chi^2 = 292.59$, $p < 0.0001$: Figure 3.6). Post-hoc analysis revealed that there were significantly less crawlers in spring compared to winter ($p < 0.0001$) and autumn ($p < 0.0001$).

There was no relationship between the number of crawlers and the soil pH, moisture, or organic matter content (all $p < 0.05$),

Number of adults

Our GLM revealed a significant effect of season on the number of adults. (df = 2, $\chi^2 = 67.35$, $p < 0.0001$: Figure 3.6). Post-hoc analysis revealed that there were significantly more adults in autumn compared to both spring ($p < 0.0001$) and winter ($p = 0.0042$)

Additionally, our linear regression revealed a weak but significant negative relationship between the number of adults and the soil pH ($r^2 = 0.29$, $F = 16.57$, $p = 0.0002$) however, this relationship was lost when an outlier (a root sample with approximately 23 times the mean number of adults calculated for all root samples) was removed ($p < 0.5$). There was a weak but significant negative relationship between the number of adults and soil moisture ($r^2 = 0.16$, $F = 8.08$, $p = 0.0068$: Figure 3.7) which held with the removal of the outlier ($p = 0.0027$). There was no relationship between the number of adults and the soil's organic matter content ($p = 0.85$).

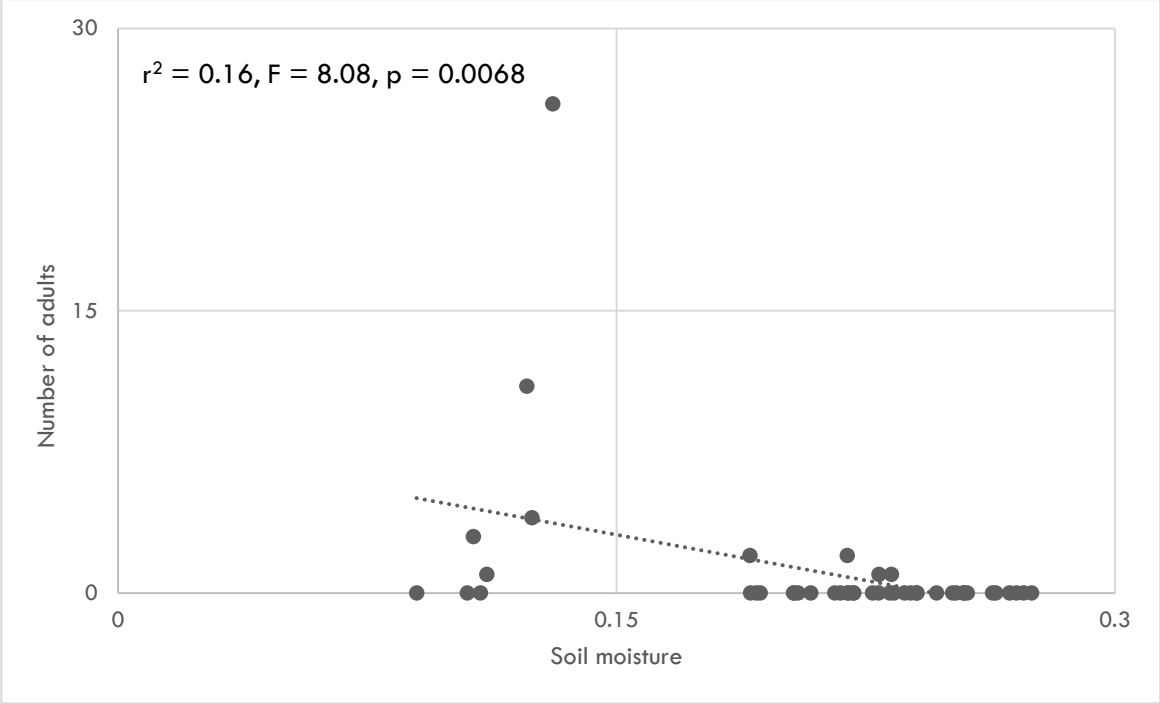


Figure 3.7: There was a significant negative relationship between the number of adults and soil moisture. This relationship holds when the outlier (root with 26 adults) is removed ($r^2 = 0.19$, $F = 10.15$, $p = 0.0027$)

Number of total insects

Our GLM revealed a significant effect of season on the total number of insects. ($df = 2$, $\chi^2 = 288.92$, $p < 0.0001$: Figure 3.6). Post-hoc analysis revealed that there were significantly more insects in autumn compared to both winter ($p = 0.0050$) and spring ($p < 0.0001$) and significantly more insects in winter compared to spring ($p < 0.0001$).

Additionally, our linear regression revealed a significant negative relationship between the number of total insects and the soil pH ($r^2 = 0.31$, $F = 19.23$, $p < 0.0001$; Figure 3.8), but no relationship between the total number of insects and the soil's moisture or organic matter content (all $p > 0.1$).

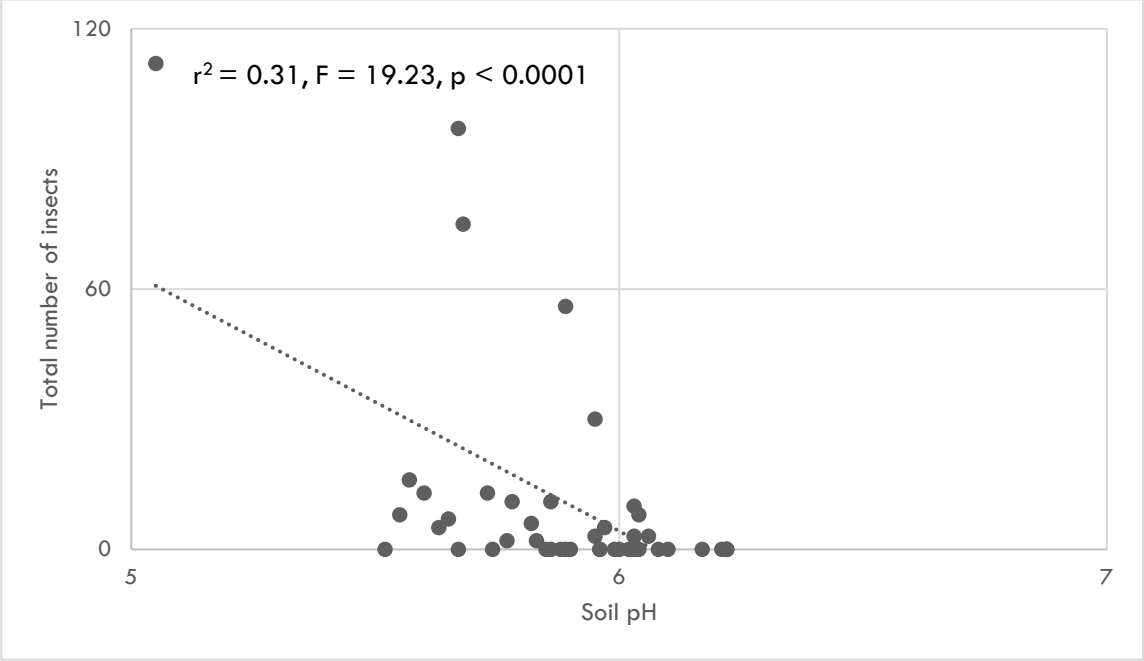


Figure 3.8: There was a significant negative relationship between the total number of insects observed on roots and the soil pH

Key Research Findings Part B: Phylloxera survival and development at overwintering temperatures

Survival and development at 25°C

When maintained at 25°C (control) individuals from all genotypes developed into adults within the first 4 weeks. Additionally, G1 and G4 phylloxera produced eggs within the first 4 weeks at this temperature, while G19 took an additional week to produce eggs.

Survival and development at 4°C

At 4°C, no G1 or G19 individuals developed into crawlers. A single G4 individual maintained at 4°C developed into a crawler but died before developing further. No individuals developed further when maintained at 25°C following the 4 weeks at 4°C.

Survival and development at 9°C

At 9°C, a single G1 individual, but no G4 or G19 individuals developed into crawlers. The G1 crawler died before developing further. No individuals developed further when maintained at 25°C following the 4 weeks at 9°C.

Survival and development at 14°C

At 14°C,40% of G1 and G19 individuals and 80% of G4 individuals developed into crawlers. These crawlers survived the four weeks at this temperature but did not develop into adults at this temperature. Two weeks after being maintained at 25°C. 32% of G1 and 40% of G4 individuals had developed into adults and were producing eggs. In contrast, G19 individuals did not develop any further than the intermediate stage and died following their transfer to the control temperature.

Comparison of survival and development at winter temperatures

Our generalized linear model (GLM) revealed a significant effect of temperature (df = 2, $\chi 2 = 72.32$, $p < 0.0001$) but not genotype ($p > 0.9$) or their interaction ($p > 0.9$) on the number of crawlers to develop at four weeks. Our post-hoc analysis revealed that across all genotypes the number of crawlers that developed at 14°C was significantly higher compared to the number of crawlers that developed at either 4°C (G1: $p = 0.013$, G4: < 0.0001, G19: $p = 0.002$) or 9°C (G1: $p = 0.013$, G4: < 0.0001, G19: $p = 0.002$: Table 3.5). As no individuals developed further than crawlers at any of the winter temperatures, our GLM revealed no significant effect of temperature, genotype, or their interaction on the number of adults to develop after four weeks (all $p = 1$)

Table 3.5: The mean ± SE number of individuals to develop into crawlers following four weeks at 25°C (control), 4°C, 9°C, or 14°C. Significantly more individuals maintained at 14°C developed into crawlers compared to those maintained at 4°C or 9°C, noting that the control treatment was not included in the analysis.

Genotype	4°C	9°C	14°C	Control (25°C)
G1	0	0	1.6 ± 0.67*	15.8 ± 3.61
G4	0	0	3.8 ± 0.37*	5.6 ± 3.17
G19	0	0	2 ± 0.63*	2 ± 0.63

Our GLM revealed a significant effect of temperature (df = 2, $\chi 2 = 522.94$, $p < 0.0001$ and genotype (df = 2, $\chi 2 = 304.23$, $p < 0.0001$) but not their interaction ($p > 0.9$) on the number of eggs produced at seven weeks (two weeks following their transfer to control temperatures). Post-hoc analysis revealed that G1 and G4 individuals maintained at 14°C produced significantly more eggs at seven weeks compared to their counterparts maintained at either 4°C (G1: $p = 0.049$, G4: $p = 0.046$) or 9°C (G1: $p = 0.049$, G4: $p = 0.046$: Table 2), whereas G19 individuals did not produce any eggs regardless of their winter temperature treatment ($p = 1$: Table 3.6)

Table 3.6: The mean ± SE number of eggs produced following four weeks at 25°C (control), 4°C, 9°C, or 14°C and a subsequent two weeks at the control temperature. Significantly more eggs were produced by G1 and G4 maintained at 14°C compared to those maintained at 4°C or 9°C, noting that the control treatment was not included in the analysis.

Genotype	4°C	9°C	14°C	Control (25°C)
G1	0	0	39.4 ± 22.04*	14.2 ± 7.40
G4	0	0	8.2 ± 4.52*	31.8 ± 24.98
G19	0	0	0	15.4 ± 11.44

Our GLM also revealed a significant effect of temperature (df = 2, $\chi 2 = 35.15$, $p < 0.0001$) and genotype (df = 2, $\chi 2 = 13.18$, $p = 0.0009$) but not their interaction ($p > 0.9$) on the number of crawlers to develop at seven weeks. Post-hoc analysis revealed that significantly more G1 and G4 individuals maintained at 14°C developed into crawlers at seven weeks compared to their counterparts maintained at either 4°C (G1: $p = 0.038$, G4: $p = 0.002$: Table 3) or 9°C (G1: $p = 0.038$, G4: $p = 0.002$: Table 3), whereas all G19 individuals that had developed into crawlers previously, died following their transfer to the control temperatures ($p = 1$: Table 3.7).

Table 3.7: The mean ± SE number of individuals to develop into crawlers following four weeks at 25°C (control), 4°C, 9°C, or 14°C and a subsequent two weeks at the control temperatures. Significantly more G1 and G4 individuals maintained at 14°C developed into crawlers compared to those maintained at 4°C or 9°C, noting that the control treatment was not included in the analysis.

Genotype	4°C	9°C	14°C	Control (25°C)
G1	0	0	2 ± 1.05*	56.8 ± 26.25
G4	0	0	1.2 ± 0.37*	28.2 ± 27.21
G19	0	0	0	12.2 ± 8.17

Lastly, our GLM revealed a significant effect of temperature (df = 2, $\chi 2 = 39.55$ $p < 0.0001$) and genotype (df = 2, $\chi 2 = 14.81$, $p = 0.0006$) but not their interaction ($p > 0.9$) on the number of adults to develop at seven weeks. Post-hoc analysis revealed that significantly more G1 and G4 individuals maintained at 14°C developed into adults at seven weeks compared to their counterparts maintained at either 4°C (G1: $p = 0.0004$, G4: $p = 0.018$: Table 3.8) or 9°C (G1: $p = 0.0004$, G4: $p = 0.018$: Table 4), whereas no G19 individuals developed into adults at any of the winter temperatures either before or following their transfer to the control temperature ($p = 1$: Table 3.8).

Table 4: The mean ± SE number of individuals to develop into adults following four weeks at 25°C (control), 4°C, 9°C, or 14°C and a subsequent two weeks at the control temperature. Significantly more G1 and G4 individuals maintained at 14°C developed into adults compared to those maintained at 4°C or 9°C, noting that the control treatment was not included in the analysis.

Genotype	4°C	9°C	14°C	Control (25°C)
G1	0	0	1.6 ± 0.40*	2.8 ± 1.07
G4	0	0	2 ± 0.89*	2.5 ± 2.36
G19	0	0	0	1.2 ± 0.73

Discussion and Recommendations

Risk of human-assisted dispersal during winter

Taken together, our results suggest that **human-assisted dispersal of phylloxera during winter is possible**. In winter, the number of crawlers (the life stage considered to have an increased likelihood of infesting new vineyards due to their mobility above ground) was highest. This is consistent with previous research which suggests that phylloxera overwinter or hibernate as crawlers (Granett et al., 2005). This may also explain the absence of root galls during winter, as overwintering individuals may limit or stop feeding on the vine. Our laboratory studies also provide evidence of overwintering, as any crawlers that developed from eggs maintained at 14°C did not develop further until exposure to control temperatures. Although our laboratory studies suggest that at low winter temperature phylloxera crawlers may be unable to survive, we found that within the maximum winter soil temperatures in the North-East Phylloxera Infested Zone (between 12.57 and 14.25°C: unpublished data Catherine Clarke) crawlers can survive and will develop into adults shortly after their exposure to warmer temperatures. Additionally, crawlers were captured in emergence (bucket) traps deployed in the Yarra Valley between June and August 2023 (see *Improved Surveillance and Detectability of Grapevine Phylloxera)* suggesting that at least some individuals disperse above ground during winter. As the life cycle of phylloxera in Australia is asexual, the introduction of a single individual is

sufficient to infest a new vineyard and **we therefore recommend that effective disinfestation and quarantine protocols continue to be implemented in winter.** The exact mechanisms triggering the above-ground dispersal of crawlers have not been established but this knowledge may enable growers and industry to predict when an increased density of crawlers in vineyard canopies is expected and therefore predict when there is an increased risk of human-assisted dispersal. **We recommend future research is directed at establishing the mechanisms triggering the above-ground dispersal of phylloxera crawlers**.

Seasonal and genotypic variation in phylloxera population dynamics

Our observations of phylloxera population dynamics in the field are mostly consistent with those observed by Hoffman and colleagues (2016) with some small differences. In line with Hoffman and colleagues, we observed significantly more individuals in autumn (reproducing stage), high numbers of crawlers in winter (overwintering stage), and significantly fewer individuals in spring (declining stage). Unlike Hoffman and colleagues who only observed crawlers during the overwintering stage, we also recorded both eggs and adults, albeit in small numbers. Our laboratory studies suggest that at low winter temperatures (4 and 9°C) these eggs would be unlikely to develop and survive but at mild winter temperatures, some eggs would continue to develop. Differences in the observed population dynamics between studies may be due to genotypic variation. Our laboratory studies suggest that genotypes vary in their sensitivity to temperature as G1 and G4 appear to have an increased tolerance for winter temperatures compared to G19. Further previous studies using California populations suggest that the developmental threshold for phylloxera is 18°C (Turley et al., 1996), whereas our results demonstrate that at least some Australian genotypes can reach the first-instar stage (crawlers) at just 14°C. Few studies have investigated variation in the survival of different phylloxera genotypes at different temperatures outside of the context of disinfestation protocols. Yet, understanding how different phylloxera genotypes develop and survive in different climates is critical in predicting how our changing climate will influence the prevalence and distribution of different genotypes in Australia. This may have significant implications for growers replanting on resistant rootstocks, as their choice of rootstock is highly dependent on the risk of infestation from specific phylloxera genotypes. We therefore, recommend future research investigates the temperature thresholds of prevalent phylloxera genotypes and maps their predicted distributions under different climate scenarios to help future-proof the replanting efforts of growers.

The influence of soil characteristics on phylloxera population dynamics

As soil moisture increased, we observed significantly fewer root galls and adults. We also found significantly fewer individuals on roots as the soil pH increased. Although most of these relationships are weak ($r^2 < 0.3$), they have important applications for the management of grapevine phylloxera. Unfortunately, findings from field studies investigating the relationship between soil characteristics and phylloxera populations are mostly inconsistent (see Hoffman et al., 2016 and Powell et al., 2001), making it difficult to draw reliable conclusions about the mechanisms involved and their potential applications for phylloxera management. This may be due to the complexity of soil and the nature of field studies where it is not possible to control for important variables (e.g. vine age, rootstock, presence of other pests, surrounding plants, and climate). **Empirical laboratory studies are likely needed to determine which soil characteristics influence phylloxera population dynamics and whether they can limit their ability to establish, reproduce, or disperse**. As many different and interacting soil characteristics may influence phylloxera populations, **we recommend a literature review is conducted to collate previous and within-project research in this area which also considers the potential implications of any soil-based management strategy on vine health and productivity.**

Conclusion

Taken together our results suggest that **human-assisted dispersal of phylloxera during winter is possible.** Phylloxera overwinter as the most mobile life stage and although their activity may be reduced, our laboratory studies suggest that a small number of phylloxera are able to survive and develop at mild winter temperatures. Additionally, a small number of phylloxera crawlers were captured in emergence traps in the Yarra Valley between June and August 2023. The exact mechanisms triggering the above-ground dispersal of phylloxera have not been established but this project provides evidence that at least in some parts of Victoria it can occur during winter. An improved knowledge of the factors triggering above above-ground

dispersal of crawlers may allow growers and industry to better predict when an increased density of crawlers in vineyard canopies will occur and therefore predict when there is an increased risk of human-assisted dispersal. **We recommend future research investigates the mechanisms triggering above-ground dispersal of phylloxera crawlers**. Importantly, as the life cycle of phylloxera in Australia is asexual, the introduction of a single individual is sufficient to infest a new vineyard, **we therefore recommend that effective disinfestation and quarantine protocols continue to be implemented in winter.**

Our laboratory studies suggest that genotypes vary in their sensitivity to temperature as G1 and G4 appear to have an increased tolerance for winter temperatures compared to G19. Few studies have investigated variation in the survival of different phylloxera genotypes at different temperatures outside of the context of disinfestation protocols. Yet, understanding how different phylloxera genotypes develop and survive in different climates is critical in predicting how our changing climate will influence the prevalence and distribution of different genotypes in Australia. This may have significant implications for growers replanting on resistant rootstocks, as their choice of rootstock is highly dependent on the risk of infestation from specific phylloxera genotypes. **We, therefore, recommend future research investigates the temperature thresholds of prevalent phylloxera genotypes and maps their predicted distributions under different climate scenarios to help future-proof the replanting efforts of growers.**

Lastly, we found significant negative relationships between the number of root galls and adults and soil moisture, as well as the total number of insects and soil pH. **Empirical laboratory studies are likely needed to determine which soil characteristics influence phylloxera population dynamics and whether they can be manipulated to limit their ability to establish, reproduce, or disperse**. As many different and interacting soil characteristics may influence phylloxera populations, **we recommend a literature review is conducted to collate previous and within-project research in this area, which also considers the potential implications of any soil-based management strategy on vine health and productivity.**

Research Theme 4: Improved Knowledge of Phylloxera Ecology

Increased Prevalence of Leaf Galling Phylloxera in the North-East Phylloxera Infested Zone

Executive Summary

Unlike root galling phylloxera, leaf galling phylloxera are thought to have little impact on the health of the vine. As such, few studies have investigated the factors influencing their abundance. Occurrences of leaf galling phylloxera in commercial vineyards have recently increased in the North-East Phylloxera Infested Zone (PIZ) in Victoria, with a similar trend observed more widely in many parts of the world. Additionally, the G38 phylloxera genotype, which was originally characterised from inside of leaf galls, has subsequently been documented feeding and reproducing on roots. Consequently, there has been a heightened interest in leaf galling phylloxera, especially in understanding the factors that may account for the recent increase in their abundance. We investigated whether the abundance of leaf galling phylloxera in infested vineyards is influenced by vineyard practices (such as row spacing), grape variety, or rootstock by counting the number of galls on leaves collected from different vineyards in the North-East PIZ. Lastly, galls were dissected to determine whether there was any relationship between the number of galls on leaves and the level of phylloxera infestation within each gall.

No significant differences were found in the abundance of galls between grape varieties and rootstocks. This is in contrast with recent international research that demonstrated variation in the ability of different leaf galling phylloxera genotypes to develop and reproduce on different rootstocks. This research was conducted in a controlled laboratory setting, whereas the present study was conducted in commercial vineyards where unaccounted-for variables (such as the number of founding phylloxera, time since initial infestation, and the age of the vines) may be masking differences among grape varieties or rootstocks. As such we recommend **greenhouse rootstock screening assays are used to determine the susceptibility of novel rootstocks to prevalent leaf galling phylloxera genotypes, particularly those that exist in both leaf galling and root galling forms such as G38.**

We also found a positive correlation between the number of phylloxera per gall and the number of galls per leaf. Whether this is due to the length of time the leaves had been infested, or whether specific leaf characteristics are better able to support or attract leaf galling phylloxera requires further study. We recommend **future studies investigate whether variation in leaf characteristics contribute to their ability to support and/or attract leaf galling phylloxera.** These findings may help researchers developing novel grape varieties to select for leaf characteristics that are less susceptible to leaf galling genotypes.

Surprisingly, approximately 17% of the leaf galls dissected did not contain any phylloxera. We recommend **future research is directed at determining what is driving the absence of individuals inside some galls** as this information could be used to develop management strategies that help growers reduce populations of leaf galling phylloxera.

There was insufficient variation in the irrigation practices, row spacing, vine spacing, and the cardinal direction of the vines between vineyards to statistically determine whether these vineyard practices contribute to the abundance of leaf galling phylloxera. However, two rows of ornamental vines that were planted at commercial vineyards were also sampled and these rows differed from commercial vines in both their vine spacing and irrigation practices Specifically, ornamental vines were planted further apart and unlike commercial vines were not drip irrigated. Despite these differences in vineyard practices, there was no significant difference in the number of galls on leaves between ornamental vines and any of the commercial varieties sampled noting that the sample size is relatively small (n = 4 ornamental vines sampled). As commercial vineyard practices are mostly consistent between vineyards, it may be difficult to determine whether they impact the abundance of leaf galling phylloxera in a field setting. **Empirical laboratory studies are likely needed to determine whether vineyard practices influence the abundance of leaf galling phylloxera.**

Lastly, as this study did not investigate whether the increased prevalence of leaf galling phylloxera has any impact on vine health or productivity we recommend that this is investigated in future research. Although recent international research found the effects of leaf galling phylloxera on vine health to be marginal, this has not been studied in the Australian context.

Key findings and recommendations

Effect of rootstock and grape variety

- We found no significant relationship between the abundance of leaf galling phylloxera and rootstock or grape variety
- Greenhouse rootstock screening assays may be more beneficial to determining whether there is any relationship between rootstock or grape variety and the abundance of leaf galling phylloxera to account of variables such as vine age, nutrition and time since initial infestation.

Effect of viticulture practices

- Lack of variation in the viticulture practices of growers prevented an analysis of how these practices may influence the abundance of leaf galling phylloxera
- Laboratory studies are needed to determine how vineyard practices such as spacing or irrigation may influence the abundance of leaf galling phylloxera.

Relationship between the number of galls on leaves and the level of infestations within galls

- We found positive and significant correlations between the level of infestation within galls and the number of galls on leaves.
- Future studies could investigate variations in leaf characteristics that may contribute to their ability to support and/or attract leaf galling phylloxera

Absence of phylloxera within galls

- Approximately 17% of galls dissected did not contain any individuals.
- We recommend future research is directed at determining what is driving the absence of individuals inside some galls as this information could be used to develop management strategies that help growers reduce populations of leaf galling phylloxera.

Introduction

Grapevine phylloxera is the most damaging viticulture pest in Australia. This pest has a complicated life-cycle and exists in either root (radicicolae) or leaf galling (gallicoae) forms (Powell, 2012). Infestation of root galling phylloxera typically leads to vine death (Edwards, 2006). Root galling phylloxera induce nodosities on the roots of vines during feeding, which limits water update by the vine (Clarke et al., 2023) and causes rips and tears in the roots, exposing the root system to soil-borne pathogens (Edwards et al., 2006; Granett et al., 1998). Unlike root galling phylloxera and despite inducing striking galls on the surface of the leaf (Figure 1), leaf galling phylloxera are thought to have little impact on the health of grape vines (Wilmink et al., 2022). As such, few studies have investigated the factors that impact their abundance (but see Wilmink et al., 2021). However, there has been a recent rise in the prevalence of leaf galling phylloxera in vineyards in many parts of the world (Bao et al., 2015; Fahrentrapp et al., 2015), including the North-Eash Phylloxera Infested Zone (PIZ) in Victoria, Australia (personal observation – Catherine Clarke). Further, the phylloxera genotype G38, which was first characterised from inside of leaf galls in the North-East PIZ (Umina et al 2007), has recently been documented developing and reproducing on vine roots (Clarke et al., 2023). Consequently, there has been a heightened interest in leaf galling phylloxera, especially in the factors that may account for the recent increase in abundance. Recent international research suggests that factors such as temperature, rootstock,

and their interactions with phylloxera genotypes, can influence the abundance of leaf galling phylloxera (Wilmink et al., 2021) but this has not been studied in the Australian context,

We investigated whether the abundance of leaf galling phylloxera in infested vineyards is influenced by vineyard practices (such as vine spacing, irrigation, row spacing and the cardinal direction of the vines), grape variety, or rootstock by counting the number of galls on leaves collected from different vineyards in the North-East PIZ. Additionally, galls were dissected to determine whether there was any relationship between the number of galls on leaves and the level of phylloxera infestation within each gall.

Project Objectives

This project aimed to investigate environmental- and rootstock-related factors that may account for the increased prevalence of leaf galling phylloxera in vineyards and the associated risks to industry.

Additionally, genotype mapping of leaf galling phylloxera in the North-East Phylloxera Infested Zone (PIZ) was conducted and is reported below in the following section: *Genetic diversity of leaf galling phylloxera in the King Valley, North-Eastern Victoria*)

Methodology

Field surveys

Leaf galling phylloxera surveys were conducted in 2021, 2022 and 2023 during summer and autumn (Table 4.1). All surveys were carried out in commercial vineyards in the North-East Victoria Phylloxera Infested Zone (PIZ). A vineyard was defined as a single viticultural operation, owned and operated by an individual or company. All vineyards surveyed were planted on soil with over 80 years of grapevine history except for two rows of ornamental vines. The distance between vines, the distance between rows, the cardinal direction, the irrigation practice, grape variety, and rootstock for each vine sampled were recorded. Two rows of ornamental vines that had been established at two vineyards were also sampled.

Table 4.1: Cardinal direction, the distance between rows, the distance between vines, and the irrigation practices of vineyards studied. There is insufficient variation in these practices to perform a robust or meaningful analysis to determine if these factors influence the abundance of leaf galling phylloxera. Samples collected from vineyards marked with asterisks were from single rows of ornamental vines.

Vineyard ID	Cardinal direction	Distance between rows (m)	Distance between vines (m)	Drip irrigated?	Number of vines sampled	Number of leaves sampled	Number of galls dissected
1	NS & EW	2.5–3	1.5	Yes	7	26	131
2	NS	3	1.5	Yes	1	3	15
3	NS	3	1.1	Yes	6	20	100
4	NS	2.5	1.5	Yes	6	23	115
5*	NS	NA	3	No	3	9	45
6*	NS	NA	3	No	1	6	30

Between three and eight leaves with recognisable phylloxera galls (Figure 4.1) were excised from the terminal shoots of vines and placed in zip-lock plastic bags. The plastic bags were placed in an esky with ice packs and then transported to the laboratory where they were stored at 4°C until they could be processed.



Figure 4.1: Image of leaf galls on a vine leaf collected from a vineyard in the King Valley, North-East Phylloxera Infested Zone

Gall dissections

The number of galls on each leaf were recorded. Up to six galls on each leaf were then randomly selected and were then carefully dissected and examined under a 10-x dissecting microscope. The number of phylloxera eggs, nymphs, and adults within each gall was recorded (Figure 4.2). Individuals within the gall were subsequently placed into individual Eppendorf tubes (1.5 ml) with 100% ethanol and stored at 4°C for genotyping (described below in *Genetic diversity of leaf gall grapevine phylloxera in the King Valley, North-Eastern Victoria*

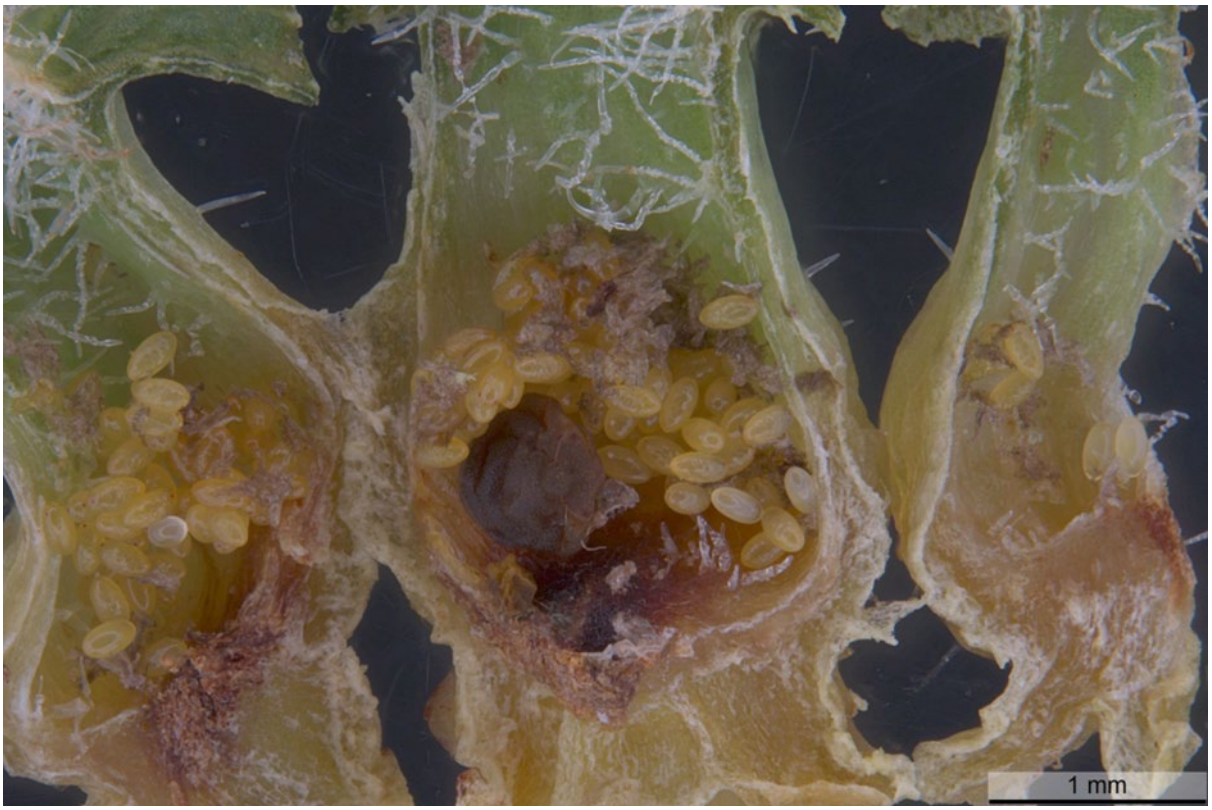


Figure 4.2: Dissected leaf galls containing phylloxera of different life stages.

Statistical analysis

All statistical analyses were performed in JMP (Version 17, SAS institute). To limit pseudo-replication the mean number of eggs, nymphs, adults, and total insects from all galls dissected from each leaf was calculated. We then performed a series of linear regressions to determine if there was any correlation between the level of gall infestation (mean number of eggs, nymphs, adults, or total insects within galls) and the number of galls on the vine leaves. As the mean number of eggs, nymphs, adults, and total insects inside the galls was significantly and positively correlated with the number of galls on vine leaves (see results below), and as the proportion of galls dissected on each leaf varied, we only included the number of galls on vine leaves in our analysis.

To determine whether variation in the number of galls could be explained by rootstock or grape variety we performed two separate restricted maximum likelihood models (REML), which both included leaf ID as a random effect.

There was insufficient variation in the irrigation practices, row spacing, vine spacing, and cardinal direction between vineyards to statistically determine whether these factors contribute to the abundance of leaf galling phylloxera (Table 4.1). Although there were differences in vine spacing and irrigation practices between the ornamental and commercial vines sampled (Table 4.1), the difference in grape variety and rootstock would confound any statistical test. Nonetheless, differences between the ornamental and commercial grape varieties are discussed.

Key Research Findings

Gall dissections

In total, 428 galls were dissected and the individuals of different life-stages inside each gall were recorded. Almost all galls dissected (n = 356) contained individuals from all life-stages. Approximately 17% of galls contained no individuals from any life-stage (n = 72). Many of the galls containing no individuals originated from the same leaves. A subset of these empty galls had a black interior (n = 25) that differed from the galls containing live phylloxera. All galls that were found to have a black interior were collected from a single vineyard and from vines of the same grape variety (Cabernet) and rootstock (Schwarzmann).

Relationship between gall infestations and gall number.

There was a significant positive correlation between the mean number of eggs ($r^2 = 0.31$, F-ratio = 37.86, df = 1, p <0001; Figure 4.3), the mean number of nymphs ($r^2 = 0.22$, F-ratio = 24.26, df = 1, p <0001; Figure 4.4), the mean number of adults ($r^2 = 0.18$, F-ratio = 18.21, df = 1, p <0001; Figure 4.5) and the mean total number of insects ($r^2 = 0.34$, F-ratio = 46.63, df = 1, p <0001; Figure 4.6) and the number of galls on vine leaves.

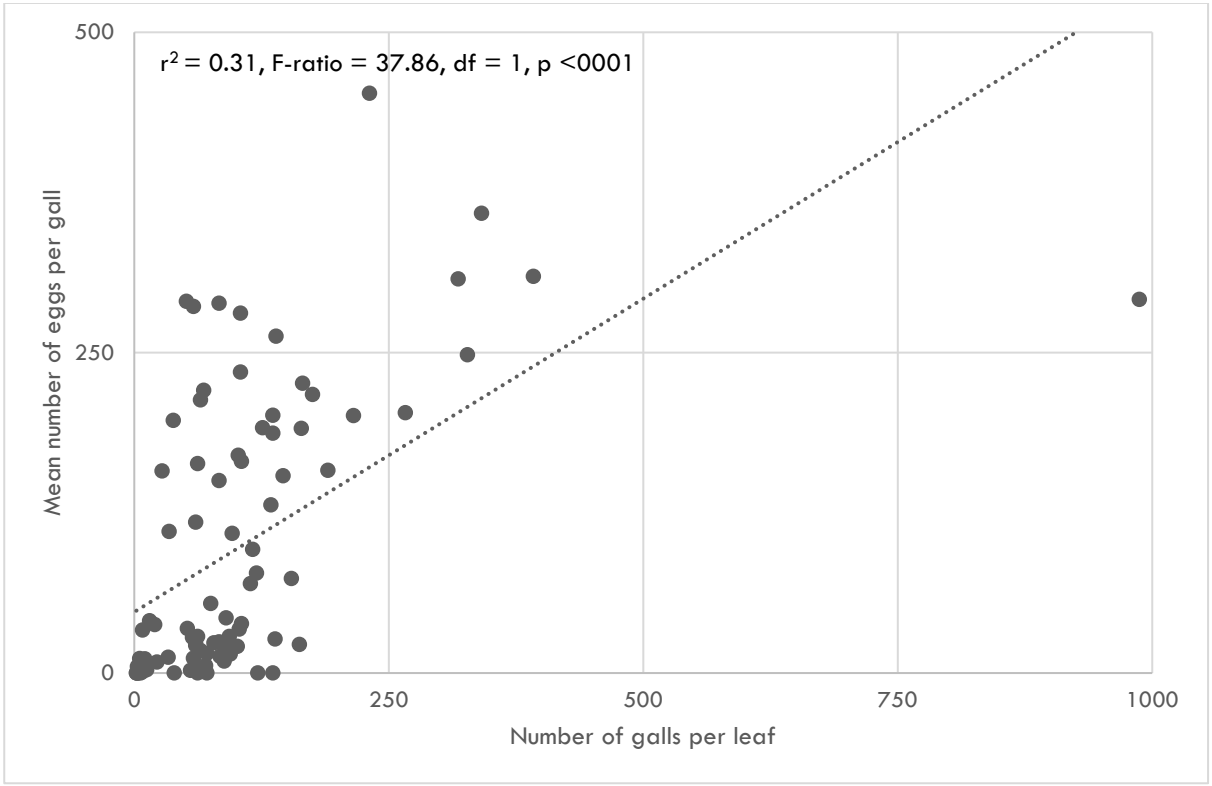


Figure 4.3: There was a significant and positive correlation between the mean number of eggs per gall and the number of galls per leaf.

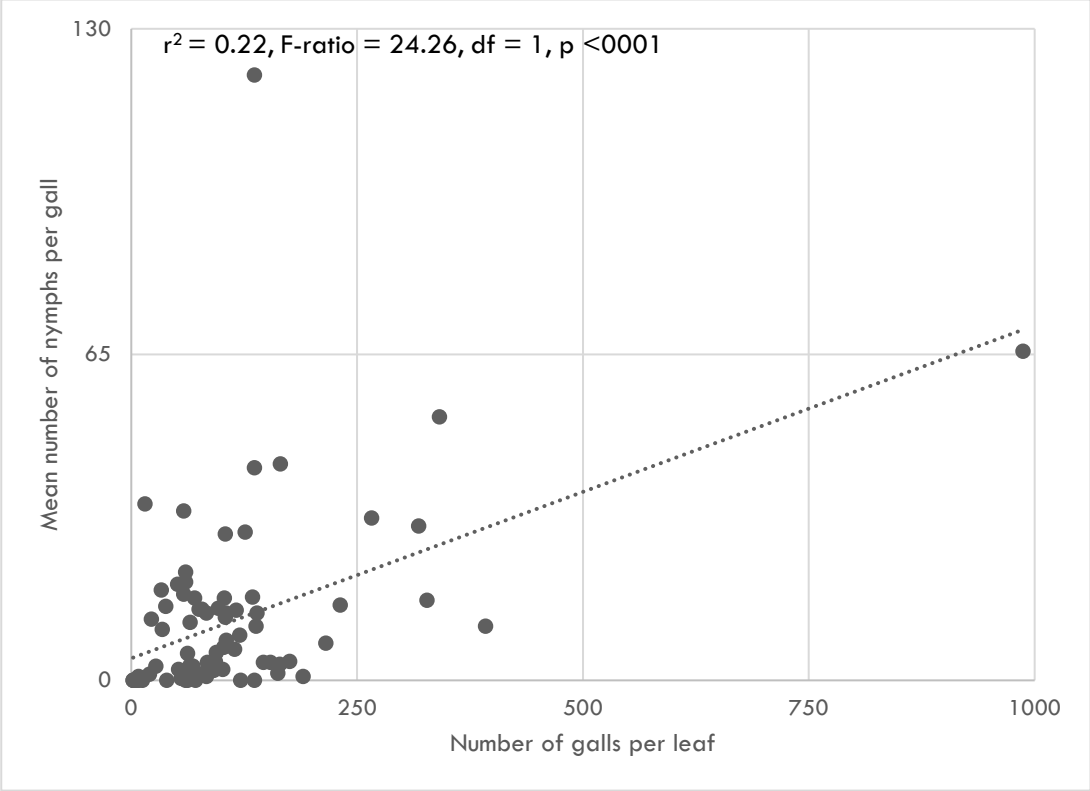


Figure 4.4: There was a significant and positive correlation between the mean number of nymphs per gall and the number of galls per leaf.

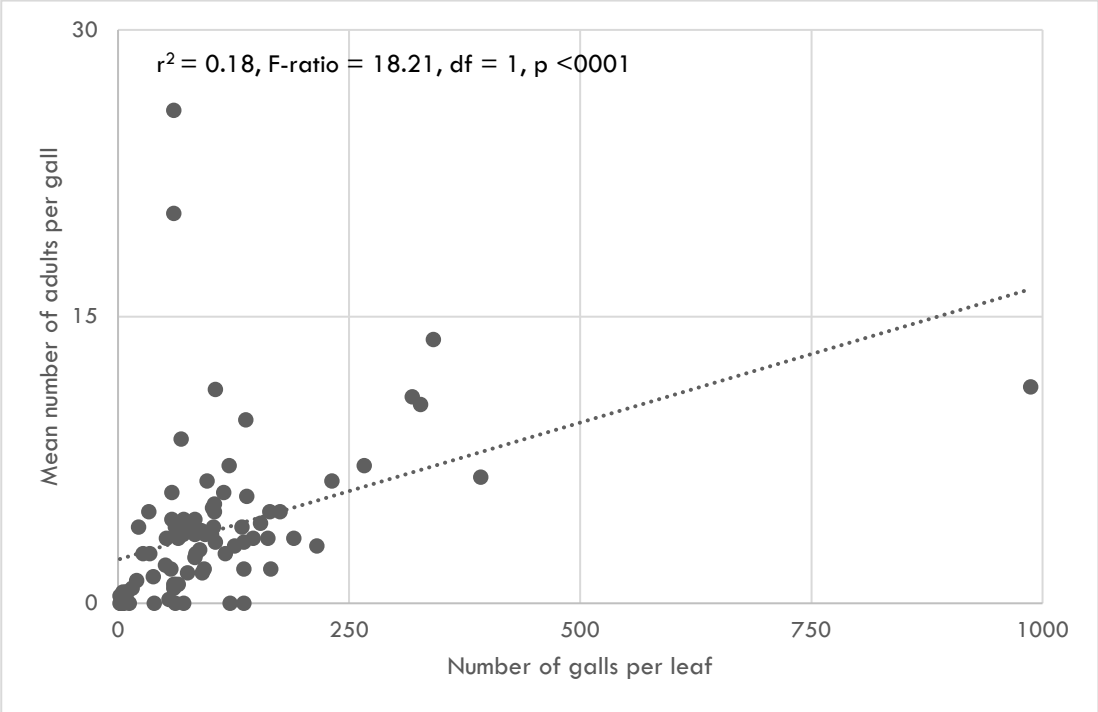


Figure 4.5: There was a significant and positive correlation between the mean number of adults per gall and the number of galls per leaf.

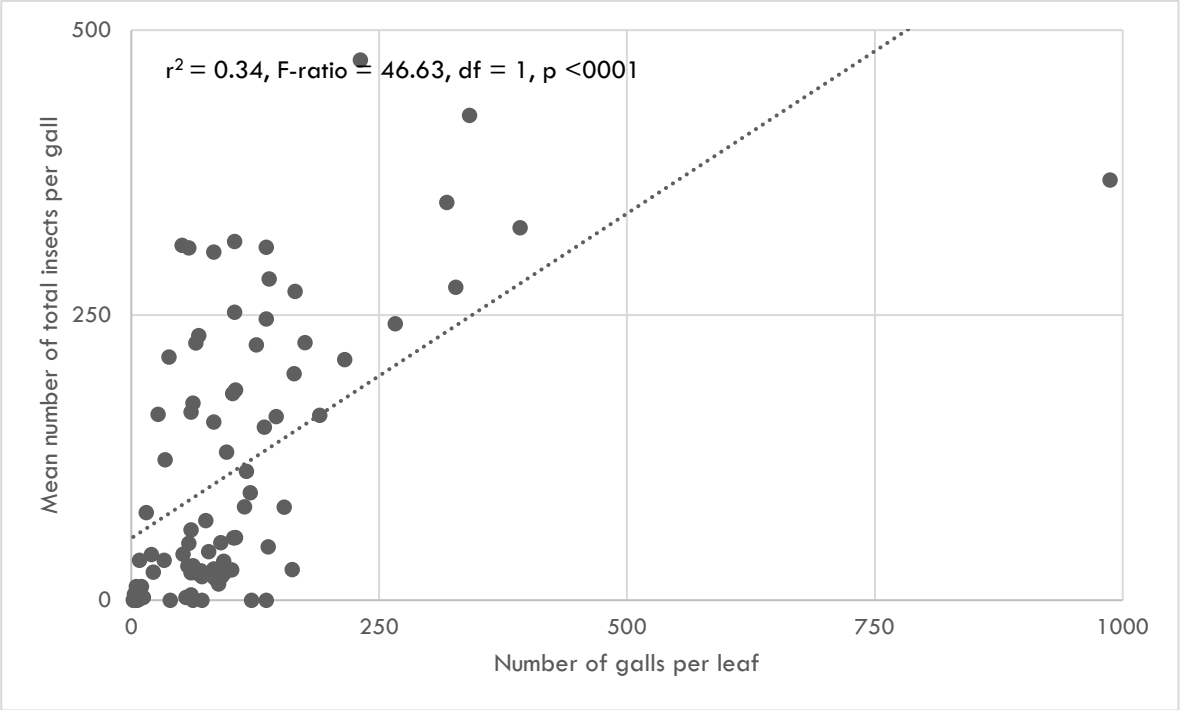


Figure 4.6: There was a significant and positive correlation between the mean number of total insects per gall and the number of galls per leaf.

Effect of rootstock on gall number

Our REML model revealed no significant effect of rootstock on the number of galls on vine leaves (df = 2, F-ratio = 1.38, p = 0.25; Figure 4.7). Leaf ID contributed to 2.05% of the variation in the number of galls on leaves (p =0.57).

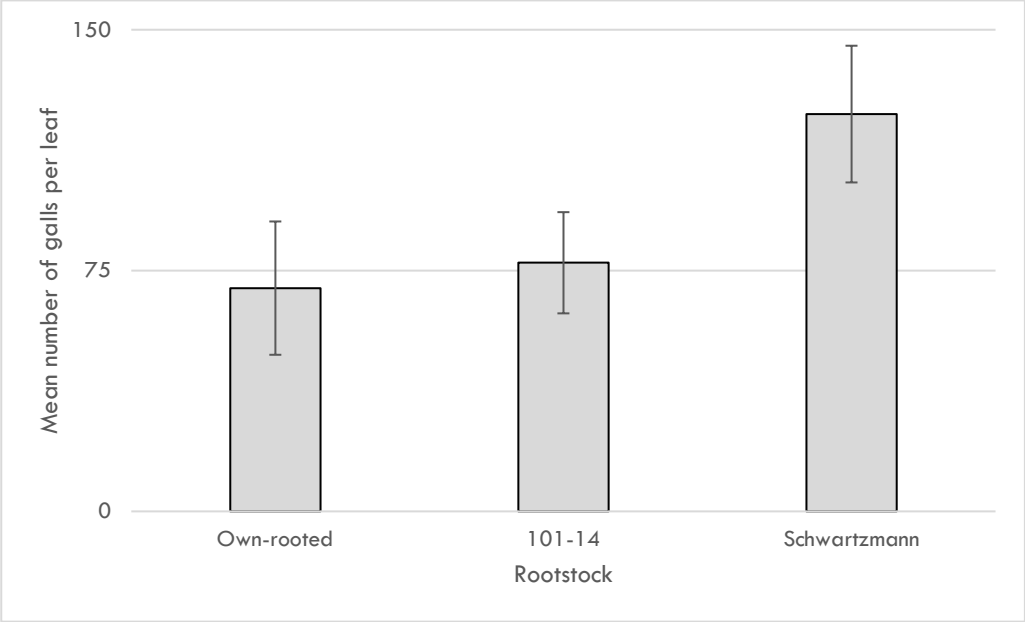


Figure 4.7: Our REML model revealed no significant effect of rootstock on the number of galls (p = 0.25)

Effect of grape variety on gall number

Our REML models revealed no significant effect of grape variety on the number of galls on vine leaves (df = 3, F-ratio = 0.57, p = 0.63; Figure 4.8). Leaf ID contributed to 2.43% of the variation in the number of galls of leaves (p = 0.56).

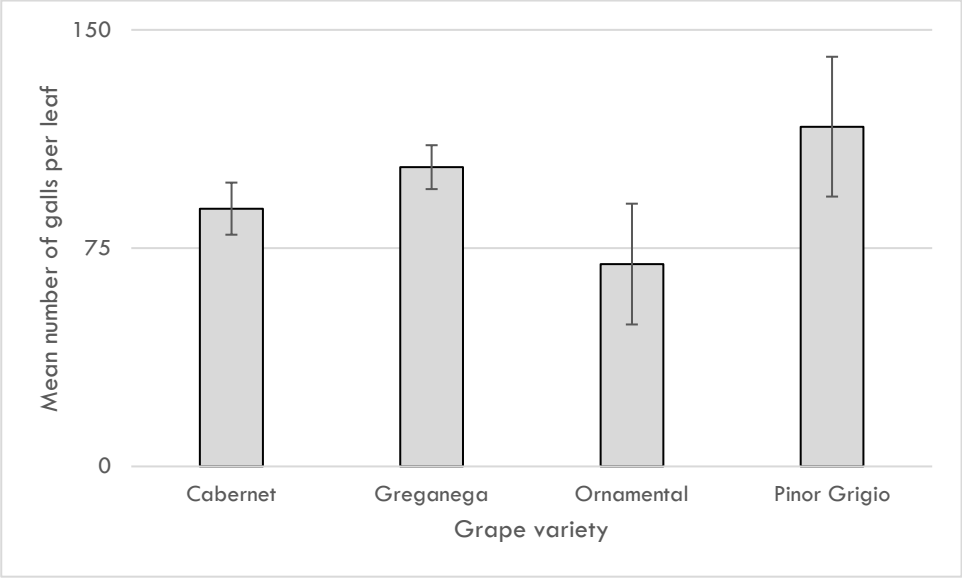


Figure 4.8: Our REML model revealed no significant effect of grape variety on the number of galls (p = 0.63)

Effect of vineyard practices on leaf galling phylloxera

There was insufficient variation in the irrigation practices, row spacing, vine spacing and cardinal direction between samples collected from commercial vine varieties to statistically determine whether these factors contribute to the abundance of leaf galling phylloxera (Table 4.1).

Vineyard practices did differ between commercial varieties and the two rows of ornamental vines sampled. Commercial vines were planted closer together (between 1.1 – 1.5m) compared to ornamental vines (3m). Commercial varieties were also drip irrigated, while ornamental vines were not. Despite these differences in vineyard practices, there was no significant difference in the number of galls on leaves between ornamental vines and any of the commercial grape varieties (Figure 4.8) sampled.

Discussion and Recommendations

Our data suggests that rootstock and grape variety have little impact on the abundance of leaf galls in the vineyards surveyed. Recent international research has demonstrated variation in the ability of leaf galling phylloxera to establish on different rootstocks (Wilmink et al., 2021). Importantly, this research was conducted in a controlled laboratory setting, whereas the present study was conducted in commercial vineyards where unaccounted-for variables (such as the number of founding phylloxera, time since initial infestation, and the age of the vines) may be masking differences among grape varieties or rootstocks. As such, we recommend **rootstock screening assays are used to determine the susceptibility of novel rootstocks to prevalent leaf galling phylloxera genotypes particularly those that exist in both leaf galling and root galling forms such as G38.**

Our analysis revealed statistically significant positive correlations between the level of phylloxera infestation within galls and the number of galls on leaves. Leaves with a higher number of galls might have been

infested for a greater length of time, but this needs to be studied. Galls on such leaves may contain individuals from multiple generations who either develop and reproduce inside their original gall or leave and form new galls on the same leaf. Microsatellite analysis of phylloxera collected from different galls on the same leaf revealed that different genotypes do coinhabit the same leaf and even the same gall (see *Genetic Diversity of Leaf Gall Grapevine Phylloxera in the King Valley, North-Eastern Victoria phylloxera* below for details). Assuming, asexual reproduction, these individuals could not have originated from the same gall. Alternatively, differences in leaf-characteristics may contribute to variation in either i) the ability of the leaf to support larger populations of leaf galling phylloxera or ii) the attractiveness of the leaf to the mobile life-stages of phylloxera (crawlers). This knowledge could be used to help select for vine characteristics that are less susceptible to leaf galling genotypes. We recommend that **future studies investigate variations in leaf-characteristics that may contribute to their ability to support and/or attract leaf galling phylloxera.**

Interestingly, approximately 17% of the galls dissected contained no individuals. Many of these galls originated from the same leaf and a subset of these had a black interior. Whether the absence of individuals within these galls is due to plant defences against phylloxera or predation from natural enemies (e.g., general predators, viruses, fungi, or other microbes) is unknown. Galls with a black interior were collected from a single vineyard, and it is possible that a natural predator of phylloxera was present in this vineyard, but not others. Regardless, understanding what is driving the absence of individuals in some galls could help growers reduce populations of leaf galling phylloxera. We recommend **future research is directed at determining what is driving the absence of individuals inside some galls**.

Although the lack of variation in vineyard practices prevented a statistical analysis of their impact on the abundance of leaf galling phylloxera, the irrigation practices and vine spacing did differ between the two rows of ornamental vines and the commercial vines samples. There was no significant difference between the number of leaf galls between grape varieties, suggesting that vine spacing, and irrigation practices are unlikely to contribute to the abundance of leaf galling phylloxera in vineyards. The mobile life-stages of grapevine phylloxera can disperse up to 100m in a year (King and Buchanan, 1986), and the vines sampled during these surveys were between 1.1 and 3m apart. As such, natural dispersal of phylloxera between all vines surveyed is possible. Given the consistency in vineyard practices (i.e. row and vine spacing and irrigation), it may be difficult to determine whether these practices impact the abundance of leaf galling phylloxera in a field setting. **Empirical laboratory studies are likely needed to determine how vineyard practices may influence the abundance of leaf galling phylloxera.**

Lastly, as the impacts of leaf galling phylloxera on vine health have not been studied in the Australian context and have received little attention internationally (but see Wilmink, et al 2022), **we recommend the impacts of leaf galling phylloxera on vine health is investigated.** Whether the increased prevalence of leaf galling phylloxera impacts the health and/or productivity in Australian vineyards is unknown. This knowledge is needed to determine the extent to which resources are directed at managing the leaf galling forms of phylloxera.

Conclusion

Our results suggest that rootstock and grape variety have little if any impact on the abundance of leaf galling phylloxera in the North-East PIZ. This finding is inconsistent with international research (Wilmink et al., 2021).

We therefore recommend greenhouse rootstock screening assays are used to determine the susceptibility of novel rootstocks to prevalent leaf galling phylloxera genotypes.

The lack of variation in the viticulture practices (i.e. vine and row spacing and irrigation) of the vineyards prevented a statistical analysis of how these practices may influence the abundance of leaf galling phylloxera.

Empirical laboratory studies are needed to determine how vineyard practices such as spacing or irrigation may influence the abundance of leaf galling phylloxera.

We found positive and significant correlations between the level of infestation within galls and the number of galls on leaves. Whether this is due to the length of time the leaves had been infested or whether specific leaf-characteristics are better able to support or attract leaf galling phylloxera requires further study.

Future studies could investigate variations in leaf characteristics that may contribute to their ability to support and/or attract leaf galling phylloxera as this may help select for vine characteristics that are less susceptible to leaf galling genotypes.

Approximately 17% of galls dissected did not contain any individuals.

We recommend future research is directed at determining what is driving the absence of individuals inside some galls as this information could be used to develop management strategies that help growers reduce populations of leaf galling phylloxera.

The prevalence of leaf galling phylloxera in commercial vineyards is increasing globally, and the emergence of new leaf galling genotypes in Australia (presented below: *Genetic diversity of leaf gall grapevine phylloxera in the King Valley, North-Eastern Victoria*) means **we need a better understanding of the impacts of leaf galling phylloxera on vine health and productivity in the Australian context.**

Management tools that target leaf galling phylloxera are also needed, to support growers currently managing vineyards with leaf galling phylloxera infestations, and to future-proof vineyards against changes in the behaviour of known Australian genotypes.

Genetic Diversity of Leaf Gall Grapevine Phylloxera in the King Valley, North-Eastern Victoria

Executive summary

A recent Agriculture Victoria Research study detected 36 genotypes from phylloxera collected from roots in the King Valley (DED1701 2020), with observations noting an increase of leaf galling grape phylloxera from the same area. The current study aimed to survey and genotype leaf galling grape phylloxera from the King Valley to compare with this previous root phylloxera study. This new survey revealed a total of 67 phylloxera genotypes in leaf galls from the King Valley, with 41 new and 26 already known from previous work. Matching genotypes were found in some samples from roots and leaves collected from the same vineyard/area across the two studies, and also from the same vine when simultaneously sampled in the current study. However, a higher-than-expected genetic diversity of grape phylloxera present on leaves revealed that some particular genotypes are able to live on both roots and leaves, while others appear to be restricted to either, with this pattern linked to genotypic diversity present at sites, surveillance effort and level of vineyard infestation. Importantly, more than one genotype was found across most vineyards, plants, leaves and even within single galls. These results highlight the importance of conducting localised surveillance to update the genotype distribution in a vineyard. This in turn will assist growers with selecting the appropriate rootstock based on the local grape phylloxera genotype populations.

Key findings and recommendations

Leaf gall grapevine phylloxera genotypes

- Across all vineyards surveyed, 67 phylloxera genotypes were characterised from inside leaf galls
- 41 of these genotypes had not previously been characterised
- Phylloxera of different genotypes were characterised on the same leaf and even within the same gall
- Local surveillance to update the genotype distribution plays a critical role in supporting the replanting efforts of growers

Leaf galls on ornamental vines

- Leaf galls were recorded on both commercial vines and ornamental vines
- Future research could investigate whether ornamental vines present a risk to the spread of phylloxera outside of the current quarantine boundaries

Introduction

The previous survey of the King Valley was conducted during 2018-2019 on phylloxera collected from roots (DED1701 2020). This survey detected 36 distinct genotypes in total, with 32 newly characterised and 4 already known from previous work (Corrie et al. 2002). The current study focused specifically on obtaining genotypes from leaf galling phylloxera to complement the root samples obtained during the previous survey.

The aim of the current study was to examine the genetic diversity in the same region through collection of grape phylloxera specimens from grapevine leaves from multiple King Valley vineyards, and use these results to update the known distribution of genetic variation of grape phylloxera in the King Valley, the Alpine Valley and NE PIZ vineyards. This will better inform targeted surveillance approaches (based on genotype presence), and post-incursion management decisions — such as the selection of genotype-specific grape phylloxera-resistant rootstocks across this and other PIZs.

Genotypes in the current study were identified using DNA microsatellites, which are still currently the only method available for identification of grape phylloxera genotypes in Australia (Corrie et al. 2002, Corrie et al. 2003, Corrie et al. 2004, Hoffmann & Corrie 2003, Umina et al. 2007, Agarwal et al. 2020).

Project objectives

The aim of this project was to genotype 600 x leaf galling grape phylloxera (approx.) from the King Valley and the surrounding North-East Grape phylloxera Infested Zone.

Methodology

Vineyard selection

Four vineyards, located in the King Valley (CHE-2, CHE-1, HAN-1, WHF-1), the Alpine Valley (WHO-1) and in the North East (NE) Victoria PIZ (RUT-1), were selected based on recent infestations, previous reports (DED1701 2020) and grower contacts sourced from networks in the wine industry (Table 4.2).

Prior to sampling, growers were contacted by phone for a brief overview of the vineyard operations. A vineyard was defined as a single viticultural operation, owned and operated by an individual company or person. Samples were collected in 2022, 2023 and 2024 between January and March when grape phylloxera leaf gall populations are relatively high (Table 4.2).

Table 4.2: Localities and vineyards for 2022-2024 leaf gall samples.

Region	Locality	Vineyard name	Block (vine cultivar)	Date of sample collection
King Valley	Cheshunt	CHE-1	Cabernet	11/03/2022
			Garganega	11/03/2022
			Cabernet	15/02/2024
		CHE-2	Creek (Chardonnay)	16/02/2022
			Pinot Grigio	16/02/2022
	Hansonville	HAN-1	Ornamental	21/01/2022
	Whitfield	WHF-1	C1 (Pinot Grigio)	24/01/2022
			F1	24/01/2022
North-East Victoria PIZ	Rutherglen	RUT-1	S (ornamental)	21/02/2023
			N (ornamental)	21/02/2023
Alpine Valley	Whorouly	WHO-1	Shed (unknown)	23/03/2022

Sample collection

At each vineyard (Figure 4.9), we surveyed vines for the presence of leaf galls by walking up rows identified by growers as having symptomatic vines. When an infested vine was identified, we collected between three and eight leaves with recognisable phylloxera galls from the terminal shoots of vines and placed them in zip-lock plastic bags. The plastic bags were placed in an esky with ice packs and then transported to the

laboratory where they were stored at 4°C until they could be processed at either Rutherglen or AgriBio centres.

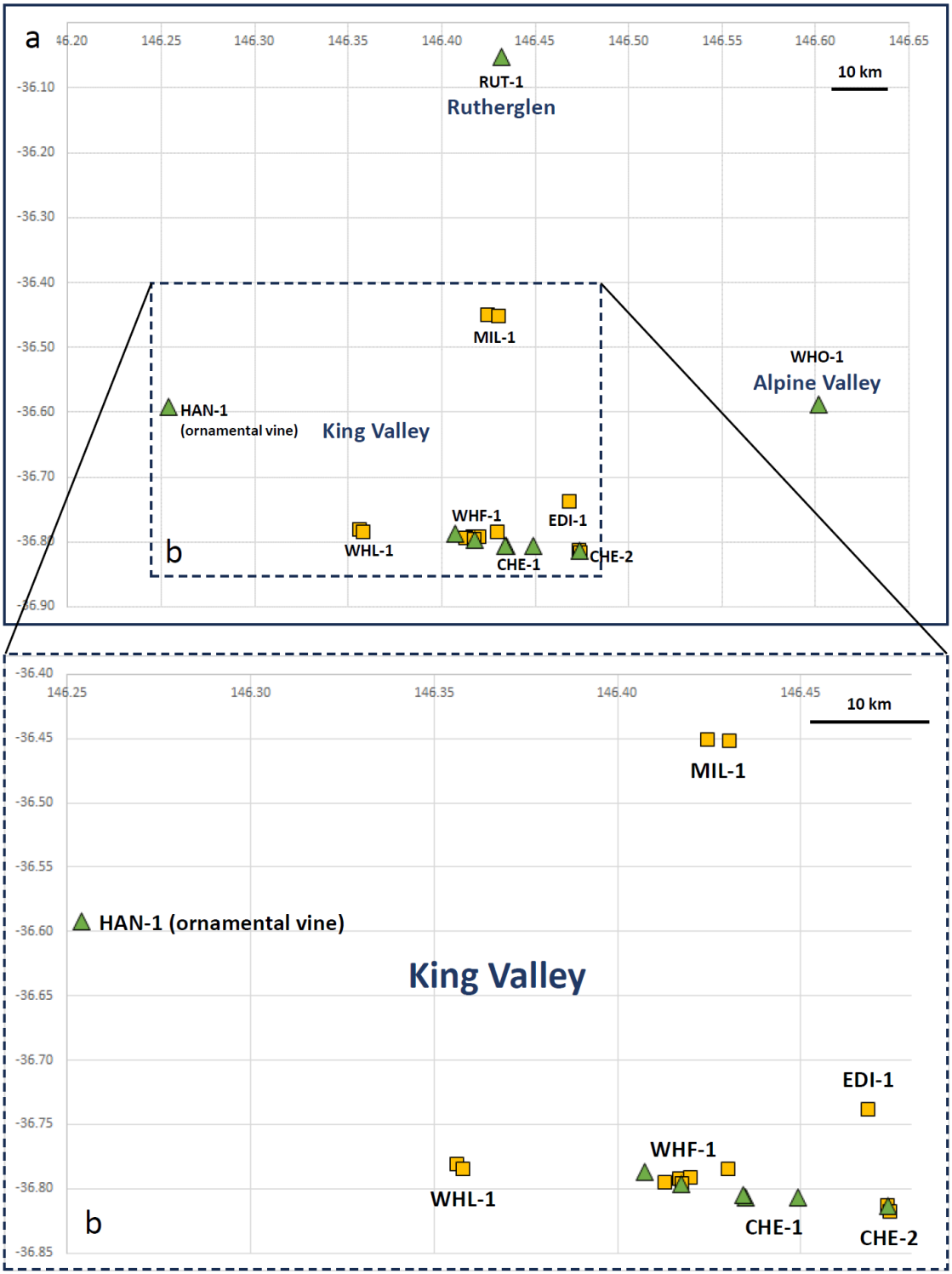


Figure 4.9: Collection localities of leaf gall phylloxera (this study, green triangles) and previous root survey (DED1701 2020, yellow squares) samples from the King Valley (b, inset) and surrounding area (a).

Once at the Rutherglen or Agribio centres, galls were opened with a scalpel and all stages of grape phylloxera extracted from the gall (Figure 4.10), placed in a tube containing absolute ethanol and kept at -20oC for long-term preservation. In year 1 and 2, a single individual grape phylloxera from each gall was preserved for DNA extraction and microsatellite analyses. In order to determine if more than one genotype could coincide within a single gall, in year 3 i.e., 2024, we collected 3 individuals per gall for microsatellite analyses.

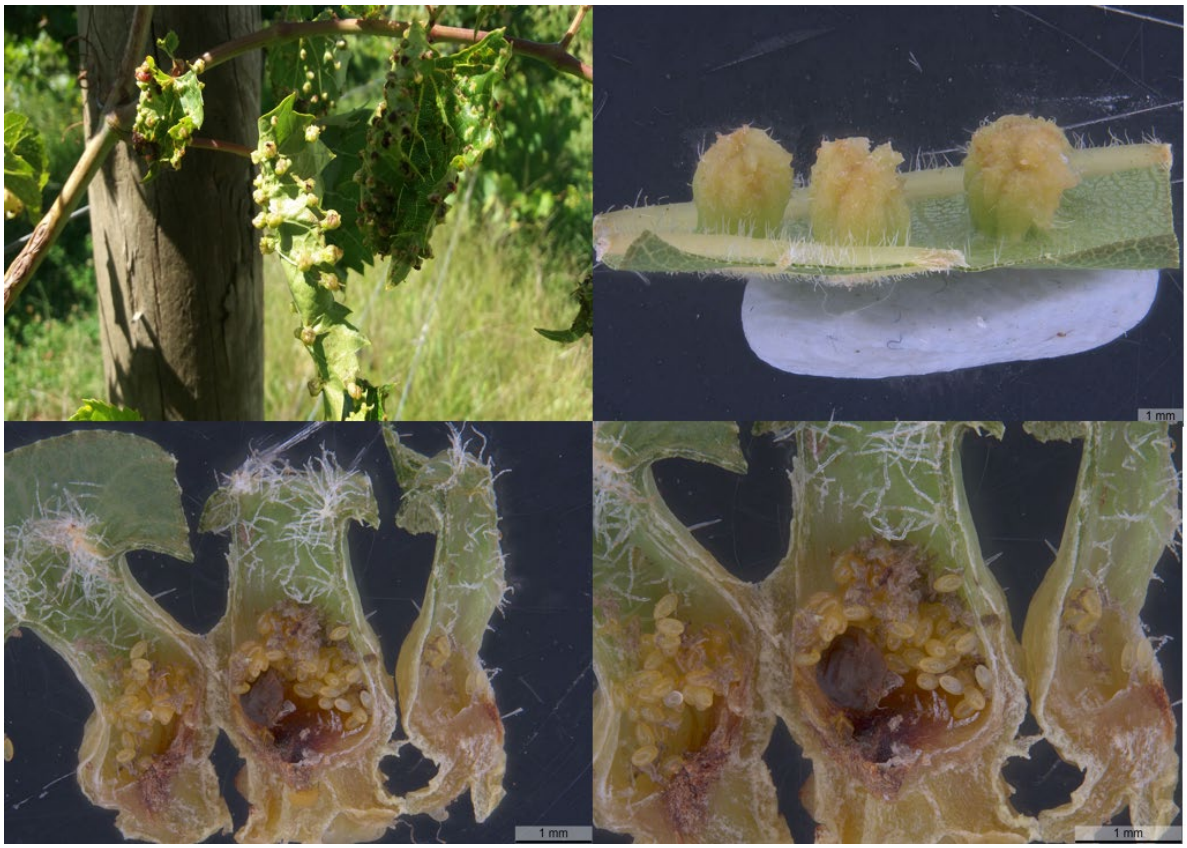


Figure 4.10: Grapevine with leaf galls (upper) and leaf gall dissection (lower). Source: Agriculture Victoria.

For most sites and years, the sampling from each vineyard followed Table 4.3. The number of individual grape phylloxera tested per vine was generally 15 individuals (i.e., 3 x leaves/vine; 5 x galls/leaf; 1 x individual grape phylloxera/gall). Some root samples were also taken simultaneously to the leaf gall samples in some vineyards (Table 4.3).

Table 4.3: Number of phylloxera genotyped per site.

Sites	Number of phylloxera genotyped
General strategy per vineyard / time	**3 x leaves per vine**
	5 x galls per leaf
	1 x individual per gall
2022 Whorouly, WHO-1 vineyard,	**1 x vine**
'Shed' block,	**3 x leaves per vine**
leaf and roots sampled simultaneously.	**5 x galls per leaf**
	1 x individual per gall
	1 x individual from the roots from the same vine
2022 Cheshunt, CHE-1 vineyard,	**3 x vines**
Cabernet block,	**3 x leaves per vine**
leaf and roots sampled simultaneously.	**5 x galls per leaf**
	1 x individual per gall
	1 x individual from the roots from the same vine
2024 Cheshunt, CHE-1 vineyard,	**3 x vines**
Cabernet block,	**Up to 3 x leaves per vine**
leaf and roots sampled simultaneously.	**Up to 6 x galls per leaf**
	Up to 3 x individuals per gall
	Up to 7 x individuals from the roots from the same vine

Microsatellite Genotyping and Analysis

Microsatellite genotyping was performed following a laboratory protocol (Blacket et al. unpublished) that screens the six grape 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. Genotyping of fsa files was performed using the microsatellite plugin in Geneious R11 (https://www.geneious.com).

Key research findings

Overall sampling and genotypes detected

A total of 37 vines were sampled with 530 galls from 112 leaves inspected (Table 4.4). A total of 625 grape phylloxera were genotyped for six microsatellites (as above). Of the 625 samples tested, 112 did not yield a successful (i.e., complete genotype) result, so were excluded from analyses. Thus, the final data analysed was 513 (497 from leaves and 16 from roots; Table 4.4).

Table 4.4. Vineyards, vines, leaves and galls sampled in 2022-2024.

Region	Locality	Vineyard	No. vines	No. leaves	No. galls	No. grape phylloxera from leaf galls	No. grape phylloxera from roots	Total no. grape phylloxera genotyped	Total no. grape phylloxera used in analysis
King Valley	Cheshunt	CHE-1	20	59	293	367	20	387	285
		CHE-2	6	18	79	79	0	79	71
	Hansonville	HAN-1	3	6	30	30	0	30	30
	Whitfield	WHF-1	6	17	75	75	0	75	74
North East Victoria PIZ	Rutherglen	RUT-1	3	9	37	37	0	37	36
Alpine Valley	Whorouly	WHO-1	1	3	16	16	1	17	17
			39	**112**	**530**	**604**	**21**	**625**	**513**

Overall, we detected a total of 76 genotypes, with 27 genotypes known from previous work (G20 to G115) and 49 new genotypes (G116 to G166). In the leaf gall category, the most abundant genotype found was G94 (7.4% of all samples), followed by G95 (6%), G96 (5.6%) and G113 (5%). The remaining genotypes represented less than 5% of the samples (Appendix 6, Table 1). In the root category, the most abundant genotype was G95 (37.5%), followed by G20 (19%), G88 and G111 (12.5%). The remaining genotypes G38, G160 and G115 represented 6.3%.

The full list of genotypes and their frequencies by tissue type (i.e., leaf galls/roots) is provided in Appendix 6, Table 1.

Diversity within a vineyard

Distinct count of genotypes

Both high and low levels of genetic diversity were observed per vineyard (Table 4.5). The vineyard with the highest observed genetic diversity i.e., highest number of distinct genotypes was the CHE-1 vineyard with 53 distinct genotypes (31 new and 22 ‘old’) on leaf galls and 6 distinct genotypes (1 new and 5 ‘old’) on roots. All 6 ‘root’ genotypes were also found on the leaves.

However, the genetic diversity of leaf galls expressed as a function of sample size (which is related to level of vineyard infestation) in the CHE-1 vineyard has a ratio of 0.20 (20%), revealing that CHE-1 actually has an equally high level of genetic diversity as HAN-1, CHE-2 and RUT-1 vineyards (all around 20%), followed by WHF-1 (11%) and WHO-1 (6%), (Table 4.5).

Table 4.5: Distinct count of genotypes and ratios by vineyard.

Tissue	Vineyard	Distinct Count of GENOTYPES	Sample size	Ratio (genotypes/sample size)
Leaf	HAN-1	6	30	0.20
	WHO-1	1	16	0.06
	CHE-2	14	71	0.20
	RUT-1	7	36	0.19
	WHF-1	8	74	0.11
	CHE-1	53	270	0.20
Root	WHO-1	1	1	1.00
	CHE-1	6	15	0.40

Genotypes Vineyard 1: HAN-1

Six genotypes (Table 4.6) were detected on the ornamental vine sampled at HAN-1 (6 x leaves, 5 x galls/leaf, 1 x phylloxera/gall). Some leaves (Appendix 7, Figure 1) showed a high level of genetic diversity with up to 3 different genotypes detected in a single leaf e.g., leaf#3, while others had one genotype only e.g., G131 in leaf#5. Some genotypes were found in more than one leaf e.g., G130 found in 4 x leaves while others were found in one leaf only e.g., G93 found only in leaf#4.

The genotype G129 was also detected in CHE-1 vineyard. The remaining 5 x genotypes G93, G130, G131, G132, G133 were found nowhere else (Appendix 6, Table 2).

Table 4.6: HAN-1 samples genotypes and frequency

Genotype	Total no. of grape-phylloxera
G129	5
G130	9
G131	7
G132	5
G133	1
G93	3

Genotypes Vineyard 2: WHO-1

1 x genotype was detected on the one vine sampled at WHO-1 (3 x leaves, 5 x galls/leaf, 1 x phylloxera/gall plus one root sample) (Table 4.7). All 3 x leaves sampled had the same genotype G153. Simultaneous collections from the roots (1 x root phylloxera sample) showed a different genotype G38 (Appendix 7, Figure 2). These genotypes were found nowhere else (Appendix 6, Table 2).

Table 4.7: WHO-1 samples genotypes and frequency.

Genotype	Tissue	Total no. of grape-phylloxera
G153	leaf galls	16
G38	roots	1

Genotypes Vineyard 3: CHE-1

Fifty-three genotypes were detected in CHE-1 (various sampling strategies and years), (Table 4.8). G95 was the most abundant genotype. Some vines showed a high level of genetic diversity with up to 8 different genotypes detected in one vine e.g., year: 2022, Cabernet block, Row 1, Vine #12 (Appendix 7, Figure 6A), while other vines had one genotype only, year: 2022, Garganega block, Row 3, Vine #15 (Appendix 7, Figure 6B). Genotype results for each block sampled per year are shown in Appendix 7, Figures 6A to 6E.

The genotypes G20, G92, G94, G95, G96, G97, G112, G118, G128, G129, G138 and G140 were also detected in WHF-1, CHE-2 and HAN-1 vineyards (Appendix 6, Table 2).

Table 4.8: CHE-1 samples genotypes and frequency.

Genotype	Total no. of grape-phylloxera	Genotype	Total no. of grape-phylloxera
G101	2	G140	3
G102	3	G145	1
G103	1	G146	1
G104	5	G155	4
G107	7	G156	3
G108	11	G157	1
G110	2	G158	2
G111	5	G159	1
G112	7	G160	23
G113	1	G161	1
G115	18	G162	9
G116	6	G163	1
G117	3	G164	9
G118	2	G165	3
G119	3	G166	2
G120	2	G20	1
G121	1	G84	7
G122	3	G88	17
G123	1	G90	1
G124	1	G91	1
G125	4	G92	3
G126	1	G94	10
G127	1	G95	28
G128	4	G96	6
G129	11	G98	7
G137	1	G99	15
G138	4		

In 2022 and 2024, roots were simultaneously sampled along with leaf galls. The 2022 data, which included one root sample/vine, showed in some cases different genotypes in the roots e.g., Cabernet block, Row 1, Vine #12 (Appendix 7, Figure 6A), and in other cases the same genotypes in the roots and leaves e.g., Cabernet block, Row 2, Vine #26 (Appendix 7, Figure 6A). The 2024 data included more root samples and showed same genotypes in both rots and leaves (Appendix 7 Figures, 7A, 7B, 7C).

In addition, in 2024, up to 3 x grape phylloxera/gall were examined for microsatellites, with data showing high genetic diversity within a gall with up to 3x distinct genotypes detected (Appendix 7, Figures, 7A, 7B, 7C).

Genotypes Vineyard 4: CHE-2

14 x genotypes were detected at CHE-2 (6 x vines, 3 x leaves/vine, 5 x galls/leaf, 1 x phylloxera/gall), (Table 4.9). Some vines showed a high level of genetic diversity with up to 6 different genotypes detected e.g., vine

#4 in row 3 (Appendix 7, Figure 3A), while other vines had one genotype only e.g., vine#26 in row 4 (Appendix 7, Figure 3B).

The genotypes G94, G95, G97, G112, G118, G138, G140 were also detected in CHE-1 vineyard and the genotype G96 in CHE-1 and WHF-1 vineyards (Appendix 6, Table 2).

Table 4.9: CHE-2 samples genotypes and frequency.

Genotype	Total no. of grape-phylloxera
G112	2
G114	3
G118	2
G138	4
G139	1
G140	2
G141	4
G142	1
G143	5
G144	1
G94	27
G95	2
G96	14
G97	3

Genotypes Vineyard 5: RUT

Seven genotypes were detected on the 3x vines sampled in RUT-1 (3 x vines, 3 x leaves/vine, 5 x galls/leaf, 1 x phylloxera/gall), (Table 4.10). Out of 9 x leaves sampled, only 2 x had mixed genotypes with one leaf having 4 x genotypes, while the remainder leaves had one genotype only (G147) which was the most abundant genotype found in this vineyard (Appendix 7, Figure 4).

All 7 x genotypes were found nowhere else (Appendix 6, Table 2).

Table 4.10: RUT-1 samples genotypes and frequency.

Genotype	Total no. of grape-phylloxera
G147	22
G148	8
G149	1
G150	1
G151	1
G152	2
G154	1

Genotypes Vineyard 6: WHF

8 x genotypes were detected in WHF-1 (6 x vines, 3 x leaves/vine, 5 x galls/leaf, 1 x phylloxera/gall), (Table 4.11). Some vines showed a high level of genetic diversity with up to 4 different genotypes detected e.g., Vine #4, row 64 (Appendix 7, Figure 5A), while the Vine #3, row 64 had one genotype only G113, the most abundant genotype in this vineyard (Appendix 7, Figure 5A). This was also observed in the F1 block (Appendix 7, Figure 5B).

The genotypes G20, G92 and G128 were also detected in CHE-1 vineyard while the genotype G96 in CHE-1 and CHE-2 vineyards (Appendix 6, Table 2).

Table 4.11: WHF-1 samples genotypes and frequency.

Genotype	Total no. of grape-phylloxera
G113	24
G128	10
G135	7
G20	7
G34	14
G92	2
G96	8
G97	2

Diversity between vineyards

Most genotypes (n= 64) were detected at a single site (Table 10). However, twelve genotypes were detected at multiple sites (Table 4.12). Six genotypes were detected in both leaves and roots in the current study (Table 4.11).

Table 4.12: Summary of presence of genotypes in leaves and roots, and at single or multiple sites. (Note, complete data is provided in Appendix I, Table 2).

Plant Tissue Type	1 site	2 sites	3 sites
Leaves only	x 59	x 9	x 1
	(G34, G84, G90, G91, G93, G98, G99, G101, G102, G103, G104, G107, G108, G110, G113, G114, G116, G117, G119, G120, G121, G122, G123, G124, G125, G126, G127, G130, G131, G132, G133, G135, G137, G139, G141, G142, G143, G144, G145, G146, G147, G148, G149, G150, G151, G152, G153, G154, G155, G156, G157, G158, G159, G161, G162, G163, G164, G165, G166)	(G112, G118, G128, G129, G138, G140, G92, G94, G97)	(G96)
Leaves and Roots	x 4	x 2	0
	(G111, G115, G160, G88)	(G20, G95)	(-)
Roots only	x 1	0	0
	(G38)	(-)	(-)

Diversity within vine

Between one and fourteen different genotypes were detected from single vines in the current study (Table 4.13). Multiple genotypes within vines were detected at all sites, with the highest diversity observed in the site which was most heavily surveyed due to having a high level of gall infestations (CHE-1), (Table 4.13).

Table 4.13: Summary of the number of genotypes detected from galls per vine at each site. (Note, complete data is presented in Appendix I, Table 3).

	Number of Genotypes Detected per Vine									
Vineyard	1	2	3	4	5	6	7	9	10	14
HAN-1						1				
WHO-1	1									
CHE-2	3				2	1				
RUT-1	2					1				
WHF-1	3	1	1	1						
CHE-1	4	2	2	4	3		1	1	1	2

Diversity within leaf

47% of leaves had more than 1 genotype. An average of 2.1 genotypes were observed within individual leaves, with the number of genotypes ranging from a minimum of 1 to a maximum of 12, with a standard deviation 1.6 (Appendix 6, Table 4).

Diversity within gall

For most sites and years, the number of grape phylloxera processed were 1x sample/gall except for the two following sampling events:

- 2022 Cheshunt, CHE-1, Cabernet block where 2x samples/gall were processed (for one gall only) (Appendix 7, Figure 6A)
- 2024, Cheshunt, CHE-1, Cabernet block where up to 3x samples/gall were processed for up to 6x galls/leaf (Appendix 7, Figure 7A, 7B, 7C).

Grape phylloxera genotype diversity within a gall ranged between 1 and 3 distinct genotypes.

Comparison with 2018-19 root study

One vine from the following vineyard was sampled in the 2018-19 root study and the current leaf gall one, with results showing the same G94 genotype was found in both studies from the roots (2018) and the leaves (2022, figure 3A).

Vineyard CHE-2, Creek block, row 3, vine 1:

- 2018 roots: G94 (2x)
- 2022 leaves (Appendix 7, Figure 3A): G94 (8x), G97 (2x), G118 (1x), G138 (2x), G139 (1x)

Commercial vs ornamental Vitis vinifera

Our study shows that Vitis vinifera used in an ornamental setting is a suitable host for grape phylloxera, at least on the leaves.

In HAN-1 vineyard, 5 distinct genotypes were found on 6 leaves of the ornamental vine (Appendix 7, Figure 1). One genotype from this ornamental vine (G129), detected 5 x times was also found in the CHE-1 vineyard in 2022 Garganega block (Appendix 7, Figure 6B), row 3, vine 68), 11 x times (Appendix 6, Table 2). The remaining 'HAN-1' genotypes were found nowhere else.

In RUT-1 vineyard, 7 distinct genotypes were found on 3 ornamental vines (all unique to this vineyard) (Appendix 6, Table 2).

Discussion and Recommendations

This new survey revealed a total of 67 phylloxera genotypes in leaf galls from the King Valley, with 41 new and 26 already known from previous work. Matching genotypes were found in some samples from roots and leaves collected from the same vineyard/area across the two studies, and also from the same vine when simultaneously sampled in the current study. However, **a higher-than-expected genetic diversity of grape phylloxera present on leaves revealed that some particular genotypes are able to live on both roots and leaves, while others appear to be restricted to either, with this pattern linked to genotypic diversity present at sites, surveillance effort and level of vineyard infestation.**

Whether ornamental vines present a risk to the wine industry should be considered further. Non-commercial gardeners growing vines may be unaware of phylloxera or may not readily recognise symptomatic vines.

Conclusion

Importantly, more than one genotype was found across most vineyards, plants, leaves and even within single galls. **These results highlight the importance of conducting localised surveillance to update the genotype distribution in a vineyard.** This in turn will assist growers with selecting the appropriate rootstock based on the local grape phylloxera genotype populations.

Research Theme 5: A Conservation Biocontrol Approach

A Conservation Biocontrol Approach

Executive Summary

The "Conservation Biological Control of Phylloxera" report explores the potential of biological control strategies for managing grapevine phylloxera, a significant pest in vineyards. Current control methods rely heavily on chemical treatments and phylloxera-resistant rootstocks, with limited exploration of biological alternatives.

Project Objectives:

- Conduct field surveys to identify potential phylloxera predators in Victorian vineyards.
- Assess the efficacy of commercial aphid predators in laboratory settings for potential use in Inundation Biological Control.

Methodology:

Field surveys were conducted in Victorian vineyards, focusing on phylloxera-infested roots and leaves to identify resident predatory arthropods. Laboratory trials tested the predation capabilities of ladybirds (*Harmonia octomaculata*) and green lacewing larvae (*Mallada signatus*) on phylloxera eggs and crawlers.

Key Findings:

Field surveys identified 56 arthropod specimens from 24 families, with predators comprising 38% of the total taxa.

Predators included spiders, ladybeetles, lacewings, ants, and predatory mites, primarily found on grapevine leaves.

Laboratory feeding trials showed ladybird larvae as effective predators, consuming all phylloxera crawlers within an hour. Ladybird adults and lacewing larvae also predated phylloxera eggs, with ladybirds showing higher predation rates.

Discussion and Recommendations:

The study highlighted the potential of both Conservation and Inundation Biological Control strategies. Field surveys cataloged a modest diversity of phylloxera predators, suggesting opportunities for enhancing these populations. Laboratory trials confirmed the predation capacity of commercially available aphid predators, though their practical deployment requires further investigation. Future research should focus on validating predator-prey associations through direct observation or molecular analysis and exploring large-scale application methods for these biocontrol agents.

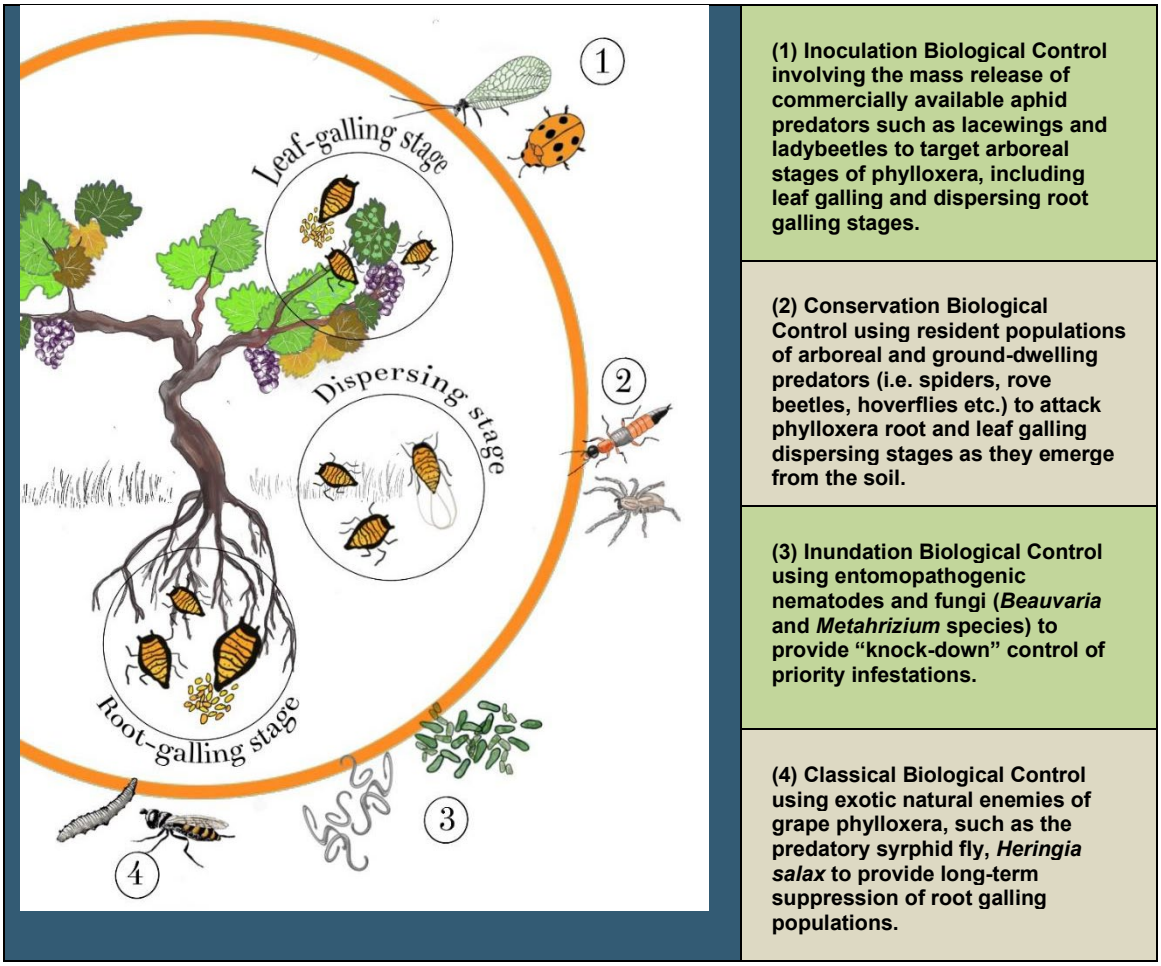
Conclusion:

Biological control presents a promising alternative for sustainable phylloxera management. Enhancing resident predator populations and utilizing commercial aphid predators could assist in reducing the prevalence of dispersing stages of phylloxera. Further research is essential to optimize these strategies and ensure their effectiveness in diverse vineyard conditions.

Introduction

Many successful Integrated Pest Management (IPM) systems are built upon the foundation of effective biological control. However, in the case of grape phylloxera, biological control has been relatively neglected compared to the emphasis on chemical control methods and the development of phylloxera-resistant rootstock. Although there have been some instances of success observed abroad, particularly in laboratory and field trials utilizing entomopathogenic fungi, other avenues of biological control remain largely unexplored.

A literature review conducted for the Wine Australia project, DED 1701 – Integrated management of established grapevine phylloxera, aimed to evaluate the viability of biological control strategies for grape phylloxera in Australia (Kwong, 2020). This review encompassed various approaches, including the release of exotic natural enemies from the pest's native habitat (Classical Biological Control), the introduction of commercially reared predatory insects, mites, or parasitoids (Inoculation Biological Control), the utilization of entomopathogenic organisms like nematodes and fungi (Inundation Biological Control), and the promotion of resident native predators through habitat enhancement (Conservation Biological Control) (see Box 5.1)



Box 5.1: A multi-pronged 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. The different biological control approaches can also be applied depending on the management objective of existing or new infestations. For instance, Inoculation and Inundation methods could be applied where a knock-down approach is required for the rapid suppression of new infestations, while Classical and Conservation Biological Controls could be adopted for the long-term suppression of persistent and widespread infestations.

The review revealed significant potential for further development in each of these approaches. For example, Classical Biological Control, utilizing a predatory syrphid fly (*Heringia salax*), stands out due to its documented effectiveness in targeting both adult and juvenile stages of root galling grape phylloxera in its native environment (Riley, 1874, Walsh and Riley, 1869). Further investigations, including prey-range studies and field surveys in North America, could identify additional natural enemies suitable for release in Australia.

Globally, substantial progress has been made in developing biopesticides for Inundation Biological Control against various soil-dwelling pests, employing entomopathogenic fungi and nematodes. Surprisingly, little research has focused on biopesticides for grape phylloxera, aside from European studies demonstrating the efficacy of the fungal pathogen, Metarhizium (Kirchmair, et al 2007), Initial screening of commercial products against Australian strains of grape phylloxera, along with exploration of indigenous entomopathogenic organisms, presents opportunities for further research (Korosi, 2019).

While over 30 predatory insects and mites that have been identified attacking grape phylloxera worldwide, Australia lacks comprehensive knowledge regarding predation of the pest, despite similar predator

populations resident in Australian vineyard ecosystems (Kwong, 2020, Retallack et al., 2019). Recent studies on Conservation Biological Control in Australian vineyards highlight the potential of native plant species to attract diverse insect predators (Retallack, 2019), potentially offering tailored strategies against grape phylloxera at different life stages and infestation types.

In this study, we sought to determine the potential for Inundation and Conservation Biological Control strategies for grape phylloxera. Firstly, we conducted field surveys in several Victorian vineyards to identify resident populations of predatory arthropods found on phylloxera-infested roots and leaf galls and assessed them for their suitability for Conservation Biological Control. We then conducted laboratory feeding trials using commercially available aphid predators (lacewings and ladybirds) to confirm their efficacy in predating upon phylloxera eggs and therefore their potential for use in Inundation Biological Control.

Project Objectives

The aim of this project was to:

1. Conduct field surveys for phylloxera predators and assess their potential for Conservation Biological Control of phylloxera in Victorian vineyards.
2. Conduct laboratory feeding trials with commercial aphid predators to assess their potential for Inundation Biological Control.

Methodology

Field surveys for phylloxera predators

The survey for potential natural predators for phylloxera in Victorian vineyards was conducted in association with field surveys of leaf galling phylloxera (output c). A total of four sites in the King and Buckland Valleys regions were sampled for leaf galls between January to March 2022. Leaf galling samples were also collected from an ornamental vine in the King Valley. Root samples were collected from two sites in the King Valley and one site at Rutherglen. At each site, around 18 root and 45 leaf samples were collected and microscopically examined. All arthropods found were preserved in 100% ethanol for subsequent identification to family level, and categorised into different functional groups as either predators, herbivores or other (scavengers, fungivores, detrivores).

Laboratory feeding trials with commercial aphid predators

An opportunistic trial was conducted using late-instar larvae of the ladybird, *Harmonia octomaculata* in which one larva was placed into each of two Petri dishes containing ten phylloxera 1st instar crawlers. Each dish was observed under a dissecting microscope at approximately 10-minute intervals for up to one hour.

A replicated laboratory trial was then conducted using adult ladybirds, *H. octomaculata* and second instar green lacewing, *Mallada signatus* larvae to assess consumption rates of phylloxera eggs sourced from a G4 laboratory strain. For each treatment, ten phylloxera eggs were placed onto moistened filter paper in a 90mm x 15 mm size Petri dish. A single predator (either a ladybird adult or lacewing larva) was added to each Petri dish and the lids sealed with parafilm (Figure 5.1). Each treatment was replicated ten times. The eggs were inspected at 1, 2, 4 and 24 hr intervals and the number of uneaten intact eggs and hatched phylloxera crawlers were counted. A one-way analysis of variance was used to compare the mean number of eggs/crawlers remaining between ladybird and lacewing treatments.

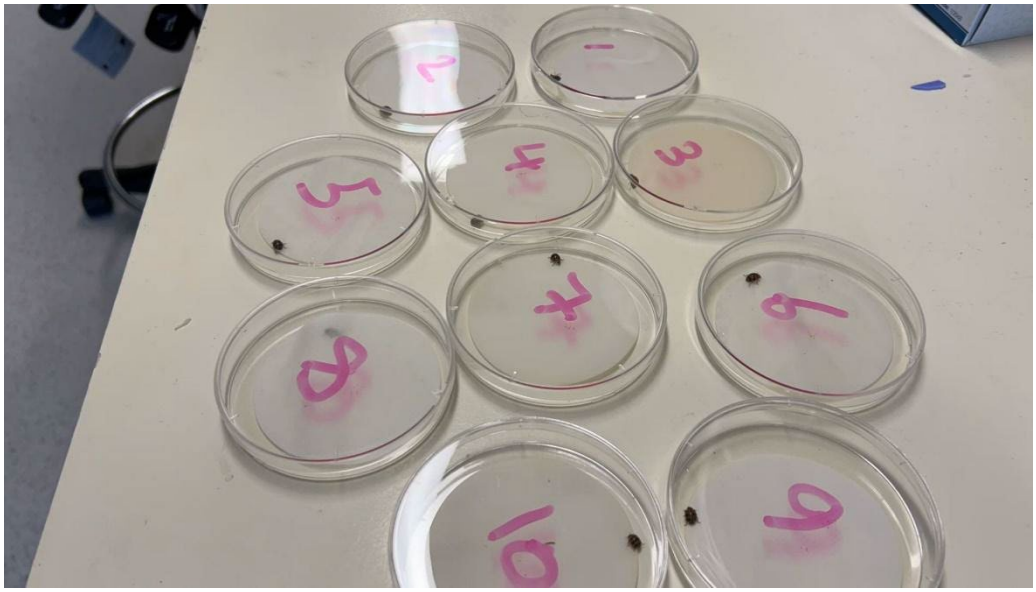


Figure 5.1: Petri dishes containing ladybird adults Harmonia octomaculata and phylloxera eggs.

Key Research Findings

Field surveys for phylloxera predators

A total of 56 arthropod specimens were collected from 225 leaf and 54 root samples, comprising 24 families from ten orders. The greatest abundance and diversity of arthropods was found on the grapevine leaves with 77% of the specimens collected from leaves compared with 20% from the roots and only 3% from within leaf galls (Table 5.1).

Table 5.1: Arthropod taxa and number of individuals associated with each plant structure (roots, leaves or phylloxera-infested leaf galls) in North-East Victorian vineyards. • = predator, □ = herbivore, □ = other.

	Roots	Leaves	Leaf Gall
Acarina (mites)			
Phytoseiidae (*Paraseiulus* sp) •		3	
Sarcoptiformes: Oribatida □		6	
Unidentified □		2	1
Aranea (spiders)			
Theridiidae •		6	
Araneidae •		1	
Linyphiidae •		3	
Ctenizidae •	1		
Hemiptera (true bugs)			
Heteroptera □		2	
Pseudococcidae □		3	1
Aphididae □	3	1	
Lygaeidae □		1	
Coleoptera (beetles)			
Coccinellidae (*Harmonia* sp) •		4	
Coccinellidae (*Hippodamia variegata*) •		1	
Scarabaeidae (larva) □	1		
Latridiidae □		3	
Unidentified □	2		
Neuroptera (lacewings)			
Chrysopidae (larvae) •		2	
Thysanoptera (thrips)			
Phlaeothripidae □		1	
Isoptera (termites) □	2		
Hymenoptera (sawflies, wasps, bees, ants)			
Formicidae (ants) •	2		
Pergidae (sawflies) □		1	
Lepidoptera (moths, butterflies)			
Unknown larva □		1	
Geometridae larva □		1	
Collembola (springtails) □		1	
Total	**11**	**43**	**2**

Of the 24 taxa, 38% were predators (n=9), 33% were herbivores (n = 8) and 29% were other (n = 7). Most of the predators were found on the leaves and comprised of spiders, ladybeetles, lacewings, ants and predatory mites (Figure 5.2). The only predatory taxa found on the roots were ants and a trapdoor spider. No predators were found within the leaf galls.

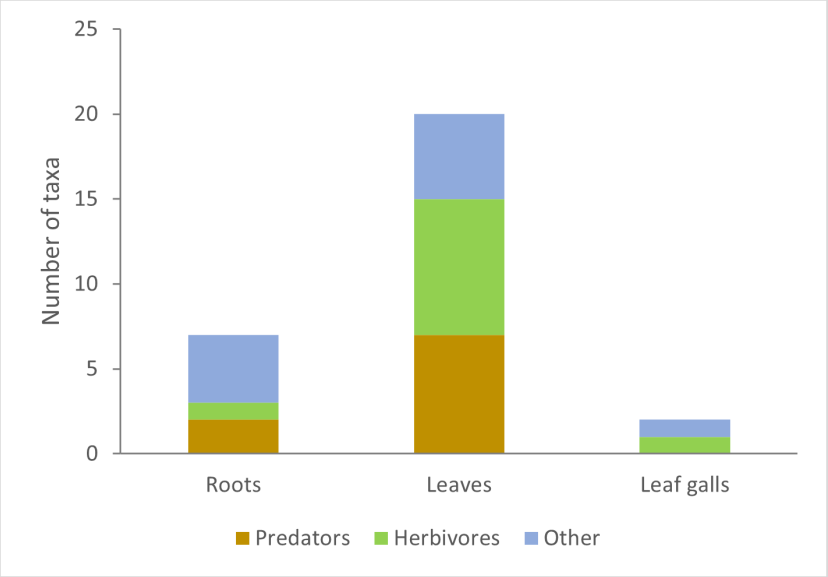


Figure 5.2: Number of predator, herbivore and other arthropod taxa collected from the roots, leaves and phylloxera leaf galls (pooled from all sites) from north-east Victorian vineyards between January to May 2022.

Laboratory feeding trials with commercial aphid predators

The opportunistic feeding trial revealed that ladybird larvae were ferocious predators of phylloxera, with all ten crawlers in each of the two dishes being consumed within one hour.

For the laboratory-feeding trial, ladybird adults and green lacewing larvae both predated upon phylloxera eggs consuming a mean total of 3.2 ± 1.0 (SE) and 1.4 ± 1.0 (SE) respectively over the 24hr period ($F_{1,18}$=1.6, *P* = 0.22). The mobility and feeding activity of the lacewing larvae was negligible over the first four hours with only three of the ten larvae consuming phylloxera eggs. In comparison, the ladybird adults were very mobile with six of the ten adults feeding on phylloxera eggs (Figure 5.3).

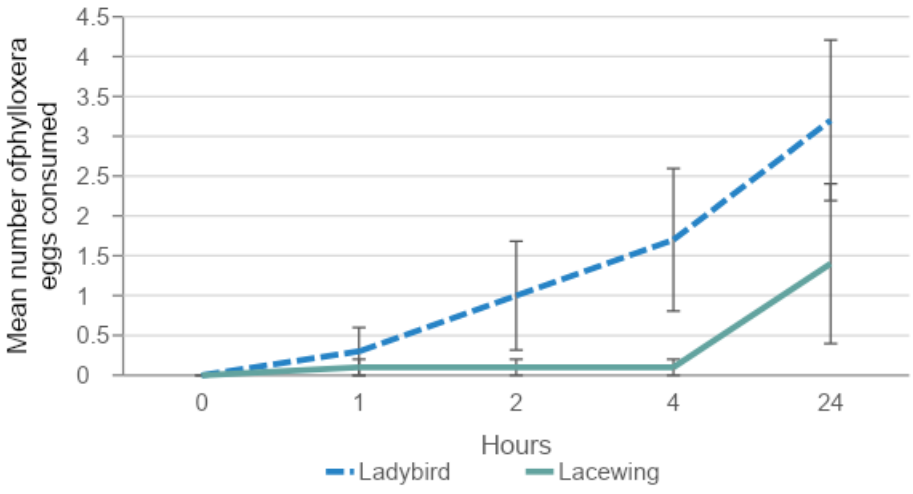


Figure 5.3: Consumption of phylloxera eggs by adult ladybirds, Harmonia octomaculata and green lacewing larvae, Mallada signatus over a 24 hour period. Means ± SE.

Discussion and Recommendations

This study conducted a preliminary investigation into the potential for conservation and inundation biological control strategies for phylloxera by firstly determining what aphid predators were found in phylloxera-infested vineyards, and secondly conducting feeding trials using two commercially available aphid predators.

The field survey identified a modest guild of arthropods found on grapevine leaves, roots and leaf galls infested with phylloxera. However, the diversity of arthropod taxa assigned to three main functional groups (i.e., predators, herbivores and 'other'), was not too dissimilar to those found in other studies on arthropod biodiversity in Australian vineyards. Retallack (2019) collected 127 arthropod taxa from *Vitus vinifera* at six sites in South Australia of which 44% were predators, 23% were herbivores and 32% were 'other', whilst in our study, 38% of the arthropod taxa collected were predators, 33% herbivores and 29% other, despite a much smaller sampling effort which was focussed on phylloxera-infested plants.

Of the 11 specimens collected from the root samples, most were soil-inhabiting detrivores or fungivores, except for several ants which may possibly be predating upon phylloxera on the roots. In comparison, there were many arthropods collected from the leaves which are known predatory arthropods and could potentially be predating upon phylloxera. These include the mites, *Paraseiulus* sp. (Acari: Mesostigmata: Phytoseiidae) and several species of spiders from the families Theridiidae, Araneidae and Linyphiidae. There were also two species of ladybirds (*Harmonia* sp and *Hippodamia variegata*) and one unidentified lacewing larva, which are specialist aphid predators.

The laboratory feeding studies using adult and juvenile ladybirds and juvenile green lacewings confirmed that these generalist aphid predators will also consume phylloxera eggs and 1st instar crawlers. In a practical sense, the arboreal nature of these predators means that they are unlikely to have an impact on root-infesting stages of phylloxera. However, it is possible that they might opportunistically predate upon the crawler stages that have emerged from the soil to disperse to other vines. More likely, these predators may have a role to play in predating upon leaf galling phylloxera, especially if the early-instar ladybird and lacewing larvae are able to enter the galls to feed on phylloxera adults, eggs and crawlers.

Conclusion

While the field surveys were instructive in cataloguing arthropod diversity and abundance, confirming the associations between predator and prey requires further evidence, such as observed predation or molecular gut content analysis (Andow and Paula, 2023). Such studies were beyond the scope of this project and should be considered for future research so that conservation biological control strategies can be developed that promote the abundance of phylloxera predators above and below-ground.

Similarly, the laboratory feeding studies demonstrated that ladybirds and lacewings could predate upon phylloxera eggs and crawlers, however further studies are needed to determine if these commercially available predators could be effectively deployed *en mass* through an inundation biocontrol approach

List of appendices

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Appendix 2: Intellectual property (this document

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Appendix 5: Interactive spreadsheet for estimating the probability of detecting phylloxera (provided)

Appendix 6: Leaf gall study data, including Appendix Tables 1 to 5 (provided)

Appendix 7: Diagrams of genotype results for each gall per vineyard (provided)

Appendix 1: Communications

The table below lists all communication efforts carried out during this project

Communication	Date	Team members	Place	Title/Activity	Target Audience
Publication	2023	C. Clarke, J. Hennken, B. Camody & P Cunningham	Australian Journal of Grape and Wine Research	Performance of six genetically diverse phylloxera strains on 5C Teleki	Scientific community
	2023	C. Clarke, K. Powell, S. Norng, B.Camody, M. Warpole & P. Cunningham	Australian Journal of Grape and Wine Research	Performance of a leaf galling phylloxera on roots of diverse *Vitis* spp. Rootstocks in North-East Victoria Australia	Scientific community
	2020	A. Agarwal, P. Cunningham, I. Valenzuela & Mark. Blacket	Scientific reports	A diagnostic LAMP assay for the destructive grapevine insect pest, phylloxera (Daktulosphaira vitifoliae)	Scientific community
Industry articles	2024	J. Henneken	Vine Health Australia	Jessi Henneken: Tackling Phylloxera	Growers and industry
	2024	C. Clarke, J. Hennken, B. Camody & P Cunningham	Wine and Viticulture Journal	Performance of genetically diverse phylloxera genotypes on 5C Teleki (V. berlandieri X V. riparia) rootstock	Growers and industry
	2024	J. Henneken & I. Valenzuela-Gonzalez	Grape Grower and Wine Maker magazine	Tech development aids phylloxera detection	Growers and industry
	2021	C. Clarke, J. Weiss & Vine Health Australia	Grape Grower and Wine Maker magazine	New phylloxera research: actions for industry	Growers and industry
	2021	C. Clarke, B. Camody & Paul. Cunningham	Wine and Viticulture Journal	Dogs sniffing phylloxera – an industry perspective	Growers and industry
	2021	C. Clarke & M. Blacket	Daily wine news	Australia's knowledge and response to grapevine phylloxera is evolving	Growers and industry
Conference presentations	2022	C. Clarke	The 18th Australian Wine Industry Technical Conference	Grapevine Phylloxera: Latest science and implications for management	Scientific community, growers and industry.

	2022	C. Clarke	Hemipteran-plant Interactions Symposium	Grapevine Phylloxera: Latest science and implications for management	Scientific community
	2022	C. Clarke & I. Valenzuela-Gonzalez	Hemipteran-plant Interactions Symposium	Phylloxera genetic diversity and implications for management	Scientific community
Seminars	2023	J. Henneken	GoTafe Wangaratta	Grapevine Phylloxera: the vine-destroying bug	Diploma of Viticulture students
	2021	C.Clarke	Australian Wine Research Institute	Integrated management of established grapevine phylloxera	Scientific community, growers and industry.
Meetings	2022	C. Clarke	Mornington Peninsula Growers meeting	The challenge of phylloxera: The latest science	Growers and industry
	2021	C. Clarke, CHPOU	State Biosecurity Meeting	Meeting with Biosecurity representatives within AVR and CHPOU	Biosecurity and industry
Training	2021-2023	J. Henneken & J. Weiss	Data collection (Yarra Valley)	Training of BAS and AVR staff in phylloxera surveillance	Biosecurity and researchers
Personal communication	2024	J. Henneken & J. Weiss	Email to BAS	Simulation models and slide deck shared with Biosecurity & Agriculture Services representatives for feedback	Biosecurity officers
	2023	J. Henneken	Email to BAS	Recommendations and data summary tables on dry heat disinfestation protocols shared with Biosecurity & Agriculture Services representatives	Biosecurity officers
	2021	C. Clarke	Email to growers	Results on phylloxera genotypes released to growers in the King Valley	Growers

Appendix 2: Intellectual Property

No intellectual property was developed as part of this research. Key outputs that can be directly adopted by growers and industry include i) simulation models for estimating the probability of detecting phylloxera and the time needed to survey a vineyard for phylloxera surveillance (Appendix 5), ii) information on the resistance of rootstocks M5512 and M5489 against G4 and G38, iii) an improved dry-heat disinfestation protocol, vi) improved knowledge of the risk of phylloxera dispersal in winter v) characterization of new phylloxera genotypes in the King Valley and vi) knowledge of the insects that may be feeding oh phylloxera in commercial vineyards.

Appendix 3: References

Improved Surveillance and Detectability of Grapevine Phylloxera

Agarwal, A., Cunningham, J. P., Valenzuela, I., & Blacket, M. J. (2020). A diagnostic LAMP assay for the destructive grapevine insect pest, phylloxera (Daktulosphaira vitifoliae). *Scientific Reports*, *10*(1), 21229.

Benheim, D., Rochfort, S., Robertson, E., Potter, I. D., & Powell, K. S. (2012). Grape phylloxera (Daktulosphaira vitifoliae)–a review of potential detection and alternative management options. *Annals of Applied Biology*, *161*(2), 91-115.

Blacket, M. J., Agarwal, A., Zheng, L., Cunningham, J. P., Britton, D., Schneider, I., & Rodoni, B. C. (2020). A LAMP assay for the detection of Bactrocera tryoni Queensland fruit fly (Diptera: Tephritidae). *Scientific Reports*, *10*(1), 9554.

Buchanan, G. A. (1987). The distribution of grape phylloxera, Daktulosphaira vitifolii (Fitch), in central and north-eastern Victoria. *Australian Journal of Experimental Agriculture*, *27*(4), 591-595.

Buchanan G. A. (1990). The distribution, biology, and control of grape phylloxera Daktulosphaira vitifolii (Fitch) in Victoria. PhD Thesis, La Trobe University, Melbourne

Clarke, C.W., P. Cunningham, B. Carmody, M. Blacket, R. Kwong, I. Valenzuela, K. Farnier, S. Tobin, J. Miyazaki, and J. Weiss, "Integrated management of established grapevine phylloxera", (Agriculture Victoria, Wine Australia and Department of Jobs, Regions and Precincts, 2020).

Clarke, C. W., Powell, K. S., Norng, S., Carmody, B. M., Walpole, M., & Cunningham, J. P. (2023). Performance of a leaf galling Phylloxera (Daktulosphaira vitifoliae) on Roots of Diverse Vitis spp. Rootstocks in North East Victoria, Australia. *Australian Journal of Grape and Wine Research*, *2023*.

Deretic, J., Powell, K.S., Hetherington, S. (2003) Assession the risk of phylloxera transfer during post-harvest handling of wine grapes. Acta Horticultureae 617, 61 – 66.

Edwards, J.P., Powell, K.S. and Granett, J. (2006) Tritrophic interactions between grapevines, phylloxera and pathogenic fungi - establishing the root cause of grapevine decline. The Australian and New Zealand Grapegrower and Winemaker 513, 33-37.

Granett, J., Omer, A.D., Pessereau, P. and Walker, A. (1998) Fungal infections of grapevine roots in phylloxera-infested vineyards. Vitis 37, 39-42.

Granett, J., Walker, M. A., Kocsis, L., & Omer, A. D. (2001). Biology and management of grape phylloxera. *Annual review of entomology*, *46*(1), 387-412.

Hauser, C. E., Giljohann, K. M., McCarthy, M. A., Garrard, G. E., Robinson, A. P., Williams, N. S., & Moore, J. L. (2022). A field experiment characterizing variable detection rates during plant surveys. *Conservation Biology*, *36*(3), e13888.

Herbert, K. S., Hoffmann, A. A., & Powell, K. S. (2006). Changes in grape phylloxera abundance in ungrafted vineyards. *Journal of economic entomology*, *99*(5), 1774-1783.

Herbert, K., Powell, K., Mckay, A., Hartley, D., Herdina, Ophel-Keller, K., ... & Hoffmann, A. (2008). Developing and testing a diagnostic probe for grape phylloxera applicable to soil samples. *Journal of economic entomology*, *101*(6), 1934-1943.

Hoffmann, M., Ruehl, E. H., Eisenbeis, G., & Huber, L. (2016). Grape root as habitat: overwintering and population dynamics of grape phylloxera (Daktulosphaira vitifoliae Fitch) in temperate climate viticulture. *Australian journal of grape and wine research*, *22*(2), 271-278.

Li, Y., & Cai, S. H. (2011). Sensitive and rapid detection of the insect pathogenic fungus Metarhizium anisopliae var. anisopliae by loop-mediated isothermal amplification. *Current microbiology*, *62*, 1400-1404.

Martoni, F., Nogarotto, E., Piper, A. M., Mann, R., Valenzuela, I., Eow, L., ... & Blacket, M. J. (2021). Propylene glycol and non-destructive DNA extractions enable preservation and isolation of insect and hosted bacterial DNA. *Agriculture*, *11*(1), 77.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic acids research*, *28*(12), e63-e63.

Powell, K. S., Korosi, G. A., & Mackie, A. M. (2007, September). Monitoring grape phylloxera populations using simple non-destructive trapping systems. In *IV International Phylloxera Symposium 816* (pp. 29-34).
Powell, K. S. (2009). Grape phylloxera: an overview. In *Root feeders: an ecosystem approach*, 96-114. Eds S.N. Johnson and G.M. Murray CAB international.
Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A., & Warman, M. L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques*, *29*(1), 52-54.

Improved Selection of Resilient Rootstocks M5512 and M5489

Clarke, C.W., Powell, K.S., Norng, S., Carmody, B.M., Walpole, M. and Cunningham, J. P. (2023a) Performance of a leaf galling phylloxera (Daktulosphaira vitifoliae) on roots of diverse Vitis spp. Rootstocks in North-East Victoria, Australia. Australian Journal of Grape and Wine Research 2023
Clarke, C.W., Cunningham, J.P., Carmody, B., Blacket, M., and Kwong, R, Integrated Management of Established grapevine Phylloxera, Agriculture Victoria, Wine Australia and Department of Jobs Regions and Precincts, Melbourne, Australia, 2020.
Clarke, C.W., Henneken, J., Carmody, B.D. and Cunningham, J.P. (2023b). Performance of sex genetically diverse phylloxera strains on 5C Teleki (V. berlandieri X V. riparia) rootstocks. Australian Journal of Grape and Wine Research
Corrie, A., Hoffmann, A. (2004) Fine-scale genetic structure of grape phylloxera from the roots and leaves of *Vitis*. Heredity 92, 118–127 (2004).
Edwards, J.P., Powell, K.S. and Granett, J. (2006) Tritrophic interactions between grapevines, phylloxera and pathogenic fungi - establishing the root cause of grapevine decline. The Australian and New Zealand Grapegrower and Winemaker 513, 33-37.
Forneck, A. "Do nodosities of phylloxera form a strong sink tissue in infected vine plants? Are nodosities symplasmically connected to the phloem?" (BOKU, Vienna, 2011).
Forneck, A. and Huber, L. (2009) (A) sexual reproduction–a review of life cycles of grape phylloxera, Daktulosphaira vitifoliae. Entomologia Experimentalis et Applicata 131(1), 1-10.
Hadju, E. (2015) Grapevine breeding in Hungary. (Elsevier, Woodhead Publishing: Cambridge, United Kingdom).
Hajdu, E. and Bakonyi, K (2006) Rootstock breeding in Hungary. Kertgazdasag Horticulture 38, 25-32.
Granett, J., Fossen, M. and McGourty, G. "Influence of grapevine stress on grape phylloxera-related fungal root infections", (2003), pp. Presentation part of : Ten-minute papers, Section F. Crop Protection Entomology.
Granett, J. and Kocsis, L. (2000) Populations of grape phylloxera gallicoles on rootstock foliage in Hungary. Vitis 39, 37-41.
Granett, J., Omer, A.D., Pessereau, P. and Walker, A. (1998) Fungal infections of grapevine roots in phylloxera-infested vineyards. Vitis 37, 39-42.
Lund, K.T., Riaz, S. and Walker, M.A. (2017) Population structure, diversity and reproductive mode of the grape phylloxera (Daktulosphaira vitifoliae) across its native range. PLOS ONE 12, e0170678.
Powell, K., and Krstic, M. (2015) Phylloxera: rootstock tolerance and resistance to different genetic strains of phylloxera. Wine and Viticulture Journal 30, 48-51.
Umina, P.A., Corrie., A, Herbert, A.S., White, V.L., Powell, K.S. and Hoffmann, A.A. (2007) The use of DNA markers for pest management - clonal lineages and population biology of grape phylloxera. Acta Horticulturae 733, 183-195.
Wine Australia, "Grapevine rootstock selector tool. https://www.wineaustralia.com/news/media-releases/grapevine-rootstock-selector-tool-upgraded", (Wine Australia. , Industry House, Cnr Botanic and Hackney Roads, Adelaide | PO Box 2733, Kent Town South Australia 5071, 2019), Vol. 2020.

Improved disinfestation procedures under different hygiene conditions

Clarke, C. W., Norng, S., Yuanpeng, D., Carmody, B. M., & Powell, K. S. (2018). Dry heat as a disinfestation treatment against genetically diverse strains of grape phylloxera. *Australian journal of grape and wine research*, 24(3), 301-304.

Clarke, C. W., Norng, S., Carmody, B. M., Yuanpeng, D., & 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*, 25(4), 396-403.
The Dirt Dude (2022). Modernisation of regulatory standards to grow and protect wine grape industry supply chain opportunities. Final Report (*Heat Room Assessment and Survey, Harvest 2022*)
King, P. D., & Buchanan, G. A. (1986). The dispersal of phylloxera crawlers and spread of phylloxera infestations in New Zealand and Australian vineyards. *American Journal of Enology and Viticulture*, *37*(1), 26-33.
Kingston, K. B., Powell, K. S., & Cooper, P. D. (2007). Characterising the root-feeding habits of grape phylloxera using electrical penetration graph. *Acta Horticulture*, 733, 33-46.
NVHSC (2009), National phylloxera management protocols (*National Vine Health Steering Committee*). Available at https://vinehealth.com.au/media/National-Phylloxera-Management-Protocol.pdf
Powell, K. S. (2008). Grape phylloxera: an overview. *Root feeders: an ecosystem approach*, 96-114.
Powell, K. S. (2012). A holistic approach to future management of grapevine phylloxera. In *Arthropod management in vineyards: pests, approaches, and future directions* (219-251). Dordrecht: Springer Netherlands.
Powell, K.S. (2017) "Risks and Management of Endemic and Exotic Phylloxera", Final Report (*Agriculture Victoria Australian Grape & Wine Authority, 2017*).
Prinzing, A. J. (2001). Use of shifting microclimatic mosaics by arthropods on exposed tree trunks. Annals of the Entomological Society of America, 94(2), 210-218.
Schortemeyer, M., Thomas, K., Haack, R. A., Uzunovic, A., Hoover, K., Simpson, J. A., & Grgurinovic, C. A. (2011). Appropriateness of probit-9 in the development of quarantine treatments for timber and timber commodities. *Journal of Economic Entomology*, 104(3), 717-731.
Terlau, J. F., Brose, U., Eisenhauer, N., Amyntas, A., Boy, T., Dyer, A., ... & Hirt, M. R. (2023). Microhabitat conditions remedy heat stress effects on insect activity. Global Change Biology.
Umina, P. A., Corrie, A. M., Herbert, K. S., White, V. L., Powell, K. S., & Hoffmann, A. A. (2007). use of DNA markers for pest management-clonal lineages and population biology of grape phylloxera. *Acta Horticulturae*, 733, 183-195.

Phylloxera population dynamics and survival over winter

Benheim, D., Rochfort, S., Robertson, E., Potter, I. D., & Powell, K. S. (2012). Grape phylloxera (Daktulosphaira vitifoliae)–a review of potential detection and alternative management options. *Annals of Applied Biology*, *161*(2), 91-115.
Deretic, J., Powell, K., & Hetherington, S. (2001, August). Assessing the risk of phylloxera transfer during post-harvest handling of wine grapes. In *Workshop on Rootstocks Performance in Phylloxera Infested Vineyards 617* (pp. 61-66).
Granett, J., Kocsis, L., Horvath, L., & Horvathne, E. B. (2005). Grape phylloxera gallicole and radicicole activity on grape rootstock vines. *HortScience*, *40(1)*, 150-153
King, P. D., & Buchanan, G. A. (1986). The dispersal of phylloxera crawlers and spread of phylloxera infestations in New Zealand and Australian vineyards. *American Journal of Enology and Viticulture*, *37*(1), 26-33.
Herbert, K. S., Hoffmann, A. A., & Powell, K. S. (2006). Changes in grape phylloxera abundance in ungrafted vineyards. *Journal of economic entomology*, *99*(5), 1774-1783.
Hoffmann, M., Ruehl, E. H., Eisenbeis, G., & Huber, L. (2016). Grape root as habitat: overwintering and population dynamics of grape phylloxera (Daktulosphaira vitifoliae Fitch) in temperate climate viticulture. *Australian journal of grape and wine research*, *22*(2), 271-278.
NVHSC (2009), National phylloxera management protocols (*National Vine Health Steering Committee*). Available at https://vinehealth.com.au/media/National-Phylloxera-Management-Protocol.pdf
Powell, K. S., Slattery, W. F., Deretic, J., Herbert, K., & Hetherington, S. (2001, August). Influence of soil type and climate on the population dynamics of grapevine phylloxera in Australia. In *Workshop on Rootstocks Performance in Phylloxera Infested Vineyards 617* (pp. 33-41).
Powell, K. S., Korosi, G. A., & Trethowan, C. J. (2007). Reducing the risk of phylloxera transfer on viticultural waste and machinery. In *IV International Phylloxera Symposium 816* (pp. 53-62).
Powell, K. S. (2008). Grape phylloxera: an overview. *Root feeders: an ecosystem approach*, 96-114.

Powell, K. S. (2012). A holistic approach to future management of grapevine phylloxera. In *Arthropod management in vineyards: pests, approaches, and future directions* (219-251). Dordrecht: Springer Netherlands

Turley, M., Granett, J., Omer, A. D., & De Benedictis, J. A. (1996). Grape phylloxera (Homoptera: Phylloxeridae) temperature threshold for establishment of feeding sites and degree-day calculations. *Environmental entomology*, *25*(4), 842-847.

Increased prevalence of leaf galling phylloxera in the North-East Phylloxera Infested Zone

Bao, L. V., Scatoni, I. B., Gaggero, C., Gutiérrez, L., Monza, J., & Walker, M. A. (2015). Genetic diversity of grape phylloxera leaf galling populations on Vitis species in Uruguay. *American Journal of Enology and Viticulture*, *66*(1), 46-53.

Clarke, C. W., Powell, K. S., Norng, S., Carmody, B. M., Walpole, M., & Cunningham, J. P. (2023). Performance of a Leaf galling Phylloxera (Daktulosphaira vitifoliae) on Roots of Diverse Vitis spp. Rootstocks in North-East Victoria, Australia. *Australian Journal of Grape and Wine Research*, *2023*.

Edwards, J.P., Powell, K.S. and Granett, J. (2006) Tritrophic interactions between grapevines, phylloxera and pathogenic fungi - establishing the root cause of grapevine decline. The Australian and New Zealand Grapegrower and Winemaker 513, 33-37.

Fahrentrapp, J., Müller, L., & Schumacher, P. (2015). Is there need for leaf galling grape phylloxera control? Presence and distribution of Dactulosphaira vitifoliae in Swiss vineyards. *International Journal of Pest Management*, *61*(4), 340-345.

Granett, J., Omer, A.D., Pessereau, P. and Walker, A. (1998) Fungal infections of grapevine roots in phylloxera-infested vineyards. Vitis 37, 39-42.

King, P. D., & Buchanan, G. A. (1986). The dispersal of phylloxera crawlers and spread of phylloxera infestations in New Zealand and Australian vineyards. *American Journal of Enology and Viticulture*, *37*(1), 26-33.

Powell, K. S. (2012). A holistic approach to future management of grapevine phylloxera. In *Arthropod management in vineyards: pests, approaches, and future directions* (pp. 219-251). Dordrecht: Springer Netherlands.

Umina, P. A., Corrie, A. M., Herbert, K. S., White, V. L., Powell, K. S., & Hoffmann, A. A. (2007). The Use of DNA Markers for Pest Management--Clonal Lineages and Population Biology of Grape Phylloxera. *Acta Horticulturae*, *733*, 183-195.

Wilmink, J., Breuer, M., & Forneck, A. (2021). Effect of temperature on host plant-specific leaf-and root-feeding performances: a comparison of grape phylloxera biotypes C and G. *Entomologia Experimentalis et Applicata*, *169*(12), 1113-1125.

Wilmink, J., Breuer, M., & Forneck, A. (2022). Effects of grape phylloxera leaf infestation on grapevine growth and yield parameters in commercial vineyards: a pilot study. *OENO One*, *56*(1), 197-208.

Genetic diversity of leaf gall grapevine phylloxera in the King Valley, North-Eastern Victoria

Agarwal, A., Cunningham, J. P., Valenzuela, I., & Blacket, M. J. (2020). A diagnostic LAMP assay for the destructive grapevine insect pest, phylloxera (Daktulosphaira vitifoliae). *Scientific Reports*, *10*(1), 21229.

Corrie, A.M., & Hoffmann, A.A. (2004). Fine-scale genetic structure of grape phylloxera from the roots and leaves of Vitis. *Heredity 92*, 118–127

Corrie, A.M., Crozier, R.H., Van-Heeswijck, R., & Hoffmann, A.A. (2002). Clonal reproduction and population genetic structure of grape phylloxera, *Daktulosphaira vitifoliae*, in Australia. *Heredity 88*, 203-211.

Corrie, A.M., Van-Heeswijck, R, & Hoffman, A.A. (2003). Evidence for host-associated clones of grape phylloxera *Daktulosphaira vitifoliae* (Hemiptera: Phylloxeridae) in Australia. *Bulletin of Entomological Research 93*, 193-201.

Hoffmann, A.A., & Corrie, A.M, (2003). A comprehensive assessment of genetic variation in grape phylloxera. *Final report to Grape and Wine Research & Development Corporation.* Project Number: LTU 01/01

Umina, P.A., Corrie, A.M., Herbert, K.S., White, V.L., Powell, K.S., & Hoffmann, A.A. (2007). The use of DNA markers for pest management - clonal lineages and population biology of grape phylloxera. *Acta Horticulturae 733*, 183-195.

A conservation biocontrol approach

Andow, D. A., & Paula, D. P. (2023). Estimating predation rates from molecular gut content analysis. Molecular Ecology Resources, 23(5), 1023-1033.

Kirchmair, M., Hoffmann, M., Neuhauser, S., Strasser, H., & Huber, L. (2007). Persistence of GRANMET®, a Metarhizium anisopliae based product, in grape phylloxera-infested vineyards. IOBC wprs Bulletin, 30(7), 137.

Korosi, G.A., (2019) Entomopathogenic fungi as potential biocontrol agents for grapevine phylloxera. PhD thesis Charles Sturt University (https://researchoutput.csu.edu.au/ws/portalfiles/portal/90531310/Whole_thesis_final_revision_GK_no_signatures.pdf) accessed 7 May 2024.

Kwong, R (2020) Biocontrol options for grape phylloxera in Australia Wine Australia - Project RDC Number: DED 1701 – Integrated management of established grapevine phylloxera (https://www.wineaustralia.com/getmedia/f92cd14f-b1df-45a6-b5ea-b100b5957bdc/DED1701-Appendix-9_Feasibility-of-Biological-Control-of-Grape-Phylloxera_October-2020.pdf) accessed 7 May 2024.

Retallack, M. J. (2019). The potential functional diversity offered by native insectary plants to support populations of predatory arthropods in Australian vineyards (Doctoral dissertation). https://hdl.handle.net/2440/120158 (accessed 7 may 2024).

Retallack, M. J., Thomson, L. J., & Keller, M. A. (2019). Predatory arthropods associated with potential native insectary plants for Australian vineyards. Australian journal of grape and wine research, 25(2), 233-242.

Riley, C. V. (1874). Insect injurious to the grape-vine. The grape phylloxera - Phylloxera vastatrix Planchon (Subord. Homoptera, Fam. Aphidae).

Thomson, L., & Hoffmann, A. (2009). Sustainable viticulture and beyond: Vineyard management to maximise beneficial invertebrates to increase the bottom line. Final report to Grape and Wine Research & Development Corporation.

Walsh, B., & Riley, C. (1869). The apple-root plant-louse. American Entomologist, 5, 81-84.

Appendix 4: Staff

A list of the staff aligned to this project is provided below:

Name	Organisation	Activities
Ali Pakniat-Jahromy	Agriculture Victoria DEECA	Technical assistance across activities (2023 – 2024)
Arati Agrawal	Agriculture Victoria DEECA (retired)	Effective surveillance and diagnostics (2021 – 2022)
Bernadette Carmody	Agriculture Victoria DEECA (retired)	Technical assistance across activities (2021 – 2022)
Catherine Clarke	Agriculture Victoria DEECA	Chief investigator (2021 – 2022)
Isabel Valenzuela-Gonzalez	Agriculture Victoria DEECA	Phylloxera ecology
Jessica Henneken	Agriculture Victoria DEECA	Chief investigator (2023 – 2024)
Joanne Dawson	Agriculture Victoria DEECA	Technical assistance across activities (2023 – 2024)
John Weiss	Agriculture Victoria DEECA (retired)	Effective surveillance and diagnostics
Mark Blacket	Agriculture Victoria DEECA	Effective surveillance and diagnostics & Phylloxera ecology
Paul Cunningham	Agriculture Victoria DEECA	Project leader
Rae Kwong	Agriculture Victoria DEECA	A conservation biocontrol approach