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Risks and Management of Endemic and Exotic Phylloxera



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ABSTRACT

Significant spread of grape phylloxera beyond its current range would be devastating for the Australian wine industry. The overall aims of this project were to minimise the risk of grape phylloxera spreading within and between quarantine boundaries and to improve phylloxera detection. The influence of temperature extremes and various chemical products on survival of several endemic phylloxera strains was examined, in the context of scientifically validating existing and developing new phylloxera disinfestation protocols. This evidence-based approach highlighted that endemic strains vary in their susceptibility to disinfestation treatments and, as a result, recommendations are provided to modify existing protocols. These modifications, once implemented, will reduce the risk of movement of phylloxera endemic strains as well as reducing the time and cost associated with implementing quarantine procedures and management options.

The use of sniffer dogs for phylloxera detection was explored for the first time through a pilot study. Results of this work highlighted that an odour-based detection approach should be further developed to improve the industry's capacity to detect phylloxera early and thus reduce its economic impact on grapevine production.

In addition to maintaining and utilising a collection of six phylloxera endemic strains in this work, a new strain was detected and added to the existing clonal collection. Further, five unidentified phylloxera including a leaf galling sample were detected and a culture started from these initial collections. This expanded clonal collection is a fundamental scientific resource which will be required to enable future research in early detection, rootstock management, quarantine development and alternative control options of grape phylloxera.

EXECUTIVE SUMMARY

This report describes the research, development and extension activities of the three year AGWA and DEDJTR co-funded project DEP 1301 conducted during 2014-2017. The main objectives of the project were to (i) maintain a clonal collection of selected endemic phylloxera strains, (ii) utilise selected genetic strains to scientifically validate a range of disinfestation treatments currently recommended in the National Phylloxera Management Protocol, (iii) develop a memorandum of understanding between AGWA and DEDJTR to allow future access to the clonal lineage collection, (iv) conduct a desk top literature survey to develop a Pest Risk Analysis document for phylloxera, (v) assess the potential for using sniffer dogs as part of an early detection approach for phylloxera, and (vi) disseminate research outputs through a variety of extension outlets.

The research activities conducted allow for future development of improved management practices following an in-depth comparison of the efficacy of temperature- and chemical-based disinfestation treatments on genetically diverse endemic phylloxera strains. Modification of existing disinfestation treatments for clothing, footwear, planting material, diagnostic samples and vineyard machinery, have all been proposed as a result of this research study and, once implemented, will reduce the risk of dispersal of phylloxera strains between quarantine zones. Most of the suggested modifications to protocols will reduce the amount of time and heat required for disinfestation, and thus reduce costs to industry. The study also highlighted that developing disinfestation treatments to reduce the risk of transfer on clothing and footwear requires further study. Screening of several novel disinfestation treatments for footwear demonstrated that many commercially available products (although effective against other pests and pathogens) are not suitable for use against phylloxera. Other treatments could potentially replace the widely-used method of using household bleach in footbaths.

A core collection of six clonal lineages were maintained and utilised throughout the project period, and when five new phylloxera detections were discovered in the North East Victoria Phylloxera Infested Zone, these were added to the collection. One of the newly detected strains, G38, was used to test the efficacy of a range of existing and suggested modifications to current disinfestation procedures. As a result of this comprehensive scientific-validation approach, at least seven genetically and geographically diverse endemic phylloxera strains have now been screened. The National Phylloxera Management Protocol (NPMP) is currently undergoing review, and results presented in this report will inform the re-evaluation. As existing phylloxera detection systems are economically costly, labour intensive and vary in their efficacy, a novel approach to phylloxera detection was conducted using sniffer dogs. Preliminary controlled environment studies using sniffer dogs highlighted that dogs had the

ability to detect odours or odour mixtures specific to phylloxera, both in isolation and in phylloxera-infested grapevine material. Further validation of an odour-based early detection approach may lead to a more efficient phylloxera detection system in the future.

Extensive consultation with biosecurity agencies, including Vinehealth Australia (VHA), Biosecurity Victoria, the Victorian Viticulture Biosecurity Committee and the National Viticulture Biosecurity committee occurred during the course of the project, particularly in relation to the research outputs from the disinfestation study. Awareness of phylloxera research activities and outputs was enhanced both nationally and internationally through (i) informing various state and national biosecurity agencies of potential modifications to phylloxera management protocols, (ii) improved inter-agency collaboration, particularly with Vinehealth Australia and Biosecurity Victoria, (iii) publication of several industry articles and web based information (through VHA), (iv) publication of peer-reviewed scientific articles and the first comprehensive Phylloxera Pest Risk Analysis paper, and (v) presentations at the Australia Wine Industry Technical Conference. In addition, the wider public was informed of selected project outputs through media articles and interviews.

Overall, this report addresses important knowledge gaps in quarantine and early detection of phylloxera, and provides recommendations to significantly develop protocols and research priorities in the management of this critically important insect pest.

INTRODUCTION

This is a report on a phylloxera research project conducted from July 2014 to June 2017. The research was co-funded by the Australian Grape and Wine Authority and the DEDJTR, Victoria.

Project Team

The core project activities were conducted by Agriculture Victoria (AV) staff located at AV Rutherglen with additional support provided by staff at AgriBio, Bundoora.

Collaborators

The project activities were carried out in close collaboration with industry and state biosecurity groups including Vinehealth Australia (formerly the Phylloxera and Grape Industry Board of South Australia) and the Victorian Viticulture Biosecurity Committee.

Adherence to Quarantine Protocols

All laboratory, glasshouse and field work was carried out under permit using specified procedures and disinfestation protocols specifically designed to prevent the spread of phylloxera. All field visits, where required, were arranged in consultation with vineyard managers. All laboratory and glasshouse work was conducted at AV Rutherglen which has quarantine approved premises and is located with the North East Phylloxera Infested Zone (PIZ).

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SECTION 1.

PHYLLOXERA CLONAL LINEAGES COLLECTION

Output: *Phylloxera clonal lineages collection maintained*

Summary

A core collection of six endemic root galling phylloxera strains was collected, maintained and utilised in a range of experiments (Sections 4-12, this report) during the course of the project. In addition, 'newly' detected less common strains were added to the collection. A total of six less common endemic strains were detected during the project and these have been maintained as a supplementary collection until further evaluation is conducted. Maintenance of a diverse clonal lineage collection of grapevine phylloxera underpins any future research activities particularly in the areas of early detection, alternative management, rootstock screening and validation of quarantine protocol.

Background

Eighty-three phylloxera genetic strains have so far been described in Australia (Umina et al, 2007). These endemic strains vary in both their genetic background and biological traits. Studies to date have shown that it is important to maintain selected endemic strains for experimental studies; particularly focusing on rootstock screening, detection, alternative control and validation of disinfestation protocols. Previously, six phylloxera strains were prioritised (Powell, 2012) for continued maintenance for this project and associated collaborative projects.

Material and Methods

Six phylloxera strains, G1, G4, G7, G19, G20 and G30 were collected from ungrafted *V. vinifera* in commercial vineyards located in Central and North East Victoria, Australia. The insects were genotyped using six nuclear DNA microsatellite markers as described in Umina et al. (2007). G1 was collected from the Yarra Valley, Central Victoria in the Maroondah PIZ and all other genetic strains were sourced from viticulture regions within the North East PIZ in Victoria as follows: G4 from the King Valley, G7, G19 and G30 from Rutherglen and G20 from the Buckland Valley.

All phylloxera strains were reared *in vitro* at Agriculture Research Victoria, Rutherglen, Australia, in a dark controlled environment growth room (25±2°C) under quarantine conditions. Insect stock cultures were maintained on excised *V. vinifera* cv. Chardonnay roots in 150 x 25mm petri dishes (Kingston 2007). The end of the roots were wrapped in cotton wool and moistened once every week with sterile water to sustain them for the provision of the insects' nutritional requirements. Where fungal growth was observed, the roots were cleaned by carefully wiping the contaminated root section with a sable-haired paintbrush moistened in 80% ethanol. Back up stock populations were also maintained on *V. vinifera* cv. Chardonnay reared under glasshouse conditions (see Powell, 2012).

Results

All six phylloxera genetic strains G1, G4, G7, G19, G20 and G30 were successfully established, maintained and subsequently multiplied during the course of the project to enable scientific validation of selected phylloxera disinfestation protocols (referred to subsequently in this report). The maintained clonal lineages were also used in other AGWA-funded research projects requiring screening of rootstocks for phylloxera tolerance.

In the third year of the project a number of additional genetic strains were detected in the North East (NE) Victoria PIZ. Preliminary evaluation of the geographic location, life-cycle characteristics and rootstock association of these strains (Table 1) highlighted the need for their inclusion in the existing phylloxera clonal lineage collection. Two of the strains were observed causing galls on roots and leaves of *Vitis* rootstocks (Figure 1). All six additional genetic strains are currently being maintained, under minimal conditions, since their original detection. Some preliminary data on the effectiveness of disinfestation treatments against one of the strains, G38, is highlighted in this report (Appendix 1). The detection of these strains is indicative of (i) changes in the distribution range of some genetic strains (ii) potential change in the life history traits of some genetic strains which highlights that further rootstock screening may be required using these strains in the future.



Figure 1. Leaf galls observed on *Vitis* rootstock leaves in the King Valley, NE Victoria in April 2017.

Table 1. Summary of new phylloxera strains detected in the North East Victoria Phylloxera Infested Zone (2015-2017).

Phylloxera genetic strain*	Date detected	Location detected	Characteristics	Comments
G38	November 2015	Whorouly, NE Victoria	Detected on roots of 10 rootstock types. 101-14 particularly susceptible	Root galling
A	April 2017	King Valley, NE Victoria	Detected on leaves and roots of rootstock	Leaf and root galling
B	April 2017	King Valley, NE Victoria	Detected on leaves and roots of rootstock	Leaf and root galling
C	April 2017	King Valley, NE Victoria	Detected on rootstock	Root galling
D	April 2017	King Valley, NE Victoria	Detected on rootstock	Root galling
E	April 2017	King Valley, NE Victoria	Detected on rootstock	Root galling

*further genetic characterisation of genetic strains A-E is being undertaken

Discussion

Maintenance of a diverse clonal lineage collection of grapevine phylloxera underpins any future research activities particularly in the areas of early detection, alternative management, rootstock screening and quarantine protocol validation. A core collection containing a minimum of six endemic phylloxera strains needs to be maintained, and supplementary additions to this core collection should be considered when 'new' detections are discovered.

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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.

SECTION 2.

MEMORANDUM OF UNDERSTANDING BETWEEN DEDJTR AND AGWA FOR RESEARCH RELATED ACCESS TO PHYLLOXERA COLLECTION

Output: ***Memorandum of understanding between AGWA and DEDJTR for research-related access to phylloxera collection***

Summary

The genetic clonal collection of grapevine lineages is a fundamental research collection, which is currently maintained under quarantine conditions by Agriculture Victoria at their research facilities located in Rutherglen in the North East Victoria Phylloxera Infested Zone (PIZ). A Memorandum of Understanding (Appendix 2) has been formalised between DEDJTR and AGWA that allows access to this collection for research purposes.

The MOU highlights conditions relating to:

- Procedure for access to the Phylloxera Collection
- Requirements for Compliance with quarantine policies
- Costs associated with access to the collection
- Suspension or revocation of access to Phylloxera Collection

SECTION 3. PHYLLOXERA RISK ANALYSIS

Output: *Phylloxera risk analysis paper*

Summary

A desktop study to review the key phylloxera risk vectors, pathways and knowledge gaps has been completed. A draft review paper, entitled **A Scientific Basis for Risk Analysis of Grapevine Phylloxera Daktulosphaira vitifoliae Fitch,* gathered information sourced from international and national scientific papers, industry reports and grey literature. All relevant phylloxera literature sourced was compiled into a bibliographic database of over 3000 references and selected references, including three review chapters from the Project Leader (Powell 2008, Powell 2012, Powell et al. 2013). The review focused on biotic and abiotic factors which influence the risk of entry, potential establishment and spread of phylloxera through both natural dispersal and human-assisted dispersal associated with common viticultural practices. It describes (i) the life-cycle and genetics (ii) the genetic diversity (iii) geographic distribution (iv) human-assisted risk vectors (risk pathways) (v) rootstocks and genetic strains (vi) environmental considerations and (vii) risk reduction options. It also highlights some of the knowledge gaps which, once addressed, would play a significant role in minimising Phylloxera risk to the Australian viticulture industry. This review also provides up-to-date scientific knowledge that will inform the development of future Import Risk Analysis and Pest Risk Analysis documentation and research priorities for grapevine phylloxera. A copy of the draft paper was circulated to Biosecurity Victoria staff for internal review prior to submission to the Victorian Viticulture Biosecurity Committee for endorsement in September 2015.

*The document is presented in its entirety as Appendix 3 in this report.

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SECTION 4.

EFFICACY OF SODIUM HYPOCHLORITE AS A DISINFESTATION TREATMENT FOR GRAPEVINE PHYLLOXERA ON FOOTWEAR AND HAND TOOLS

Output: ***Data set demonstrating effectiveness of sodium hypochlorite for phylloxera disinfestation of footwear and hand tools***

Summary

Procedure H is the recommended disinfestation protocol (NPMP; National Vine Health Steering Committee, 2009) for the movement of vineyard visitors (particularly in relation to footwear and hand tools) out of a PIZ or PRZ. Recommendations for footwear disinfestation in this procedure are to *"dip and scrub boots in 2% active sodium hypochlorite (NaOCl) for 30 seconds and rinse thoroughly in clean water after immersion*". The efficacy of this protocol was assessed under laboratory conditions against six endemic phylloxera strains. The results of the trial indicated that the current protocol is ineffective against all the strains tested and a recommendation to modify the protocol is provided.

Background

Grape phylloxera usually spreads from vineyard to vineyard via human pathways, including footwear, clothing, grape picking bins, machinery and equipment, soil, grapes, grape foliage and planting material (Deretic et al. 2003). The first instar nymph is the most dispersive life stage, as this can be present both below- (throughout the season) and above-ground (spring to autumn), and is relatively abundant and more active compared to other life stages (Powell et al. 2013). Strict quarantine procedures restricting movement of people, equipment, soil and other potentially infested materials between vineyards are in force and phylloxera has primarily been contained within a few geographic regions in Victoria and NSW. Quarantine protocols involve thoroughly disinfesting and cleaning all items that come into contact with infested vineyards to minimise the risk of spread to non-infested vineyards. Under the Plant Health and Plant Products Act 1995 (National Vine Health Steering Committee 2009), the Australian government has declared specific areas as Phylloxera Infested Zones (PIZ) where phylloxera is present.

National Phylloxera Management Protocols are designed to restrict the movement and spread of phylloxera out of either a PIZ or a Phylloxera Risk Zone (PRZ) and prevents entry of potentially infested material into a Phylloxera Exclusion Zones (PEZ) where phylloxera is

absent (National Vine Health Steering Committee 2009). The risk that vineyard personnel and visitors inadvertently transfer phylloxera on their footwear is particularly high between September and May in Australia (Powell et al. 2003), when first instars are likely to be present both on the soil surface and in the grapevine canopy. For footwear, a disinfestation protocol of dipping footwear in a freshly prepared household bleach and water solution containing a 2% sodium hypochlorite (NaOCl) for a minimum contact time of 30 s is currently recommended (National Vine Health Steering Committee 2009). The protocol recommends a thorough rinse in clean water post treatment so as to avoid damage of footwear and eliminate the bleach odour (National Vine Health Steering Committee 2009). Small hand tools are disinfested in the same manner.

The efficacy of NaOCl, the active ingredient of household bleach, against insects and nematodes has previously been demonstrated in laboratory and field studies. Grzegorczyk and Walker (1997) prevented microbial contamination in *in vitro* cultures of grape phylloxera eggs on roots of *Vitis* species using 5.25% NaOCl. The aforementioned study, however, showed that bleach treatments required rinsing with sterile water to encourage hatching of eggs. Studies on phylloxera disinfestation by Dunstone et al. (2003) demonstrated a dose response to NaOCl in the mortality of first instars using a phylloxera population of undetermined genetic status sourced from Victoria. In that study, it was unclear if a rinse treatment was applied, and 2% NaOCl treatments achieved 100% first instar mortality after immersion for 30 s.

In the last decade there has also been an improved characterisation of the genetic diversity of endemic phylloxera strains. Eighty-three genetic strains are known to be present in Australia (Umina et al. 2007). Thus, a validation of disinfestation protocols for endemic phylloxera strains is considered a high priority. In this study, we focused on the disinfestation protocol currently recommended for footwear and hand tools (National Vine Health Steering Committee 2009). We investigated the survival of first instars of six phylloxera strains, G1, G4, G7, G19, G20 and G30, following immersion in four concentrations of NaOCl for 30, 40 and 60 s. Our specific objectives were to: (i) identify the optimal NaOCl concentration and duration required to achieve 100% first instar mortality for all six genetic phylloxera strains; (ii) investigate the effect of NaOCl treatments on first instars ability to establish feeding sites, develop into adults and reproduce; and (iii) establish the interaction between first instar survival and a water rinse treatment following NaOCl treatments.

Materials and Methods

Collection, characterisation and maintenance of phylloxera strains

Six phylloxera strains, G1, G4, G7, G19, G20 and G30 were mass-reared in vitro at Agriculture Victoria, Rutherglen, Vic., Australia, in a dark controlled environment growth room (25±2°C) under quarantine conditions. Insect stock cultures were maintained on excised *V. vinifera* cv. Chardonnay roots in 90 x 25 mm petri dishes (Kingston et al. 2007). The end of the roots were wrapped in cotton wool and moistened once every week with sterile water to keep them viable for the insects nutritional requirements. Where fungal growth was evident, the roots were cleaned by dabbing the contaminated root section with a sable-haired paintbrush slightly moistened in 80% ethanol. Eggs aged 1-4 days old were collected using an artist's paintbrush and gently placed on moistened filter paper in a 35 mm petri dish. The petri dishes were sealed with cling film (Rapfast PVC food packaging, Integrated Packaging, Australia) to create a hatching chamber and incubated in the dark at a constant temperature of 22°C. Eggs were monitored daily until hatching. Newly emerged active first instars were collected and used for all experiments.

Sodium hypochlorite as a disinfestation treatment for six phylloxera genetic strains

The effectiveness of household bleach (White King, Pental, Melbourne, Vic., Australia; active ingredient: NaOCl 42 g/L (available chlorine 4.0% m/v); sodium hydroxide 9 g/L) was investigated against six phylloxera genetic strains. Bleach was diluted in tap water to obtain NaOCl concentrations required for different treatments.

The current recommended disinfestation protocol for footwear and hand-held tools requires immersing in 2% NaOCl solution for 30 s followed by a thorough rinse in water (National Vine Health Steering Committee 2009). Thus, the effectiveness of this disinfestation protocol was validated by immersing first instars phylloxera from six genetic strains, G1, G4, G7, G19, G20 and G30, in treatments containing 2% NaOCl for 30 s followed by a 30 s water rinse to reflect the current recommended disinfestation protocol (National Vine Health Steering Committee 2009).

To examine the effect of time of immersion on survival, first instars were also treated in 2% NaOCl for a 40 and 60 s followed by a 30 s water rinse. To investigate the effect of a water-rinse following NaOCl treatment on first instars survival, insects were subjected to 2% NaOCl for 30, 40 and 60 s but a post-treatment water-rinse was omitted. The effect of increasing NaOCl concentration and time of immersion was in addition investigated by subjecting first instars to 3% and 4% NaOCl for 30, 40 and 60 s followed by a 30 s rinse in water. First instars from the six genetic strains were also subjected to a water control (0% NaOCl) treatment for 30, 40 and 60 s.

The experiments were conducted by placing newly emerged first instars in a plastic vial (5.5 cm high, 2.5 cm diameter) that was sealed with a screw top lid with a metal mesh (53 µm aperture) at the top and bottom ends (Korosi et al. 2012). The vial was immersed in the respective NaOCl solutions prepared to a volume of 500 mL in a beaker. Each treatment was conducted in separate trials with five replications of ten insects per replicate from each of the six genetic strains. After treatment, first instars were removed from the vials using a sable-haired paintbrush that was moistened in water, placed onto a filter paper in a petri dish and examined under low power microscope. Where there was observable movement or if the antenna and legs moved when stimulated with the tip of the paintbrush, the first instars were classified as surviving the treatment. Where there was no apparent movement 2 h after treatment, first instars were scored dead. The total number of first instars recovered from the vials and those that were missing was recorded.

Effect of NaOCl treatments on feeding, development and reproduction on phylloxera

To examine the effect of NaOCl on feeding, development to adults and reproduction, first instars that survived treatments were placed on excised *V. vinifera* roots. First instars from the three immersion durations (30, 40, 60 s) were pooled and placed on a single separate root piece in a 90 x 25 mm petri dish for each NaOCl concentration (i.e. 0, 2, 3, 4%) due to low numbers of insects (<30) surviving the three time treatments. Root pieces in the bioassay plates were checked once every week following treatments, and first instars that died on the roots and those that established feeding sites were counted and recorded. Dead first instars were characterised by a dark brown colour and lack of movement within 3 days post treatment. Root pieces were thereafter cleaned and checked weekly and first instars that developed to adulthood and laid eggs were counted.

Experimental design

Experiments were conducted under controlled laboratory conditions at a set temperature of 22^{o}C. Experiments consisted of two treatment factors: (i) NaOCl at five concentration values, i.e. 0% NaOCl water control, 2% NaOCl rinsed in water for 30 s after immersion (R), 2% NaOCl not-rinsed (NR) in water after immersion, 3% NaOCl (R) and 4% NaOCl (R); and (ii) time (s) at three levels; 30, 40, 60. Each experiment implemented a randomised complete block design (RCBD) with full factorial combination of NaOCl and time with five replications each. The blocking factor was replication.

Statistical analysis

Percentage first instar phylloxera survival was calculated as the number of insects surviving treatments out of the total recovered from vials multiplied by 100. Percentage survival data was subjected to ANOVA. To compare survival in different NaOCl concentration values (2, 3, and 4%) and to compare with the control treatments, liquid (NaOCl and water) were treated in factorial combination with a genetic strain by time interaction. This treatment structure was coded in GenStat as NaOCl*Time*Genetic strain with replication as the blocking factor.

The effect of post-treatment water rinse on first instar survival was only assessed for the 2% NaOCl treatments using the treatment structure 'rinse' (Yes and No) with genetic strain and time as factors. A contrast analysis, where contrasts were constructed using the treatment factors (NaOCl, time and genetic strain) was used to compare and validate the effectiveness of the recommended disinfestation protocol for footwear and small tools between the six genetic strains for 2% NaOCl treatments.

The proportion of first instars that survived treatments, established feeding sites, developed to adulthood and subsequently laid eggs was analysed using a general ANOVA with NaOCl concentration as the treatment factor and proportion of dead crawlers, those that established feeding sites and reproduced as the response variables.

Residual values were examined graphically to ensure normality and homogeneity of variances. Observations with standardised residuals greater than 3.0 were excluded from analyses. Fishers protected LSD test (P<0.05) were used to separate means where F-tests were significant. All data were analysed using GenStat 17 software (GenStat Release 16, VSN International, Hemel Hempstead, England).

Results

Effectiveness of 2% NaOCl disinfestation treatment on six phylloxera genetic strains

When subjected to the recommended treatment of 2% NaOCl for 30 s followed by a water rinse, a mean survival of 71% (± 1.6) was observed for first instars across all the six genetic strains (Table 1). Compared to the water control treatments (0% NaOCl) for 30 s, an average of 95% first instars survived treatment (Table 1).

First instars from G1 and G4 genetic strains were most susceptible to the 2% NaOCl treatment for 30 s compared to G7, G19, G20 and G30 (Table 2). Increasing the time of immersion in 2% NaOCl from 30 to 40 s followed by a water rinse impacted on survival of first instars (Table 1) with a significant decrease in survival for G4, G7, G19, G20 and G30 first instars (P<0.05) but not G1 (P=0.156) (Table 2). When immersion time in 2% NaOCl solution followed by a water rinse was increased from 30 to 60 s, first instar survival decreased significantly (P<0.01)

across all six genetic strains (Table 1) with the treatment impacting significantly on G1 and G4 first instars which had the least mean survival (Table 2). First instars from G7 and G20 strains were most resistant to the 2% treatment at the higher immersion time while G19 and G30 strains were moderately susceptible (Table 2).

Table 1. Effect of sodium hypochlorite treatments at four levels of concentrations (NaOCl [C]),water control (0% NaOCl), 2% with no rinse (2NR), 2% with rinse (2R), 3% with rinse (3R) and 4% with rinse (4R)) and three levels of time (30, 40, 60 s) on percentage mean survival of first instars of six phylloxera genetic strains, G1, G4, G7, G19, G20 and G30.

Treatments		**Phylloxera genetic strains**					
Time (seconds)	**NaOCl [C]**	***G1***	***G4***	***G7***	***G19***	***G20***	***G30***
30	0	98	95	95	97	95	95
	2NR	2	2	5	10	14	4
	2R	65	62	75	73	76	78
	3R	61	27	40	72	68	67
	4R	17	15	32	69	64	62
40	0	96	87	93	98	98	98
	2NR	0	0	2	4	7	0
	2R	57	35	64	58	64	58
	3R	31	19	32	49	53	59
	4R	4	14	28	33	48	50
60	0	92	88	92	93	98	95
	2NR	0	0	0	0	0	0
	2R	28	26	51	36	63	38
	3R	25	14	29	30	44	35
	4R	2	0	24	30	35	23
Standard Error		4.8					
F test probabilities							
Genetic strains (G)		<0.001					
NaOCl ([C])		<0.001					
Time (T)		<0.001					
Interaction (G x [c] x T)		<0.001					

Table 2. A summary of six main contrasts showing mean differences in the survival of first instars from six phylloxera strains when subjected to 2% NaOCl solution either followed by a water rinse or without a water rinse.

Contrasts	G1	G4	G7	G19	G20	G30
2R30 vs 2R40						
Estimate	7	27	11	14	12	20
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	0.156	<0.001	0.043	0.006	0.015	<0.001
2R30 vs 2R60						
Estimate	36	36	24	37	13	40
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	<0.001	<0.001	<0.001	<0.001	0.009	<0.001
2R40 vs 2R60						
Estimate	29	9	14	22	1	20
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	<0.001	0.024	0.011	<0.001	0.850	<0.001
2R30 vs 2NR30						
Estimate	62	60	70	62	61	74
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2R30 vs 2NR40						
Estimate	65	62	73	68	68	78
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2R30 vs 2NR60						
Estimate	50	41	63	56	67	58
SE	4.2	3.3	4.2	4.1	3.8	3.9
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Estimated value is the difference between the respective treatments, 2% NaOCl solution and time of immersion 30, 40 and 60 s of first instars across the six genetic strains. R, water rinse for 30 s after immersion; NR, treatments where a water rinse after immersion in NaOCl solutions was omitted. SE= Standard Error.

Effect of water-rinsing following 2% NaOCl treatments on survival of first instars from six phylloxera genetic strains

Omitting a water rinse after immersion in 2% NaOCl resulted in a significant impact on survival of phylloxera first instars with a significant genetic strain and time interaction (*P*<0.001) (Table 1).

When immersed in 2% NaOCl for 30 s without a water rinse afterwards, at least 2% (range 2–5%) first instars from G1, G4, G7 and G30 genetic strains survived treatments compared to 10 and 14% of G19 and G20, respectively (Table 1). In contrast, an average of 68% (range 61–78%) of first instars treated in 2% NaOCl for 30 s followed by a water rinse survived treatments (Table 2).

When immersion time in 2% NaOCl without a water rinse afterwards was increased from 30 to 40 s, none of the first instars from G1, G4 and G30 genetic strains survived the treatment compared to a survival of 2, 4 and 7% for G7, G19 and G20 first instars, respectively (Table 1). In contrast, 56% (range 35–64%) of the first instars immersed in 2% NaOCl for 40 s followed by a water rinse, survived the treatment (Table 1).

When subjected to 2% NaOCl for 60 s without a water rinse afterwards, none of the first instars across the six genetic strains survived the treatment (Table 1). In contrast, an average of 40% (range 28–63%) first instars survived in 2% NaOCl when immersed for 60 s with a water rinse afterwards (Table 1).

Survival of first instars phylloxera in 3% and 4% NaOCl

Survival of first instars immersed in 3 and 4% NaOCl solutions followed by a water rinse varied with a significant interaction between the six phylloxera genetic strains, NaOCl concentration and the time insects were exposed to treatments ($P < 0.001$) (Table 1).

When immersed for 30 s in 3% NaOCl, survival of first instars from G4 and G7 genetic strains was 27 and 40%, respectively, and significantly lower than that of first instars from G1, G19, G20 and G30 strains where survival was greater than 61% (range 61–72%) (Table 1). When immersed for 30 s in 4% NaOCl, first instar survival for G1 and G4 genetic strains was 17% and 15%, respectively, and was significantly lower than that of first instars from G7, G19, G20 and G30 where survival was greater than 32% (range 32–69%) (Table 1).

When immersed for 40 s in 3% NaOCl, survival of G1, G4 and G7 first instars ranged between 19–32% and was significantly lower than that of first instars from G19, G20 and G30 genetic strains where survival ranged between 49–59% (Table 1). When immersed for 40 s in 4% NaOCl, G1 and G4 first instars were most susceptible with a significantly low survival of 4 and 14%, respectively. First instars from G20 and G30 genetic strains were least susceptible with 48 and 50%, respectively, surviving the treatment (Table 1). First instars from G7 and G19 were moderately susceptible to 4% NaOCl when immersed for 40 s with 28 and 33%, respectively, surviving the treatment (Table 1).

When immersed for 60 s in 3% NaOCl, G4 first instars were most susceptible with 14% surviving treatments compared to G1, G7, G19, G20 and G30 where survival ranged from 25–44% (Table 1). With immersion time of 60 s in 4% NaOCl, only G4 first instars did not survive the treatment (Table 1). First instars from G1 genetic strain were also highly susceptible when immersed in 4% NaOCl for 60 s with only 2% surviving the treatment (Table 1) compared to G7, G19, G20 and G30 phylloxera which were least susceptible with survival of 28% (range 23-35%) (Table 1).

Compared to the water control (0% NaOCl) where at least 87% of first instars survived treatments, survival of first instars in 3 and 4% was much lower with an average of 36% insects across the six genetic strains and combined time of immersion surviving the latter treatments (Table 1).

Effect of immersion time in NaOCl treatments on survival of grape phylloxera

Survival of first instars in NaOCl treatments that were followed by a water rinse was significantly reduced the longer the insects were exposed to treatments (for the combined four NaOCl concentration values combined) (P<.001) (Figure 1a). Across all genetic strains, a mean of 45% (±2.9) first instars survived treatments when immersed for 60 s, compared to 55% (±2.6) and 66% (±2.3) that were immersed for 40 and 30 s, respectively (Figure 1a).

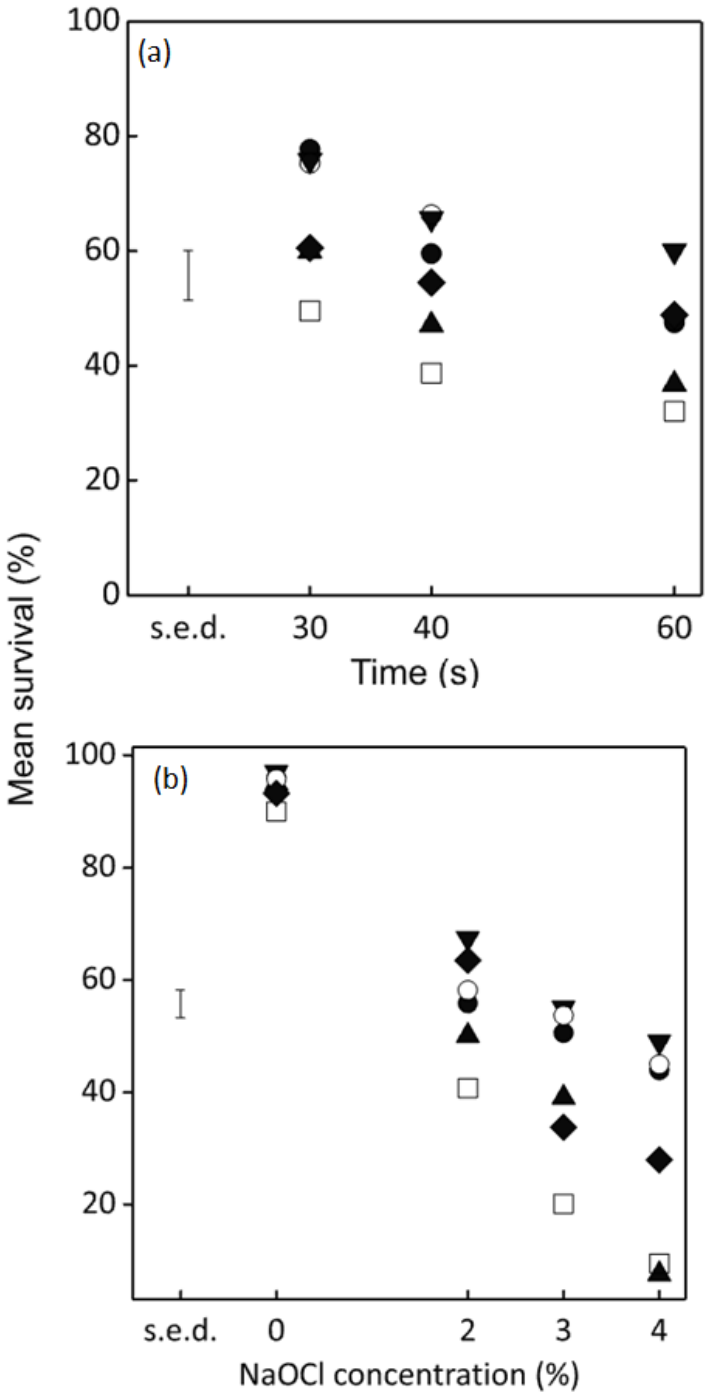


Figure 1. Effect of increasing (a) treatment duration in sodium hypochlorite (NaOCl) solutions and (b) NaOCl concentration, on first instar survival of six grape phylloxera strains, G1 (▲), G4 (□), G7 (♦), G19 (●), G20 (▼) and G30 (○). Data presented are means of first instars combined for the three concentration values and three immersion duration times for treatments that were followed by a 30s water rinse. s.e.d, standard error of difference between means.

Effect of NaOCl concentration on survival of grape phylloxera

Survival of first instars in NaOCl treatments that were followed by a water rinse was high with the water control (0% NaOCl) and the lowest NaOCl concentration (2%) used (P<0.001) (Figure 1b). Survival of first instars markedly reduced as the NaOCl concentration increased (Figure 1b). With the three immersion durations combined across the six genetic strains, 95% (± 0.7) first instars survived in the water control (0% NaOCl) compared with 55% (± 1.9) in 2% NaOCl. A mean of 42% (±2.0) and 31% (±2.3) of first instars treated in 3 and 4% NaOCl, respectively, survived treatments (Figure 1b).

Establishment and development of phylloxera strains post – NaOCl treatments

First instars that survived NaOCl treatments and were placed on roots established feeding sites and subsequently laid eggs, with varying degrees of success between the four NaOCl concentration values. There were no significant trends between the six genetic strains (P>0.05) (data not shown). Overall, NaOCl treatments negatively impacted on first instars survival on excised roots with an average of 38% (range 23-49%) establishing feeding sites compared to 78% that were subjected to water control treatments (Table 3). Of first instars that survived treatments, 4% (range 4-26%) died on excised roots before establishing feeding sites and this pre-mature mortality significantly differed between the NaOCl concentration treatments (P=0.03). Of those immersed in 2 and 3% NaOCl, 8% prematurely died before establishing feeding sites, but these were not statistically different from individuals that survived the water control treatments (Table 3). With first instars that survived 4% NaOCl treatment, 26% that were placed on roots died before establishing feeding sites (Table 3). When treated in 2 and 3% NaOCl, 43 and 49% first instars established feeding sites compared to 23% that survived in 4% NaOCl (Table 3). Of the first instars that established feeding sites, none of those treated in 4% NaOCl reproduced. However, 19 and 10% first instars that were treated in 2% and 3% NaOCl, respectively, laid eggs (Table 3).

Table 3. Effect of sodium hypochlorite treatments on the establishment and development of grape phylloxera.

NaOCl (%)	No. of first instars placed on roots	Proportion of first instars dead (%)	Proportion of first instars that established feeding sites (%)	Proportion of first instars that laid eggs (%)
0	163	4 a	78 c	60 d
2	141	8 a	49 b	19 c
3	57	8 a	43 b	10 b
4	55	26 b	23 a	0 a
df		20	20	20
P		0.03	<0.001	<0.001
SE		7.3	1.4	4.2

There were no statistical differences between the genetic strains, hence data presented are means (± SE) pooled across the six genetic strains. NaOCl, sodium hypochlorite. Values followed by the same letter are not significantly different (Fishers protected LSD test $P<0.05$)

Conclusions

Sodium hypochlorite has been used in numerous industrial applications and plays an important role in disinfestation of a variety of insect pests (Grzegorczyk and Walker 1997, Jacups et al. 2013, Mackay et al. 2015). Household bleach is routinely used for disinfestation of footwear and small tools in vineyards against grape phylloxera (National Vine Health Steering Committee 2009). The current study represents a systematic comparison of six phylloxera genetic strains and their relative susceptibility to treatments in sodium hypochlorite, the active ingredient of household bleach. The overall aims of this study were: (i) to validate the recommendation of dipping footwear and hand held tools in 2% NaOCl for 30 s followed by a water rinse as a disinfestation protocol for use in vineyards (National Vine Health Steering Committee 2009); (ii) to determine the optimal concentration of NaOCl and minimum exposure period required to achieve 100% mortality across all the six genetically diverse phylloxera strains selected; and (iii) to investigate the effect of omitting a water rinse on phylloxera survival. Results showed that more than 50% of first instars across the six genetic strains survived after 30 seconds immersion in 2% NaOCl followed by a 30 s rinse in water. These findings differ from a previous study by Dunstone et al. (2003) where 100% mortality was achieved when first instars were immersed in 2% NaOCl treatments at a minimum contact time of 30 s. The genetic strain of phylloxera used in the previous study was, however, not provided and it is also unclear if a water rinse was included in the treatments.

Results from this study further showed that increasing contact time and NaOCl concentration followed by a water rinse reduced first instar survival, but did not achieve 100% mortality. Survival was directly proportional to the increase in time of immersion as well as the NaOCl concentration and this was consistent across the six genetic strains. Results indicate that a concentration above the recommended 2% NaOCl does not yield satisfactory disinfestation and an exposure time longer than 60 s may be required to achieve close to 100% mortality.

Omitting a water-rinse phase following exposure to 2% NaOCl is a significant result from this study and represents a critical step in the disinfestation process as it significantly reduced first instar survival with differences between the six genetic strains and time of exposure to the treatment. To achieve 100% mortality, immersion for 60 s in 2% NaOCl without the water rinse afterwards is optimal across all genetic strains. This modified treatment has also been proven to be effective against the endemic phylloxera strain G38 (Appendix 1, this report).

When dissolved in water, NaOCl ionises to the sodium ion (Na+) and the hypochlorite ion (ClO-) forming hypochlorous acid (HOCl). Hypochlorous acid is responsible for damaging the microbes' cell membranes, proteins and nucleic acids through an oxidative degradation process upon contact (McDonnell and Russell 1999). Thus, in this study, omitting a water-rinse step allows HOCl more time to penetrate and react with the insect cuticle causing mortality.

The finding that rinsing of footwear and vineyard tools in water after treatment in NaOCl enhances survival is clearly an impedance to phylloxera quarantine management efforts. Different types of footwear and equipment that have crevices where phylloxera may hide could potentially make disinfestation treatments less effective, especially when a lower NaOCl concentration and less time the insects are exposed to treatments are applied. The effect of a water rinse following NaOCl disinfestation treatments is likely compromised when soil, mud as well as other contaminants are trapped on footwear and hand held tools. A possible negative influence of soil and mud is adherence to footwear and tools, thus impeding contact between grape phylloxera and NaOCl. Treatments with NaOCl require more time to penetrate into crevices and soil contaminants where first instars are present, and thus omitting the water-rinse step would markedly increase the contact time with HOCl.

Results from this study showed differences in susceptibility to NaOCl by the six phylloxera strains tested. Differences in susceptibility to disinfestation treatments using heat were observed for the G1 and G4 phylloxera genetic strains (Korosi et al. 2012), suggesting physiochemical and structural differences between strains. A potential variability in the cuticle structure could be attributed to the difference in response to the treatments by the six

phylloxera genetic strains used in this study. Further studies to investigate the cuticular difference between phylloxera genetic strains would be informative in this respect.

Although our study validated the efficacy of NaOCl as an effective disinfestation treatment, the relatively high survival rates at 2% NaOCl concentration followed by a water rinse and the ability of the surviving insects to develop to fecund adults suggests an urgent need to modify the current phylloxera disinfestation protocol. Powell et al. (2003) showed that first instars originating from radicicolae populations are the most abundant life-stage and are the active dispersive stage of phylloxera. Powell et al. (2003) further showed that the first instar populations dispersing at the soil surface are greater than those actively moving along the vine trunk and into the canopy. The risk of transferring first instars through soil particles attached on footwear from one vineyard to another are, therefore, high. More aggressive phylloxera strains (Forneck et al. 2016) would reproduce much faster causing a rapid rise in populations from a few first instars that would be surviving NaOCl treatments. In this study, the proportion of first instars that survived NaOCl treatments and went on to establish feeding sites and reproduce were not robust enough to give a statistical separation that would verify differences in survival between the six genetic strains. Future research focusing on post-chemical treatments may provide further information on the virulence status between different genetic strains.

Whilst immersing first instars in 2% NaOCl without a water rinse was an effective treatment at 60 s in the laboratory, several operational limitations should be considered if this dose and time are to be used as a recommended disinfestation treatment. Household bleach applications may be unsuited in certain situations as direct exposure to NaOCl can cause damage to footwear, hand-held equipment and skin if users come into contact with solutions (Lambert et al. 2000). Furthermore, there can be concerns of exposure to chlorine released into the air and hypochlorite on treated surfaces and thus the usage of NaOCl could compromise occupational health and safety standards (Agar 2006). Despite these concerns, the use of household bleach has its advantages because it is relatively inexpensive, readily available and most people are already familiar with it and more likely to accept its use.

Recommendations

This study highlights the need to recognise that genetic variability between phylloxera strains influences the efficacy of NaOCl as a disinfestation treatment. The findings, therefore, highlight the need to consider the phylloxera genetic background when developing and recommending disinfestation procedures for footwear and small tools in different PIZs. Our results highlight that current disinfestation protocol requires modification by: (i) omitting the water-rinse after

2% NaOCl treatments; and (ii) increasing time of immersion in 2% NaOCl from 30 to 60 s in order to achieve 100% mortality across genetic strains (Table 4). The challenge posed, however, is that of NaOCl odour and residues and subsequent damage to footwear and hand-held tools. There is, therefore, a pressing need to screen alternative disinfestation treatments to NaOCl or the use of surfactants to enhance penetration of NaOCl through soil and other contaminants adhering onto footwear and vineyard tools. Some authors have previously used ethanol as an alternative disinfestation treatment under research conditions for grape phylloxera eggs (Kellow et al. 1999) and first instars (Powell, 2006), but further investigations are required to determine the efficacy of ethanol as well as other potential treatments against different phylloxera strains. Potential alternative disinfestation treatments are discuss later in this report (Section 11).

Table 4. Current disinfestation protocol and suggested modification to sodium hypochlorite disinfestation protocol for movement of vineyard visitors out of a PIZ or PRZ vineyard (NPMP; Procedure H (1, 2)).

Current protocol **(National Vine Health Steering Committee, 2009)**	**Effective disinfestation procedure based on experimental results**
When leaving the vineyard, disinfest footwear as follows: Scrub the boots with the scrubbing brush to remove mud – preferably in water and detergent	
Dilute chlorine with water in a tub to give a 2% active sodium hypochlorite concentration and dip and scrub boots in the freshly prepared solution for a minimum of 30 seconds.	Dilute **household bleach** with water in a tub to give a 2% active sodium hypochlorite concentration and dip and scrub boots in the freshly prepared solution for a minimum of **60** seconds.
Rinse thoroughly in clean water after immersion	**DO NOT** rinse in clean water **immediately** after immersion
Wash and disinfest snips, small tools etc. with a 2% active sodium hypochlorite solution	Wash and disinfest snips, small tools etc. with a 2% active, **freshly prepared,** sodium hypochlorite solution **for a minimum of 60 seconds.**

*Suggested modification in bold – any proposed modification must be ratified by the relevant biosecurity body.

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SECTION 5.

EFFICACY OF HOT WATER AND STEAM TREATMENT AS DISINFESTATION TREATMENTS FOR GRAPEVINE PHYLLOXERA ON MACHINERY AND GRAPE BINS

Output: ***Dataset demonstrating the effectiveness of steam and hot water immersion as a disinfestation treatment for phylloxera on viticulture machinery and equipment.***

Summary

There are currently two recommendations in the National Phylloxera Management Protocol ((NPMP) National Vine Health Steering Committee, 2009) for the disinfestation of vineyard machinery and grape bins using either steam or hot water immersion. Procedure G (NPMP; Movement of vineyard equipment out of a PIZ or PRZ) currently recommends either (a) *that steam be applied above 100℃ as indicated by a jet of clear invisible steam between steam outlet and the visible condensate cloud and steam must contact all surfaces until the surface is left dry, not wet with condensate or (b) fully immerse in water at 70℃ minimum, and hold in water for at least 2 minutes after it has reached 70℃*. Both treatments were tested against six phylloxera strains and resulted in 100% phylloxera mortality. The results demonstrated that whilst the recommended disinfestation procedures were effective, there was potential to modify the procedures and these potential modifications are discussed.

Background

One of the current disinfestation protocols for use against grape phylloxera on vineyard machinery and vehicles is to apply steam above 100ºC as indicated by a jet of visible steam (See the National Phylloxera Management Protocol (NPMP) Procedure G (a) - *Movement of vineyard equipment out of a Phylloxera Infested Zone (PIZ) or a Phylloxera Risk Zone (PRZ))* (National Vine Health Steering Committee, 2009). Another heat-based protocol is to immerse grape harvesting bins as per Procedure G (b) *Movement of vineyard equipment out of a Phylloxera Infested Zone (PIZ) or a Phylloxera Risk Zone (PRZ))* (National Vine Health Steering Committee, 2009) in hot water treatment at 70ºC for 120 s (National Vine Health Steering Committee, 2009). There is no published data demonstrating the efficacy of these techniques against phylloxera, although the treatments are used widely to disinfest against other pests. Steam, applied as heat in a vapour state, is routinely used in nurseries and greenhouse crop production systems to control plant pathogens, insect pests and weeds.

Steam treatment is widely used to disinfest insect pests in fruits and vegetables (Shellie and Mangan 2000, Neven et al. 2012, Samtani et al. 2012). Hot water immersion is also effective, practical and relatively inexpensive to disinfest many arthropods of quarantine importance (Graham 2007, EPPO 2009, Gramaje et al. 2013). Hot water treatment (HWT) is also effective in managing a number of important pests and pathogens on grapevine propagating and planting material including seeds, roots and also harvesting and storage equipment.

Materials and Methods

Collection, characterization and maintenance of phylloxera strains

Six phylloxera strains, G1, G4, G7, G19, G20 and G30 were collected from ungrafted *V. vinifera* in commercial vineyards located in Central and North East Victoria, Australia. The insects were genotyped using six nuclear DNA microsatellite markers as described in Umina (et al. 2007). G1 was collected from the Yarra Valley, Central Victoria in the Maroondah PIZ and all other genetic strains were sourced from North East PIZ in Victoria, G4 from the King Valley, G7, G19 and G30 from Rutherglen and G20 from the Buckland Valley.

All phylloxera strains were reared *in vitro* at Agriculture Victoria, Rutherglen, Australia, in a dark controlled environment growth room (25±2°C) under quarantine conditions. Insect stock cultures were maintained on excised *V. vinifera* cv. Chardonnay roots in 150 x 25mm petri dishes (Kingston 2007). The end of the roots were wrapped in cotton wool and moistened once every week with sterile water to sustain them for the insects' nutritional requirements. Where fungal growth was observed, the roots were cleaned by carefully wiping the contaminated root section with a sable-haired paintbrush moistened in 80% ethanol. Using a fine artist's paintbrush moistened in sterile distilled water, 1-4 days old eggs were collected and gently placed on moistened filter paper in a 90 x 25mm dish. The petri dishes were sealed with cling film (Rapfast PVC food packaging, Integrated Packaging, Australia) to create a hatching chamber and incubated in the dark at a constant temperature of 22°C. Eggs were monitored daily and newly emerged active first instars were collected and used for all experiments.

Pressurised steam treatments

Experiments consisted of three treatments factors (i) distance from which steam was projected at two levels: 8 (Figure 1a) and 28 cm (Figure 1b) (ii) treatment duration at three levels, 10, 20, 30 seconds, and (iii) six phylloxera strains, G1, G4, G7, G19, G20 and G30. Each experiment employed a randomised complete block design (RCBD) with a full factorial combination of temperature, time and genetic strains. To determine the effect of increasing

the distance from which steam was projected on steam disinfestation efficiency, a plastic funnel with a height of 92 cm (Figure 1c) was used to project steam for 10, 20 and 30 s, but for G1 phylloxera only. The treatments were replicated five times with 10 insects each per replication. A triple action steam cleaner (H20 SteamFX™ Model No. KB-000A, Thane International, Inc. China) was used to release steam under pressure (Figure 1a). A nozzle was attached at the narrow end of each plastic funnel while the wide end was placed over a filter paper (70mm in diameter) on a petri dish (90 x 25mm) where first instars were placed (Figure 1a). The steam unit was preheated for 30 s to allow steam to be emitted steadily before subjecting the insects to treatments. A mercury thermometer and a Gemini data logger (Tiny Tag Explorer™, Hastings Data Loggers, Australia) monitored the surface temperature of the petri dish where first instars were placed. First instars were examined immediately after treatments and classified alive if there was observable leg and/or antennal movement and dead if there was no apparent movement when gently manipulated with the tip of the paint brush up to 2 h after treatment. Counts of live or dead first instars were recorded for each treatment.

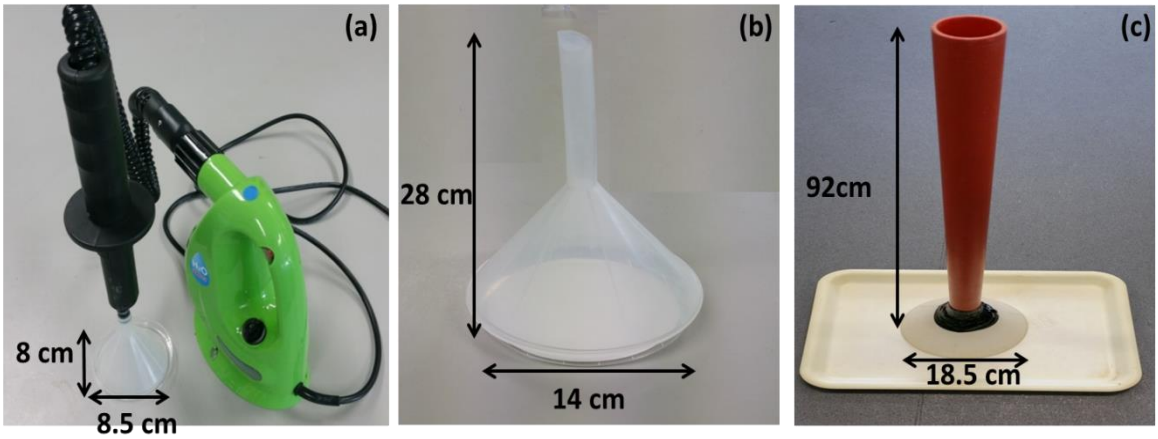


Figure 1. Experimental set up with a triple action steam cleaner (a) (H20 SteamFXTM Model No. KB-000A, Thane International, Inc. China) that was used to release steam under pressure from a distance of (a) 8 (b) 28 and (c) 92cm.

Hot water immersion treatments

The experiment consisted of three treatment factors (i) water set at six temperature levels 22°C (room temperature), 40, 45, 50, 60 and 70°C (ii) treatment duration at two levels, either 60 or 120 s, and (iii) six phylloxera strains, G1, G4, G7, G19, G20 and G30. For each Temperature x Time x Genetic strain combination, 10 insects from each genetic strain were

placed in a plastic vial (5.5 cm high, 2.5 cm diameter) that was sealed with a screw top lid made up of a metal mesh (53µm aperture) at the top and bottom ends (Korosi et al. 2012). The vial was immersed a water bath (SB-35 EYELA Tokyo Rikakikai Co. Ltd) at the respective temperature for either 60 or 120 s. The treatments were replicated five times in a randomised complete block design (RCBD) with temperature, time and genetic strain in full factorial combinations.

First instars were removed from the vials using a fine artist's paintbrush soon after treatments, placed onto a damp filter paper and examined under a low magnification microscope. First instars were classified alive if there was observable leg and/or antennal movement and dead if there was no apparent movement when gently manipulated with the tip of the paint brush up to 2 h after treatment. First instars were counted and recorded as either dead or alive for each treatment vial.

Establishment and development of phylloxera after hot water treatment

First instars that survived HWTs were placed on excised *V. vinifera* Chardonnay roots to examine the effect of heat treatment on establishment of feeding sites, development to adults and subsequent reproduction. First instars that survived from hot water treatments at the two immersion periods (60 and 120 s) were pooled onto a single root piece in a 90 x 25 mm petri dish for each temperature (i.e. 40 or 45^{o}C). Bioassay root pieces were examined three days post-treatment and thereafter, checked weekly. First instars that died on the roots post treatments, those that established feeding sites, developed to adults and laid eggs were counted and recorded.

Statistical analysis

No statistical analysis was required for the steam experiments as all treatments were 100% effective and therefore all survival data were zero.

With respect to the HWT trial the percentage first instar survival, calculated as numbers of insects surviving treatments out of the total recovered from treatment vials multiplied by 100, was subjected to an Analysis of Variance (ANOVA) in GenStat (GenStat Release 16, 2015). To compare survival, temperature was treated in a factorial combination with genetic strain by time interaction. Treatment structure was coded in GenStat as temperature*time*genetic strain, with replication as the blocking factor.

Percentage of first instars that survived treatments, established feeding sites, developed to adults and subsequently laid eggs was analysed using ANOVA (unbalanced design using regression) with temperature as the treatment factor. Percentages of dead first instars, those

that established feeding sites and oviposited were the response variables. Residual values were graphically examined to ensure normality and homogeneity of variances. Observations with standardised residuals greater than 3.0 were excluded from analyses. Fishers protected Least Significant Difference (LSD) test ($p<0.05$) were used to separate means where F-tests were significant.

Results

Pressurised steam treatments

All steam treatments, regardless of duration and distance, resulted in 100% first instar mortality (N=1800). No survival was observed when steam was projected from a distance of 24cm for 20 and 30 s, across the six genetic strains, and at 92cm for G1 phylloxera. Similarly, 100% of first instars across the six genetic strains did not survive treatments when subjected to steam from a minimum distance of 8 cm and exposure period of 10 s. Preliminary experiments showed that maximum temperatures of 102, 98 and 90°C were reached when steam was projected at a distance of 8, 24 and 92 cm, respectively, after a minimum exposure period of 10 s (Figure 2). This highest temperature was reached approximately 4 s after steam exposure and was maintained for the longest duration until treatments were completed (Figure 2).

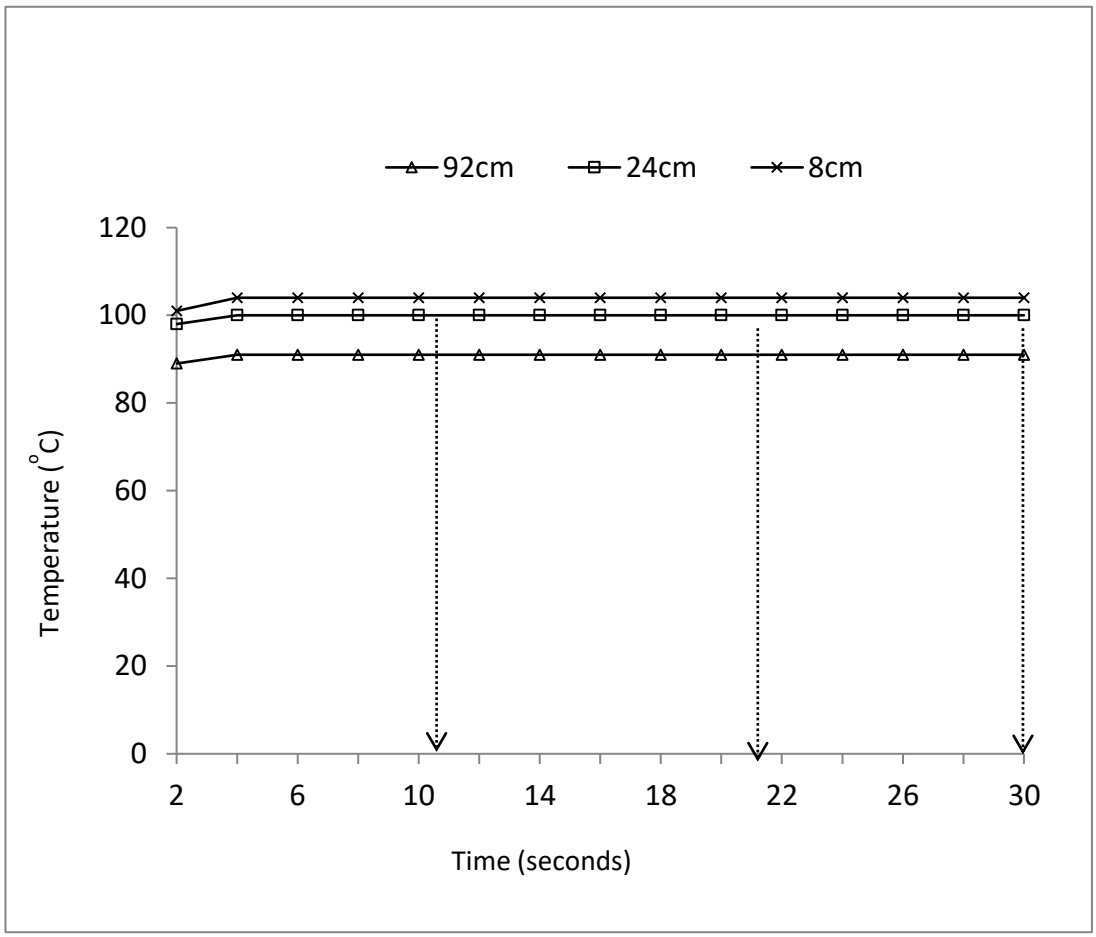


Figure 2. **Temperature records during treatments at three distances (8 and 24cm across six genetic strains (G1, G4, G7, G19, G20 and G30) and 92cm for G1 only) from which steam was applied for 10, 20 and 30s. Drop down arrows indicate maximum temperatures reached at 10, 20 and 30 seconds.**

Hot water immersion

None of the first instars survived treatments when subjected to hot water at 50, 60 and 70ºC for 60 and 120 s across the six genetic strains (Figure 3). Significant genetic strain x temperature x time interactions were observed for survival of first instar grape phylloxera (Figure 3) when subjected to hot water immersion. These interactions were attributed to no survival of first instars at the highest temperatures and duration of treatments (Figure 3). Temperature showed a significant negative effect on first instar survival (Figure 3) as the water temperature increased (Figure 3). This latter result was consistent across all six genetic strains (Figure 3). Treatment duration also significantly affected survival with percentage decrease in first instars surviving treatments as time of immersion increased ($p< 0.05$; Figure 3).

When subjected to HWT at 40 and 45ºC for 60 and 120 s, however, a proportion of first instars across the six phylloxera genetic strains survived treatments (Figure 3) with a significant

interaction between genetic strain x temperature x time ($p<0.05$). On average, 50 and 20% first instars across all genetic strains survived HWTs at 40 and 45^{o}C, respectively, compared to 99% survival at control room temperature 22^{o}C (Figure 3). At both 40 and 45^{o}C, first instar survival decreased as time of immersion increased, a trend that was consistent across all six phylloxera genetic strains ($p<0.001$; Figure 3).

At 45^{o}C for 120 s, first instars from G4, G7 and G20 phylloxera were most susceptible with observed survival of 4, 7, 5%, respectively, compared to 10% survival observed for G1, G19 and G30. When subjected to hot water at 45^{o}C for 60 s, G20 first instars were the least susceptible with only 13% first instars surviving treatments compared to 51 and 40 % survival observed for G19 and G30 phylloxera, respectively, which were most susceptible to the HWT (Table 1).

Survival of first instars in hot water set at 40^{o}C for 120 s averaged 32% (range 28-37%) across the six genetic strains. When subjected to HWTs at 40^{o}C for 60 s, first instars of G4 phylloxera were least susceptible with 84% surviving treatments compared to an average of 65% (range 61-73%) of G4, G7, G19, G20 and G30 phylloxera.

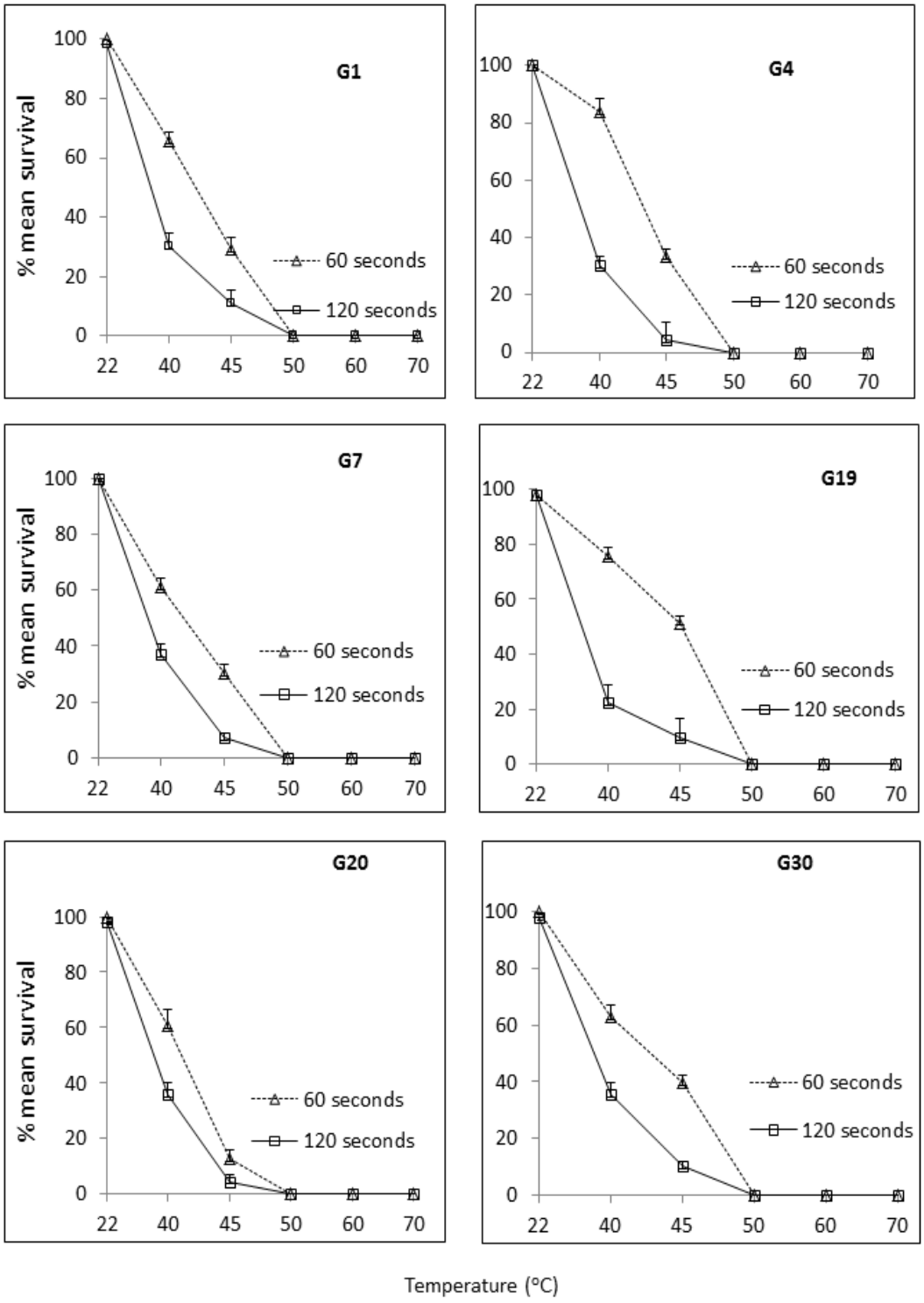


Figure 3. First instar survival of six phylloxera genetic strains (G1, G4, G7, G19, G20 and G30) following immersion in water set at six temperatures 22ºC (room temperature), 40ºC, 45ºC, 50ºC, 60ºC and 70ºC for 60 and 120 seconds.

Establishment and development of first instar phylloxera after hot water treatment

First instars that survived HWT at 22, 40 and 45ºC and were placed on excised *V. vinifera* roots established feeding sites and, subsequently developed to the adult stage and laid eggs. Establishment of feeding sites, premature mortality or reproduction was not significantly different between phylloxera genetic strains (P>0.05). Temperature had an effect on first instar premature mortality on root pieces and establishment of feeding sites (P<0.05) but not reproduction (p>0.05). Premature mortality of first instars immersed in hot water at 45ºC was higher (51%) compared to those immersed in hot water at 40ºC (32%) and at room temperature (11%) (Table 1). Dead first instars were characterised by a brown dark colour and lack of movement within three days following treatment. 64 and 67% of first instars across the six phylloxera genetic strains subjected to 40 and 45ºC, respectively, established feeding sites compared to 91% subjected to water at room temperature (Table 1). There was no significant difference in reproduction on excised roots between first instars across the six genetic strains (p>0.05) when subjected to HWT at 22, 40 and 45ºC (Table 1).

Table 1. Predictions from regression model on the response of first instars following hot water treatments at 22, 40 and 45ºC. Figures are percentage of first instars that died after treatments, established feeding sites and reproduced across the six genetic strains, G1, G4, G7, G19, G20 and G30.

Temperature (ºC)	Mean number of first instars placed on roots	% premature mortality	% of first instars that established feeding sites	% of first instars that laid eggs
22	42	11	91	89
40	34	32	64	79
45	25	51	67	71
P		0.02	<0.001	0.30
S.E		12	6	11

Conclusions and Recommendations

Disinfestation of machinery using steam

The experimental results demonstrate that all the steam treatments tested were effective across all strains of phylloxera and resulted in 100% mortality. The results therefore scientifically validate the existing recommendation in the National Phylloxera Management

Protocol (National Vine Health Steering Committee, 2009) of "*applying steam above 100°C as indicated by a jet of visible steam"* by demonstrating 100% mortality across all six genetic strains tested. The results also demonstrate that "*applying steam at a minimum of above 84°C for at least 10 seconds at a distance of at least 8 cm"* also achieves 100% mortality across genetic strains so there is potential to modify the exiting protocol accordingly (Table 3). This modified treatment has also been proven to be effective against the endemic phylloxera strain G38 (Appendix 1, this report). Any modification to the protocol would need to be endorsed by the appropriate state and national regulatory committees.

Disinfestation of grape bins using hot water immersion

The experimental results demonstrate that the genetic variability between phylloxera strains influences the efficacy of hot water immersion as a disinfestation treatment particularly at 40 and 45°C at both 60 and 120s duration. These temperatures/durations of hot water immersion reduce phylloxera survival, but are not 100% effective and thus could not be recommended. The results scientifically validate the existing recommendation in the National Phylloxera Management Protocol (National Vine Health Steering Committee, 2009) of "*fully immersing in water at 70℃ minimum, and holding in water for at least 2 minutes after it has reached 70℃"* by demonstrating 100% mortality across all six genetic strains tested. The results also demonstrate that "*fully immersing in water at 50℃ minimum, and holding in water for at least 1 minute after it has reached 50℃"* also achieves 100% mortality across genetic strains so there is potential to modify the existing protocol accordingly (Table 3). This modified treatment has also been proven effective against the endemic phylloxera strain G38 (Appendix 1, this report). Any modification to the protocol would require endorsement by the appropriate state and national regulatory committees.

Table 3. **Current disinfestation protocol and suggested modification to steam disinfestation protocol for movement of vineyard equipment out of a PIZ or PRZ (NPMP; Procedure G (a)).**

Current protocol (National Vine Health Steering Committee, 2009)	**Effective disinfestation procedure based on experimental results**
Steam applied must be above 100°C as indicated by a jet of clear invisible steam between steam outlet and the visible condensate cloud	**Steam applied must be a MINIMUM of 84°C for at least 10 seconds at a distance of at least 1 metre**
Steam must contact all surfaces until the surface is left dry not wet with condensate	**Steam must contact all surfaces**

*Suggested modification in bold – any proposed modification must be ratified by the relevant biosecurity body.

Table 4. Current disinfestation protocol and suggested modification to steam disinfestation protocol for movement of vineyard equipment out of a PIZ or PRZ (NPMP; Procedure G (b)).

Current protocol (National Vine Health Steering Committee, 2009)	Effective disinfestation procedure based on experimental results
Fully immerse the item in water at 70ºC minimum, and hold in water for at least 2 minutes after it has reached 70ºC.	Fully immerse the item in water at **50ºC** (**after** water temperature **has reached 50ºC)** and hold item in water for at least **1 minute.**

*Suggested modification in bold – any proposed modification must be ratified by the relevant biosecurity body.

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SECTION 6.

EFFICACY OF DRY HEAT TREATMENT AS A DISINFESTATION TREATMENT FOR GRAPEVINE PHYLLOXERA ON VINEYARD MACHINERY

Output: ***Dataset demonstrating the effectiveness of dry heat as a disinfestation treatment for phylloxera on viticulture machinery.***

Summary

The National Phylloxera Management Protocols (NPMP; National Vine Health Steering Committee, 2009) recommends a disinfestation protocol for use against phylloxera on vineyard machinery (particularly grape harvesters) *Procedure G - Movement of Vineyard equipment out of a PIZ or PRZ*) using "*dry heat at 45^{o}C for 75 minutes or 40^{o}C for 120 minutes"*. This protocol was previously scientifically validated (Korosi et al. 2012) against two endemic strains (G1 and G4) of grape phylloxera and found to be effective against both strains. In this study we compared the efficacy of the two available dry heat protocols against six endemic phylloxera strains. The results indicate that the protocol of 45^{o}C for 75 minutes is effective against all six strains. However, the protocol of 40^{o}C for 120 minutes is only 100% effective against four strains, G1, G4, G7 and G19. A small proportion of G20 and G30 first instars survived this treatment but did not develop to reach the adult stage. It is recommended that if 40^{o}C is to be maintained as an alternative treatment to 45^{o}C then the duration of treatment at 40^{o}C should be extended from 120 minutes to 135 minutes to ensure 100% mortality across diverse phylloxera genetic strains.

Background

Dry heat treatment is recommended as a disinfestation treatment for vineyard machinery and equipment against phylloxera. Items to be disinfested are first washed down and then held in a suitable room until it has reached either 40^{o}C for 2 hours or 45 for 75 minutes. In a previous study it has been shown that both these treatments are effective against two highly virulent phylloxera strains G1 and G4 (Korosi et al. 2012). This experiment validated the effectiveness of the dry heat recommended treatments against six endemic phylloxera strains - G1, G4, G7, G19, G20 and G30.

Material and Methods

Eggs of G1, G4, G7, G19, G20, G30 phylloxera were collected from excised roots using a small artist's paint brush and placed on moistened filter paper in a 90 x 25mm petri dish. The petri dishes were sealed with cling film (Rapfast PVC Food Packaging, Integrated Packaging, Australia) to create a hatching chamber and incubated in the dark at a constant temperature of 22°C. The eggs were monitored daily and newly emerged active first instars were collected and used for all experiments.

A specially designed screw-top desiccator 13 cm (h) x 9.5 cm which contained saturated magnesium chloride solution was used as a desiccator chamber (Figure 1a). The experiments used ten newly emerged first instars of G7, G19, G20 and G30 phylloxera and 20 first instars of G1 and G4 (G1 and G4 are the most virulent of the six genetic strains and hence, 20 first instars were used for the experiments because insects were readily available). First instars were placed in a plastic vial (5.5 cm high, 2.5 cm diameter) and positioned on a plastic mesh (diameter 9.5 cm) that was placed on top of a cylindrical plastic container (5.5 cm(h) x 4.5 cm (d)) (Figure 1b). The plastic container was partially filled with saturated magnesium chloride, to maintain a 30% relative humidity level (Buchanan, 1990) and placed at the centre of the desiccator chamber which held the treatment vials.

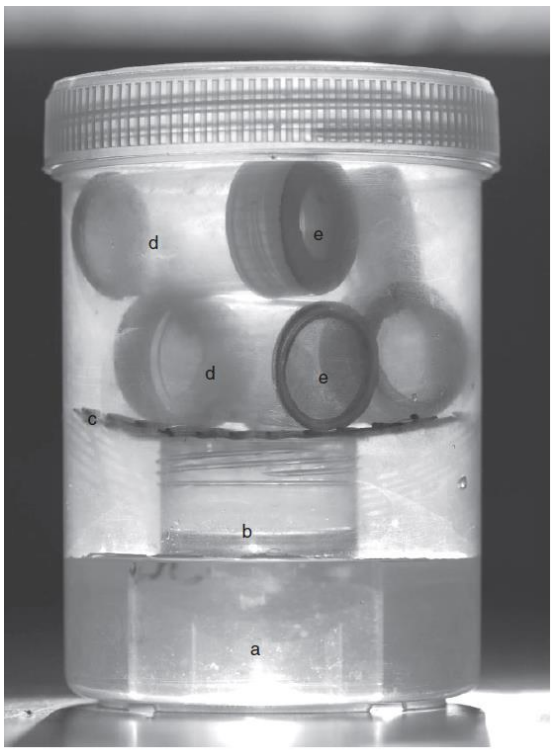


Figure 1. Set up with container with (a) saturated magnesium chloride solution (b) plastic vial that supported (c) a wire mesh where (d) plastic vials with phylloxera were placed during the experiments.

First instars across the six phylloxera genetic strains were subjected to 35, 40 and 45ºC for 75, 90 and 120 mins. As a proportion of first instars of G20 and G30 phylloxera survived in 40°C for 120 m, a further treatment of 135 mins at 40ºC was conducted. The experiment was replicated five times for the three temperatures and time durations across the six phylloxera genetic strain in full factorial combination.

Post treatment survival and development

Following treatments, first instars were removed from the vials using a fine artist's paintbrush, placed onto a damp filter paper and examined under low power magnification microscope. First instars were classified alive if there was observable leg and/or antennal movement and dead if there was no apparent movement when gently manipulated with the tip of the paintbrush up to 2 h after treatment. First instars were counted and recorded as either dead or alive for each treatment vial.

A proportion of first instars from G7, G19, G20 and G30 phylloxera that survived treatments were placed on excised *V. vinifera* cv Chardonnay roots to examine the effect of dry heat on establishment of feeding sites, development to adults and subsequent reproduction. First instars that survived heat treatments at the four exposure periods (75, 90, 120 and 135 minutes) were pooled onto a single root piece in a 90 x 25 mm petri dish for each temperature, i.e. 35 or 40ºC. Bioassay root pieces were examined every day for three days post-treatment and thereafter, checked and cleaned weekly. First instars that established feeding sites, prematurely died on excised roots and reproduced were counted and recorded.

The percentage of surviving insects was analysed using an analysis of variance (ANOVA) in GenStat (GenStat Release 16.1 2015). Fishers protected Least Significant Difference (p= 0.05) were used to separate means where F-tests were significant. Percentage of first instars that survived treatments, established feeding sites, developed to adults and subsequently laid eggs was analysed using ANOVA (unbalanced design using regression) with temperature as the treatment factor.

Results

Efficacy of dry heat treatments

The upper thermal limit for 100% mortality was achieved at 45°C when first instars were subjected to the treatment for 75 minutes and this was consistent across the six phylloxera genetic strains (Figure 2). At 40ºC for 2 h, 100% mortality was reached after 120 minutes exposure for G1, G4, G7, G19 phylloxera and at 135 minutes for G20 and G30.

There was a significant difference across the six genetic strains ($p<0.001$) in response to both temperature and time when first instars were exposed to treatments. First instar survival reduced as temperature and time of exposure increased, a trend that was consistent across the six phylloxera genetic strains (Figure 2).

First instar survival decreased as temperature and time of exposure to treatments increased, a trend that was consistent across the six phylloxera genetic strains ($p<.001$; Figure 2). First instars from G1 and G4 were most susceptible to increasing temperatures compared to G7, G19, G20 and G30 ($p<.001$) (Figure 2). At 35ºC, survival of G1 and G4 averaged 10% compared to 59% (range 51 – 65%) for G7, G19, G20 and G30 phylloxera (across the combined times of exposure). At 40°C, survival of G1 and G4 averaged 4% compared to 40% (range 23 – 75%) for G7, G19, G20 and G30 at 75 m with no survival when exposed for 120 m for G1, G4, G7 and G19 (Fig 1). First instars from G19 were overall, more resistant to heat treatments, with 58% survival observed (across the combined temperatures) compared to less than 46% of G1, G7, G19 first instars that survived (Figure 2). G20 and G30 phylloxera responded similarly to the heat treatments (Figure 2).

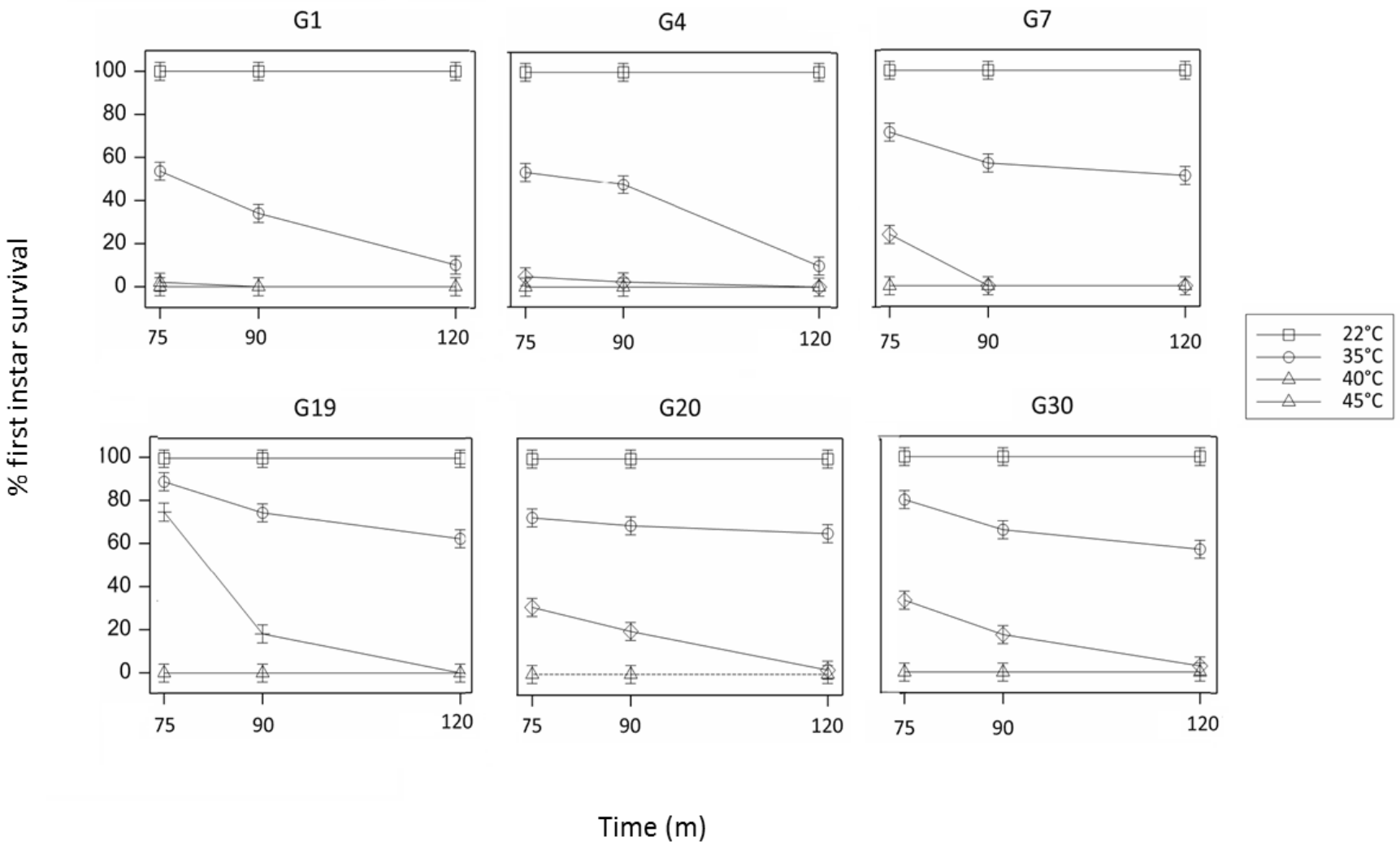


Figure 2. First instar survival of six phylloxera genetic strains, G1, G4, G7, G19, G20 and G30, when subjected to dry heat at 35, 40, 45°C for 75, 90, 120 minutes. Upper thermal limit at 40°C for G20 and G30 phylloxera was reached after 135 minutes (data not shown on graph).

Post-treatment development

For G7, G19, G20 and G30 that survived the dry heat treatments (35 and 40°C at combined time of exposure), temperature had a significant effect ($p<0.001$) on first instars that established feeding sites on *V. vinifera* and those that reproduced ($p<0.001$; Figure 3) without significant differences between genetic strains ($p>0.05$). Of first instars that survived treatments at 35 and 40°C, 18% and 35% established feeding sites, respectively (Figure 3). Premature mortality of first instars on *V. vinifera* roots did not significantly differ with temperature ($p<0.05$) with an average of 16% first instars dying for the combined temperature treatments, 22, 35 and 40°C. First instars that were subjected to heat treatments at 40°C and developed to adults did not reproduce on excised *V. vinifera*. However, 14% (range 7-20%) of first instars that were subjected to 35°C treatments and developed to adults reproduced (Figure 3).

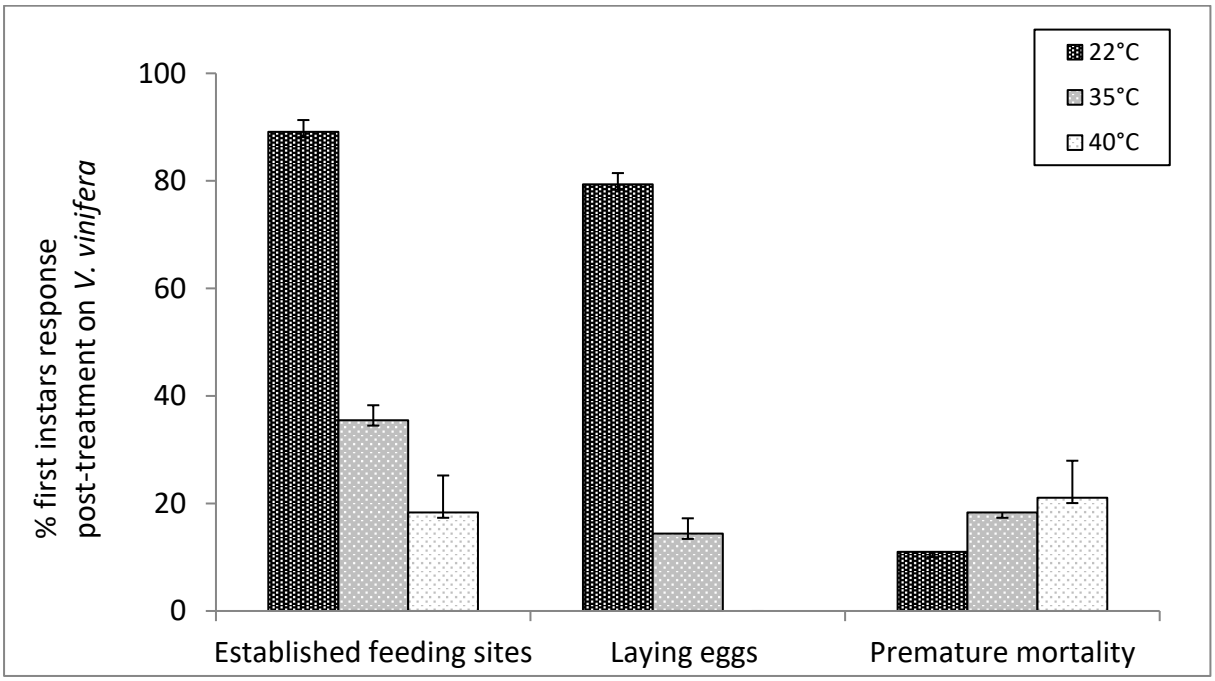


Figure 3. Response on *V. vinifera* of first instar phylloxera following dry heat treatment at 22 (control room temperature), 35 and 40°C, combined across G7, G19, G20 and G30 genetic strains and treatment duration, 75, 90 and 120 minutes.

Discussion and Recommendations

Disinfestation of grape bins using hot water immersion

The experimental results demonstrate that the genetic variability between phylloxera strains influences the efficacy of dry heat as a disinfestation treatment, particularly at 35 and 40°C, over a range of time durations from 75 mins to 120 mins. Our results highlight that dry heat is an effective disinfestation treatment against six selected endemic trains of phylloxera. However, temperature thresholds differ between endemic strains. A temperature of 35°C for up to 2 hours is ineffective as a disinfestation treatment against any of the six phylloxera strains tested in this study, as survival can be observed between 10-60%. The latter temperatures/durations of dry heat reduce phylloxera survival, but they are not 100% effective across all genetic strains tested and therefore could not be recommended.

The results scientifically validate one of the existing recommendations in the National Phylloxera Management Protocol (National Vine Health Steering Committee, 2009) for machinery disinfestation of "*Holding the machinery in the hot room for a minimum of 75 minutes after the machinery has reached 45^{o}C"* by demonstrating 100% mortality across all six genetic strains tested. This treatment was also shown to be effective against the endemic phylloxera strain G38 (Appendix 1, this report).

Treatment at 40^{o}C for 120 minutes was effective against the four phylloxera strains G1, G4, G7 and G19 as well as endemic phylloxera strain G38 (Appendix 1, this report). However, at 40^{o}C for 120 minutes, two phylloxera strains, G20 and G30 (both currently restricted to North East Victoria) do survive at low levels (<5%) but do not develop to adulthood and cannot therefore reproduce. Therefore the existing recommendation in the National Phylloxera Management Protocol (National Vine Health Steering Committee, 2009) for machinery disinfestation of "*Holding the machinery in the hot room for a minimum of 2 hours minutes after the machinery has reached 40^{o}C"* is not 100% effective across all six genetic strains. However, when the treatment time at 40^{o}C was increased to 135 minutes, 100% mortality was achieved. As a precautionary measure it would be advisable to modify the current dry heat protocol accordingly (Table 2). Any modification to the protocol would need to be endorsed by the appropriate state and national regulatory committees.

Table 2. Current disinfestation protocol and suggested modification to dry heat disinfestation protocol for movement of vineyard equipment out of a PIZ or PRZ (NPMP; Procedure G(c)).

Current protocol **(National Vine Health Steering Committee, 2009)**	**Effective disinfestation procedure based on experimental results**
Hold in the hot room for a minimum of:	
EITHER 75 minutes after the machinery has reached 45^{o}C	EITHER 75 minutes after the machinery has reached 45^{o}C
OR 2 hours after the machine has reached 40^{o}C	OR 2 hours **and 15 minutes** after the machine has reached 40^{o}C

*Suggested modification in bold – any proposed modification must be ratified by the relevant biosecurity body.

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SECTION 7.

EFFICACY OF HOT WATER IMMERSION AS A DISINFESTATION TREATMENT FOR GRAPEVINE PHYLLOXERA ON PLANTING MATERIAL

Output: ***Dataset demonstrating the effectiveness of hot water immersion as a disinfestation treatment for phylloxera on grapevine planting material.***

Summary

The efficacy of a hot water treatment (HWT) disinfestation protocol (procedure B (NPMP); National Vine Health Steering Committee 2009) for grapevine planting material (rootlings and cuttings) were scientifically validated by testing against six endemic phylloxera strains (G1, G4, G7, G19, G20, G30) under laboratory conditions. Existing protocols for HWT disinfestation of planting material are (i) *54℃ for 5 minutes* or (ii) *50 for 30 minutes.* However, prior to this study there was no published data to confirm the effectiveness of these protocols against endemic phylloxera strains. In this study, first instars attached to feeding sites on *V. vinifera* were subjected to hot water at 40, 45, 50 and 54°C for 5 and 30 minutes. Results indicate that current HWT recommendations of either 54°C for 5 minutes or 50 for 30 minutes are effective, with 100% mortality observed against all seven strains tested. A reduction in treatment temperature to 45°C (for 5 minutes) was effective against only three (G1, G4 and G7) of the six phylloxera strains tested. However, at 45°C for 30 minutes, 100% mortality was achieved across all six phylloxera strains. In contrast, when the HWT temperature was dropped to 40°C for either 5 or 30 minutes, over 80% phylloxera survival occurred across all six genetic strains. The results highlighted the need for further studies to determine the impact of temperature of different phylloxera strains. The results were reported to the Victorian Viticulture Biosecurity Committee and the National Viticulture Biosecurity Committee.

Background

Existing protocols (see NPMP Procedure B - *Movement of grapevine cuttings and rootlings from a PIZ or PRZ into a PRZ or PEZ* (National Vine Health Steering Committee, 2009)) for HWT disinfestation of grapevine planting material are (i) 54°C ± 1°C for 5 minutes or (ii) 50°C ± 1°C for 30 minutes. Previous studies (Buchanan et al. 1996) using Australian sourced phylloxera populations, with unspecified genetic lineage, reported that 52°C for 5 minutes was an effective treatment, resulting in 100% mortality. However, no further studies on this HWT procedure have been published to confirm its effectiveness against endemic phylloxera

strains. In this study, six endemic phylloxera strains G1, G4, G7, G19, G20 and G30 attached to grapevine root material were treated in hot water at temperature ranges of between 40 to 54°C (40, 45, 50 and 54°C) for 5 and 30 minutes .

Materials and Methods

Six phylloxera genetic strains G1, G4, G7, G19, G20 and G30 were established on excised *Vitis vinifera* cv. Chardonnay roots and subjected to hot water treatment immersion in a temperature controlled water bath.

For each genetic strain, phylloxera eggs were placed on an 8cm excised *V. vinifera* root and incubated at 23ºC until hatching and establishment of feeding sites on the root pieces. Roots with 10 first instar crawlers that had established feeding sites were selected and carefully wrapped in a fine mesh (Figure 1a). To simulate a bundle of cuttings/rootlings as used in the disinfestation of grapevine material (Procedure B), 20 grapevine cuttings were bundled around each mesh-wrapped *V. vinifera* root piece that contained feeding phylloxera (Figure 1b). The bundles were weighted with a stir bar magnetic rod which ensured complete submersion in water during treatments and immersed in a hot water bath (Figure 1c), set at 40, 45, 50, or 54ºC for 5 or 30 minutes. Treatments were replicated three times for each temperature, time of immersion and six phylloxera genetic strains.

Following treatments, the bundles were immediately immersed in cold water for 30 seconds to stop the heat reaction. Phylloxera that were alive or dead after the experiments were counted and recorded. The root pieces were subsequently placed in a 150 x 25mm petri dish, incubated at 23ºC and monitored daily for the first 4 days and weekly thereafter.

Phylloxera that were alive soon after treatments and those that survived to adulthood on excised *V. vinifera* after incubation at 23ºC were counted and recorded. Phylloxera adults that laid eggs or that died on excised *V. vinifera* four weeks after treatment were also counted and recorded.

Percentage of surviving insects was analysed using an ANOVA in GenStat (GenStat Release 16.1, 2015). Fishers protected LSD (p= 0.05) were used to separate means where F-tests were significant. Percentage of first instars that survived treatments, established feeding sites, developed to adults and subsequently laid eggs was analysed using ANOVA (unbalanced design using regression) with temperature as the treatment factor.



Figure 1. (**a) Excised *V. vinifera* root with first instar phylloxera that have established feeding sites and wrapped in a fine mesh (b) treatment bundle surrounded by grapevine cuttings and (c) water bath with replicate cages used for experiments.**

Results

In this study, temperature and duration of immersion significantly influenced phylloxera first instar survival ($p<0.001$). Hot water immersion as currently recommended i.e. 54°C for 5 minutes and 50°C for 30 minutes (Procedure B, NPMP), was 100% effective for disinfestation of first instars across all six genetic strains (Figure 2).

Over 90% of phylloxera first instars, regardless of genetic strain, survived following treatments in water heated to 40°C at both immersion durations, 5 and 30 minutes. At 45°C, disinfestation was effective across the six genetic strains when first instars were immersed for 30 minutes. However, survival was observed for G19, G20 and G30 phylloxera when first instars were immersed in water heated to 45°C for 5 minutes.

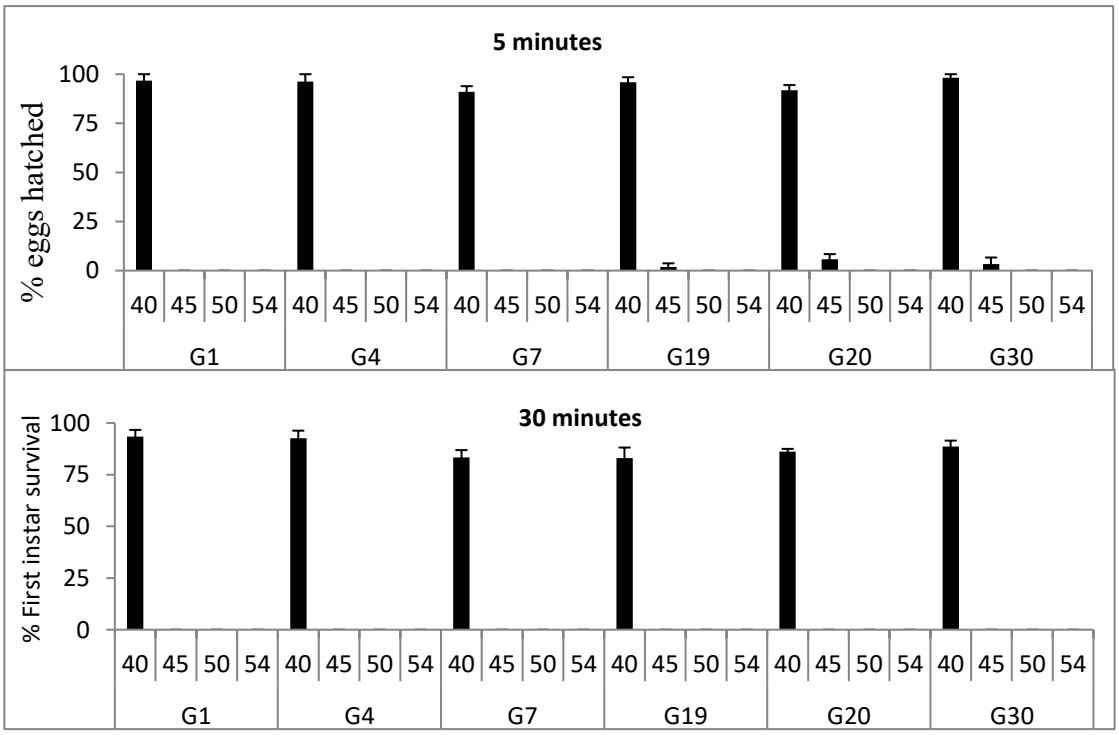


Figure 2. Survival of phylloxera that were established on excised *V. vinifera* roots following immersion in a water bath set at 40, 45, 50 and 54°C for 5 and 30 minutes. Figures are percentage of first instar survival of six phylloxera genetic strains as indicated.

Development and reproduction of first instar phylloxera post hot water treatment

A proportion of first instars subjected to hot water immersion at 40 and 45ºC survived on excised *V. vinifera* roots and subsequently developed to the adult stage and reproduced. There were significant differences between phylloxera genetic strains (p=0.005), temperature (p<0.001) but not time of immersion (p<0.05). Overall, up to 80% of first instars developed to adults and laid eggs when subjected to hot water heated to 40ºC at both 5 and 30 minutes (Figure 2). First instars that survived treatments in hot water heated to 45ºC for 5 minutes did not develop to adults. Reproduction of G19 phylloxera was most impacted by hot water treatment at 40°C with 53% of adults laying eggs compared with 78 and 79% adults of G4 and G30 phylloxera, that reproduced (Figure 3).

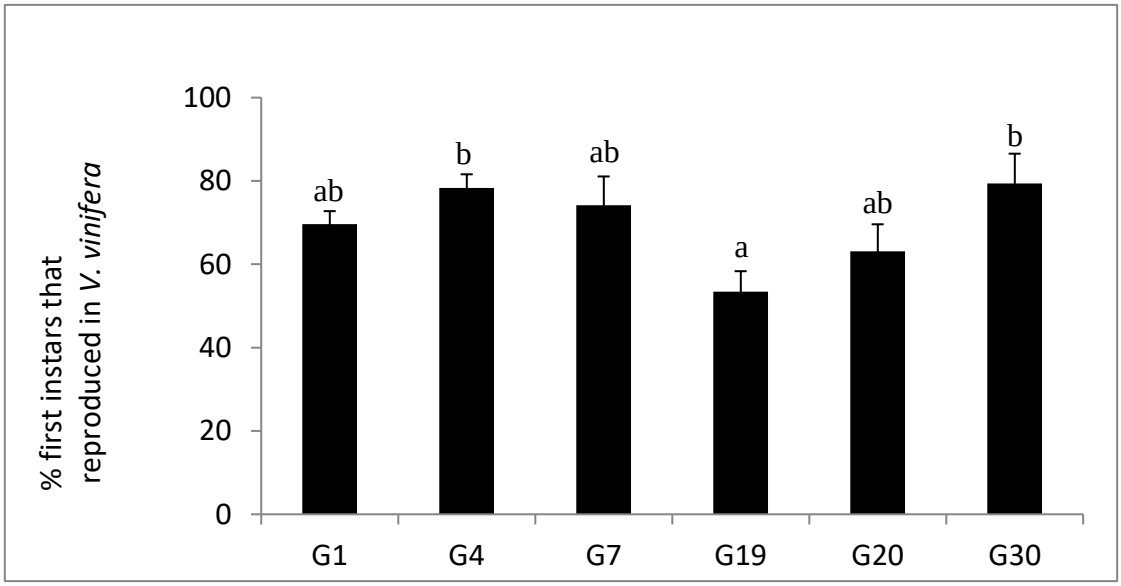


Figure 3. Proportion of first instars that developed to adult stage and successfully reproduced following hot water treatment at 40ºC. Values on the bars followed by the same letter(s) on the graph are not significantly different (Fishers protected LSD test P<0.05)

Conclusions and Recommendations

Our results highlight that hot water treatment at the current recommendations of either 54 for 5 minutes or 50ºC for 30 minutes are both 100% effective across the six endemic phylloxera strains tested. Both treatments were also proven to be effective against the endemic phylloxera strain G38 (Appendix 1, this report). However, temperature thresholds differ between endemic strains. A temperature of 45ºC for 5 minutes is not 100% effective as a disinfestation treatment against three phylloxera strains (G19, G20 and G30) tested in this study, as 10% survival was observed. However, surviving insects in water at 45°C did not develop to the adult stage, and therefore did not reproduce. When the same temperature

immersion duration was extended to 30 minutes, 100% mortality was achieved against all strains.

At 40ºC for both 5 and 30 minutes, survival was over 80% across all strains and of those first instars that survived, over 50% were able to develop to the adult stage and reproduce. The current recommended treatments of 54ºC for 5 minutes or 50ºC for 30 minutes are effective against all six strains. However, 50ºC for 5 minutes or 45°C for 30 minutes are also as effective across all strains so there is potential to modify the treatment protocol (Table 2), provided the equipment used in the operation is monitored carefully to ensure the correct lethal temperatures and durations are reached.

Table 2.Current disinfestation protocol and suggested modification to hot water disinfestation protocol for movement of grapevine cuttings and rootlings from a PRZ or PEZ (NPMP; Procedure B).

Current protocol (National Vine Health Steering Committee, 2009)	**Effective disinfestation procedure based on experimental results**
Cuttings/rootlings must be hot water treated immediately prior to dispatch as follows :	
EITHER at 50ºC +/-1ºC for 30 minutes	EITHER at **45**ºC +/-1ºC for 30 minutes
OR at 54º C +/-1ºC for 5 minutes	OR at **50**ºC +/-1ºC for 5 minutes
The hot water treatment procedure MUST ensure that ALL material reaches the required temperature for the specified time.	

*Suggested modification in bold – any proposed modification must be ratified by the relevant biosecurity body.

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GenStat Release 16.1 (2015) Copyright ©. VSN International Ltd, Hemel Hempstead.

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SECTION 8.

EFFICACY OF HOMOGENISATION AS A DISINFESTATION TREATMENT FOR DIAGNOSTIC SAMPLES

Output: ***Dataset demonstrating the effectiveness of homogenisation as a novel disinfestation treatment for phylloxera on diagnostic samples.***

Summary

Currently, smoke taint samples (as whole grapes) are treated as diagnostic samples and are subjected to freezing at -18°C for 24 hours prior to transportation (NPMP; Procedure C (a)) from a PIZ or a PRZ to a PEZ. However, experiments assessing the survival of phylloxera first instars at -20°C (Section 10, this report) have only just been completed and the minimum storage requirement would be 12 hours. If laboratory analysis is required quickly then a more rapid alternative to cold storage would be advantageous. Alternative disinfestation treatments are therefore required to enable movement of smoke taint samples rapidly between phylloxera zones. The efficacy of a homogenisation procedure for wine grapes was tested under laboratory conditions against multiple phylloxera genetic strains. Results indicate that when subjected to this treatment, phylloxera first instars are physically damaged to a point where no whole live phylloxera, regardless of genetic strain, survive the homogenisation procedure. This finding could be used to modify the procedure for transferring whole grape samples, from smoke affected properties within a PIZ to laboratories located in a PEZ, for smoke taint analysis. In contrast to a cold treatment (which requires 24 hours storage), a simple rapid homogenisation procedure could be utilised as an alternative method to ensure that 100% phylloxera mortality is achieved prior to removing potentially phylloxera-infested smoke taint samples to a receiving laboratory. The results of this trial and recommendations have been reported to the Victorian Viticulture Biosecurity Committee. The project leader has highlighted the need to conduct further testing to refine the procedure based on sample size and duration and speed of homogenisation.

Materials and Methods

A GRINDOMIX GM 20™ knife mill (Retsch, Hann, Germany) that is used for grinding and homogenizing foods and feeds was used as a homogenizer for all experiments (Figure 1a). The homogenizer contained two sharp blades that rotated in the center of the grinding container (Figure 1b) which were run on a 50/60 Hz motor. The homogenizer had three standardized settings (see below):

1. 0.010 seconds x 2000rpm
2. 0.20 seconds x 5000rpm
3. 3 seconds x 10, 000rpm

100g of fresh Golden Globe grapes were placed in the grinding container which was then connected to the homogenizer. First instars of G1 phylloxera were placed on the surface of the grapes using an artist's paintbrush. The grapes and the first instars were centrifuged at the three standard settings (see above).

One hundred first instars were used for each treatment. Following the complete cycle, the homogenate was poured into a clean 150 x 20mm petri dish and examined for surviving insects under low power microscope.

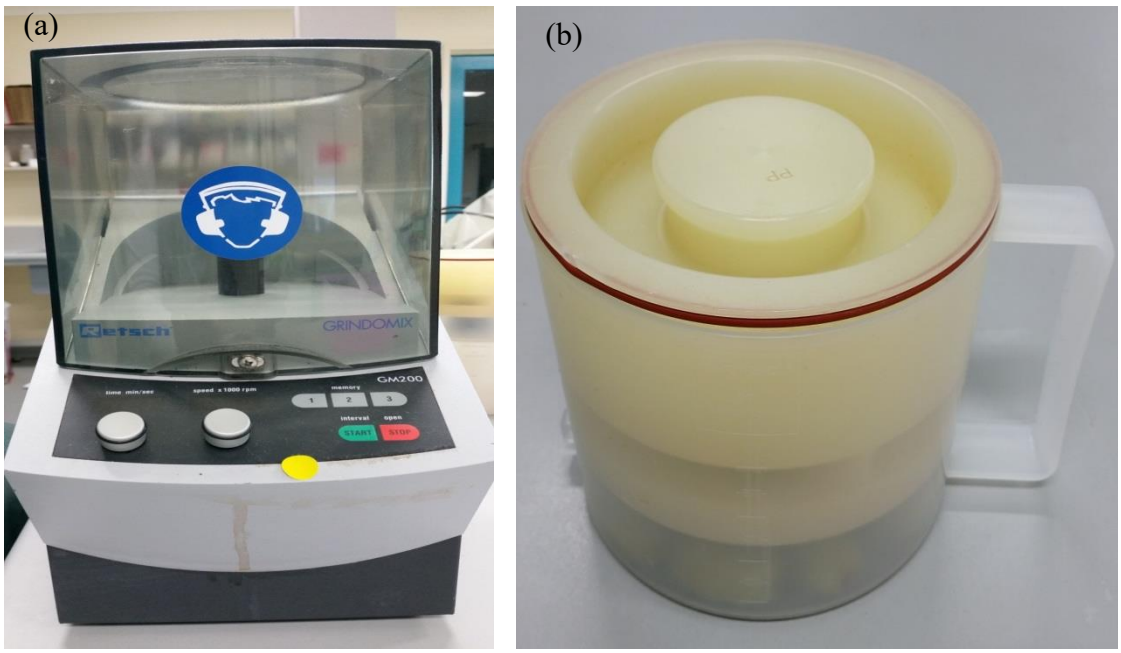


Figure 1. GRINDOMIX GM20 knife mill (a) homogeniser and (b) grinding container used for homogenisation of phylloxera infested grapes.

Results

After treatment, all homogenised samples were examined for the presence of whole first instar nymphs. No whole samples were detected and therefore no first instars survived treatment at all three homogeniser settings (Table 1).

Table 1. Centrifuge speed, time of homogenisation and expected fineness and records of first instars found.

Treatment Duration (seconds)	Centrifugal setting (rpm)	Expected fineness	Numbers of whole first instars surviving treatment
10	x 2000	<300µ	0
20	x 5000	<300µ	0
33	x 10000	<300µ	0

Conclusions and Recommendations

In high fire danger regions, grapes can be exposed to smoke and become tainted with various odours. Smoke taint results in the production of free volatile phenols, which can affect grape flavour and, during the fermentation process, can impact on wine quality. Studies on smoke taint often require grape samples to be collected from smoke affected vineyards. These vineyards can be, and often are, located in either phylloxera infested or phylloxera risk zones (PIZ or PRZ), whilst the laboratories which conduct the analysis for smoke taint are located in Phylloxera Exclusion Zones (PEZ). If grapes are removed from phylloxera infested vineyards they can present a quarantine risk, as first instars have been detected on grape bunches in commercial vineyards (Powell et al., 2000). Generally, movement of whole wine grapes from PIZs or PRZs to a PEZ is prohibited (NPMP; National Vine Health Steering Committee, 2009). However, when whole grape movement is required for smoke taint analysis they can be moved under protocol and permit (NPMP, 2009) as they are classified as diagnostic samples. There are various disinfestation procedures to allow this movement, but only one of these is practical for smoke taint analysis samples as it retains the integrity of the sample. The disinfestation procedure predominantly used is storage of grapes at “-18°C for 24 hours”. Upon receiving smoke-tainted grape samples at the diagnostic laboratory, the samples are normally homogenised prior to analysis.

We have demonstrated in this study that a potential alternative approach is available which would ensure that whole phylloxera cannot be transferred on grapes if the samples are first homogenised prior to dispatch to the receiving analytical laboratory. As the process is purely a physical, rather than a chemical, disinfestation of the insects, it would not taint the whole grapes. Before incorporating this disinfestation process (Table 2) in the NPMP, further research is required to (i) compare frozen and homogenised grape samples to determine if these disinfestation processes affect the subsequent smoke taint analysis; (ii) standardise the disinfestation procedure (with respect to duration and speed of homogenisation); and (iii) establish the efficacy of the homogenisation treatment for large sample sizes (as sometimes grape samples of up to 2 kg are homogenised).

Table 2. Current disinfestation protocol and suggested modification to movement of smoke taint grape samples out of a PIZ or PRZ.

Current protocol* National Vine Health Steering Committee (2009).	**Effective disinfestation procedure based on experimental results**
*No current protocol for homogenisation for grape samples	**Homogenise 100 g samples at:**
	EITHER 10 seconds at x 10000 rpm
	OR 20 second at x 5000 rpm
	OR 30 seconds at x 2000 rpm

*Suggested modification in bold – any proposed modification must be ratified by the relevant biosecurity body.

References

National Vine Health Steering Committee (2009). National Phylloxera Management Protocol movement procedures for phylloxera risk vectors from a Phylloxera Infested Zone (PIZ). http://www.vinehealth.com.au/bio-security/phylloxera-regulations/**:** 32pp.

SECTION 9.

EFFICACY OF ALTERNATIVE DISINFESTATION TREATMENTS FOR PHYLLOXERA ON VINEYARD CLOTHING

Output: ***Dataset demonstrating the effectiveness of novel disinfestation procedures and hot water treatment for phylloxera on clothing.***

Summary

There are no current recommended disinfestation protocols for clothing in the NPMP (National Vine Health Steering Committee, 2009), despite the fact that clothing is a recognised risk of phylloxera transfer (Powell, 2015). First instars of root galling grape phylloxera are present above ground in the spring and summer months, both on the soil surface and in the canopy. Vineyard workers are therefore likely to inadvertently transfer phylloxera on their clothing when they are working close to the canopy during standard vineyard operations.

Two potential ways in which clothing could be disinfested were investigated: (i) a desk top analysis was conducted to examine the potential effectiveness of both warm or hot water treatment through washing using a standard domestic washing machine and (ii) use of a chemical insecticide spray treatment for clothing (particularly hats). Temperature in a domestic washing machine is variable depending on manufacturer's instructions and is therefore difficult to standardise in an experimental setting. Temperatures range from 32-53°C (warm) and from 54°C and above (hot), with duration of the washing cycle also varying. Washing temperatures of commonly-used domestic washing machines were therefore compared to data from hot water disinfestation protocols (tested against six phylloxera strains). Analysis of the data set indicated that phylloxera first instars from six endemic strains are likely to survive a warm water treatment (as phylloxera survival was observed at 40°C and 45°C for up to 30 minutes duration) but not a hot water treatment wash, as no phylloxera strain survived in hot water at 54°C for 5 minutes. A draft recommended disinfestation protocol for clothing was suggested to disinfest clothing in hot water wash (54°C or above) for a minimum of 5 minutes.

Analysis, and comparison with data in Section 7 of this report, indicates that a hot water wash for clothing – provided temperatures reach at least 54°C for 5 minutes or 50°C for 30 minutes - is likely to prove effective as a disinfestation treatment for phylloxera first instar larvae. However, it is important to refer to the manufacturer's guidelines on temperature and duration settings for the hot wash cycle as these vary markedly depending on the model of the machine.

The use of an insecticide spray for five seconds is also an effective treatment, provided the treatment contact with the insect for at least five seconds. When using insecticide sprays it is important to refer to the MSDS documentation to examine the potential health and safety risks associated with its use.

Background

Grape phylloxera can be inadvertently transferred between vineyards by people on their clothing and footwear. There are specific disinfestation protocols for footwear in the National Phylloxera Management Protocol document (NPMP *Procedure H Movement of vineyard visitors out of a PIZ or PRZ vineyard*; National Vine Health Steering Committee 2009) and chemical alternatives for footwear disinfestation treatments have been highlighted (Section 11, this report). However, there are no disinfestation protocols specifically designed to minimise the risk of phylloxera transfer on other clothing items in the NPMP other than to "change, wash or discard (if disposable) clothing before entering the next vineyard" (National Vine Health Steering Committee, 2009). Although washing is recommended, the protocol does not specify either the temperature or duration of the wash procedure. It has already been demonstrated that phylloxera can survive for up to 21 days in cold water (at 10°C) (Powell et al., 2014). Hot water treatment as a disinfestation procedure for either machinery or grapevine material is already recommended in the National Protocols (National Vine Health Steering Committee, 2009) and we have demonstrated earlier in this report that the recommended procedures are effective, resulting in 100% mortality across six genetic strains of phylloxera.

We therefore conducted a literature review to determine the temperature ranges and wash cycle durations of common brands of domestic washing machines, in order to determine their efficacy against phylloxera on clothing when compared to our earlier experiments demonstrating the efficacy of HWT. We also examined the efficacy of a commonly used household insecticide spray for disinfestation of phylloxera on hats and small hand tools. The use of insecticides to manage phylloxera in the vineyard situation is not widespread and there are no effective insecticide recommendations in Australia due to the difficulties in finding an upwardly and downwardly mobile systemic (Benheim et al. 2012). The potential for using an insecticide spray as a disinfestation treatment for clothing (particularly hats) has not previously been scientifically validated although it has been previously suggested as a potential treatment (Peter Hackworth, PGIBSA, pers. comm.).

In this section we report on potential alternative disinfestation methods for reducing the risk of grape phylloxera on clothing by using (i) a hot water wash cycle or (ii) an insecticide spray as effective disinfestation methods.

Materials and Methods

Insects

Eggs of G1, G4, G7, G19, G20, and G30 phylloxera were collected from excised roots using a small artist's paint brush and placed on moistened filter paper in a 90 x 25mm petri dish. The petri dishes were sealed with cling film (Rapfast PVC Food Packaging, Integrated Packaging, Australia) to create a hatching chamber and incubated in the dark at a constant temperature of 22°C. The eggs were monitored daily and newly emerged active first instars were collected and used for all experiments.

Insecticide treatment

First instar phylloxera were subjected to a spray with Mortein Fast Knockdown Multi-Insect Killer (Reckitt Benckiser (Australia) Pty Limited, Sydney) which contains two main insecticidal ingredients (a.i. 1.1g/kg Esbiothrin and 0.5g.kg Permethrin). For each of the six phylloxera genetic strains, 10 first instars were placed on a filter paper and the spray nozzle directed to the insects for 5 seconds at a distance of 8 cm. The treatment was replicated five times for each of the six genetic strains.

After treatment, first instars were transferred to a clean filter paper using a sable-haired artist's paintbrush and examined under a low power microscope. Where there was observable movement or if the antenna and legs moved when stimulated with the tip of the paintbrush, the first instars were classified as surviving. Where there was no apparent movement two hours after treatment, first instars were scored dead. The total number of first instars recovered from the petri dishes and those that were missing were also recorded.

Assessment of potential efficacy of washing machine treatments on grape phylloxera survival on clothing

A literature review was conducted on 12 common brands of domestic washing machines to determine the most suitable wash cycle for effective disinfestation of first instar grape phylloxera. The review focused on washing machine temperature settings, duration of wash cycle, estimated load and the efficacy of hot water wash in achieving mortality against pathogens and insect pests.

Results

Efficacy of insecticide spray treatment on grape phylloxera

When first instar phylloxera were subjected to a five second insecticide pray treatment, 100% mortality was achieved across the five replicates and the six genetic strains (Table 1).

Table 1. Effect of a 5 second spray treatment of an insecticide (a.i. 1.1g/kg Esbiothrin and 0.5g.kg Permethrin) on survival of six genetic strains of first instar grape phylloxera.

Phylloxera genetic strain	G1	G4	G7	G19	G20	G30
% first instar survival	0 (N=50)	0 (N=50)	0 (N=50)	0 (N=50)	0 (N=50)	0 (N=50)

Potential efficacy of washing machine cycles as disinfestation treatment for grape phylloxera survival on clothing

Temperature ranges

A review of manufacturer's documentation of 12 selected common domestic washing machines revealed three basic temperature settings: (i) a cold temperature setting, categorised at ≤ 30°C (ii) warm temperature setting at ≥ 31 and (iii) a hot temperature setting at > 55°C. For the 12 washing machines reviewed, the average temperature is 26°C (range 15-30°C), 50°C (range 40-60°C) and 80°C (range 60-95°C) for a cold water setting (Table 1), warm setting (Table 2) and hot water temperature setting (Table 3), respectively. The temperature reached by individual washing machines is dependent on load size, fabric type, machine brand, water pressure, amount of detergent used, load balancing and the wash option selected (reference 1). The water temperature is also dependent on whether hot water is in use from the tap at the time of washing or whether the water is heated by the internal machine heater.

From earlier studies, and based on recommendations in the NPMP (NVHSC, 2009), there are two potential options for the use of water treatment for phylloxera. Effective disinfestation can only be achieved with a hot water immersion at a minimum temperature of either 54°C for 5 minutes or 50°C for 30 minutes (Section 7, this report).

Crucial information from the literature review is that these temperatures and durations may not be reached for some washing machines. Both the cold and warm wash cycle would not be effective for disinfesting phylloxera on clothing, as the temperature settings for cold and warm wash cycles are on average 30°C and 40°C, respectively. These temperatures are unable to cause 100% mortality in phylloxera (Section 11, this report).

Although anecdotally a hot wash is often considered a suitable disinfestation treatment for clothing, as it should reach the critical temperatures of 50°C for 30 minutes or 54°C for 5 minutes, there appears to be a wide variation in critical temperature settings between washing machines. An investigation at a 60°C setting has been conducted for six of the 12 washing machines in this review, namely, Bosch (2), Hoover (3), Miele (4), Panasonic (5), Samsung (6) and Whirlpool (7) (Figure 1; reference 8). The investigations showed that only Samsung and Panasonic reached temperatures of slightly above 60°C. Bosch, Miele and Whirlpool reached 59, 56 and 54°C, respectively (Figure 1). The maximum water temperature was only 43°C for the Hoover model DYN8163D8P-80 (3), and the washing cycle records for the water temperature were below 40°C for most of the washing cycle (Figure 1; reference 8).

This review found that choice of temperature while washing clothes is largely cost-dependent. The higher the washing temperature above 30°C, the higher the costs incurred. For instance, the running cost of the 30°C program is reduced by about 46% compared with the 40°C setting (reference 9).

Table 1. Cold wash cycle temperatures of for 12 domestic washing machines according to manufacturer's recommendations.

Washing machine brand name	Model and capacity (kg)	Temperature setting (°C)	Duration of wash (mins) (min - max)	Recommended load (kg) (min - max)
Bosch (2)	WAW32640AU 8.5kg	30	Automated	3.5
Hoover (3)	DXC 27/1-AUS 8kg	20 30	Automated 14-44	5-8 1 – 2.5
Miele (4)	WKH 130 WPS 8kg	30	38-69	2-3
Panasonic (5)	NA-148VS4 10kg	30	15-40	2
Samsung (6)	WA11M8700 11kg	Temperature (not specified)	Automated	Load sensor
Whirlpool (7)	FSCR80410 7.5kg	15 20 30	60 60 60	3 3 3 - 7
Ariston (10)	AQ104D 69D 10kg	20 30	Automated	9 1 - 6
Electrolux (11)	EWF14922 9kg	30	Adjustable	1.5
Fisher and Paykel (12)	WH8560F 8.5kg	30	Automated	2
LG (13)	WD14070SD6 9.5kg	20 30	30 30	3 3
Siemens (14)	WM16Y890AU 8kg	30	40-137	2 - 8
Simpson (15)	SWF14843 8kg	30	Automated	3

Table 2. Warm wash cycle temperatures of for 12 domestic washing machines according to manufacturer's recommendations.

Washing machine brand name	Temperature setting (°C)	Duration of wash (mins) (min – max)	Recommended load (kg) (min – max)
Bosch	40	Automated	1 - 6
Hoover	40	59	1 - 8
Miele	40	Automated	2 - 8
Panasonic	40	49 - 225	2 - 10
Samsung	Warm wash setting not available	Warm wash setting not available	Warm wash setting not available
Whirlpool	40	60 -230	1 - 7
Ariston	40	Automated	2.5 - 10
Electrolux	40	Adjustable	2 - 9
Fisher and Paykel	40	Automated	2 - 8.5
LG	40	Automated	5.5 - 6.5
Siemens	40	Quick 40°C for 15 minutes otherwise automated	2 - 6
Simpson	40	Automated	2 - 4

Table 3. Maximum temperatures at a setting of 55°C and above for 12 commonly available washing machines, according to manufacturer's documentation.

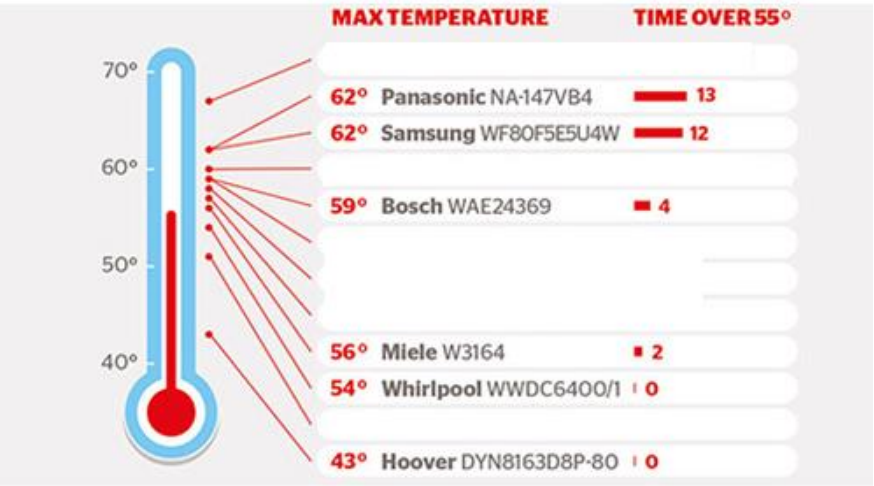
Washing machine brand name	Temperature setting (°C)	Duration of wash (mins) (min – max)	Recommended load (kg) (min - max)
Bosch	60 80 90	Automated	2 - 7 0 (Drum clean only) 5 - 8.5
Hoover	60 90	Automated	1.5 - 5.5 5 - 8
Miele	60 75 90	Automated	2 - 8 0 (Drum clean only) 8
Panasonic	60	142 - 225	5 - 10
Samsung	Hot (not specified)	Automated	Load sensor
Whirlpool	60 95	80 - 140 160	3 - 7 7
Ariston	60 90	Automated	5 - 10 10
Electrolux	60 90	Adjustable	3 - 9 9
Fisher and Paykel	60 90	Automated	2 - 8.5 4 - 8.5
LG	60 95	Automated	5.5 - 6.5 8
Siemens	60 90	147-236 137	2 - 8 5 - 8
Simpson	60 90	Automated	3.5 - 8 7 - 8

Wash cycle durations

The literature review showed that the average duration for a cold, warm and hot wash cycle is 50 minutes (range 14-137 mins), 108 minutes (range 40-230 mins) and 152 minutes (range 80-236 mins), respectively. Washing cycle duration is largely dependent on the cold, warm and hot temperature setting. Of the 12 washing machines reviewed, seven had an automated setting. The manuals indicated that the duration of the wash varied according to load size, cycle selection based on fabric, water flow rate and water pressure (reference 1). In addition, unexpected events in a wash cycle such as unbalanced loads and overuse was specified to lengthen the washing duration (reference 1). In some washing machines (e.g. Samsung), the program time after the load-sensing phase is completed can be shorter than that estimated (reference 6).

Actual washing time also differs slightly from the displayed washing cycle duration. The displayed time for most washing machines includes the time needed for the water to fill up and drain out and varied depending on water pressure, drainage and load size (reference 1).

In addition, the duration at some temperature settings may not be maintained for the entire washing cycle for some washing machines. A comparative study of five washing machine brands (i.e. Bosch, Hoover, Miele, Panasonic, Samsung and Whirlpool) showed that duration of wash cycle at 60°C varied with the make of the machine (reference 8). The study showed that the duration at which water temperatures were held above 55°C at a 60°C setting was 12 and 13 minutes for Samsung and Panasonic washing machines, respectively. For Bosch, Hoover, Miele and Whirlpool machines, the time the water temperature was held above 55°C was less than 4 minutes (range 0-4 mins) (Figure 1; reference 8).



Source: Stevens (2016) (reference 8)

Figure 1. Selected washing machines tested at a 60°C washing cycle, maximum temperature reached and the length of time each washing cycle spent above 55°C.

Pathogens and insects

Studies on the efficacy of washing temperature to kill pathogens and bed bugs have been documented (Rutala and Weber, 1997; Patel et al. 2006; Naylor and Boase, 2010; Tano and Melhus, 2014). For clothes infested with bed bugs, washing and drying the clothes on the hottest temperature the fabric can safely withstand is recommended (Naylor and Boase, 2010). To only kill bed bugs without the need to wash clothes, putting infested items in a dryer for 30 minutes on high heat has been shown to be effective (Naylor and Boase, 2010).

Previous work showed that bacteria and viruses die at the temperature of at least 60 °C (references 1, 3) but not 40°C (Tano and Melhus, 2014). The median washing time to kill bacteria at 60°C is 15 min (range 13–17 min) and for a full washing circle 30 min (range 30–33 min). Following the washing cycle with a drying process contributes significantly to a reduction in bacteria numbers and the potential to carry over into the next washing cycle (Rutala and Weber, 1997; Naylor and Boase, 2010; Tano and Melhus, 2014). Tano and Melhus (2014) showed that without tumble drying, there was a risk of contaminating test samples with bacteria during the next washing process. A 75 or 90°C washing cycle is effective against bacteria, viruses and bed bugs (reference 8; Naylor and Boase, 2010; Tano and Melhus, 2014). To save energy, however, it is possible to use a washing temperature of 60°C followed by tumble drying session for 30 minutes (Naylor and Boase, 2010; Tano and Melhus, 2014). The use of a bleach-based product and washing powder could also significantly contribute to mortality of heat–resistant bacteria (reference 8) and possibly insects. Bleach is already a recommended as disinfestation procedure against phylloxera (Clarke et al., 2017) on footwear. However the concentration required (2% sodium hypochlorite) may damage clothing. There are no published studies that have examined the impact of washing powder detergent on grape phylloxera.

Conclusions and Recommendations

Disinfestation of phylloxera on clothing using an insecticide spray

The efficacy of an Esbiothrin/Permethrin based insecticide spray was tested against six endemic strains of phylloxera. This treatment has also been proven to be effective against the endemic phylloxera strain G38 (Appendix 1, this report). The particular formulation used was effective at a spray duration of 5 seconds resulting in 100% mortality across all strains. Insecticide could therefore be considered as a disinfestation option (Table 5) for small articles of clothing such as gloves or hats and even hand tools (e.g. pruning shears). However, it also important to consider health and safety regulations concerning the use of insecticides and MSDS information should be examined prior to use.

Disinfestation of phylloxera on clothing using a hot wash

From earlier studies there are two potential options for the use of hot water treatment (based on studies on hot water immersion of grapevine cuttings) of phylloxera. According to experiments in this study (Section 7), a minimum hot water immersion of 50°C for 30 minutes or 54°C for 5 minutes would be required for effective disinfestation for phylloxera. Across the range of domestic washing machine appliances reviewed it was evident that there is large variation in both high temperatures and durations of hot wash cycles and not all brands

examined met the HWT disinfestation requirements. Therefore, although a potential disinfestation requirement is suggested based on the use of hot water (Table 5) it is with the provision that it is the responsibility of the operator to check the manufacturer's guidelines for the particular washing machine brand to ensure that the appropriate temperature and duration of cycle is reached.

Table 5. Current disinfestation protocol and suggested modification for movement of vineyard visitors out of a PIZ or PRZ (NPMP; Procedure H (3)).

Current disinfestation protocol for clothing (excluding footwear) (National Vine Health Steering Committee, 2009).	**Effective disinfestation procedure based on experimental results***
Change, wash or discard (if disposable) clothing before entering next vineyard)	Change or discard (if disposable) clothing before entering next vineyard)
	OR Wash clothing on a hot wash cycle using a domestic washing machine ensuring that the water temperature reaches EITHER 54°C for 5 minutes OR 50°C for 30 minutes.**
	OR spray small articles of clothing with a fast knockdown insecticide spray for at least 5 seconds at a minimum distance of 8 cm**

* Suggested modification in bold – any proposed modification must be ratified by the relevant biosecurity body**.**

** Refer to manufacturers instruction manual for HOT WASH Cycle temperature and duration; a.i. 1.1g/kg Esbiothrin and 0.5g.kg Perme.

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SECTION 10.

EFFICACY OF COLD TREATMENT FOR DISINFESTATION OF GRAPE PHYLLOXERA ON DIAGNOSTIC SAMPLES

Output: ***Data set demonstrating the effectiveness of cold disinfestation treatment of phylloxera on diagnostic samples.***

Summary

The recommended protocol (NPMP; National Vine Health Steering Committee, 2009) for the movement of diagnostic samples of grapevine material from a PIZ or a PRZ into a PEZ includes *"freezing to -18^{o}C for 24 hours, and packed in dry ice for transport"* (*National Phylloxera Management Protocol Procedure C - Disinfestation procedure (a)*). Additionally, in some research instances, diagnostic samples are moved under permit and stored at 5°C prior to analysis. There are no published studies that validate the effect of either 5°C or -18^{o}C against genetically diverse endemic phylloxera strains. In this study, temperatures of 4°C and 20°C were tested against six endemic strains of phylloxera and recommendations for modification of the disinfestation procedure are discussed.

Background

The current cold treatment disinfestation method applies to diagnostic samples of parts of the grapevine and vineyard soil samples. The recommended procedure is freezing samples to -18^{o}C for 24 hours before transportation from a PIZ or PRZ to a PEZ. In addition, under a special state permit, some diagnostic samples (e.g. soil) can be moved between quarantine zones without any heat treatment requirement (Giblot-Ducray et al. 2016). There is no published data which demonstrates the efficacy of either -18 or 4^{o}C as an effective disinfestation treatment for grape phylloxera. Previous studies have examined the effect of -16^{o}C temperature on adults, eggs and crawlers on a leaf galling strain of phylloxera from Rutherglen, but the genetic strain used was not characterised (Buchanan *et al*., 1996). G1 phylloxera has been shown to survive for up to 8 days at 5°C when immersed in water (Korosi et al. 2009).

Our study firstly validated the effectiveness of cold treatments against first instars and secondly, examined egg viability of six phylloxera genetic strains.

Materials and methods

Effect of cold treatment on first instar phylloxera survival

Eggs of G1, G4, G7, G19, G20 and G30 phylloxera were collected from excised roots (held in the clonal collection (see Section 1) using a small artist's paint brush and placed on moistened filter paper in a 90 x 25mm petri dish. The petri dishes were sealed with cling film to create a hatching chamber and incubated in the dark at a constant temperature of 22°C. The eggs were monitored daily and newly emerged active first instars were collected and used for experiments.

For each of the six phylloxera genetic strains, 10 first instars were transferred into a 90 x 25mm petri dish and placed on a filter paper. The petri dish was sealed with cling film and first instars subjected to temperature treatments of -20, 4 and 22^{o}C (control) for 6, 12 and 24 hours. The experiment was replicated five times for all temperatures and time durations across the six phylloxera genetic strains in full factorial combination.

Following treatments, first instars were removed from the petri dishes using a fine artist's paintbrush, placed onto a damp filter paper and examined under low power binocular microscope. First instars were classified alive if there was observable leg and/or antennal movement and dead if there was no apparent movement when gently manipulated with the tip of the paint brush up to 2 hours after treatment. First instars that survived treatments, those that died and were missing were counted and recorded.

Effect of cold treatments on phylloxera egg viability

Eggs of G1, G4, G7, G19, G20 and G30 phylloxera were collected from excised roots using the same procedure as above. For each genetic strain, 10 eggs were placed on a filter paper. The eggs were subjected to -20, 4 and 22^{o}C for 6, 12 and 24 hours and the experiments replicated five times for both temperatures and time durations across the six phylloxera genetic strain in full factorial combination. Eggs that hatched within seven weeks after treatments were counted and recorded.

Percentage of surviving first instars (calculated as surviving first instars out of total insects recovered after treatments multiplied by 100) was analysed using an ANOVA. Percentage egg hatch (calculated as number of eggs that hatched out of ten multiplied by 100) was analysed using ANOVA. Fishers protected LSD (p= 0.05) were used to separate means where F-tests were significant. Data were analysed in GenStat (GenStat Release 16.1, 2015).

Results

Effect of cold treatments on first instar phylloxera survival

Decreasing chilling temperature and time of exposure significantly impacted survival of first instars ($p<0.001$) (Figure 1a, b), with reduced survival as temperature decreased and time of exposure increased. The results validated the disinfestation recommendation of -20ºC for an exposure period of 24 hours, with 100% mortality achieved across the six genetic strains, but also showed that no first instars survived at -20°C for 12 hours.

First instars across the six genetic strains survived treatments when subjected to 4ºC for 6, 12 and 24 hours (Figure 1). Results showed a significant difference in response to chilling temperature between phylloxera genetic strains ($p<.001$) with differences observed when first instars were subjected to 4ºC. First instars of G1 phylloxera were most susceptible to treatments, with 54% survival compared to over 60% (range 64-88%) survival from G4, G7, G19, G20 and G30 strains. At room temperature (22°C) survival was over 90% across all strains (Figure 2).

Effect of cold treatments on phylloxera egg viability

Egg hatchability was influenced by both temperature and time of exposure across the six genetic strains ($p<.001$) (Figure 2). None of the eggs hatched when subjected to -20ºC for 12 and 24 hours. However, 6% of G30 phylloxera eggs subjected to -20°C for 6 hours hatched within seven days following treatments (Figure 2). Eggs that did not hatch were characterised by dark coloration within four days of exposure to cold treatments compared to eggs in the control treatments which were a light yellow coloration until hatching (Figure 3).

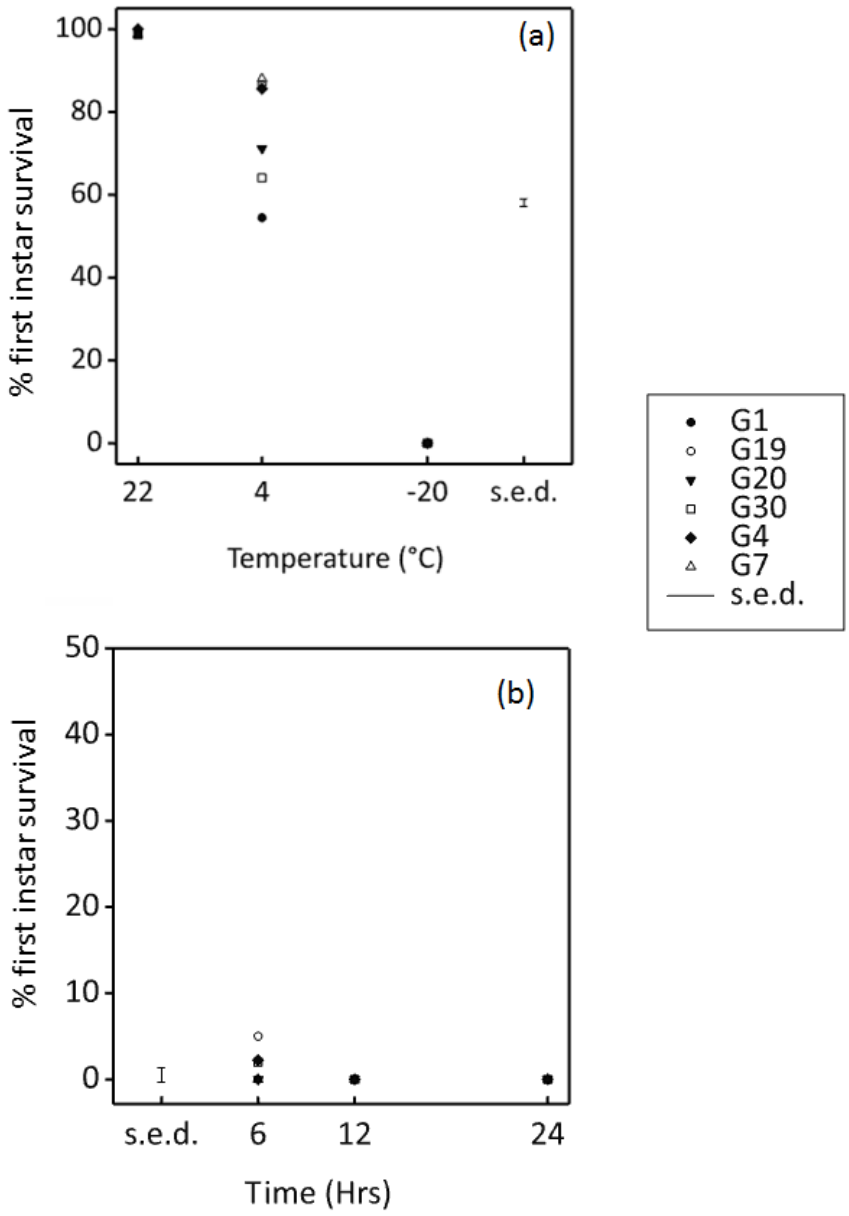


Figure 1. Effect of (a) chilling temperatures on six phylloxera genetic strains and (b) time of exposure on first instars survival of six grape phylloxera strains at -20°C. Data presented are percentage means of first instars combined for the three exposure durations.

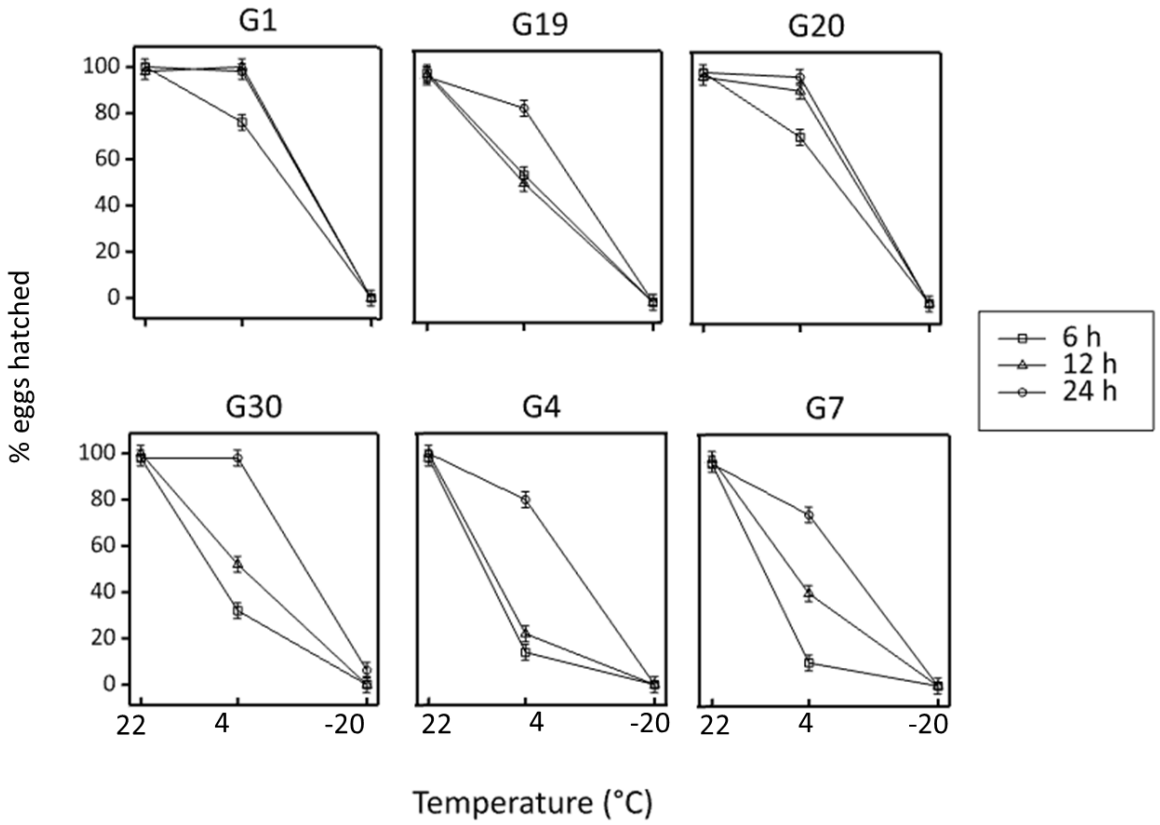


Figure 2. Effect of temperature and time of exposure on % egg hatching of six phylloxera genetic strains.

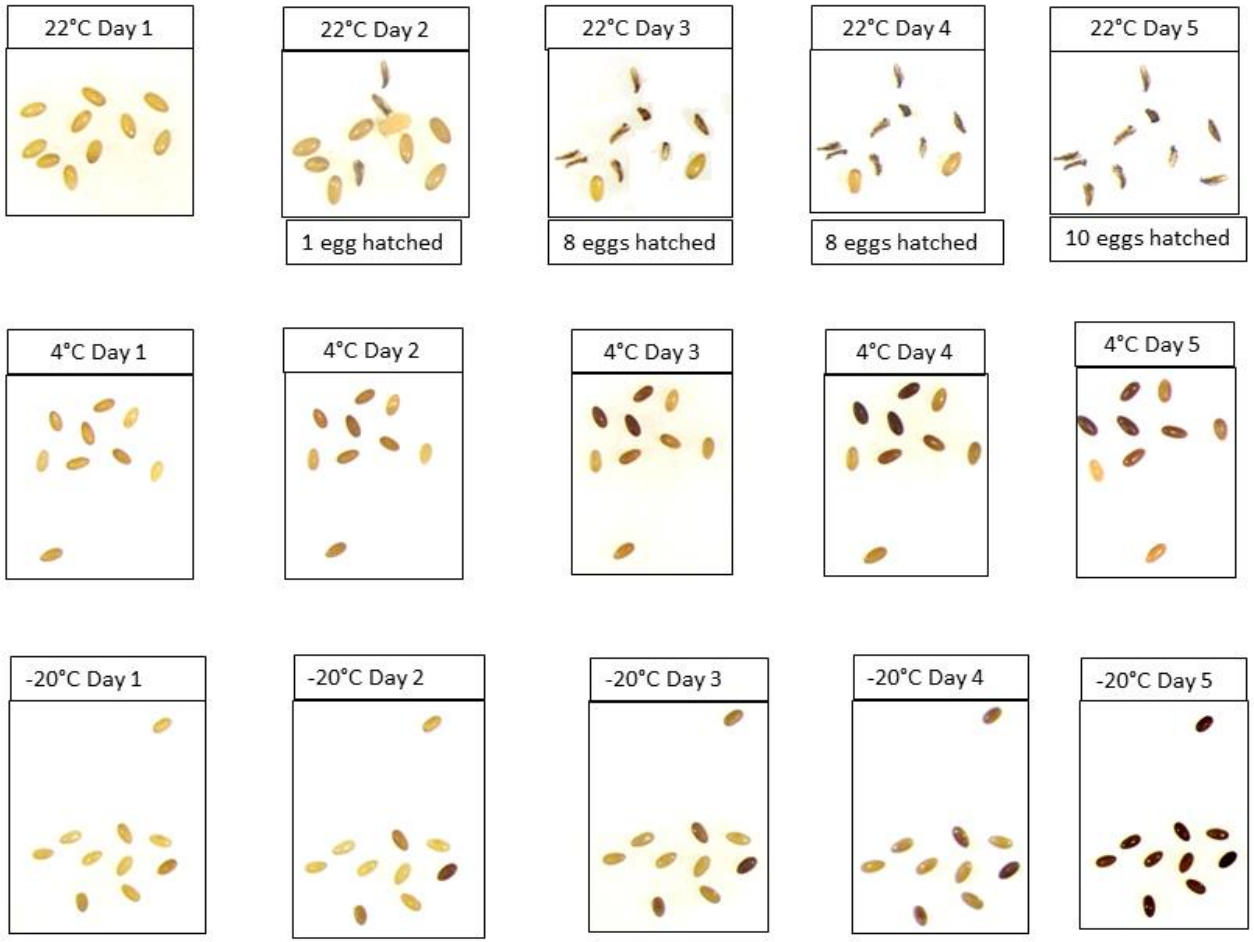


Figure 3. Characteristics of G1 phylloxera eggs five days following a 24 hours treatment at -20, 4 and 22°C.

Conclusions and Recommendations

The current recommendation for transport of diagnostic samples (Procedure C; NPMP; National Vine Health Steering Committee, 2009) is "*freezing to -18°C for 24 hours; and packed in dry ice for transport"*. Previous studies have examined the effect of -16ºC on adults, eggs and crawlers on a leaf galling strain of grapevine phylloxera population from Rutherglen. However, the genetic strain used was not characterised in that study. The study showed that 100% mortality of the three life stages was achieved after 24h at -16ºC (Buchanan et al. 1996). In our study, we used -20°C and 4°C as cold treatments, as these temperatures represent the average temperature settings of laboratory freezers and refrigerators, respectively. At -20°C for 12 hours, 100% mortality of first instar nymphs and eggs across six strains of phylloxera was achieved. In addition, this treatment proved to be effective against the endemic phylloxera strain G38 (Appendix 1, this report).

If this disinfestation procedure were to be used, then the additional protocol of transferring samples on dry ice (dry ice or frozen carbon dioxide has a surface temperature of -78.5ºC) would not be required. However, at 4ºC, first instars across the six genetic strains survived after 24 hours. In addition, this treatment has been proven to be ineffective against the endemic phylloxera strain G38 (Appendix 1, this report). Therefore using 4°C as a disinfestation treatment is not recommended because viable phylloxera would potentially remain in the diagnostic sample.

Table 1. Current disinfestation protocol and suggested modification to cold temperature disinfestation protocol for movement of diagnostic samples from a PIZ or PRZ vineyard into a PRZ or PEZ (NPMP; Procedure C (a)).

Current protocol (National Vine Health Steering Committee, 2009)	Suggested modification to protocol*
-18°C for 24 hours, and pack in dry ice for transport	**Treat sample at -20°C (laboratory freezer) for 12 hours**
**4°C	**Not suitable as a disinfestation treatment**

**4°C is not a national protocol but transport of some diagnostic samples without heat treatment is allowed by some states under state quarantine regulations.

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SECTION 11.

EFFICACY OF ALTERNATIVE DISINFESTATION TREATMENTS FOR FOOTWEAR AND HAND TOOLS

Output: ***Dataset demonstrating the effectiveness of alternative chemicals as disinfestation treatments for phylloxera on footwear and hand tools.***

Summary

As a potential alternative to the use of household bleach for disinfestation of footwear and small hand tools, a range of chemical disinfestation treatments were tested against six phylloxera strains. Of the six different products tested, only methylated spirits at 30 seconds duration showed comparable efficacy to household bleach treatments of 2% NaOCl for 60 s.

Background

Sodium hypochlorite (NaOCl) is currently recommended for use as a disinfestation treatment for grapevine phylloxera (NPMP; National Vine Health Steering Committee, 2009). The current recommended disinfestation protocol for footwear and hand held tools requires "*immersion in 2% NaOCl solution for 30 s followed by a thorough rinse in water* (National Vine Health Steering Committee, 2009). However recent studies (see Section 4, this report; Clarke et al. 2017) have highlighted that the disinfestation procedure should be modified to *2% NaOCl solution for 60 s with no water rinse* to be effective across a diverse range of phylloxera strains. However, there are health and safety concerns around the use of sodium hypochlorite, particularly as the duration of immersion recently recommended is longer than previously recommended. Therefore, alternative disinfestation treatments are required. In this study, six different products were evaluated as alternatives to NaOCl treatment for phylloxera disinfestation in footwear and handheld tools.

Materials and Methods

Eggs of G1, G4, G7, G19, G20, and G30 phylloxera were collected from excised roots using a small artist's paint brush and placed on moistened filter paper in a 90 x 25mm petri dish. The petri dishes were sealed with cling film to create a hatching chamber and incubated in the dark at a constant temperature of 22°C. The eggs were monitored daily and newly emerged active first instars were collected and used for all experiments.

Newly emerged first instars were placed in a plastic vial (5.5 m high, 2.5 m diameter) and subjected to six treatment solutions for 30 and 60 seconds followed by a rinse in water for 30 s. The treatment solutions were, Biopest, ethanol, methylated spirits, Phytoclean, Pulse Penetrant, and Virkon (Table 1). Similarly, a second treatment without a water rinse afterwards was conducted. Treatment solutions were prepared to a volume of 500ml in a beaker at the manufacturer's recommended dose specified for disinfestation of insect pests and pathogens (Table 1). For ethanol and methylated spirit where no specifications were available, solutions were used at 80% and without dilution (Table 1). Each treatment was conducted in separate trials with five replicates of 10 insects each using the six genetic strains.

After treatment, first instars were removed from the vials using a sable-haired paintbrush that was moistened in water, placed onto a clean filter paper in a petri dish and examined under low power microscope. Where there was observable movement of antenna and legs when stimulated with the tip of the paintbrush, the first instars were classified as surviving. Where there was no apparent movement two hours after treatment, first instars were scored dead. The total number of first instars recovered from the vials and those that were missing were also recorded.

Percentage first instar phylloxera survival was calculated as the number of insects surviving treatments out of the total recovered from vials multiplied by 100. Percentage survival data was subjected to ANOVA. To compare the efficacy of the potential alternative disinfestants with the current recommendation (2% NaOCl for 30 s) and the effective treatment (2% for 60 s without water rinse afterwards - see section 4), first instar survival was analysed using ANOVA. Data were analysed in GenStat Release 16.1 (2015).

Results

Overall the six phylloxera genetic strains responded differently to treatments (Figure 1). Time of exposure to treatments influenced survival with a high survival recorded at 30 seconds compared to 60 seconds immersion. Of the six chemical products tested, only methylated spirits at 30 seconds (and 60 seconds) was effective in achieving 100% mortality across all the six genetic strains (Figure 1). This treatment was also effective against the endemic phylloxera strain G38 (Appendix 1, this report). Ethanol was 100% effective against G1, G4 and G7 phylloxera (and G38 – refer to Appendix 1, this report) but not against G19, G20 and G30 where average survival was 7, 18 and 55%, respectively (Figure 1).

Treatments with the recommended dilution using Biopest, Pulse Penetrant, Phytoclean and Virkon followed by a water rinse or without the water rinse did not achieve 100% first instar

mortality across the six phylloxera strains. However, first instar survival was reduced as time of immersion in treatment solutions was increased (p=0.02) (Figure 2).

Table 1.Disinfectant products trialled against six genetic strains of grape phylloxera.

Brand Name	Active ingredient	Source	Manufacturers recommended concentration for pathogens and insects	Evidence disinfestations treatment use
Virkon[1]	49.8% potassium peroxymonosulfate	DuPont Animal Health Solutions, Suffolk, UK	1% (w/v)	Used in orchards against viruses, bacteria and fungi, Avian Influenza, Salmonella and Campylobacter
Phytoclean (Sanitiser)[2]	100g/L benzalkonium chloride	Robjulz Pty Ltd. Australia	10% (v/v)	Disinfection of common nursery surfaces from various plant pathogens
Pulse Penetrant[3]	1020g/L olydimethyl siloxane	Nufarm Australia Limited, Laverton, Victoria	0.2% (v/v)	Recommended for use against mealy bugs, leafhoppers, psyllids in cotton.
Biopest[4]	815 g/L paraffinic oil	SACOA Pty Ltd. Western Australia	1% (v/v)	Recommended for use against aphids and mites in low infestations
Ethanol[5]	Ethyl alcohol	Wilmar BioEthanol, Yarraville, Victoria	*80% (v/v)	Recommended for diagnostic sample movement for phylloxera
Methylated spirit[6]	95% Ethyl alcohol	Recochem Inc. Lytton, QLD	*undiluted	No recommendations

*No manufacturer's recommendation for pests and pathogens. Numbers 1-6 refer to reference sources

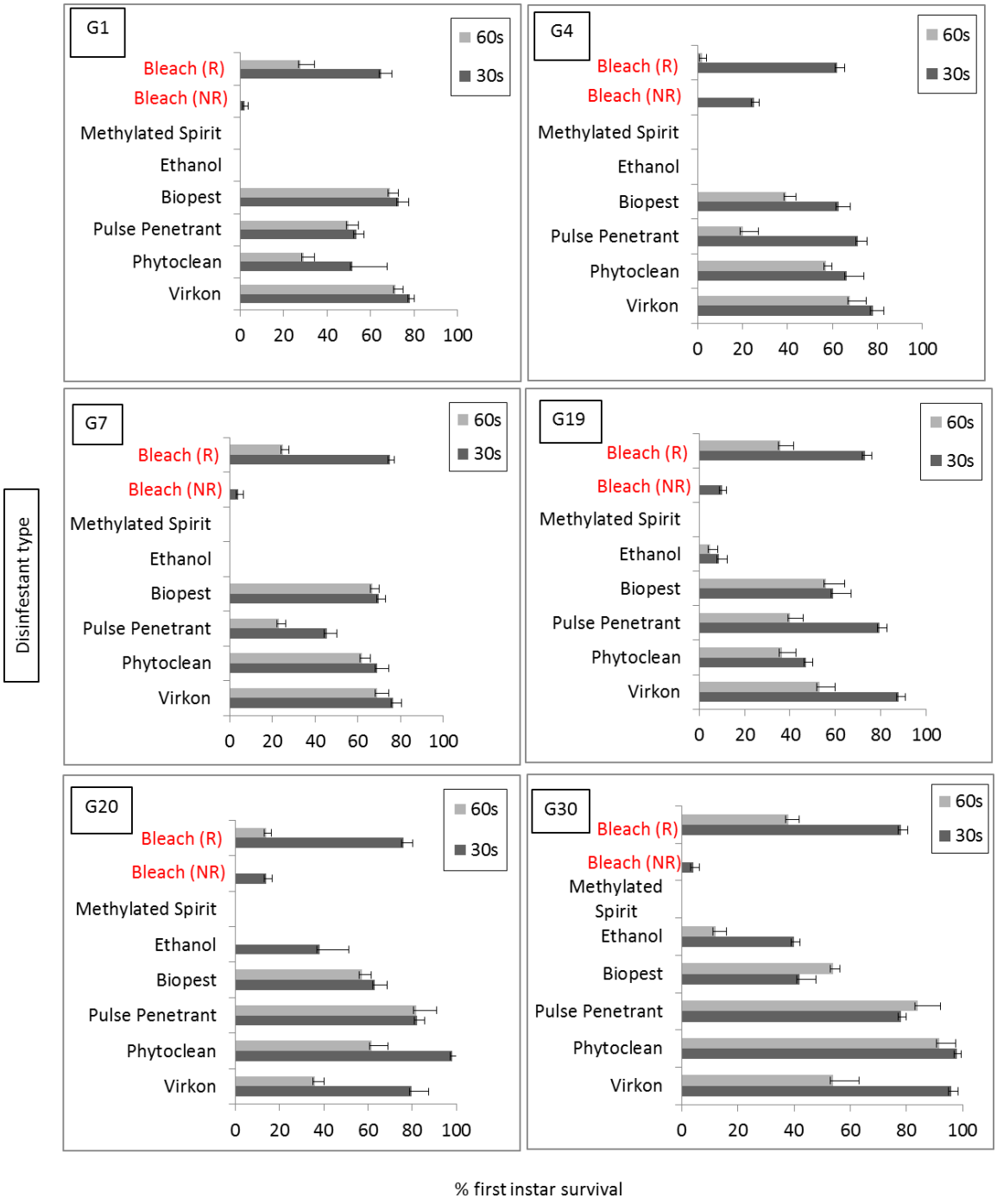


Figure 1. Effectiveness of Methylated spirit, Ethanol, Biopest, Pulse Penetrant, Phytoclean and Virkon against six phylloxera genetic strains when subjected to treatments at 30 and 60 seconds followed by a water rinse compared to the recommended treatment with household bleach (a.i sodium hypochlorite) highlighted in red. R = Water rinse after treatments, NR= Not rinsed in water after treatments.

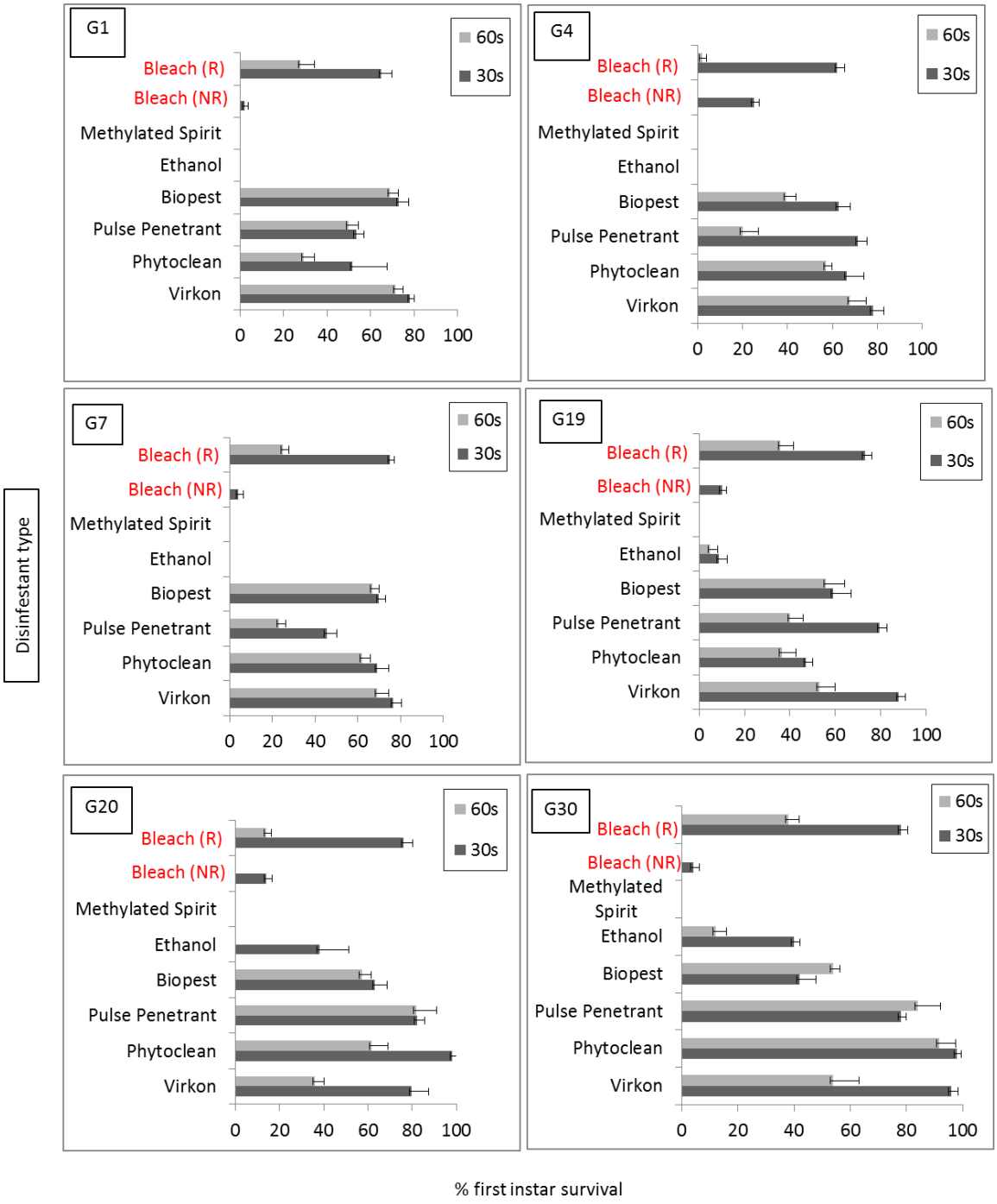


Figure 2. Effectiveness of Methylated spirit, Ethanol, Biopest, Pulse Penetrant, Phytoclean and Virkon, without a water rinse afterwards, in the disinfestation of six phylloxera genetic strains when subjected to the treatments for 30 and 60 seconds. Sodium hypochlorite (Bleach), the nationally recommended treatment is highlighted in red. R = Water rinse after treatments, NR= Not rinsed in water after treatments

Conclusions and Recommendations

Of the six potential alternative chemical disinfestation treatments tested, only methylated spirits was 100% effective across all strains at both 30 and 60 seconds immersion. Phytoclean is commonly used as a disinfestation product in footbaths but it had minimal effect on phylloxera even after treatments were not rinsed in water afterwards. The active ingredient of Phytoclean is benzalkonium chloride, which is a known bactericide. Although Phytoclean has been used to surface sterilise insect eggs (Connell, 1981), there is no evidence that it has insecticidal properties. Virkon, with potassium peroxymonosulfate as the active ingredient, has bactericidal and virucidal properties and is sometimes recommended for disinfestation of vineyard machinery (Anon, 2009) but again there is no evidence of insecticidal properties.

Both Pulse Penetrant and Biopest, although having insecticidal properties to a range of Hemipteran pests, were relatively ineffective when compared to NaOCl and methylated spirits and would not therefore be suitable disinfestants.

Ethanol at 70% concentration (v/v) is a recommended fixative to devitalise diagnostic samples (NPMP; Movement of diagnostic samples from a PIZ or PRZ into a PRZ or PEZ, National Vine Health Steering Committee, 2009) however the time of sample immersion is not specified in the protocol. In our studies, we immersed phylloxera in an 80% ethanol solution and a proportion of G19, G20 and G30 strains survived, even when immersed for up to 60 seconds.

The only treatment that was 100% effective across all strains was undiluted methylated spirits. This product could therefore be considered as a potentially suitable alternative to NaOCl (see Table 2). However, as with all disinfestation treatments, health and safety issues need to be considered. For example, methylated spirits is highly flammable compared to NaOCl. Further screening should be conducted to determine the efficacy of other potential disinfestation chemicals.

Table 2. Current disinfestation protocol and suggested modification to sodium hypochlorite disinfestation protocol for movement of vineyard visitors out of a PIZ or PRZ vineyard (NPMP; Procedure H (1, 2)). Recommendations from this section have been combined with those from Section 4.

Current protocol National Vine Health Steering Committee (2009).	Effective disinfestation procedure based on experimental results
When leaving the vineyard, disinfest footwear as follows: Scrub the boots with the scrubbing boots to remove mud – preferably in water and detergent	
Dilute chlorine with water in a tub to give a 2% active sodium hypochlorite concentration and dip and scrub boots in the freshly prepared solution for a minimum of 30 seconds.	Dilute **household bleach** with water in a tub to give a 2% active sodium hypochlorite concentration and dip and scrub boots in the freshly prepared solution for a minimum of **60** seconds.
	OR place freshly prepared undiluted methylated spirits in a tub and dip and scrub boots in the solution for a minimum of 30 seconds.
Rinse thoroughly in clean water after immersion.	**DO NOT** rinse thoroughly in clean water **immediately** after immersion.
Wash and disinfest snips, small tools etc. with a 2% active sodium hypochlorite solution.	Wash and disinfest snips, small tools etc. with a 2% active, **freshly prepared,** sodium hypochlorite solution **for a minimum of 60 seconds.**
	OR with freshly prepared undiluted methylated spirits for a minimum of 30 seconds.

*Suggested modification in bold – any proposed modification must be ratified by the relevant biosecurity body.

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SECTION 12.

THE USE OF SNIFFER DOGS FOR PHYLLOXERA DETECTION – A PILOT STUDY

Output: *Demonstration of potential efficacy of sniffer dogs for phylloxera detection under controlled conditions.*

Summary

Over the last decade, phylloxera detection methods have proven to be labour intensive, costly, have low sensitivity, and are relatively slow in terms of their detection rate. A review of the available methods for phylloxera detection, including molecular, chemospectral fingerprinting, remote sensing and trapping techniques, has recently been published by the Project Leader (see Benheim et al. 2012). None of the existing methods are cost effective for industry and the relevant biosecurity agencies. A pilot study was therefore conducted to determine the potential for using sniffer dogs for detection of grapevine phylloxera, based on the enhanced ability of dogs to detect odours given off by either insect pests or their host plants. Studies were conducted under controlled laboratory conditions and preliminary indications suggest that dogs can recognise odours produced by either phylloxera in isolation or phylloxera-infested roots. Further research into this area is discussed and recommendations are made accordingly.

Background

Sniffer dogs have been used for a variety of insect pests, including bed bugs (Vaidyanathan, and Feldlaufer, 2013), termites (Brooks et al. 2003), and the biosecurity pest, the red imported fire ant (Lin et al. 2011).

Detection of grapevine phylloxera is difficult due to the insect's small size, asexual life cycle and predominantly subterranean habitat. Although a variety of detection approaches for phylloxera are available, including visual observation of phylloxera-induced root and foliage damage, soil DNA testing and the use of emergence traps, we still do not have an efficient and reliable early detection system for area-wide surveillance of this pest. In most instances, once phylloxera has been detected, significant damage to the grapevine root system has already occurred, resulting in yield losses and considerable risk of further spread of the insect. Previous studies have shown that grapevine leaf chemistry is affected by the presence of phylloxera on the root system, and the production of particular chemicals are up-regulated or

down- regulated in a host plant which has phylloxera feeding on the root system (Benheim et al, 2012). Given the production of these chemicals, a detection approach focusing on specific host plant “identifier” chemicals is worth investigating.

Odour detection dogs could be used to recognise the presence of volatile chemicals that are emitted either directly from phylloxera insects or grapevine roots when attacked by phylloxera, provided they can discriminate between phylloxera (target) and non-phylloxera material (non-target). Dogs have a highly sensitive olfactory system, and when exposed to a target odour using an appropriate training regime, can learn to recognise these odours under variable environmental conditions when the target odour is present amongst non-target odours.

This short-term pilot study was designed to determine if trained detection dogs could identify the presence of odours related to (i) phylloxera life stages or (ii) grapevine roots infested with phylloxera.

Two dogs were trained to detect grape phylloxera using a Pavlovian conditioning training method supplemented with a food and play reward system. The ability of dogs to discriminate between target odours (i.e. phylloxera) and non-target odours was tested in two trial scenarios (i) using a known number of G1 phylloxera first instars and eggs with no host *Vitis* material and (ii) using various numbers and life-stages of G1 phylloxera established on *Vitis vinifera* roots.

Materials

Dogs

Two Labrador Retrievers, Glory (fox red colour) and Mamba (black colour), bred by the Credible Canines Pty Ltd, Brisbane were used in the study. Both dogs originated from the same litter and were 20 months old at trial commencement. Dogs were handled throughout the training period by Mr Craig Murray (Senior Dog Trainer at Credible Canines Pty Ltd, QLD). The dogs’ wellbeing during the course of the study was regulated by guidelines endorsed by Agriculture Victoria’s Agricultural Research and Extension Animal Ethics Committee.

Phylloxera

G1 phylloxera were mass-reared *in vitro* at Agriculture Victoria, Rutherglen, Victoria in a dark controlled environment growth room (25±2°C) under quarantine conditions on excised roots of *V. vinifera* Chardonnay in 90 x 25 mm petri dishes (Kingston et al. 2007). The end of the roots were wrapped in cotton wool and moistened once every week with sterile water to keep them viable for the insects nutritional requirements. Where fungal growth was evident, the

roots were cleaned by dabbing the contaminated root section with a sable-haired paintbrush slightly moistened in 80% ethanol. In trial one, newly emerge first instars and eggs were removed using an artist's paintbrush and gently placed on moistened filter paper in a 35 mm Petri dish. The Petri dishes were sealed with clingfilm (Rapfast PVC food packaging, Integrated Packaging, Australia) around the edge, and a fine mesh on the top of petri dishes allowed odours to permeate in the atmosphere. In trial two, excised root bioassay plates heavily infested with phylloxera were used. Prior to use, all plates were incubated in the dark at a constant temperature of 22°C.

Methods and Results

Pre Trial – Non Target Preconditioning

Over a four-week period, both dogs were preconditioned at Credible Canines Pty Ltd, Brisbane, to any non-phylloxera material used in the trial. This included plastic Petri dishes, cotton wool, glue, fine mesh, filter paper and PVC containers. This preconditioning stage was critical as it reduced the likelihood that dogs would respond to non-target odours used in the experimental study.

Trial One - Olfactory Imprinting for Phylloxera

An olfactory learning method was developed using either phylloxera alone (Trial 1a) or phylloxera infested grapevine roots (Trial 1b) over a period of one week as follows:

(a) Both dogs were tested with 100 first instar phylloxera and 100 G1 phylloxera eggs, which were placed on moist filter paper in vented petri dishes. The Petri dishes were placed in vented screw top PVC containers.

(b) Both dogs were tested with G1 phylloxera-infested grapevine root pieces (containing >200 phylloxera of all life stages), which were placed on moist filter paper in vented petri dishes. The Petri dishes were placed in vented screw top PVC containers.

In both trials an olfactory learning method was used whereby both dogs were exposed to the containers with either (i) only phylloxera eggs and crawlers (Figure 1) or (ii) grapevine roots infested with all life stages of phylloxera. The process of conditioning the dogs to the positive phylloxera target odour and vessels involved exposing the dogs repeatedly to the target odour source to form a scent association in a positive and motivating scenario. Using this method, the dogs were rewarded with either food or play activity (e.g. ball games) each time they

recognised the target odour source. This recognition of a positive target to gain reward is a form of Pavlovian association (Hilliard, 2003).

The olfactory process to imprint the odour (associated with phylloxera presence) was continued for several days with multiple rapid-fire repetitions. By the end of week, both dogs had been given 169 exposures to the positive phylloxera vessels and both showed behaviours that were driven and motivated. However as the dogs became too motivated (which can be counter-productive) control exercises were introduced which included (i) on-lead control from vehicle to scent lab and start of search command and (ii) calming or minor control exercises.

Once dogs had been olfactory imprinted to phylloxera insects only (Trial 1a) the trial conditions were modified to incorporate grapevine roots infested with grape phylloxera (Trial 1b), as this is more likely 'in field' scenario and plant odours may also be involved in the olfactory detection response.



Figure 1. Initial Phase of Olfactory Imprinting using phylloxera in a sealed vessel.

Following the initial olfactory learning phase which used only single containers, a single wooden rack system (Figure 2) was introduced, followed by multiple containers (Figure 3), so that the dogs could leave the handler and go to the target odour vessel, demonstrate an indicative behaviour of a positive odour and be quickly and repetitiously exposed to the target odour.



Figure 2. Incorporation of rack system to allow dog to focus on phylloxera odour rather than handler.

The next olfactory learning phase was incorporation of additional scent racks and continuation of repetitive training to challenge the dogs to discriminate the target odour vessels correctly (Figure 4) and prepare them for the ever-increasing changes to the training process which is required before completion of blind testing olfactory recognition. During this phase, multiple styles of reward and reward scheduling rates were included to maintain and build the dogs motivational drives.



Figure 3. Incorporation of multiple containers to begin discrimination process.



Figure 4. Concealment of pots to challenge target odour discrimination.

Trial 2 – Scent discrimination - Stage 1

Once a strong level of target odour detection had been achieved (in Trial 1a and 1b) a process of scent discrimination (Trial 2) was carried out to train the dogs to distinguish between target and non-target odours. This process was on a constant scale of increasing difficulty, commencing with a smaller number of non-targets and gradually increasing the number and variety of non-targets.

In the second week, sample containers included (i) grapevine roots with all phylloxera life stages included (ii) grapevine roots without phylloxera, (iii) blank PVC vessels and (iv) PVC vessels with petri dishes including filter paper and cling wrap (but no phylloxera), which allowed the introduction of multiple non-target and target discrimination. An average of 50 exposures using this experimental set-up were conducted on a daily basis (Table 1). Over five consecutive days the position of positive targets and non-targets was changed randomly so that the dogs could only predict the phylloxera positive position by correct olfactory recognition (as opposed to vessel position) (Figure 5). By the end of week two, the dogs still showed interest in some non-target vessels regularly but would keep checking until the positive target had been located, sometimes this would occur on several non-targets, but the dogs would only be rewarded on correct positive target location. Rapid-fire repetitions were still employed to enhance the olfactory learning process.



Figure 5. Utilising double racks and multiple odour containers with four non-target and one phylloxera positive (Mamba) – starting strong indication.

Trial 2 – Scent discrimination - Stage 2 – Location change

To date all the trials had been conducted in one laboratory location at AVR Rutherglen. As dogs sometimes become acclimatised to working in one location it was decided move the trials to another building location, still on AVR Rutherglen site. This location change provided a new challenge for the dogs as they now had to deal with a change of working environment and associated distractions, which included different lighting conditions and strong winds entering the building. The wind was particularly a challenging variable as it could potentially displace any positive odour which would make discrimination more difficult. Testing under these conditions showed that only one dog (Glory) adapted well to the changed conditions (Figure 6), whereas the second dog (Mamba) lost motivation and started to struggle with scent discrimination in the new environment.



Figure 6. Working in workshop – multiple non targets and change of environment to challenge the dog to strongly discriminate. Glory showing strong indication with back turned to handler.



Figure 7. Glory precisely and strongly indicating on positive phylloxera sample whilst discriminating against multiple non-target vessels.

Trial 3 - Blind Testing

Conscious and unconscious bias is a common constraint to sniffer dog research (Johnen et al. 2017). To avoid any potential bias from the dog handler a third blind testing trial was conducted in the final week (week 5). In this blind testing process, in contrast to the previous trial (which were conducted by the trained dog handler) AVR Rutherglen staff organised random placement of phylloxera-positive and phylloxera-negative samples. Two sets of scent racks were set up, each containing six possible scent vessels with only one in each set containing phylloxera on roots. Prior to each test, the dog and its handler were moved away from the test arena (building) and called in after vessel placement had been made. Four tests were conducted on a single day. Blind testing was only conducted using a single dog, Glory (Table 1).

Blind Test no 1: Glory correctly displayed a behavioural response of dropping over the container housing the Phylloxera positive (target) (Figure 7).

Blind Test no 2: Glory walked past all vessel several times before displaying a behavioural response that correctly indentified the target sample which contained phylloxera infested roots.

Blind Test no 3: Glory displayed a behavioural response of dropping over the container housing the Phylloxera positive (target)

Blind Test no 4: Glory quickly went along the entire scent rack, only momentarily stopping with enough body language to indicate that she had correctly located the target sample (at position 3). She then continued to position no 5 (non-target) and after a short period moved back to position 3 and *gave a stronger indication of the Phylloxera positive* and dropped over the sample to gain a reward.

Table 1. Summary of sniffer dog efficacy trials.

Day	Exposures	Dog 1 (Mamba)	Dog 2 (Glory)
Olfactory imprinting - Trials 1 and 1b			
1-5	169	NR	NR
Target Scent Discrimination - Trial 2			
6	50	NR	NR
7	48	NR	NR
8	48	NR	NR
9	14	NR	NR
10	10	30%	80%
11	16-20	*50%	90%
12	3	-	NR
13	9	-	100%
14a	2	-	100%
14b		-	25%
15	8	-	100%
16	5	-	100%
Blind Trials - Trial 3			
17	4	-	100%

*At this point Dog 1 was discontinued from trial involvement due to poor discrimination. NR=No response

Discussion

The aim of this pilot study trial was to determine the potential for using sniffer dogs for the detection of grape phylloxera. There are no universal guidelines for conducting odour trials using sniffer dogs and therefore we designed this pilot study in collaboration with a qualified dog handler who has over 20 years' experience in the use of sniffer dogs for detecting biosecurity pests and diseases.

The trial highlights the complexities and variables when using dogs in this type of scenario. The first complexity is sample size. The number of dogs used in sniffer dog trials varies

between 1-10 and is generally around four (Johnen et al., 2013), but is dependent on the number of dog handlers available and their training procedure and techniques. Only two dogs were available for use in the trial described here and therefore replication and statistical analysis is not possible with our dataset. In addition, the dog which showed relatively poor discrimination between phylloxera-positive and phylloxera-negative samples was discounted from further study midway through the trial. However, the remaining dog was able to effectively (80-100%) discriminate between phylloxera-infested roots and non-target samples.

There are many variables that could influence this type of study. For example, environmental conditions were clearly a factor. Even though the trials were conducted in on-site farm buildings, strong winds at one location are likely to have affected odour dispersal, which may have made it difficult for one of the dogs to detect the odour. Detection sensitivity will vary between dogs, being dependent on genetic factors such as the dogs' olfactory system and temperament. Although both dogs in this study were from the same litter, it was clear that their temperaments differed. The genotypes of both the host plant and the insect could also have influenced the trials. Only one phylloxera genetic strain, G1, and one root type, *Vitis vinifera* cv. Chardonnay were used in this pilot study and further trials should include multiple phylloxera genetic strains.

Although no attempt was made to collect volatiles for either phylloxera or phylloxera-infested roots in this study, it is likely that some odour is being produced – otherwise the dogs would not be able to distinguish between infested and uninfested samples.

This pilot study was conducted under controlled conditions using either phylloxera alone or phylloxera-infested grapevine root. In a more 'real' situation of detection efforts in the field, the phylloxera would be present within a vineyard setting, where insects will be predominantly below ground on attached roots. In this situation, the 'odour signature' that the dogs are detecting may be modified or degraded such that the rate of changes to an odour signature, (called biotransformation (Göth et al. 2003)), will depend on many factors in the soil including humidity, temperature, presence of microorganisms and likely other physiochemical properties of the soil which also influence the abundance of grape phylloxera on the roots.

Although in this trial we did not examine which chemical components of the odour (volatiles) were emitted either by phylloxera or infested roots, it will be important to characterise these odours for their potential use with other technologies. Electronic-nose (e-nose) technologies are capable of real-time, rapid, and sensitive analysis of odours, using a hand-held device. Recent landmark advances in e-noses have paved the way to apply these devices across many fields, including the early detection of plant pests and diseases. E-nose technology is currently being examined for detection of horticultural pests of biosecurity significance (e.g.

Queensland fruit fly). E-nose technology could be trialled for phylloxera detection if key-volatile identification is conducted to enable sensor development. Volatile analysis could also be used to develop prototype synthetic blends for training sniffer dogs outside phylloxera-infested regions.

Recommendations

The targeted study has provided promising results, but it is by no means definitive. We have shown clear proof of concept that dogs can discriminate phylloxera both in the absence and presence of grapevine roots. More comprehensive testing is now required to evaluate (i) the ability of dogs to identify phylloxera infestation (e.g. compared to mechanical or other pest damage to roots) (ii) the sensitivity of dogs to detect low phylloxera infestation levels (iii) the sensitivity of dogs to detect different phylloxera strains on different grapevine cultivars and (iv) detection efficacy under field conditions. Furthermore a comparative study should be conducted under field conditions to determine the relative efficacy and cost of sniffer dogs compared with other detection approaches including e- nose, DNA testing, emergence trapping and visual surveillance.

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SECTION 13.

INDUSTRY ARTICLES RELATED TO THE PROJECT DELIVERABLES

Output: *Industry, media and scientific publications relating to project outputs.*

Summary

A number of media, industry and scientific articles were published in the course of the project.

Industry articles

In collaboration with Vinehealth Australia eight industry articles highlighting research outputs particularly phylloxera disinfestation and detection using sniffer dogs were made available on the Vinehealth Australia website.

http://www.vinehealth.com.au/media/Vinehealth-Footwear-and-Small-Hand-Tools-Disinfestation-Protocol-White-A3.pdf

http://www.vinehealth.com.au/sniffing-enemy/

http://www.vinehealth.com.au/meet-phylloxera-guru-kevin-powell/

http://www.vinehealth.com.au/dr-kevin-powells-phylloxera-fast-facts/

http://www.vinehealth.com.au/grape-phylloxera-strains-matter/

http://www.vinehealth.com.au/media/WBM-Phylloxera-Strains-Matter-MayJune-2017.pdf

http://www.vinehealth.com.au/media/GGWM-Feb-2017-Vinehealth-Australia-Phylloxera-Update-article.pdf

http://www.vinehealth.com.au/60-seconds-safe-shoes/

Media Articles

In addition, a number of media articles and interviews were conducted during the course of the project, in relation to the sniffer dog component of the project:

- Dogged phylloxera pest may meet its match. *The Weekly Times*. May 3, 2017.
- Sniffer-dogs-brought-in-to-protect-the-grapevines. *Shepparton News*. May 9, 2017.
- More grapevines at risk. *Lilydale & Yarra Valley Leader.* May 16, 2017.
- Man's best friend may be phylloxera's worst enemy. *The Vine*. July 2017. (In press).

Media Interviews

Two radio interviews were conducted with the Project Leader in relation to the sniffer dog component of the project:

- ABC Goulburn Murray Rural Report. Phylloxera sniffer dogs. May 2017.
- ACE Radio, Gippsland. Phylloxera sniffer dogs. May 2017.

Conference Papers/Presentations

Three conference papers were presented at National Conferences:

Clarke C.W., Yuanpeng, D., Carmody, B.M., Powell, K.S. (2016) Does soil type influence the establishment and development of grape phylloxera (*Daktulosphaira vitifoliae* Fitch) on grapevine *Vitis vinifera* L? Combined Australian Entomological Society/New Zealand Entomological Society Conference, Melbourne, November 2016.

Powell, K.S. (2016) Endemic strains of the biosecurity pest grape phylloxera: How can we effectively manage them? Combined Australian Entomological Society/New Zealand Entomological Society Conference, Melbourne, November 2016.

Powell, K.S. (2016) Endemic strains of grapevine phylloxera and rootstocks. 16th Australian Wine Industry Technical Conference, Adelaide, July, 2016.

Peer Reviewed Scientific Papers

Three scientific papers were produced during the project and two more articles are currently in preparation for submission:

Clarke, C.W., Wigg, F., Norng, S., and Powell, K.S. (2017). Effectiveness of sodium hypochlorite as a disinfestation treatment against genetically diverse strains of grape phylloxera *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae). Australian Journal of Grape and Wine Research, doi: 10.1111/ajgw.12288.

Clarke, C.W., Norng, S., Yuanpeng, D., Carmody, B., and Powell, K.S. (2017) Efficacy of steam and hot water disinfestation treatments for genetically diverse strains of grape phylloxera *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae) on viticulture equipment and machinery. Australian Journal of Grape and Wine Research (under review).

Clarke, C.W., Norng, S., Yuanpeng, D., Carmody, B., and Powell, K.S. (2017) Efficacy of hot water disinfestation treatments for genetically diverse strains of grape phylloxera *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae) on grapevine (*Vitis* spp) planting material. Australian Journal of Grape and Wine Research (in prep).

Clarke, C.W., Norng, S., Yuanpeng, D., Carmody, B., and Powell, K.S. (2017) Validating the effectiveness of dry heat as a disinfestation treatment against six genetically diverse phylloxera strains. Australian Journal of Grape and Wine Research (in prep).

Forneck, A., Powell, K.S., and Walker, A.M. (2016) Scientific Opinion: Improving the Definition of Grape Phylloxera Biotypes and Standardizing Biotype Screening Protocols. American Journal of Enology and Viticulture. doi: 10.5344/ajev.2016.15106.

APPENDIX 1: SUPPLEMENTARY SCREENING OF G38 PHYLLOXERA TO RECOMMENDED DISINFESTATION TREATMENTS.

NPMP Protocol	Disinfestation procedure	Life stages/Treatments	% G38 phylloxera survival
Procedure B – Movement of grapevine cuttings and rootlings	Hot water immersion	*First instars* 50°C for 30 m 54°C for 5 m	 0% (N=30) 0% (N=30)
Procedure C- Movement of diagnostic samples	Cold storage	*First instars at -20°C* 6 h 12 h 24 h *First Instars at 4°C* 6 h 12 h 24 h *Eggs at -20°C* 6 h 12 h 24 h *Eggs at 4°C* 6 h 12 h 24 h	 0% (N=50) 0% (N=50) 0% (N=50) 96% (N=30) 81% (N=50) 34% (N=50) 0% (N=50) 0% (N=50) 0% (N=50) 96% (N=50) 90% (N=50) 15% (N=50)
Procedure G-Movement of vineyard equipment out of a PIZ or PRZ vineyard	Steam Hot water immersion Dry heat	*First instars* 10 s at a distance of 8 cm 50°C for 60 s 40°C for 120 m 45°C for 75 m	 0% (N=50) 0% (N=50) 0% (N=50) 0% (N=50)
Procedure H- Movement of vineyard visitors out of a PIZ or PRZ vineyard	Bleach (a.i. sodium hypochlorite) Alternative treatments to bleach	*First instars* 2% for 30 s (with rinse) 2% for 30 s (without rinse) 2% for 60 s (without rinse) Undiluted methylated spirits 30 s 60 s Ethanol 30 s 60 s	 0% (N=50) 0% (N=50) 0% (N=50) 0% (N=50) 0% (N=50) 15% (N=50) 3% (N=50)
Vineyard clothing	Insecticide spray	Mortein multi-purpose insect killer spray for 5 s	0% (N=50)

APPENDIX 2: MEMORANDUM OF UNDERSTANDING

Memorandum of Understanding

for

Research-related access to Phylloxera Collection

between

The State of Victoria through the Department of Economic Development, Jobs, Transport and Resources

and

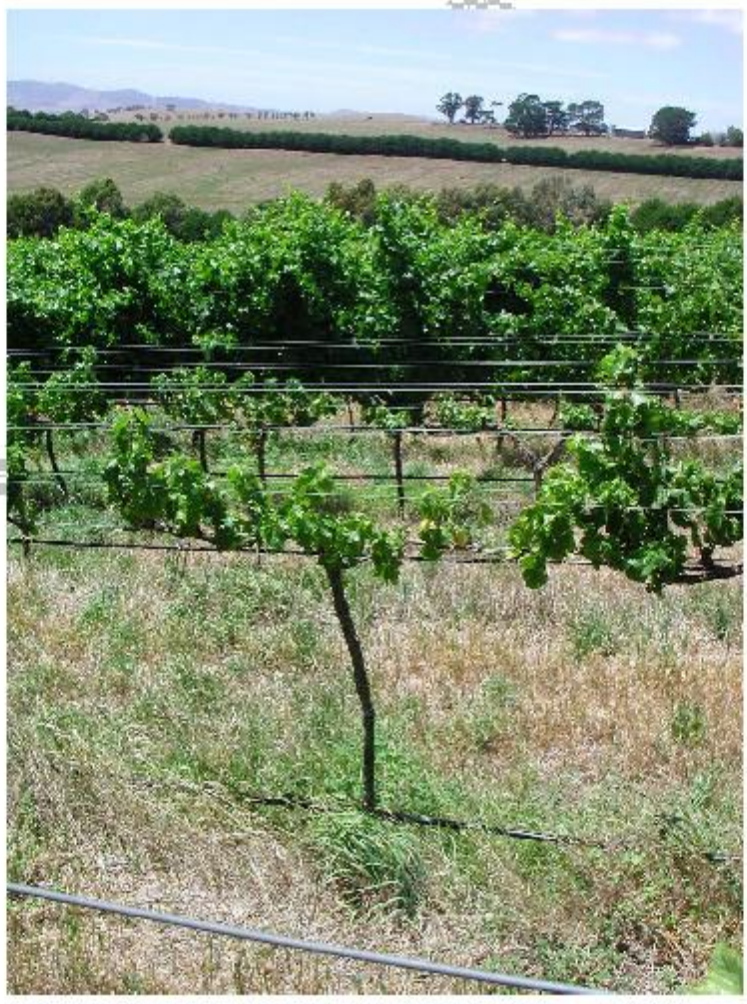
Australian Grape and Wine Authority

*Supplied as a supplementary pdf file to AGWA

APPENDIX 3: PEST RISK ANALYSIS PAPER*

A Scientific Basis for Risk Analysis of Grapevine Phylloxera *Daktulosphaira vitifoliae* Fitch

Assoc. Professor Kevin S. Powell, Principal Research Scientist, Biosciences Research, Department of Economic Development, Jobs, Transport and Resources, 124 Chiltern Valley Road, Rutherglen, Victoria 3685.



*Attached as a supplementary Appendix

Appendix 4- List of Acronyms

a.i	active ingredient
AGWA	Australia Grape and Wine Authority
AVR	Agriculture Victoria Research
DEDJTR	Department of Economic Development Jobs and Transport
rpm	reps per minute
HOCl	Hypochlorous Acid
HWT	Hot Water Treatment
NPMP	National Phylloxera Management Protocol
PEZ	Phylloxera Exclusion Zone
PIZ	Phylloxera Infested Zone
PRZ	Phylloxera Risk Zone
PVC	Polyvinyl chloride
RCBD	Randomised Complete Block Design
VHA	Vinehealth Australia

APPENDIX 5: STAFF

Assoc. Prof. Kevin Powell -Project Leader/Chief Investigator.

Dr Catherine Clarke – Research Scientist

Ms Bernadette Carmody – Technical Officer