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CESAR Centre for Environmental Stress and Adaptation Research

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Grape and Wine Research and Development Corporation

Early detection and alternative management of phylloxera in ungrafted vineyards



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **2.2.3**

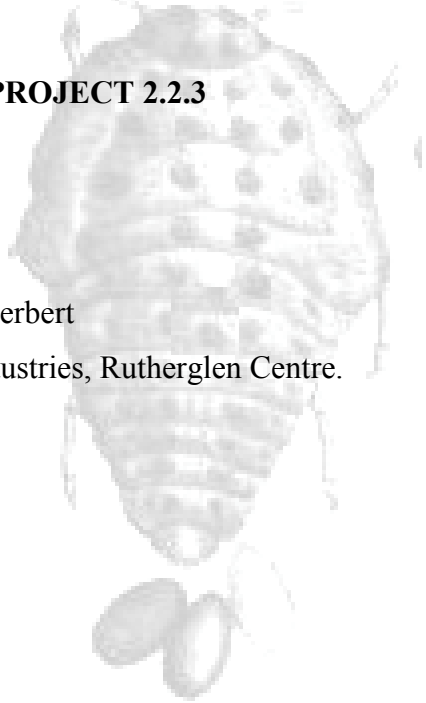
Principal Investigator: **Kevin Powell and Karen Herbert**

Research Organisation: **Department of Primary Industries, Rutherglen**

Date: **April, 2005**

EARLY DETECTION AND ALTERNATIVE MANAGEMENT OF PHYLLOXERA IN UNGRAFTED VINEYARDS

A FINAL REPORT OF PROJECT 2.2.3



AUTHORS:

Kevin Powell and Karen Herbert

Department of Primary Industries, Rutherglen Centre.

April, 2005.

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This report was compiled by Kevin Powell and Karen Herbert.
Department of Primary Industries, Rutherglen Centre
RMB 1145
Chiltern-Valley Road
Rutherglen, Victoria, 3685
Australia

Phone: +61 2 6030 4500
Facsimile: +61 2 6030 4600
Email: kevin.powell@dpi.vic.gov.au

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Executive Summary

Grape phylloxera, *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae), is a devastating pest of cultivated grapes, *Vitis vinifera* L., worldwide. One component of this report explores factors influencing the abundance and distribution of phylloxera populations in order to maximise the effectiveness of short-term chemical control strategies. The second component which examines novel molecular methods for the early detection of phylloxera infestations is also presented.

Field monitoring of phylloxera populations indicated high levels of insect variation between the major SE Australian phylloxera-infested grape-growing regions, making it very difficult to accurately predict peaks in phylloxera emergence. This variability was not associated with soil temperature variables. Within regions, however there were concordant insect numbers for different trapping techniques as well as a consistency in insect emergence within grape varieties in a given region.

An assay for assessing systemic insecticide effectiveness on the suppression of phylloxera populations was developed. Results show that insecticide usage resulted in reduced phylloxera numbers and improved vine vigour and has the potential to be successfully trialed for phylloxera suppression in the field.

The performance of common and less common phylloxera genotypes (as originally defined by Corrie et al. (2002)) were compared. Significant differences in the numbers of phylloxera life stages were found and, in addition, plant variables such as leaf area were significantly influenced by the phylloxera genotype present.

Finally, two molecular approaches for phylloxera detection are presented. The first compares the expression of four plant defence genes as markers of phylloxera infestation. Non-specific expression levels were found making this method unsuitable. The second approach presents progress towards a diagnostic DNA soil probe. Three conserved gene regions were targeted. Greater than 98% homology between phylloxera and aphids was found which requires further work focussing on the genetic separation of these two groups so that a phylloxera-specific probe can be further developed.

Background (as provided in original proposal)

The Australian Viticulture Industry recognises that grape phylloxera is the major insect pest constraint to long-term sustainable production. Phylloxera outbreaks and infestations in Victoria have highlighted the industries vulnerability and the need for vigilance, awareness and on-going research into early detection and phylloxera management. The use of grafted grapevines and phylloxera-resistant rootstocks is currently the only effective method known to control phylloxera. Over 85% of Australian vineyards are planted to ungrafted *Vitis vinifera* which is susceptible to phylloxera. Due to the cost of rootstock material, shortage of supply and the extra cultural management required for grafted vines, the proportion of total vineyard area in Australia planted as own-rooted vines is unlikely to decrease appreciably over the next 20 years.

A critical factor in the management of phylloxera in Australia is the early detection of infestations to ensure that appropriate quarantine measures are in place and control methods are implemented before irreversible damage to an infested vineyard or vine-growing region occurs. Current methods of detection are based on a combination of aerial and ground surveying which by their very nature are costly, time consuming and not fully effective. A phylloxera infested vineyard may go unnoticed for 2-3 years before an infestation is recognised using these techniques, by which time there is irreversible and economically significant damage to the vines and a greater risk for a breakdown in quarantine.

In order to minimise the effects of phylloxera management strategies must be applied as soon as an infestation is detected. To date this has consisted solely of replanting with resistant rootstocks. A potential alternative approach could be the use of either novel chemical insecticides or biofumigants, enabling continued production at economically sustainable levels without the need or delaying the need for replanting. Conventional chemical control methods have been trialed previously in Australia, Europe, South Africa and the USA, and although they appeared to reduce phylloxera populations none have prevented grapevine decline due to the damage caused by the insect. One of the main restrictions to the development of these new methods has been a lack of knowledge of the insect-plant interaction, and subsequent inability to target control methods for maximum effect.

Infestations detected in the King Valley in the mid-90s highlighted the vulnerability and economic impact of phylloxera on ungrafted vineyards. Unfortunately since the first King Valley outbreak new infestations are being located every year with the latest occurring in the Buckland Valley in 2004. Direct costs of replanting with resistant rootstocks can be more than $20,000+ per hectare and shortages of rootstock material for widespread replanting are inevitable. The indirect costs of quarantine restrictions and education of vineyard personnel in disinfestation procedures add further to the economic burden imposed by phylloxera.

As the majority of Australian vines are ungrafted and susceptible to phylloxera it is essential to rapidly identify infestations as soon as possible and implement appropriate management strategies to prevent irreversible damage to the vines. This can be achieved through:

- Development of diagnostic techniques for early detection of phylloxera and thereby enable the application of control methods before irreversible vine decline occurs.

- Identifying the physiological changes that occur in the grapevine when infested with phylloxera and determine the mechanisms by which vine growth and grape production are reduced.

- Determining the key period in the growing season when the phylloxera population is most susceptible to control agents by identifying critical population growth phases and their relationship with the grapevine growth phases.

- Assessing the feasibility of alternative/novel methods of control, which may include the use of systemic chemical insecticides and biofumigants, utilising a targeted approach based on the results of insect population studies.

Project Aims and Performance Targets

This is a report on phylloxera research and extension activities carried out form April 2000 to December 2005. The work was funded by the CRCV, GWRDC, the Phylloxera and Grape Industry Board of South Australia and the Department of Primary Industries, Victoria.

There were two main objectives to the project:

1. Develop diagnostic techniques to improve early detection of phylloxera in the field (sub-project 1)

2. Evaluate alternative and novel methods of phylloxera control including the use of systemic insecticides (sub-project 2)

The overall aim of the research program was to develop new methodologies for early detection of phylloxera infestations and identify and develop new methods for the control of these infestations.

The components of this project were:

1. Development of diagnostic techniques to improve early detection of phylloxera

2. Identification of gene/protein markers to enable early infestation of phylloxera to be detected.

3. Investigation of the physiological basis of the effects of phylloxera on the grapevine leading to induction of the gene/protein markers.

4. Definition of the key periods when phylloxera is most vulnerable to chemical control agents

5. Screening selected chemical insecticides for efficacy towards phylloxera.

6. Development of recommendations for industry on alternative management options.

Project Team

The projects core activities were conducted by a PhD student based primarily at the DPI Rutherglen centre with additional support provided by academic co-supervisors located at the Universities of Adelaide and La Trobe (CESAR). In addition collaborative support was provided by staff at DPI, Bundoora and SARDI. The project activities were carried out in close collaboration with growers in North-East and Central Victoria.

Adherence to Quarantine Protocols:

All field and laboratory work was carried out under permits that specified procedures and disinfestation protocols designed to prevent the spread of phylloxera by project staff. Field visits were arranged in close consultation with vineyard managers and equipment, vehicles, clothing and footwear was cleaned and disinfested according to recommendations in the National Phylloxera Management Protocols. Field collected samples were stored and transported in sealed containers under permit to the appropriate laboratory facilities for analyses.

The outputs and performance targets for the project were as shown in following table:

Outputs	***Performance targets***
Appointment of PhD student	Start of PhD candidature.
PhD student literature review and research plan	Completion of literature review and research plan.
Sub-project 1: Early detection of phylloxera infestations	
Identification of potential marker genes/proteins	Completion of preliminary leaf collection, and screening for induction of genes/proteins.
Validation of potential marker genes/proteins	Completion of more extensive leaf collection, and screening for induction of genes/proteins.
Information on grapevine response to phylloxera infestation	Description of molecular/physiological events associated with phylloxera infestation and induction of leaf marker genes/proteins.
Diagnostic technique for early detection of phylloxera infestations	Development of diagnostic technique and application to selected lab and field samples.
Sub-project 2: Alternative management strategies	
Identification of potential chemical insecticides for control of phylloxera	Completion of glasshouse based chemical screening trial of two chemical insecticides.
Determine key insect and vine growth phases where chemical applications would be most effective against phylloxera	Completion of population dynamics experiment at selected field site in King Valley.
Validation of potential chemical control agents in field situation over successive seasons	Complete first season of field trail testing of chemical insecticides.
Recommendation for alternative control strategy for phylloxera	Development of recommendations for alternative phylloxera control based on laboratory and field trials.
PhD Thesis	Completion of PhD thesis.

Molecular approaches for the early detection of phylloxera

Objective 1:
Develop diagnostic techniques to improve early detection of phylloxera in the field (sub-project 1)

Introduction

This chapter will present two different molecular approaches for phylloxera detection. The first approach explores the feasibility of using potential differences in the expression levels of plant defence pathogenesis proteins (PR) proteins as indicators of phylloxera infestation. The expression of PR proteins from the PR-2, PR-3 and PR-5 families in response to phylloxera infestation was investigated. The second approach was aimed at developing a diagnostic DNA probe for the detection of phylloxera in soil. Genomic sequences from within 3 conserved gene regions, the Elongation Factor (EF), the inter transcriptional spacer regions (ITS) and ribosomal RNA regions (rRNA), were amplified to generate potential phylloxera-specific DNA regions that could be analysed for DNA probe suitability.

Material and Methods

Approach 1:

PR protein expression in infested and uninfested grapevines

A glasshouse pot-trial, focussed on comparing the gene expression levels of 4 different PR-proteins from the leaves of infested and uninfested *Vitis vinifera.* The assumption made with this study was that a stress-response caused by phylloxera feeding on the root system would be systemic and therefore similarly detected in the leaves of the plant. Leaf tissue was the preferred tissue type as continual harvesting from vines was possible throughout the experiment without disturbing the active phylloxera populations on the vine root systems. If this technique was to be potentially used as a routine assessment of phylloxera infestation, leaf tissue would be the preferred tissue type due to the greatly reduced labour inputs required to collect leaf tissue compared to root tissue.

1. Pot trial establishment and leaf harvesting procedure

Twelve, 1-year old Sauvignon Blanc (*Vitis vinifera cv*. Sauvignon Blanc clone H5V10) rootlings were potted in 20cm pots as described on page 77. The study comprised 3 treatments: 4 vines infested with the G4 phylloxera genotype, 4 vines infested with G29 genotype and 4 vines that were not infested. Treated vines were infested with 500 eggs as described on page 77. The study was conducted in a temperature controlled glasshouse using a random trial design as outlined on page 77.

Leaves were harvested from vines at 7, 30, 45 and 150 days following infestation. At each sampling two vines from the same treatment were selected at random for leaf harvesting. A single sink and source leaf were removed from each vine. For the purposes of this the study the sink leaf was determined as the 3rd leaf inwards along the primary cane from the growing tip and the source leaf was described as the 3rd oldest leaf inwards from the opposite end of the primary cane. Leaves were cut at the petiole using secateurs, immediately placed in a 50ml falcon tube and immersed in liquid nitrogen and stored at −80°C.

2. RNA extraction method

Previous studies (Kellow 2000), have found the extraction of RNA from grapevine tissue difficult due to high concentration of phenolic compounds which can interfere with the extraction of nucleic acids. After some preliminary trials, a hexadecyltriethylammounium bromide (CTAB) RNA extraction method, modified from Chang et al (1993) was adopted. This method was originally designed to extract RNA from pine trees also known for their high polyphenol content. RNA extractions were performed using pooled material from sink and source leaves from each vine. Leaf tissue was ground to a fine powder under liquid nitrogen in a mortar and pestle prior to extraction. Diethylpryocarbonate-treated (DEPC) water was used for all buffer solutions which where autoclaved prior to use.

4-5g of ground leaf tissue was added to 20mls of extraction buffer [(2% CTAB, 2% polyvinylpyrrolidione K 30 (PVP), 100mM Tris-hydrochloric acid (Tris-HCl) (pH 8.0), 25mM ethylenediaminetetraacetic acid (EDTA), 2.0M sodium chloride (NaCl)], 0.5g/L spermidine) warmed to 65°C, mixed via inversion and incubated at 65°C for 3

minutes to allow for the complete disruption of the plant cell membranes. Three extractions with equal volumes of chloroform:isoamyl alcohol (42:1) were performed (mixed via inversion 100 times) and separated by centrifugation at 3500 rpm for 20 minutes at 4°C. One-quarter volume of 10M lithium chloride (LiCl) was then added to the supernatant, inverted 100 times and left to precipitate overnight at 4°C and centrifuged at 4000rpm for 30 minutes. The RNA pellet was resuspended in 500µl SSTE buffer [(1.0M NaCl, 0.5% sodium dodecyl sulphate (SDS), 10mM Tris-HCl (pH 8.0) and 1mM EDTA (pH 8.0)] heated to 50°C, transferred to a 2ml microfuge tube and extracted with one equal volume of chloroform:isoamyl alcohol (42:1). Following centrifugation (10,000rpm for 10 minutes at 25°C), two volumes of cold absolute ethanol (1ml) was added to the aqueous phase, precipitated for 2 hours at –20°C and centrifuged for 20 minutes at 13,000rpm. Supernatant was removed with the excess ethanol blotted dry using a Kimwipe™. The RNA pellet was then air-dried for 10 minutes and resuspended in 30µl RNAse-free water.

RNA yield and quality was determined by loading a 1:10 diluted aliquot onto an RNA 6000 Nano LabChip® and running on a Agilent™ 2100 Bioanalyser.

3. Northern blot hybridisation method

Northern blotting was carried out with 4 cDNA probes (see Table 1) kindly provided by Ian Dry (CSIRO, Plant Industries, Adelaide). 4µg RNA was loaded for each sample onto 4 replicate gels.

3(a) Transfer of RNA to nylon membranes

Gels were rinsed in 2 × sodium chloride/sodium citrate (SSC) buffer for 5 minutes and then blotted onto positively charged nylon membrane (Ambion™) according to the manufacturer's instructions. The membrane was then rinsed briefly in 2 × SSC and exposed to UV light using a UV straterlinker (at 120 mJ) to immobilise the DNA and then immediately rinsed in double distilled water. Filters were used only once.

3(b) Hybridisation conditions

Pre-hybridisation, hybridisation and washes were carried out in glass hybridisation tubes in a Hybaid™ rotating incubation oven. The incubation temperature for pre-hybridisation and hybridisation was 42°C.

3(c) Pre-hybridisation procedure

Filters were pre-hybridised in 20mls of pre-warmed Denhardt's hybridisation solution [50% (w/v) formamide, 4 × SSC, 5 × Denhardt's solution (Eppendorf), 5% (w/v) dextran sulfate, 0.5% (w/v) SDS and 0.1 mg/ml denatured fish sperm DNA] and incubated for 2 hours.

3(d) Probe labelling and hybridisation procedures

cDNA probes were labelled using a Rediprime™ II random prime labelling system kit (Amersham™), according to the manufacturer's instructions, except that the radioactive isotope used was a α-^{32} dCTP (Geneworks™). 20mls pre-warmed Denhardt's hybridisation solution was added and incubated overnight in a rotating oven (Hybaid™). Membranes were then washed once in 2 × SSC and 0.1% (w/v) SDS for 10 minutes, and once in 0.1 × SSC and 0.1% SDS (w/v) for 5 minutes, both at 42°C, placed in sheet protectors and double sealed using a heat sealer before 2 days exposure on an erased phosphor screen.

cDNA	Clone/ Genbank Assession Number	Reported expression in *V.vinifera*
VvTL1	AF003007	Not expressed in grapevine roots (Tattersall et al. 1997, Kellow et al. 2004). Slight gene induction detected in leaves upon powdery mildew infection (Jacobs et al. 1999).
VvTL2	Y109920	Expressed in grapevine roots (Kellow et al. 2004). Pronounced inductions recorded from *V.vinifera* leaves infected with powdery mildew (Jacobs et al. 1999).
VvChi3	Z68123	Expression in grapevine roots unknown. Inductions recorded from *V.vinifera* leaves infected with powdery mildew (Jacobs et al. 1999).
VvGlub	AF053750	Expression in grapevine roots unknown. Pronounced inductions recorded from *V.vinifera* leaves infected with powdery mildew (Jacobs et al. 1999).

Table 1. cDNA clones used as probes in northern hybridisations. All clones refer to *Vitis* sp.

Approach 2:

Developing a DNA probe for the detection of phylloxera in soil

1. Genomic DNA extractions

Mature adults belonging to either G1, G3, G4, G19, G20 genotypes were sourced from the field and stored in 100% ethanol prior to extraction. These genotypes were verified by Paul Umina (La Trobe University, Victoria) using the microsatellite marker classification technique originally developed by Corrie et al (2002). Additional insects of genotypes different to the above were kindly provided by Kathy Viduka and Angela Corrie (CESAR, La Trobe University, Victoria). Single insect DNA extractions were performed using a chelating resin method outlined by Walsh et

al. (1991). Briefly, insects were placed in sterile 0.5ml tubes and air-dried to remove any excess ethanol. 5µl of proteinase K (14mg/ml; Boehringer Mannheim) was pipetted onto the insect before finely grinding it with a pestle. 100µls of 5% Chelex® 100 (Biorad) pre-warmed approximately to 50°C was then added, followed by incubation at 55°C for 12 hours, then 95°C for 5 minutes. The tube was then centrifuged for 5 minutes at 14,000rpm to pellet the resin and stored at –20°C until required. The aqueous phase containing the DNA was used as template for all subsequent PCR reactions.

2. Gene amplification derived from universal primers

The Elongation Factor (EF), the inter-transcriptional spacer regions (ITS) and ribosomal RNA regions (rRNA) were the 3 regions chosen for this study. These individual regions were amplified using the universal primer combination pairs listed in Table 2. The PCR reaction mixture comprised 2mM $MgCl_2$, 200µM dNTPs (Invitrogen), 10pmol appropriate primer pair, 2.5µl 10 × PCR Buffer, 1.0U Taq polymerase (Gibco) and 5µl template solution. A 5µl aliquot of the amplification product was visualised following electrophoresis on 1.8% agarose gels and stained with ethidium bromide. Excess primers and nucleotides were removed from the remaining PCR products using Qiagen™ clean up kit according to the manufacturer's instructions. One half of the eluted DNA product was sequenced. Amplification products were sequenced in both directions by a commercial company (Macrogen, Seoul, Korea).

Universial primer name	Sequence (5' to 3')	Position	Amplified region	Source
2880	CTGGTTGATCCTGCCAGTAG	5'-3'	18S	von Dohlen (1996)
B	CCGCGGCTGCTGGCACCAGA	3'-5'	18S	von Dohlen (1996)
EF-F	TCCGGATGGCATGGCGAGAATATG	EF0-5	Elongation Factor (EF)	Palumbi (1996)
EF-R	ATGTGAGCAGTGTGGCAATCCAA	EF2-3	EF	Palumbi (1996)
D2F	AGTCGTGTTGCTTGATAGTGCAG	5'-3'	D2 region of 28S rRNA	Campbell et al. (1993)
D2R	TTGGTCCGTGTTTCAAGACGGG	3'-5'	D2 region of 28S rRNA	Campbell et al. (1993)
WD3a	GACCCGTCTTGAAACACGGA	5'-3'	D3 region of 28S rRNA	Whiting et al. (1997)
WD3b	TCGGAAGGAACCAGCTACTA	3'-5'	D3 region of 28S rRNA	Whiting et al. (1997)
ITS4	CGAGTATCGATGAAGAACGAGC	5.8S	ITS2	Navajas et al. (1994)
ITS5	ATATGCTTAAATTCAGCGGG	28S	ITS2	Navajas et al. (1994)
CSA	GTCGTAACAAGGTTTCCGT	18S	ITS1	Schlötterer (1998)
CSB	CGATGAAGAACGCAGC	ITS1	ITS1	Schlötterer (1998)
CSC	GCTGCGTTCTTCATCG	5.8S	ITS2	Schlötterer (1998)
CSD	GTTAGTTTCTTTTCCTCC	28S	ITS	Schlötterer (1998)

Table 2. Universal primers used in polymerase chain reaction amplification of the EF, 18S, 28S and ITS2 DNA regions studied.

3. Gene amplification targeting phylloxera-specific PCR products

Sequence data for the six major genotypes were aligned using the BioEdit™ sequence alignment editor software package for each of the gene regions. Consensus phylloxera sequence was compared with sequence obtained from the Genbank database search using the BLAST feature Version 2.2.10 (Altschul et al 1997). Sequences with homology above 95% were downloaded and aligned using BioEdit™. Additional nematode and aphid sequence was also included. Regions of low sequence homology were identified for phylloxera-specific primer design. Potential phylloxera-specific primers, typically of 17-22 bp length were designed to incorporate a minimum of 2 unique bases and to amplify a region containing a minimum of 30 phylloxera-specific base pairs. The program Netprimer™ was used to further optimise primer pairs to decrease the likely hood of secondary structure formation and to optimise the melting temperatures.

4. Validation of potential phylloxera-specific primers

The testing procedure to determine the specificity of the new primers to phylloxera sequence was conducted using a series of two PCR reactions for each organism tested. The first PCR tested DNA viability using universal primers (from Whiting et al 1997) and the second PCR tested the phylloxera primer specificity. For samples that gave a positive result using universal primers but no product with phylloxera-specific primers, it was assumed that the given primers were not complimentary to the sequence of the given organism tested. The organisms tested were divided into the following categories:

4(a) Commonly-found vineyard organisms

These included: Wasps (Order Hymenoptera); Honey bees (*Aphis melifera*); Fruit flies (*Drosophila melogaster*); Thrips (Order Thysanoptera); Caterpillars (Order Lepidoptera); and springtails (Order Collembola), collected via the emergence trapping system (refer page 54) during the 2003 vintage from a number of vineyards in the Rutherglen and King Valley regions.

4(b) Nematode species

The following nematode species, kindly provided by Herdina (SARDI, Adelaide, South Australia) were tested: Stem nematode (*Ditylenchus dipsaci*); Cereal cyst nematode (*Heterodera avenae*); and two root-lesion nematodes (*Pratylenchus neglectus*) and (*P. thornei*). Note, that while not all the above nematode species are widely present in Australian vineyards, they were particularly targeted because they are soil-dwelling parasites, and, in the case of *P. neglectus* and *P. thornei*, cause root lesions on the grapevine root system.

4(c) Mite species

Bud mite (*Colomerus vitis*); blister mite (*Eriophyes vitis*); and rust mite (*Calepitrimerus vitis*), specimens, kindly provided by Melissa Carew, (CESAR, La Trobe University, Bundoora, Victoria) were tested. These 3 mite species are widely distributed throughout vineyards across Australia.

4(d) Common aphid species

The following aphid species were used and kindly provided from the collections of Paul Sunnucks and Isabel Valenzuela (LaTrobe University, Bundoora): pea aphid (*Acyrtosiphon pisum*); spotted alfalfa aphid (*Therioaphis trifolii*); blackberry-cereal aphid (*Sitobion fragariae*); bird cherry-oat aphid (*Rhopalosiphum padi*); rice root aphid (*Rhopalosiphum rufiabdominalis*). honeysuckle aphid (*Hyadaphis foeniculi*); gall-forming aphids (*Pemphigus* sp.); potato aphid (*Macrosiphum euphorbiae*); cotton aphid (*Aphis gossypii*); peach-potato aphid (*Myzus persicae*); grass and cereal aphid (*Metapolophium dilhodum*); ornate aphid (*Myzus ornatus*); and the rose aphid (*Macrosiphum rosae*).

5. Analysis

The programs Sequencer™ version 3.1.2 (Gene Codes Corporation) and ClustalX (Thompson et al 1997) were used to check and align all sequence data. Distance analysis was constructed using the neighbour-joining method outlined by Saitou and Nei (1987) in the PAUP* program (Swofford 1998). Confidence values for the branches were obtained using Rzhetski and Nei's (1993) bootstrapping method, again in PAUP*. Bootstrap tests were performed with 10,000 iterations.

Results

Approach 1:

PR protein expression in infested and uninfested grapevines

The RNA extraction method used gave good yield and high purity levels for 21 of the 24 samples extracted (Figure 1 and Table 3). RNA yields ranged between 281 to 900ng/µl and with RNA purity ratios between 0.9 and 2.0 (Table 3). There were 3 samples shown in Figure 1 where the RNA had clearly degraded, as there were no characteristic 18S or 28S RNA bands present. These samples were not used in the northern blots.

The levels of VvTL1 and VvTL2 expression were generally consistent between treatments and sampling periods (Figure 2). Phylloxera infestation appears not to affect the expression of either of these two PR genes. This result differs to the findings of Kellow (unpublished) who found differences in VvTL2 induction between infested and uninfested *V.vinifera* varieties. The levels of PR expression for VvChi3 and VvGlub are lowest at 7 days following infestation (Figure 2). Following this time point the expression patterns are greater but inconsistent between treatment types for both transcripts. For example, VvChi3 expression is highest in the uninfested treatments when compared to the infested treatments for 2 of the four sampling intervals. VvGlub expression is higher for infested treatments for 3 of the 4 sampling intervals. Of particular contrast is the VvGlub expression measured at 150 days following infestation. At this time point a clear difference between infested and uninfested treatments can be seen, with greatest expression seen in the G4 treatment compared to the G29 treatment (Figure 2).

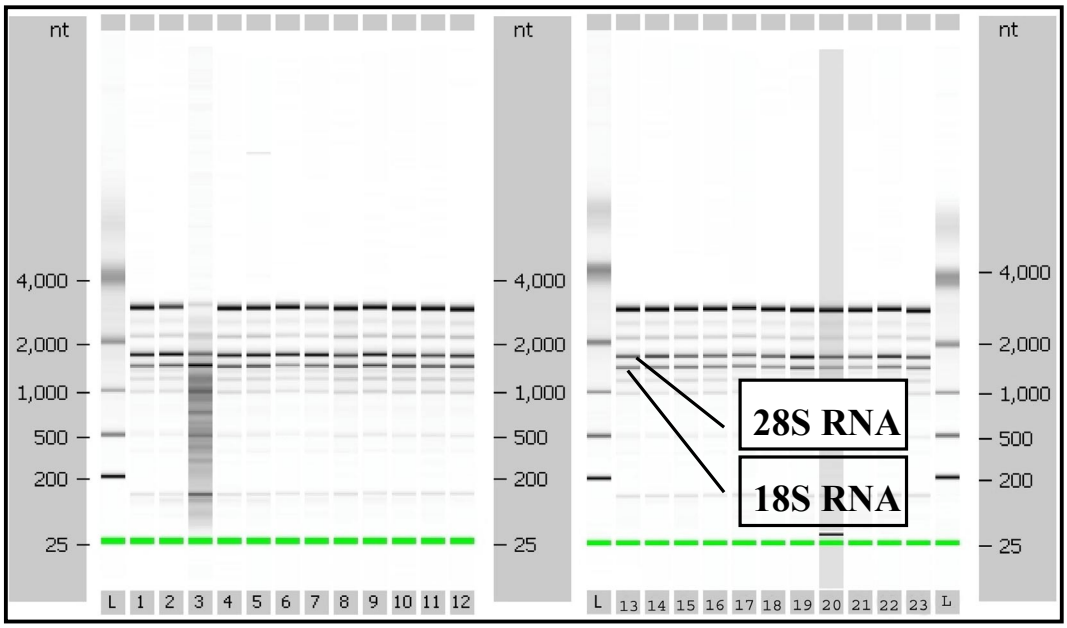


Figure 1. Electrophoresis gel results obtained from 1:10 diluted RNA samples determined by the Agilent™ 2100 Bioanalyser.. The presence of both 18S and 28S RNA indicates that the RNA has not been degraded. Sample numbers 3, 20 and 24 were discarded.

Lane Number (refer Figure 5)	Treatment	Days post infestation	RNA concentration (ng/ul)	RNA ratio (28S/18S)
1	Uninfested	7	482.6	1.3
2	Uninfested	7	607.5	0.9
3	Infested - G29	7	463.6	0.2
4	Infested - G29	7	760.4	1.3
5	Infested - G4	7	513.8	1.2
6	Infested - G4	7	459.1	1.3
7	Uninfested	30	561.1	1.0
8	Uninfested	30	715.8	1.7
9	Infested - G29	30	480.3	1.3
10	Infested - G29	30	633.5	1.5
11	Infested - G4	30	455.5	1.7
12	Infested - G4	30	915.4	1.6
13	Uninfested	45	642.4	1.9
14	Uninfested	45	758.8	1.6
15	Infested - G29	45	385.5	1.9
16	Infested - G29	45	630.0	1.8
17	Infested - G4	45	471.6	1.0
18	Infested - G4	45	249.1	9.0
19	Uninfested	150	585.3	1.8
20	Uninfested	150	976.8	1.8
21	Infested - G29	150	771.4	1.3
22	Infested - G29	150	399.8	2.0
23	Infested - G4	150	281.1	1.1
24	Infested - G4	150	not measured	not measured

Table 3. Summary of RNA concentrations and 28S/18S ratios as calculated using an Agilent™ 2100 Bioanalyser from 1:10 RNA dilutions.

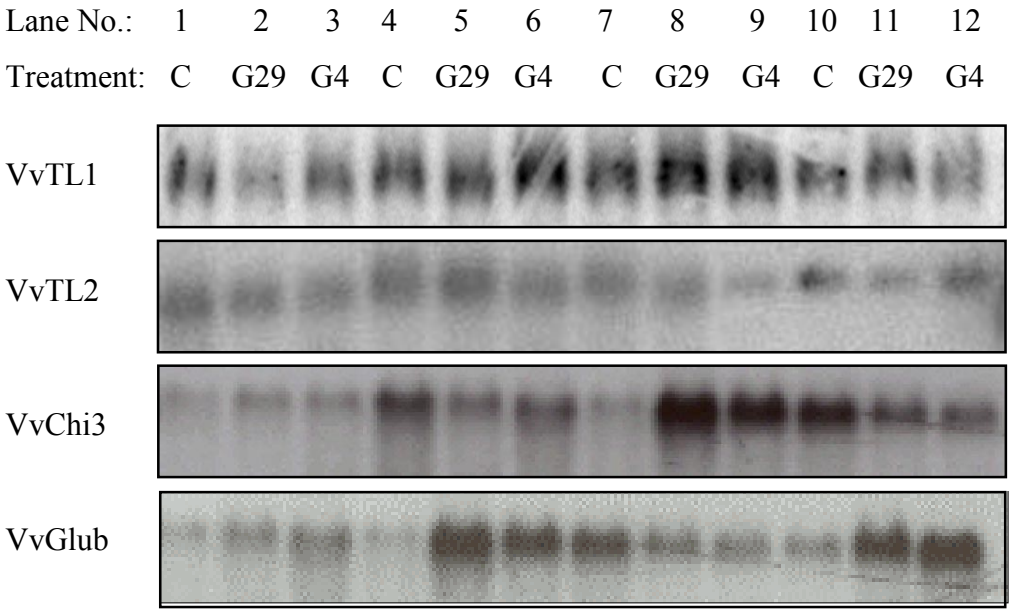


Figure 2. Induction of grapevine PR genes in *V.vinifera* leaves following infestation with phylloxera on the root system. C, control (uninfested) treatments, G29 and G4, phylloxera infested treatments. Lanes 1-3 were sampled 7 days following infestation, Lanes 4-6: 40 days following infestation, Lanes 7-9: 45 days following infestation, and 10-12: 150 days following infestation. 4µg total RNA was loaded per track.

Approach 2:

Developing a DNA probe for the detection of phylloxera in soil

PCR conditions for the universal primers listed in Table 2 are summarised in Table 4. The gene sequence regions obtained from the universal primer combinations '2880/B' (von Dohlen, 1996) and 'D2-F/D2-R' (Campbell et al. 1993) had greater than 98% homology to members of the Aphidoidea, making these sequences unsuitable (Figure 3). No phylloxera ITS PCR amplification product was obtained in this study (Table 4). While an ITS PCR product was initially recorded using the ITS primers from Navajas et al. (1994), a BLAST of the consensus sequence found this sequence to be a fungal ITS sequence and it was assumed that these primers preferentially bound to suspected fungal contaminants in the DNA template. Nucleotide BLAST comparisons of the PCR amplification products from the EF region using 'EF-F/EF-

R' (Palumbi 1996) and from the 28S RNA using the 'WD3a/WD3b' (Whiting et al 1997) primer combinations revealed sufficient single nucleotide base pair differences to enable the potential design of phylloxera-specific primers (Table 4). Forward and reverse primers for both EF and and 28S D3 regions were designed taking into account these nucleotide differences as evidenced in Figure 3 based on the 5 closest organism matches identified from the BLAST search.

Primer combination	Amplified region	PCR conditions	Product obtained	Diversity in phylloxera sequence?
2880/B	18S	94°C for 3 min; 30 cycles of (94°C for 30s, 55°C for 30s and 72°C for 1 min); 72°C for 7 min.	yes	no
**EF-F/EF-R	EF	93°C for 3 min; 35 cycles of (94°C for 30s, 54°C for 1 min and 72°C for 1 min); 72°C for 7 min.	yes	yes
D2F/D2R	28S rRNA - D2 region	96°C for 1 min; 29 cycles of (94°C for 1min, 50°C for 1 min and 72°C for 2 min); 72°C for 7 min.	yes	no
**WD3a/WD3b	28S rRNA - D3 region	94°C for 3 min; 35 cycles of (94°C for 30s, 52.5°C for 30s and 72°C for 1 min); 72°C for 7 min.	yes	yes
ITS4/ITS5	ITS2	94°C for 5 min; 35 cycles of [94°C for 30s, 57°C for 30s and 72°C for 1 min + 1sec/cycle]; 72°C for 7 min.	yes	no
CSA/CSB	ITS1	94°C for 3 min; 35 cycles of [94°C for 30s, (47°-60°C) for 30s and 72°C for 1 min]; 72°C for 7 min.	no	n/a
CSC/CSD	ITS2	94°C for 3 min; 35 cycles of [94°C for 30s, (47°-60°C) for 30s and 72°C for 1 min]; 72°C for 7 min.	no	n/a

Table 4. Universal primer amplification conditions and results for selected gene regions. Note that the sequence obtained from primers marked with an asterisk was used for the design of phylloxera-specific primers.

(A) EF forward

```
PHYLLOXERA    TCG TGG TTT TGT TGC TGG AG
PLATYPALPUS   ... ... ..A ... A.. ... TC
STOMAPHIS     ... ... ..A ... A.. ... TC
PHEROCERA     ... ... ..A ... A.. ... TC
TRAMA         ... ... ..A ... A.. ... T.
STENOMPHRALE  ... ... ..A ... A.. ..C TC
```

(B) EF reverse

```
PHYLLOXERA    GGA AAG GCT GAC GGT AAA TC
PLATYPALPUS   ... ... .T. ... ... ... AT
STOMAPHIS     ... .TT .G. T.. TTG ..C C.
PHEROCERA     ... ... .T. ... ... ... AT
TRAMA         ... ..T ... ..A ..A ... AT
STENOMPHRALE  ... ... CT. ... ... ... AT
```

(C) D3 forward

```
PHYLLOXERA    GTC GCC GCG CGC TAA TTA C
CALOPTERYX    ..G CG. ... AT. .T. C.. G
EPIOPHLEBIA   A.G CG. ... AT. .T. ... G
OPHIOGOMPHUS  ..G CG. ... AT. .T. ... G
TIMEMA        g.. TG. ... AT. AT. ... G
MANTIS        ..G TG. ... AT. .T. ... G
```

(D) D3 reverse

```
PHYLLOXERA    GTC GGC TTC GGC CGT CC
CALOPTERYX    TC. ..G ... ... .A. .G
EPIOPHLEBIA   T.G A.T ... CAT ..C .G
OPHIOGOMPHUS  C.G A.T ... .AT ..C AG
TIMEMA        C.. ..T G.. .AG ..C AG
MANTIS        CC. ..G ... ... ... .G
```

Figure 3. Phylloxera-specific primers designed from the elongation factor (EF) and 28S, D2 (D3) rRNA regions. BLAST comparisons of these phylloxera sequences with the top 5 closest matched organisms are shown, illustrating single nucleotide differences. The organisms listed are: *Platypalpus sp.* (dance fly); *Stomaphis malloti* (aphid); *Pherocera niger* (fly); *Trama rara* (aphid); *Stenomphrale teutankhamen*i (fly); *Calpteryx aequabilis* (damselfly), *Epiophlebia superstes* (dragonfly); *Ophiogomphus severus* (dragonfly); *Timena knulli* (stick insect); and *Mantis religiosa* (mantid).

PCR amplification using the newly designed phylloxera-specific primers for the EF and 28S regions gave positive amplification for the 10 phylloxera genotypes tested. In the case of the EF-primers, there were 5 different phylloxera sequences obtained and 2 different genetic variants determined for the 28S D3 primers (for sequences refer to Appendix 5). Both primers did not positively amplify product from the common vineyard organisms tested, as well as the nematode and mite species tested (data not presented). Positive amplification, however, was recorded from a small number of aphid species tested. With respect to the EF primers, positive amplification was recorded for *Sitobion fragariae, Rhopalasiphum padi* and *Hyadaphis foeniculi* (Figure 4b), whereas the 28S D3 primers positively amplified. DNA belonging to *Sitobion fragariae, Myzus religiosa*, *Macrosiphum euphorbiae*, and *Pemphigus* sp. (Figure 4c). The DNA viability for all 13-aphid species are confirmed using universal primers from Whiting et al (1997) in Figure 4(a). Neighbourhood-joining (N-J) analysis (Figures 5 and 6) combining the above phylloxera and aphid sequences as well as those organisms identified from the original BLAST comparisons revealed the very close sequence homology of particular aphid species to phylloxera. 13 different taxa in total were used in the EF N-J tree (Figure 5), and based on 318 informative characters. Eight different taxa were used for the 28S D3 N-J tree (Figure 6), based on 43 different informative characters. Sequence alignments used for organisms used in Figures 5 and 6 are shown in Appendix 5.

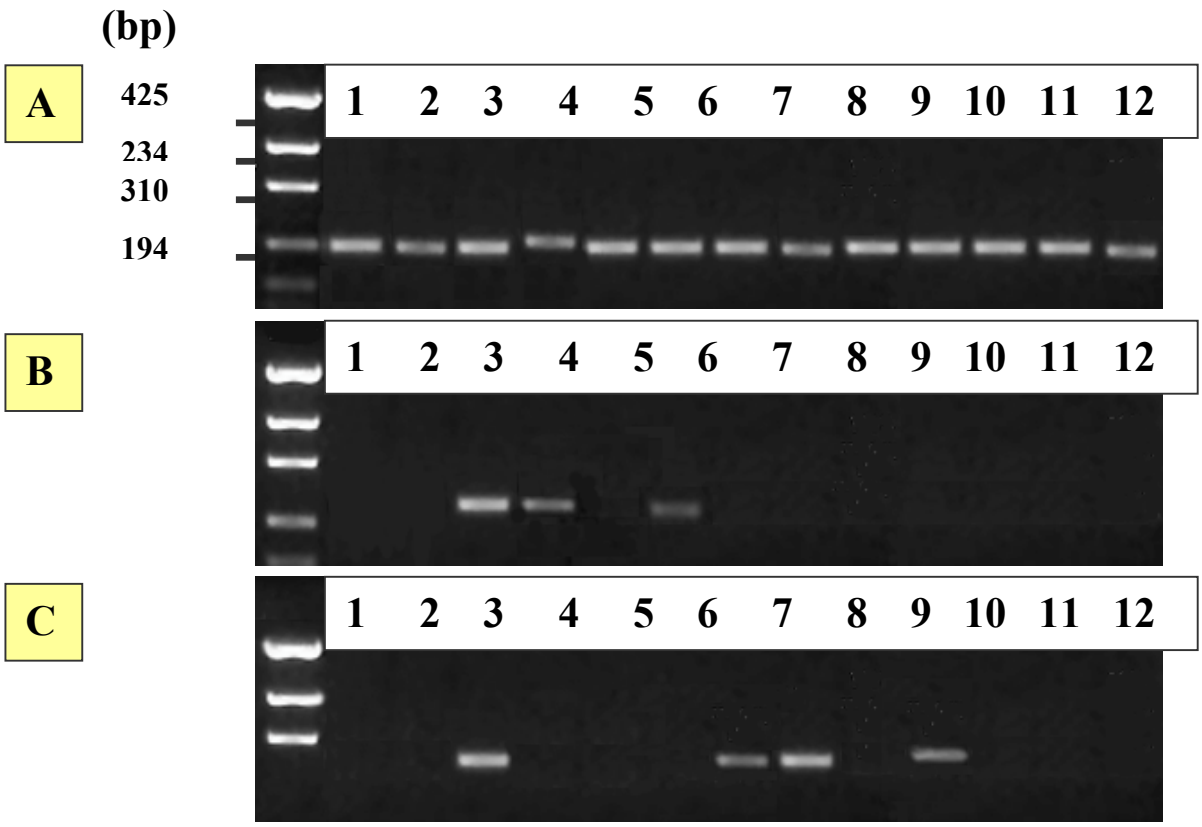


Figure 4. PCR amplification of aphid DNA illustrating amplification using universal primers from Whiting et al (1997) (Figure 8a), as well as for some as in some species positive amplification using potential phylloxera-specific primers spanning the EF (Figure 8b) and 28S D3 rDNA (Figure 8c) regions. Lane 1, *Acyrtosiphon pisum*; Lane 2, *Therioaphis trifolii*; Lane 3, *Sitobion fragariae*; Lane 4, *Rhopalosiphum padi*; Lane 5, *Rhopalosiphum rufiabdominalis*; Lane 6, *Hyadaphis foeniculi*; Lane 7, *Pemphigus* sp.; Lane 8, *Macrosiphum euphorbiae*; Lane 9, *Aphis gossypii*; Lane 10, *Myzus persicae*; Lane 11, *Metapolophium dilhodum*; Lane 12, *Myzus ornatus*; and Lane 13, *Macrosiphum rosae.* Molecular weight standard is ΦX174RF HaeIII.

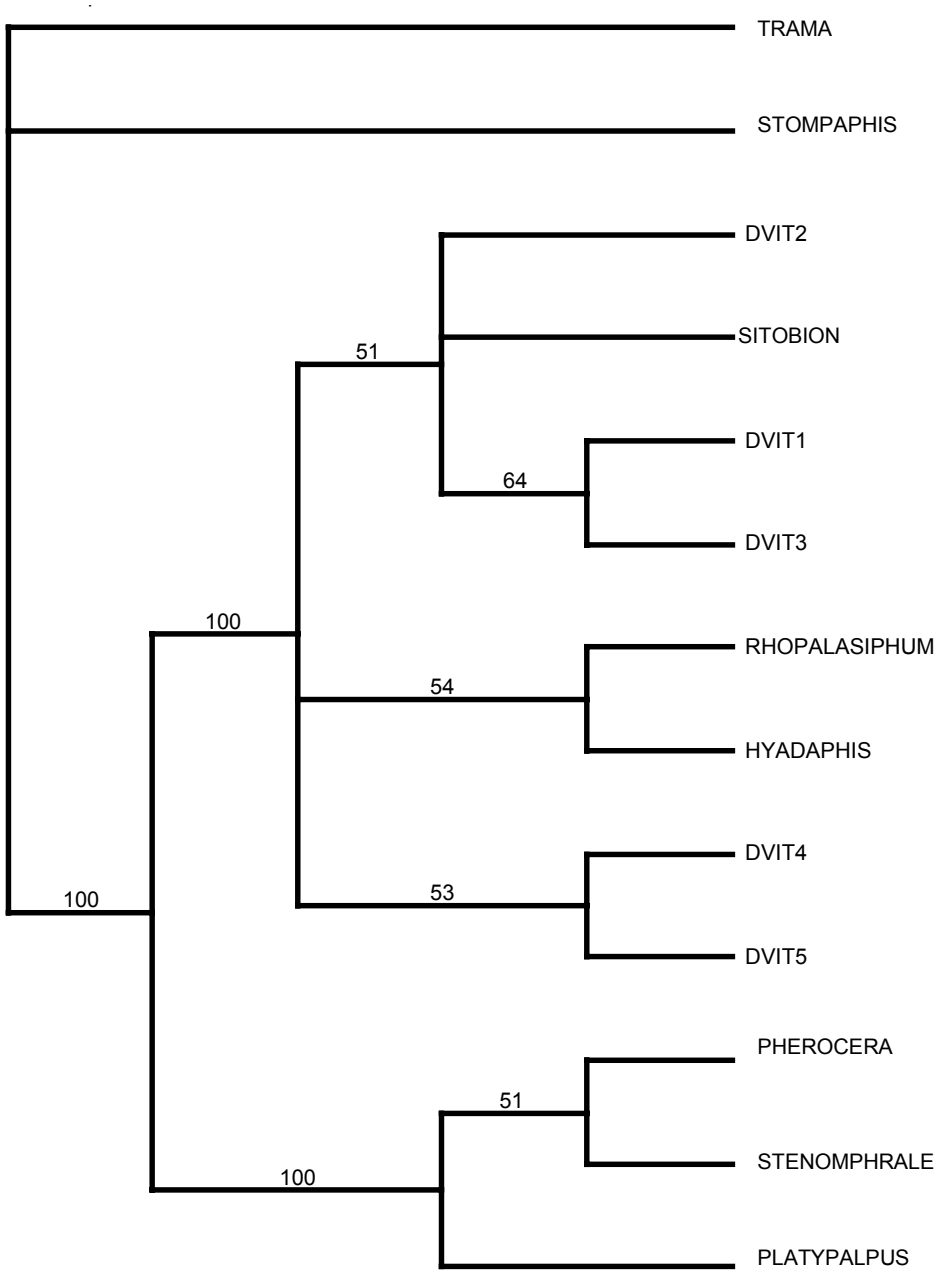


Figure 5. Consensus tree from neighbourhood-joining analysis of *Daktulosphaira vitifoliae* elongation factor DNA sequences (DVIT1-DVIT5), with closest organisms identified using BLAST comparisons (*Trama rara*, *Stomaphis malloti*, *Pherocera niger*, *Stenomphrale teutankhameni* and *Platypalpus sp.),* and with aphid species that positively amplified using the same EF primers (*Sitobion fragariae*, *Rhopalasiphum padi* and *Hyadaphis foeniculus*). Bootstrap values are shown.

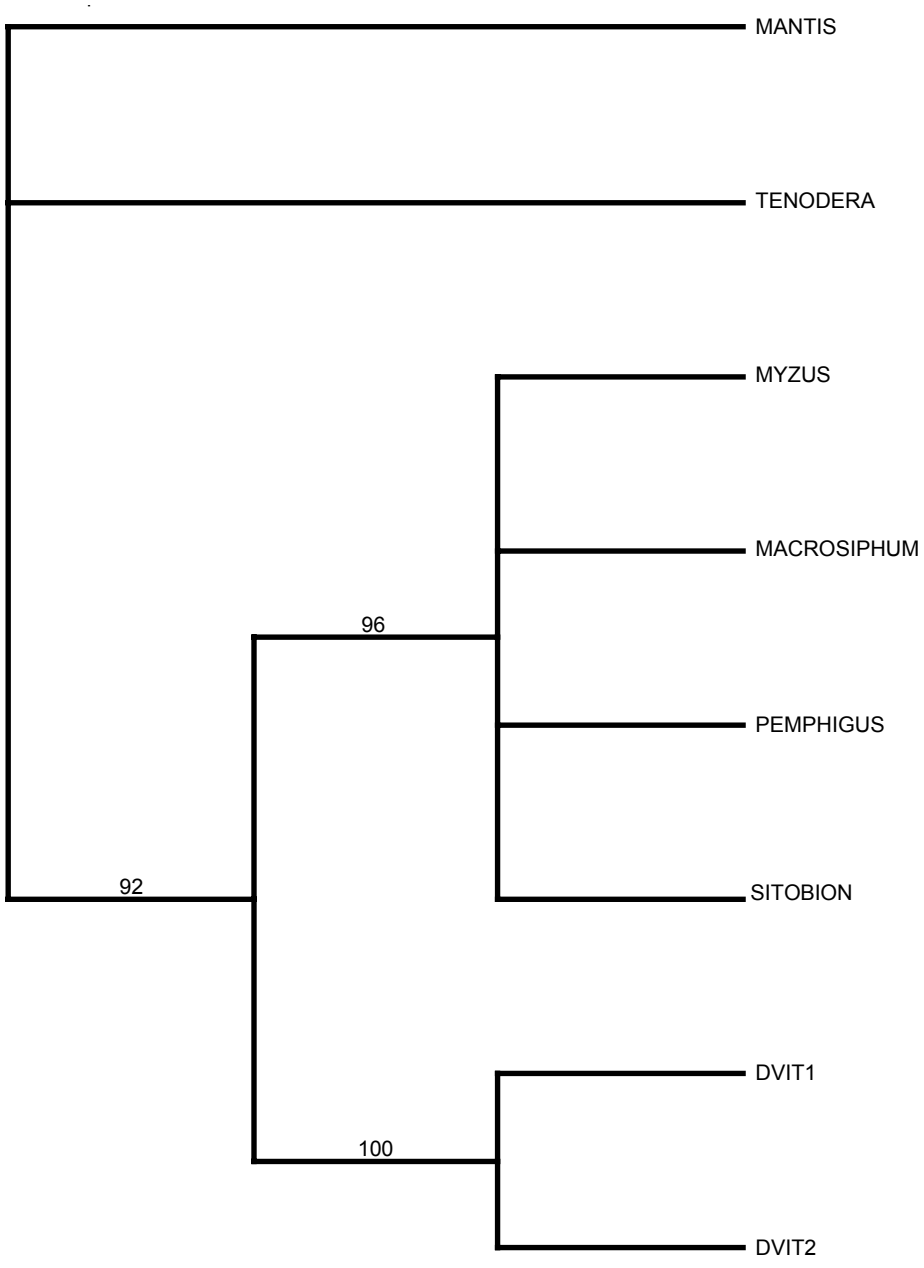


Figure 6. Consensus tree from neighbourhood-joining analysis of *Daktulosphaira vitifoliae* 28S rRNA sequences (DVIT1-DVIT2), with closest organisms identified using BLAST comparisons (*Timena knulli* and *Mantis religiosa),* and with aphid species that positively amplified using the same 28S primers (*Myzus ornatus*, *Macrosiphum euphorbiae*, *Pemphigus* sp. and *Sitobion fragariae*). Bootstrap values are shown.

Discussion

Of the four different genes analysed in this study putatively encoding defence-related transcripts, no conclusive differences in transcript levels were observed in leaf tissue in response to phylloxera attack on the root system. In particular for the VvTL2 transcript, no difference in expression was detected across the time-course, contradictory to previous experiments of Kellow (2000). There is some evidence to suggest the VvGlub and VvChi3 expression levels in phylloxera-infested leaves are greater than in uninfested leaves. This results supports the finding of Jacobs et al (1999) who found VvGlub expression to be greatest in leaves of grapevines detected with powdery mildew, when compared to uninsulated grapevines.

The alteration in gene expression that constitute the defence-related response of higher plants to biological and environmental stresses are very complex (Cutt and Klessig, 1999). A single metabolite or class of metabolites present in a plant will not comprise the only defence system. A wide variety of defence-related compounds may be present – in particular tannins, polyphenols, proteases and chitinases are very widely distributed even in species which contain other major secondary metabolites such as cyanogenic glucosides, glucosinolates and alkaloids (Bennet and Wallsgrove, 1994). There are also physical defence mechanisms, secondary thickening, cuticular waxes, leaf hair and other structural factors known to protect plants (Kollatakudy and Kollar, 1983).

Limited information is currently available regarding the physiology, biochemistry and molecular biology of inducible defence mechanisms in grapevines. To date, most effort has been focussed on fungal infections, such as the induction of β-1,3 glucanases in grapevine leaves following application of salicyclic acid or infection with *Botrytis cinerea* (Renault et al 1996). Giannakis et al (1998) reported a correlation between the combined activities of chitinase and β-1,3,glucanase of a range of grapevine cultivars and their observed field resistance to powdery mildew.

The activation of diverse sets of PR genes in response to diverse pathogens and forms of stress makes it difficult to determine whether single PR genes are the result of a specific stress situation. The expression levels of the defence-related genes selected

for this study may have indirectly incurred by the presence of phylloxera. It is likely that PR expression might have much more general character, regardless of the kind of stress. Hon et al (1995) suggests that all PR proteins primarily were engaged in a defence against microorganisms. The developmental regulation of PR genes as proposed by Cutt and Klessig (1999), also implies the roles of PR genes in the physiology of healthy plants. While the expression of the various physiological and biochemical defence responses in plants is known to be coordinately regulated (Lamb et al, 1989, Graham and Graham, 1991), it is widely believed that there are multiple transduction mechanisms for defence gene activation (Repka, 2001). Hence, a phylloxera-specific PR response might be unlikely to occur which is supported in the current study.

For greater than 10 years, a series of increasingly advanced and powerful technologies have evolved for the manipulation and analysis of DNA. Because most of these require only small amounts of DNA, these technologies have enabled researchers to examine in greater detail and depth the genetic structure of natural insect populations (Black, 1991). The most powerful techniques are those such as PCR, which enable the analysis of individual insects. This is particularly noted for phylloxera, which in itself is a very tiny aphid and like other soil-borne pathogens, is difficult to quantify because is may be distributed in patches and present in very low numbers.

Parts of the rRNA and EF genes are strongly conserved even among distantly related species (Vossbrinck et al 1987). For example Lupoli et al (1990) used a mosquito probe (which was designed from variable rRNA coding regions) to detect aphid DNA. A similar finding was found in the current study where, using a PCR approach, there was insufficient genetic difference to separate phylloxera from 4 aphid genera. More sensitive genetic techniques need to be used to refine the primer specificity so that aphid DNA does not produce 'false positive' results. . Overall the phylloxera-specific probe approach by its very nature, because it relies on expression of the target organisms DNA, is more reliable than the PR approach as a potential detection system. Because it targets the insects DNA, sampling protocols could be modified to examine not only detection in soil samples but also in trap samples.

Effects of phylloxera genotype on *Vitis vinifera* damage intensities

Objective 1:
Develop diagnostic techniques to improve early detection of phylloxera in the field (sub-project 1)

Objective 2:
Evaluate alternative and novel methods of phylloxera control including the use of systemic insecticides (sub-project 2)

Introduction

The aim of this study was to test if different phylloxera genotypes had varied effects on *V.vinifera* host plant performance. A glasshouse assay of performance was used to test the performance of two separate phylloxera genotypes (G1 and G4) and a genotypes sourced from Rutherglen (G29 and G46). By measuring vine and nutrient parameters as well as numbers of phylloxera-life stages, effects on vine performance as well as phylloxera numbers were assessed.

Materials and Methods

Twenty four, 1-year old Sauvignon Blanc (*Vitis vinifera cv*. Sauvignon Blanc clone H5V10) rootlings were sourced from a commercial nursery in April 2002 and were potted into autoclaved 20cm plastic pots using a sterilised soil-perlite composite (80% potting mix, 20% perlite). Vines were kept in a shade-house for 9 months and were transferred early January 2003 to a controlled temperature glasshouse prior to phylloxera inoculation. The trial was conducted over an 8-month (245-day) period in a temperature-controlled glasshouse cycling between 24 °C ± 2 °C (600-1800 h) and 20 °C ± 2 °C (1800-600 h). This temperature range was optimal for both vine and phylloxera development during the course of the trial. Dataloggers monitored temperate at 15-minute intervals and indicated that the temperature range was maintained throughout the course of the trial. An automatic dripper watering system

was established. Vines were watered until the pots just started to drain (equivalent to field capacity) once a day, with adjustments made as water use increased during the season. Growth lights were on for 12 hours each day (600 – 1800 h), with an additional spike from 000 – 100 h to offset dormancy due to changes in day length. A portable dehumidifier was also used to maintain relative humidity levels between 60 and 70%.

A randomised design was used to examine the effects of genetically distinct phylloxera genotypes (Corrie et al, 2002) effects on *V. vinifera* host performance. The trial comprised of 4 treatments: 6 vines infested with G4 phylloxera, 6 vines infested with G1 phylloxera and 6 vines infested with a mixed population of G29 and G46, plus 6 control vines that were not infested. The four treatments were randomised over a 2 x 4 grid, which comprised of 8 upturned wire cages, with 3 vines at random per crate. To prevent contamination of uninfested vines with phylloxera, individual 45mm x 35mm draw-string bags were used as described on page 77. G1 and G4 phylloxera populations were sourced from commercial vineyards (King Valley and Upton regions respectively) and reared *in vitro* on excised root pieces based on the method by Granett et al (1987). Two Rutherglen sourced genotypes, G29 and G46, had been previously collected during the 2000 vintage from a commercial vineyard and maintained on infected stock vines throughout the course of the study. All genotypes were verified both prior to commencement of the study (Angela Corrie, pers. comm, 2000 and Kathy Viduka pers. comm, 2002) and at completion of the study (Paul Umina, pers. comm, 2004). Each of the phylloxera infected treatments was infested at 2 different rates. Three of the 6 vines/treatment were infested with a total of 60 eggs, while the remaining 3 vines were infested with 600 eggs using the method described on page 77. One hundred phylloxera eggs were placed on moistened filter paper strips. The roots of the vines were exposed and the filter paper was placed in contact with the root system.

Life-Stage Quantification

Phylloxera assessments were conducted at the completion of the study after all the vine measurements had been taken. Vines were removed from their pots and the root system scored for level of phylloxera damage (as per Boubals, 1966) in the case of the infested vines. Uninfested vines were also examined to ensure that no contamination

had occurred. Three samples of roots approximately 1 g in wet weight were taken from the root system of each vine and washed through a 60 um mesh, with the collected filtrate containing the various phylloxera life stages washed into a screw-top plastic container containing 70% ethanol. Phylloxera life-stages were determined using a dissector microscope with 10x objective. To adjust phylloxera numbers for the amount of root tissue sampled, root pieces were oven dried and weighed. The number of phylloxera at different life stages were counted. Eggs, 1st instars (or crawlers), intermediates comprised of 2nd, 3rd, 4th instars, and adults (alates and apterous) were identified. Numbers of these life stage categories were compared among treatments using a one-way analysis of variance (ANOVA) computed with SPSS Version 11.5.

Leaf Assessments:

Six leaves were taken from each cane. The youngest mature leaf along the cane was taken, defined as the 5th leaf from the growing tip (refer Figure 7), as well as the next 5 leaves inwards along the cane. Average measurements of leaf colour, leaf chlorophyll and leaf area were obtained for the six leaves on a cane.

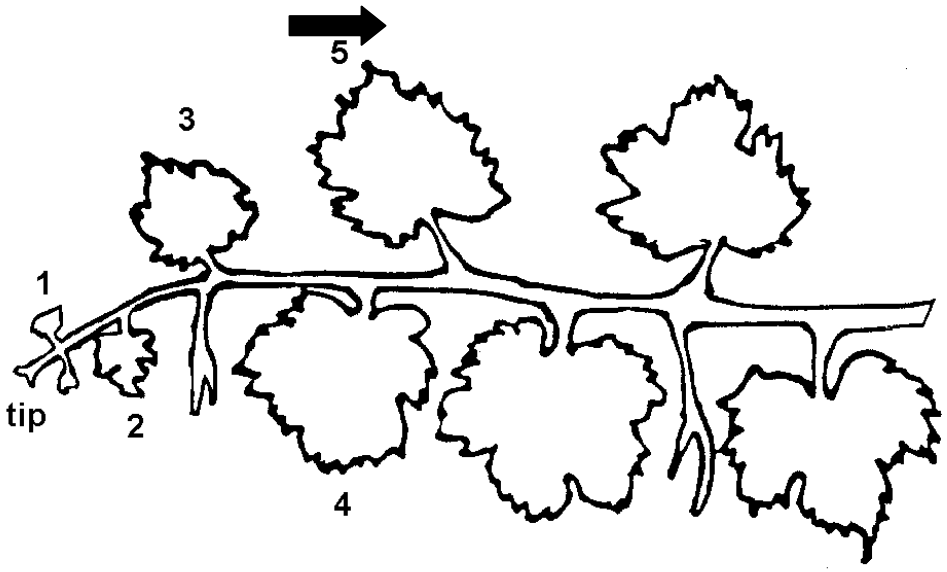


Figure 7. Diagram illustrating the position of the youngest mature leaf from the growing tip. This position was used in assessments of leaf hue° and chlorophyll content, where reading commenced at leaf 5 (marked with an arrow) and proceeded with subsequent leaves along the cane.

Leaf Colour (Hue° Angle)

To assess premature leaf yellowing in the vines and premature development of chlorotic tissue, a colour intensity assay was followed. Colour intensity was measured with a hand-held tristimulus reflectance colourimetrer (Minolta CR-200), calibrated with a white standard tile (L = 97.3; a = –0.43; b=1.91). Colour was recorded using the CIE-$L^*a^*b^*$ uniform colour space (CIE-*Lab*) where L^* indicates lightness, a^* indicates hue on a green (–) to red (+), and b^* indicates hue on a blue (–) to yellow (+) axis (Clydesdale, 1978). These three CIE-*Lab* values were further incorporated into Hue angle functions, which are used to express tissue colour, providing a single measure of colour that simulates visual judgement (Chervin, 1996). Hue angle , $H° = (\tan b/a)^{-1}$) calculations were determined for each measurement so that infested treatments could be compared to the uninfested treatments. Chlorotic leaves have lower values of H°.

Leaf Chlorophyll

Leaf chlorophyll measurements were obtained with a hand-held SPAD-502 Chlorophyll meter (Minolta™). This instrument determines the relative amount of chlorophyll present by measuring optical density differences in the chlorophyll wavelength regions, 400-500nm, and 600-700nm regions, with no transmittance in the infra-red region. Using these two transmittances, the meter calculates a numerical SPAD value (within ± 1.0 SPAD unit at room temperature) which is proportional to the amount of chlorophyll present in the given leaf. Measurements were taken by inserting the leaf and closing the measuring head (Figure 8). Two readings were taken for each leaf with the total readings for six leaves averaged to give a single chlorophyll value per vine.

Leaf Area

Total leaf area was determined for each vine using a Paton™ Electronic Planimeter. Note that all vine leaves, including those that had sprouted from pruned secondary canes were included in this assessment.

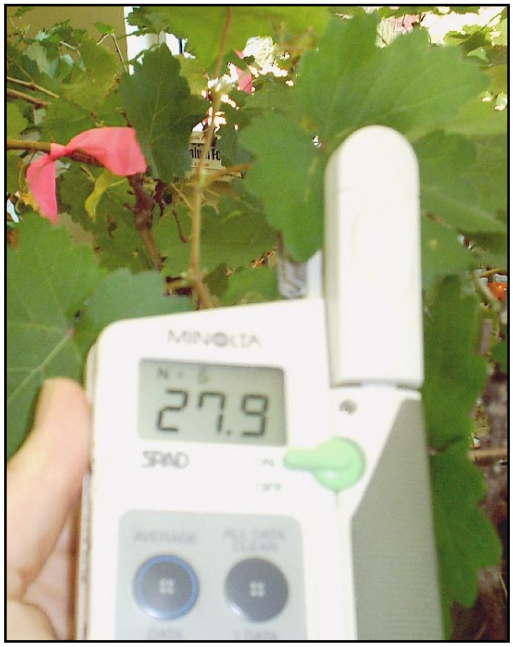


Figure 8. SPAD-502 Chlorophyll meter used to calculate the chlorophyll content of 6 grapevine leaves.

Vine assessments:

Trunk diameter measurements were taken at three separate points up the vine trunk, 5, 7 and 9 cm up from the base of the vine. Measurements were taken using a digital calliper (Vernier™). Node lengths and number were recorded from the primary cane that had been standardised to 10 nodes prior to commencement of the study. Oven dried weights for whole vine stem and root mass were also determined. Nutritional analysis of leaf blade, petiole, and roots was also undertaken.

Nutritional Analysis:

Replicate vines within each treatment, eg G4L (low) treatment, G4H (high) treatment, etc. were pooled together for the nutritional analysis in order to provide the minimum amount of tissue required for analysis. Pooled petiole, leaf blade and root material for each treatment were oven dried overnight at 50°C and then ground to a 1 mm particle size through a small cyclone mill at 2,900 rpm (Crompton Parkinson™, Model No. 5862). The ground plant components were stored in separate plastic containers and later sent to the State Chemical Laboratories (SCL), Werribee, Victoria, for nutritional analysis. At SCL, microwave digestion using nitric acid and hydrogen peroxide was undertaken, with the digests analysed by ICP-OES (inductively coupled plasma - optical emission spectroscopy) to determine total N (% w/w), as well as the exchangeable cations: P, K, S, Ca, Mg, Na, Cu, Zn, Mn, Fe and B.

Results

Life Stage Quantification

The numbers of phylloxera recorded at each life stage differed depending on genotype (Figure 13). G4 numbers were relatively higher than those of the other genotypes, particularly the G29 + G46 combination. In some cases three-fold differences existed between groups. ANOVAs (Table 5) indicate significant ($P < 0.05$) differences among the genotypes for all life cycle stages. There was also a significant difference between the high and low infestations for all three life cycle stages, although there was an interaction between infestation level and genotype for the egg and intermediate stages. In these cases the relative difference between the low and high infestations was greater for G4 than for the other genotypes. The difference was

particularly marked when compared to G29 and G46 for the intermediate stage (Figure 9). Significant ($P < 0.05$) interactions between genotypes and infestation levels were also found for egg and intermediate & life-stages (Table 5).

Leaf Assessments:

Mulitivariate analysis of variance of the leaf parameters, hue angle, leaf colour and leaf area all revealed strong effects of genotype and genotype infestation levels (Figure 14). Summaries of the statistical analyses are found in Table 5. Significant differences ($P < 0.05$) between genotype, infestation levels and the interaction between these two effects were found for all there leaf parameters (Table 5). In all cases, the uninfested 'control' treatment had the highest values (Figure 10). Infested treatments revealed varying degrees of decline in leaf parameters. G29 and G43 populations had, after the controls, the second highest values of hue angle, leaf colour and leaf area. For this population, the low and high infestation rates had similar values. This was in contrast to G1 and G4 populations, where there were marked differences in infestation rates affecting hue angle and leaf area. Vines infested with high levels of G4 recorded the lowest values in leaf chlorophyll and leaf area. The lowest values in hue angle were recorded in high infestations of the G4 and G1 genotypes.

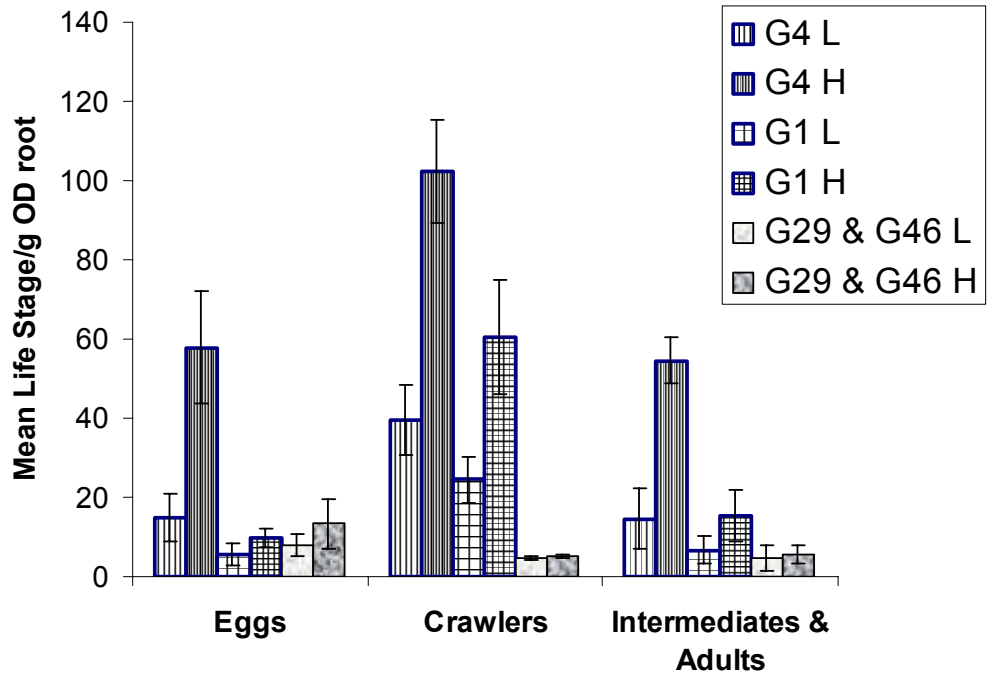


Figure 9. Mean life stages per gram oven-dried (OD) weights for the various phylloxera genotypes and infestations rates (L = low infestation and H = high infestation). Genotypes 29 and 46 were sourced from the Rutherglen PIZ, G1 from the Upton PIZ and G4 from the King Valley PIZ.

Vine Assessments:

Mean whole root weights are plotted in Figure 11a. Significant effects of genotype but not infestation levels were found, though there was an interaction between these factors (Table 6). Mean total root mass was highest for the G29 and G46 (low infestation) treatments, followed by the 'control', G4 and G1 treatments respectively. Marked levels of root decline were particularly evident for the G1 (high infestation) and G4 (high infestation) treatments. Phylloxera genotypes differed in their effects on mean stem weights (Figure 11b). The genotype, infestation and interaction terms were significant in the ANOVA (Table 6). For the infested treatments, the highest mean stem weight was seen for the G29 and G46 (low infestation) combination, whereas the lowest value was recorded for vines infested with the G1 (high infestation) treatment (Figure 11b).

Life Stage	Effect	df	MS	F	P
Egg	Genotype	2	1488.01	9.78	0.003
	High/Low Infestation	1	1397.09	9.18	0.010
	Genotype & High/Low Infestation	2	724.43	4.76	0.030
	Error	12	152.22		
1st Instar	Genotype	2	6573.30	14.36	0.001
	High/Low Infestation	1	4946.15	10.80	0.006
	Genotype & High/Low Infestation	2	1457.40	3.18	0.078
	Error	12	457.85		
Intermediate	Genotype	2	1464.54	18.25	< 0.001
	High/Low Infestation	1	1238.36	15.43	0.002
	Genotype & High/Low Infestation	2	638.43	7.96	0.006
	Error	12	80.24		

Table 5. ANOVA results for effects of genotype and high and low infestation levels on *V. vinifera*.

Neither genotype nor infestation level had any marked effects on mean trunk diameter (Figure 15c). There was a weak ($P < 0.05$) effect of genotype but no effect of infestation frequency or any interaction between these two effects (Table 6). Vines infested with G1 and G4 genotypes had similar trunk diameters to the uninfested 'control' treatment (Figure 11c). There were significant effects of genotype and infestation levels on node length and node number (Table 6). All phylloxera-infested treatments had markedly reduced mean node numbers, with the G4 (high infestation) treatment recording the lowest values (Figure 12a). Effects on mean node length were smaller than on node length, but in the same direction (Figure 12b).

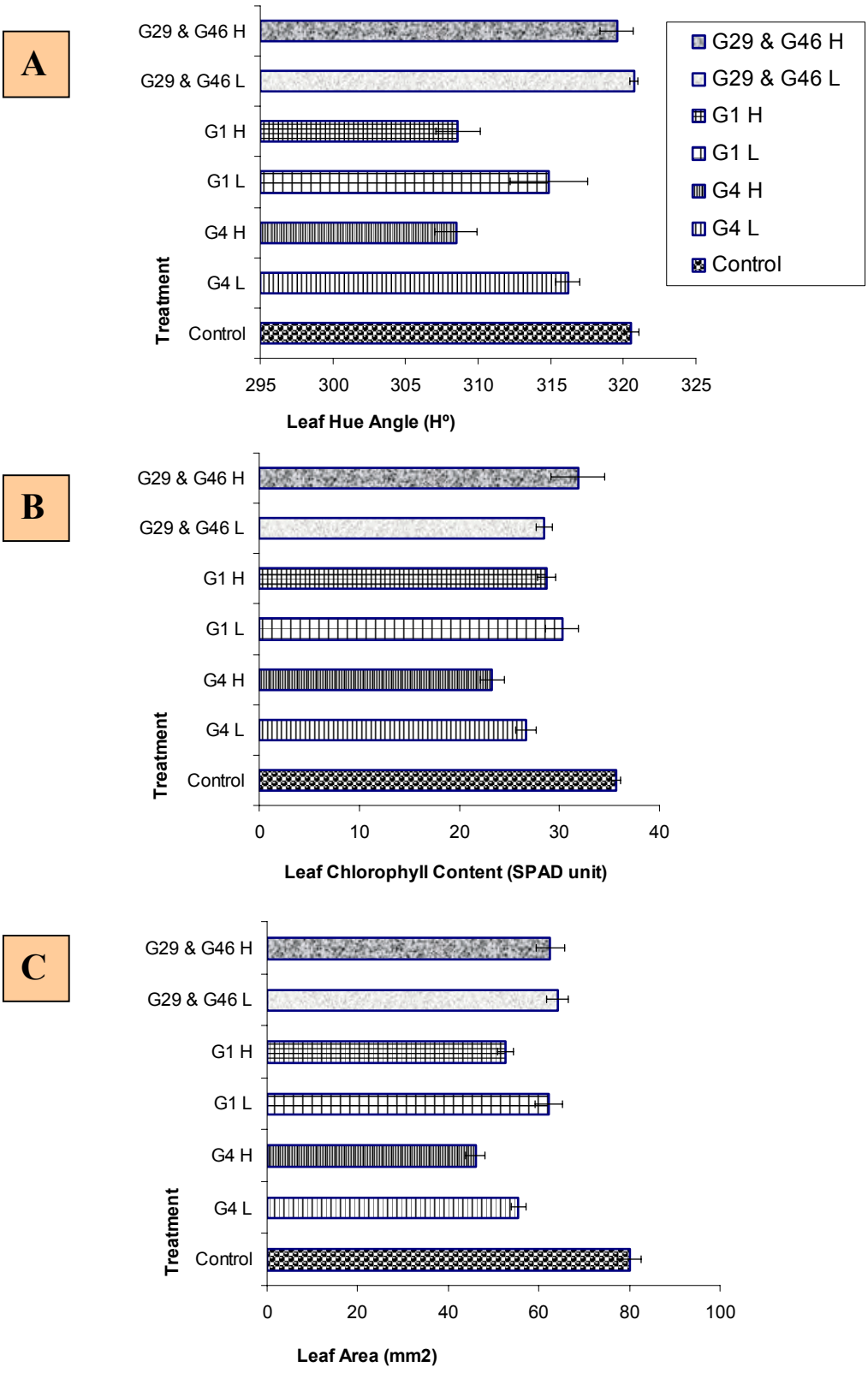


Figure 10. Leaf assessments of (A) hue angle; (B) chlorophyll content; and (C) leaf area (mm^2). The 'control' treatment represents vines that were not infested with phylloxera.

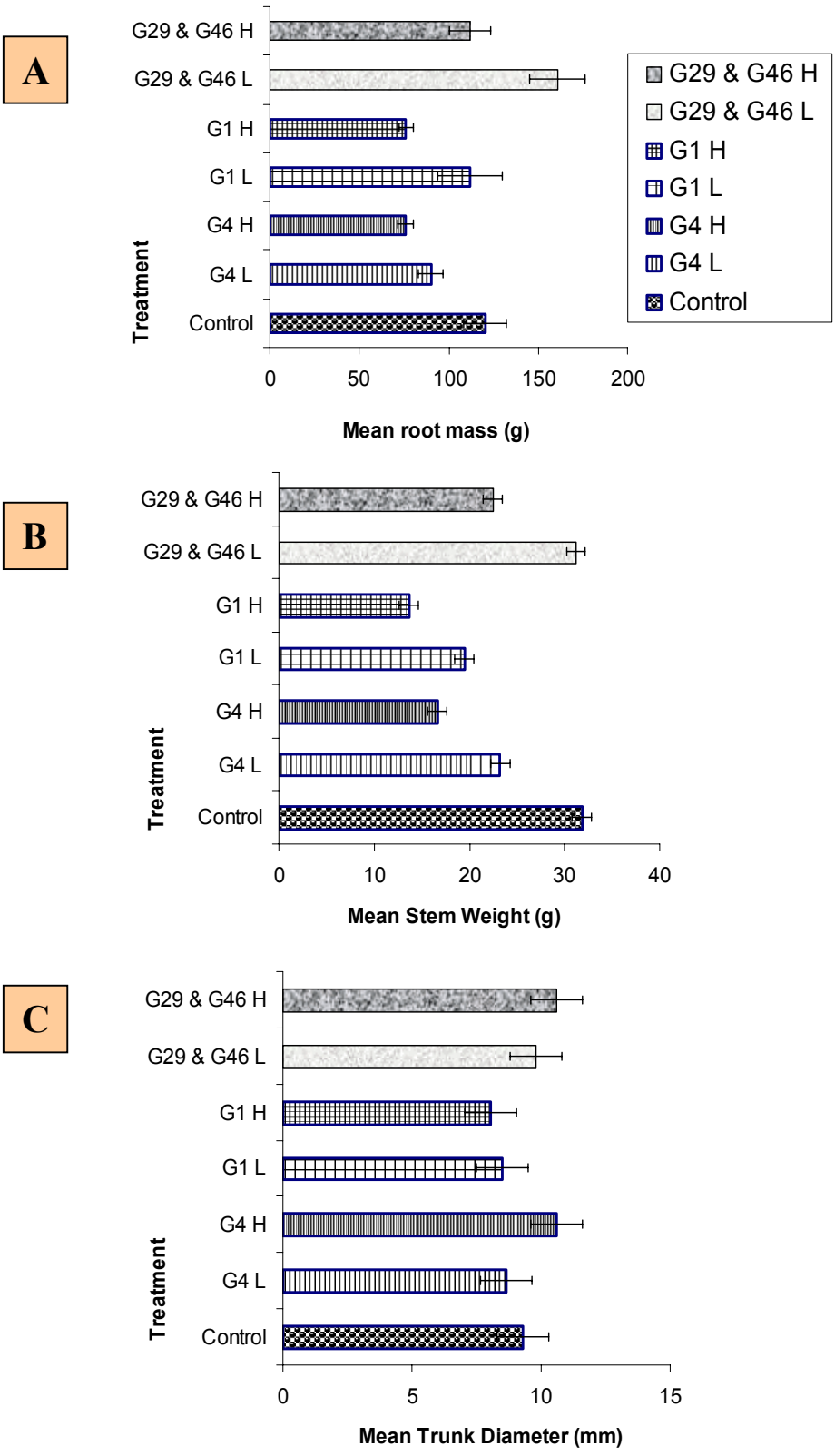


Figure 11. Mean (A) root mass, (B) stem weight and (C) trunk diameter measurements for the 7 different treatment effects.

Of the three elemental compounds tested, significant effects were only observed for nitrogen, when there were significant effects of tissue type, phylloxera genotype and the interaction between these effects (Table 7). The highest levels of all three elements were recorded in the leaf blade (Figure 13a). A weak effect of tissue type on total P levels was found but this was not due to phylloxera genotype (Table 7).

Vine Parameter	Effect	df	MS	F	P
Hue Angle (H°)	Genotype	3	107.62	7.22	0.002
	High/Low Infestation	2	203.26	19.91	< 0.001
	Genotype * High/Low Infestation	6	88.57	16.82	< 0.001
	Error	17	5.27		
Chlorophyll Content	Genotype	3	115.84	11.89	< 0.001
	High/Low Infestation	2	126.07	9.12	0.001
	Genotype * High/Low Infestation	6	64.18	6.94	0.001
	Error	17	9.25		
Leaf Area (mms^2)	Genotype	3	941.19	25.10	< 0.001
	High/Low Infestation	2	1273.37	26.04	< 0.001
	Genotype * High/Low Infestation	6	520.55	19.66	< 0.001
	Error	17	26.48		
Trunk Diameter (cm)	Genotype	3	5.74	4.92	0.010
	High/Low Infestation	2	2.85	1.72	0.203
	Genotype * High/Low Infestation	6	3.17	2.51	0.064
	Error	17	1.27		
Stem Wt (g)	Genotype	3	281.09	8.04	0.001
	High/Low Infestation	2	368.81	9.62	0.001
	Genotype * High/Low Infestation	6	178.82	6.47	0.001
	Error	17	27.62		
Root Wt (g)	Genotype	3	3548.68	4.78	0.011
	High/Low Infestation	2	3013.80	3.25	0.059
	Genotype * High/Low Infestation	6	2743.54	5.17	0.003
	Error	17	530.71		
Node Length (cm)	Genotype	3	2.54	3.85	0.025
	High/Low Infestation	2	5.28	10.86	0.001
	Genotype * High/Low Infestation	6	1.76	2.93	0.038
	Error	17	0.60		
Node Number	Genotype	3	160.56	3.90	0.024
	High/Low Infestation	2	288.89	8.35	0.002
	Genotype * High/Low Infestation	6	102.33	2.52	0.062
	Error	17	40.59		

Table 6. Summary of multivariate analysis conducted on the various vine parameters. The two studied effects were (1) Genotype effects; which refer to effects of G1, G4 and the combined genotypes G29 and G46; and (2) High/Low Infestation effects: which compares the effects of high and low infestation rates within each population.

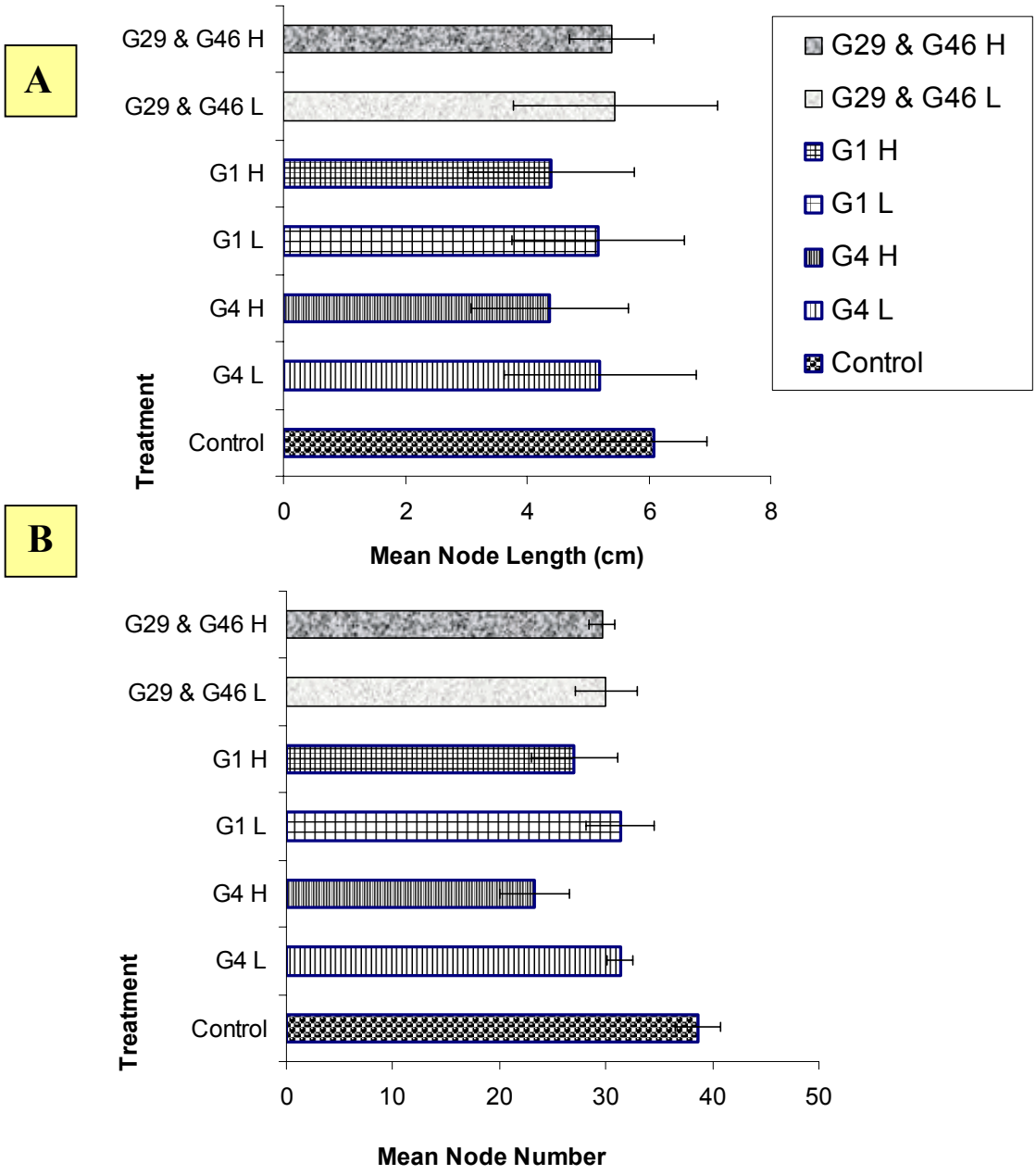


Figure 12. Node length and node number comparisons conducted on the primary cane, which were standardised to 10 nodes at the beginning of the trial.

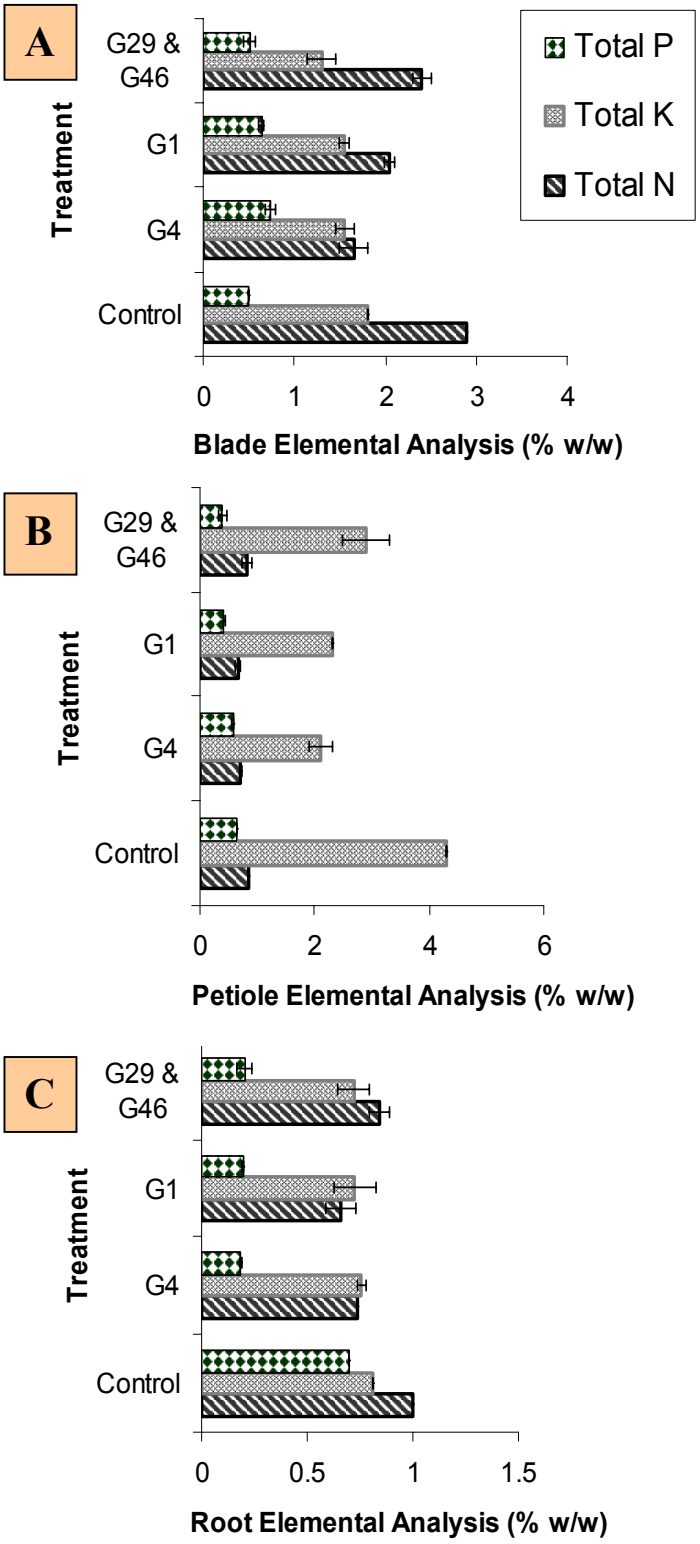


Figure 13. Elemental analysis conducted by the State Chemical Laboratories Werribee, Victoria. Due to the lack of replication for infestation levels for each population, these values were pooled together.

Vine Parameter	Effect	df	MS	F	P
Total N (% w/w)	Tissue	2	4.52	398.44	< 0.001
	Genotype	3	0.25	22.43	< 0.001
	High/Low Infestation	1	0.08	0.14	0.711
	Tissue * Genotype	6	0.09	8.27	0.003
	Error	9	0.01		
Total P (% w/w)	Tissue	2	0.21	31.36	< 0.001
	Genotype	3	0.02	2.93	0.092
	High/Low Infestation	1	0.00	0.08	0.787
	Tissue * Genotype	6	0.02	2.44	0.111
	Error	9	0.01		
Total K (% w/w/)	Tissue	2	4.03	15.56	0.176
	Genotype	1	0.71	2.73	0.347
	High/Low Infestation	1	0.71	2.73	0.347
	Tissue * Genotype	4	0.21	0.83	0.666
	Error	6	0.26	2.73	0.347

Table 7. Univariate analysis on levels of N, P and K to test for significant (P <0.05) effects of tissue (ie leaf, petiole, root), genotype and infestation levels.

Discussion

The performance of grape phylloxera measured in this study with potted grapevines indicated that the genotypes differed markedly both in terms of abundance and in terms of effects on vine hosts. High numbers of phylloxera resulted in relatively greater effects on vine health. Omer et al (1999) have previously noted that on susceptible root types (Cabernet Sauvignon and AXR # 1), the host utilisation of different phylloxera populations varied according to the attack intensities of the particular population. Increased performance and increased size of tuberosities is likely to allow for greater mobilisation of nutrients for the insect's consumption (Omer et al 1995).

Genetic studies on the spatial distribution of phylloxera genotypes within Australia by Corrie et al (2001, 2003), indicated an association of specific asexual phylloxera lineages to a particular vine host. Corrie et al (2002, 2003) proposed that the

genetically similar G1 and G4 populations, which are the most widespread genotypes in Australia, might possess characteristics that enable them to more effectively colonise vineyards comprised of *V.vinifera*. The present glasshouse results support this conjecture as G1 and G4 performed better on *V. vinifera* compared to the mixed genotypes tested. It would be worthwhile testing additional genotypes from Rutherglen and other areas on *V. vinifera.*

There are likely to be differences in the population dynamics of Rutherglen phylloxera populations compared to G1 and G4 populations on *V. vinifera* (refer Chapter 2). Very low numbers of insects were trapped throughout the phylloxera season at Rutherglen, and the vines showed no evidence of damage or yield decline. This is in contrast to the same varieties of *V. vinifera* monitored in the King Valley and Upton regions, where G4 and G1 populations respectively were present, high numbers of insects were detected from vines, and there was evidence of vine decline with associated yield loss. Other studies in the King Valley and Nagambie regions have shown a similar response (Powell et al 2003).

Most of the genotypic classes identified from the Rutherglen region are exclusive to this area (Corrie et al 2002). Reasons for this increased genetic diversity are not known but may be the result of rare sexual combination and/or multiple introductions, possibly coupled with mutation. Historical records of grapevine movement suggest that the Rutherglen region, being one the oldest viticultural areas in Australia, received extensive introductions of *Vitis* material, both, from Europe during the midy 19th century (Buchanan, 1990). Some evidence suggests that some Rutherglen genotypes are more common on phylloxera-resistant rootstocks such as ARG1 and Schwarzmann than on *V. vinifera* (Corrie et al 2003). The Rutherglen region is, with a few exceptions, planted entirely on phylloxera-resistant rootstocks (M. Campbell, pers comm.). Phylloxera genotypes from Rutherglen region may possess characteristics that better enable them to colonise rootstock varieties compared to *V. vinifera*. In contrast, G1 and G4 genotypes may be better adapted to feeding on *V. vinifera* varieties (Corrie et al 2002), accounting for the predominance of these clones in the most recently infested ungrafted vineyards in the King Valley region (G4) and Upton/Nagambie regions (G1).

Genotype G4 has not yet been found in the Rutherglen district (Corrie et al 2001), while G1 is restricted to only a few vineyards and not detected on the site used in the current study (Corrie et al 2001, Viduka et al 2003). While the exact reasons for this distribution are unknown, historic and vine-host related factors previously mentioned are likely to be involved, together with the effective implementation of quarantine. Quarantine plays an important role in preventing the cross contamination of different populations of phylloxera between infested vineyards/regions. The outcomes of this research highlight the importance of assessing the type(s) of phylloxera genotype present in a vineyard. Clearly, vineyards infested with G1 or G4 phylloxera populations may require replanting much sooner than those infested with G29 or G46 populations and (probably) other genotypes from some infested regions. Further research is needed to better understand the competitive nature of particular phylloxera genotypes to both resistant and tolerant *Vitis* species under different environmental regimes.

It is difficult to compare the findings observed in Australian vineyards to those conducted overseas because microsatellite loci used by Corrie et al (2001, 2003) to assign populations into genotypes has not yet been employed overseas. Nonetheless variation in the aggressiveness and genetic diversity of grape phylloxera populations have been reported in the USA (Granett et al 1985, 1987; Fergusson-Kolmes and Dennehy, 1993; Fong et al, 1995; Downie, 1999, 2000); Europe (Forneck et al 2001, 2002 and Yvon and Péros, 2003), South Africa (De Klerk, 1974); and New Zealand (King and Rilling, 1985, 1991). These studies, have either in the past assigned populations to particular 'biotypes', based on comparative studies of phylloxera performance on grape excised roots (refer Granett et al 2001 for the well documented case of biotype 'B' damage on AXR # 1 vineyards), or genetic analysis using amplified fragment length polymorphism (AFLP), within and between populations (Forneck et al 2002). A recent survey of phylloxera populations based on mitochondrial DNA sequences was also conducted by Downie et al (2002). None of the above overseas techniques however, can provide as detailed information on the mode of reproduction and genetic structure of a given population (Corrie et al 2002). It seems likely that genotype-vine associations as observed in Australia also occur overseas, however until a consistent method to define phylloxera populations is adopted, worldwide comparisons of phylloxera populations remain difficult.

Some early symptoms of grape phylloxera-associated vine decline previously reported are decreased cane growth, premature leaf yellowing, chlorophyll reductions, and in certain cases P deficiencies (Baldy et al 1996; Granett et al 2001). With the exception of P deficiencies, all these symptoms were detected in the current glasshouse study over a time period of 8 months. Currently, the most widely adopted method of testing the resistance on vine types to phylloxera populations involves excised root bioassays, but these overestimate grape phylloxera virulence and underestimate rootstock resistance (Granett et al 2001a). Excised root demonstrations of virulence may therefore not to be a sufficient predictor of imminent field damage (Grannet et al 2001b). The whole-vine assay used in this study may provide an alterative means to testing resistance levels, providing quantitative information on both insect fecundity as well damage intensities on *V.vinifera* and resistant *Vitis* varieties. This approach could provide a useful system to test for the resistance of all vine types to representatives of the full range of phylloxera types known to present in vineyards.

Population dynamics of grape phylloxera in ungrafted vineyards from south-eastern Australia.

Objective 2:

Evaluate alternative and novel methods of phylloxera control including the use of systemic insecticides (sub-project 2)

Introduction

This study investigates changes in the abundance of phylloxera across single and three consecutive seasons in a commercial vineyard in the King Valley region in north-east Victoria, Australia. Different trapping methods were used to determine whether phylloxera life stages vary in a predictable manner throughout the vine growth season and between seasons. Results were then compared to changes in phylloxera from two other vineyards containing different phylloxera genotypes, to test if there is variability in changes in phylloxera numbers that might be related to seasonal, regional and genotype-related effects. A specific aim of this study was to assess whether environmental and phenology-based information might predict peaks in phylloxera number as a basis for future management strategies such as the application of chemical insecticides to maximise effects on insect mortality.

Materials and Methods

1. Association between different phylloxera trapping methods

Site Selection

The study site was a commercial vineyard located 5km east of Cheshunt, within the cool-climate King Valley PIZ. Vines were planted in 1990 and phylloxera was first detected in the vineyard in May 1997. A single genotype characterised as G4 is present (Corrie et al 2002). Soil type, classified on the basis of texture and chemistry, is classified as a dystrophic brown kandosol, with the soil texture of a duplex nature comprising a sandy clay loam A horizon overlying light to medium clay subsoils (W.J. Slattery, Department of Primary Industries, Rutherglen, pers. comm. 1999).

Trial design

The study was conducted during the 2001 grape vintage. Nine adjacent rows of ungrafted *V.vinifera* L. cv. Sauvignon Blanc (clone FV5V10) vines (designated rows 1-9) were chosen for the study block. The row spacing was 3 metres and the vine spacing was 1.8 metres. Visual decline in vines attributed to phylloxera was more evident in rows 1 to 5 and was likely to be site of initial infestation of the block. The infestation was clearly spreading in a southerly (ie towards row 5) and easterly direction. A total of 12 vines were studies in the block with 3 vines per row 2, 4, 6, 8. The 3 vines selected for each row were the 18th, 26th and 34th vines positioned from the start of each row respectively. Rows 1, 3, 5, 7, and 9 were designated buffer rows where most of the root sampling was conducted (Figure 15).

Emergence Trapping Technique

The seasonal abundance of the most mobile and abundant phylloxera dispersive stage referred to as the 1st instar or 'crawler' from below the ground onto the soil surface was measured using this technique. Emergence traps (Figure 14a), consisted of translucent plastic containers (4 litre Décor™), 22 cm ×13 cm depth, open at one end and inverted onto the soil surface at a distance of 10cm from the sample vine trunk as previously described by Powell et al (2000). Traps were fixed flush with the soil surface using metal tent pegs. On emergence from the soil, phylloxera were trapped in condensation on the container sides. At fortnightly intervals commencing on the 26th October 2000 and ending on the 6th June 2001, insects were removed by washing the trap with 70% ethanol and collected in plastic containers. Traps were then rinsed with tap water and replaced. Collected insects were counted using a low power binocular microscope.

Trunk Trapping Technique

Phylloxera movement (predominantly 1st instar life stage) up and down the grapevine trunks was assessed using a trunk trapping technique as described by Powell et al (2000) involving the collection of phylloxera on tapes placed in two bands (Figure 14b). The lower band was used to collect insects moving up the vine trunk from the soil surface whilst the upper band was used to collect insects moving down the vine

trunk from the vine canopy. A 10 cm strip of grey duct tape was wrapped around each vine trunk 20cm above the soil surface and sealed at the top and bottom with a liquid sealant to prevent phylloxera moving into cracks in the vine bark. The duct tape formed a smooth surface on which the trunk traps could then be applied. Trunk traps consisted of two bands of white electrical tape wrapped around the trunks of sample vines at a distance of 25 cm above the soil surface and 20 mm apart. Tanglefoot™ was applied evenly to the centre of the electrical tape, at a width of 1 cm, using a paintbrush. Trunk traps were removed and replaced every two weeks. On removal, traps were stuck to A4 paper, covered with plastic Gladwrap™ to prevent sample contamination and insects were viewed and counted under a low power binocular microscope.



Figure 14. Emergence ((a) left) and trunk ((b) right) techniques used to monitor fortnightly movement of phylloxera from the vine roots to the soil surface (emergence) and movement of insects up and down the vine canopy (trunk).

Root sampling

Root populations were monitored monthly from October 2000 to June 2001. Samples were not taken from vines selected for trap sampling (referred to as reference vines) to avoid disturbing phylloxera populations at these vines. Instead, samples were

collected from the 8 vines surrounding the reference vines. These were the vines to the left and right of the reference vine in the same row, and 3 vines each located in the buffer rows to the top and bottom of the reference vines (Figure 15). Vines selected for root sampling were labelled 1-8 (Figure 15). The first vine to be sampled is shown in Figure 15, marked with a number "1", and sampling proceeded in a clockwise order thereafter. Sampling commenced in October 2000. At the conclusion of the study in June 2001, a total of 9 samples had been taken, with the vine shown in position "1" (Figure 15) sampled twice.

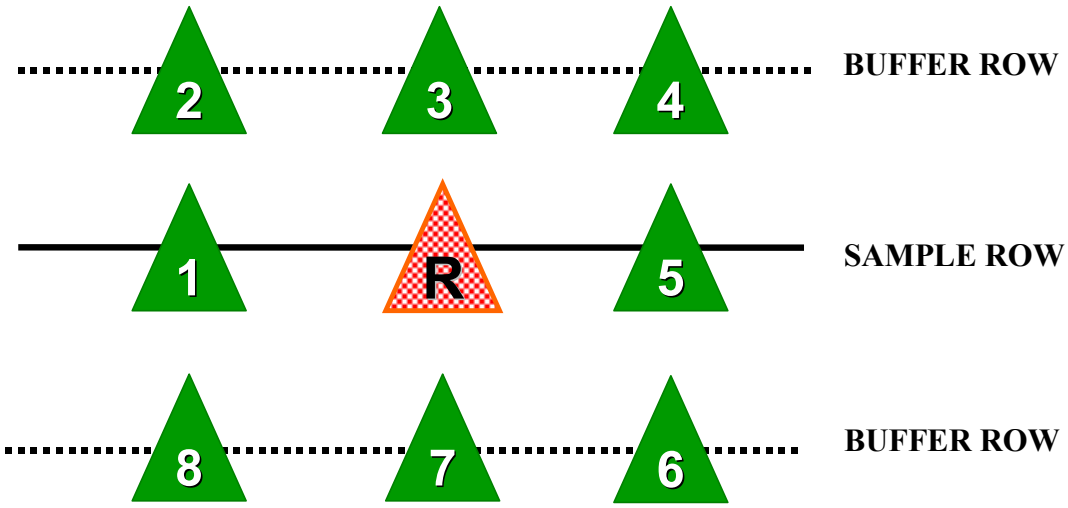


Figure 15. Diagrammatical representation of the root sampling procedure employed at a commercial vineyard in the King Valley throughout the 2001 vintage. Triangles represent individual vines located within the buffer or sample rows. Monthly root sampling was conducted around the 'reference' vines (designated with the symbol "R") within the sample rows. Reference vines were those vines selected for emergence and trunk trapping and root sampling was not conducted on these vines so that the phylloxera populations were not disturbed. Root sampling commenced at position 1 as indicated and proceeded in a clockwise direction over subsequent monthly intervals.

Root samples were collected by digging to expose the vine roots; between 2-5 g fresh root material, comprising mostly storage root and a small amount of fibrous root, was removed using secateurs. Root samples were then transported under quarantine conditions to a quarantine facility located at the Department of Primary Industries, Rutherglen, where they were washed using a sieve of 53µm aperture to collect phylloxera life stages, with the filtrate transferred to a 125ml plastic screw-top container for storage. All phylloxera life stages were counted using a low power binocular microscope. All root material was weighed, oven-dried at 50°C for 48 hours and reweighed to determine dry weight.

Analysis

Insect numbers collected with emergence and trunk trap methods were summed for the 12 reference vines for each of the 17 different sample dates and plotted to examine changes in abundance over time. For the root samples, insect life stages per gram oven-dried (OD) root were plotted for each monthly sampling period. To compare emergence and trunk data with monthly root samples, fortnightly samples were summed to produce values for each month.

To compare the numbers of phylloxera caught in different traps, Pearsons correlation coefficients were computed. In addition, a simple sign test was used to examine for consistent temporal changes in abundance between trapping methods. This test involved determining if numbers of phylloxera caught with a particular trapping method increased or decreased between sampling intervals. The sign of the change between the different types of trapping methods was compared. The number of consistent changes was compared with the expectation that inconsistent and consistent changes would occur equally frequently, using a chi-square test.

2. Emergence trapping over three consecutive seasons

The emergence trapping technique was used to examine changes over three periods because this technique captured more insects than the others tested. Emergence trapping at the Cheshunt site was continued using the same 12 reference vines described in earlier, for the 2002 and 2003 grape vintages.

To examine changes in phylloxera numbers, plots of $\log_{10}+1$ crawlers were made from the 12 traps sampled fortnightly. To compare crawler numbers across years and test for any consistent effects of vine row on crawler number, univariate analysis of variance (ANOVA) was carried out.

3. Variation in phylloxera emergence across different regions.

Site selection

Ungrafted *V.vinifera* L. cv. Cabernet Sauvignon (clone G9V3) located in the King Valley, Rutherglen and Upton regions (Figure 16) were selected for this geographic comparison. The King Valley site was located 2 km south-west of the Sauvignon Blanc vines examined in the above work. Vines had been planted in 1996 and the G4 phylloxera genotype (Corrie et al 2002) had first been identified from this block in 2001. The Rutherglen site was 5 km west from the town centre, within the north-east PIZ. Vines had been planted in the spring of 1975 (M. Campbell, pers comm) as an extension to an existing vineyard planted at the turn of the twentieth century. Phylloxera had been detected on this site since the early 1900s (M. Campbell, pers comm). A mixed population comprising of 14 different phylloxera genotypes has been recorded from the affected block, reflecting the high level of genetic diversity of phylloxera in this region (Corrie et al 2002). The two genotypes identified around the Cabernet Sauvignon vines selected for this study were G29 and G46, neither of which have been detected outside the Rutherglen and Milawa regions (Corrie et al, 2002). The soil type is classified as a mesotropic brown chromosol (Isbell, 1996), with a soil texture of a sandy clay loam in the A horizon to a fine sand to light clay in the B horizon (W.J. Slattery, Department of Primary Industries, Rutherglen, pers. comm. 1999).

The final vineyard selected was located 10 km northwest of the Upton township. Phylloxera genotype G1 (G1 and the previously mentioned G4 phylloxera predominate in infested vineyards of Australia - see Corrie et al 2002), was first detected on these 5 year old vines during April 2000. The soil-type is classified as a mixture of mottled-sodic red kurosols and acidic mesotrophic brown kandosols (Isbell, 1996), with soil textures described as sandy loam A horizons over sandy to light clay subsoils (W.J. Slattery, Department of Primary Industries, Rutherglen, pers. comm. 1999). A second ungrafted Cabernet variety, *V. vinifera* L. cv. Cabernet

Franc, clone C7115, was also monitored within the same Upton vineyard block to compare varietal differences.



Figure 16. Phylloxera infested regions and sampling sites in north-east Victoria. Areas with known phylloxera infestations are termed Phylloxera Infested Zones (PIZs); regions free from phylloxera are referred to as Phylloxera Exclusion Zones (PEZs), and Phylloxera Risk Zones (PRZs) refer to regions where the phylloxera status is unknown and currently not detected. The study sites selected were located in the King Valley, Rutherglen (which is part of the NE PIZ) and Upton PIZs (marked with a "☆ " on map). (Image used with permission from the Phylloxera and Grape Industry Board of South Australia: www.phylloxera.com.au).

Trial design

Six reference vines were selected at random for each site from a designated 100m × 100m grid. Two emergence vines were placed either side of vines where weekly sampling was conducted from late December 2002 until mid April 2003. Soil temperature at a depth of 150mm was monitored at 15 minutes intervals using data-loggers fitted with a soil probe attachment. One data logger was present in each of the sites studied with an additional logger placed in the King Valley Sauvignon Blanc study site previously mentioned.

Analysis

For each sample period, crawler numbers were summed from the 12 emergence traps at the different site and plotted to compare peaks in crawler populations and differences in the total genotype insect number. Harvest data collected from individual vines was averaged for individual varieties across the 3 regions studied. For the King Valley Cabernet Sauvignon site, the frequency of the alate and apterous life stages was also compared.

A simple sign test was again used to examine for consistent temporal changes in phylloxera emergence between varieties grown in the same region and also to compare phylloxera emergence between the 3 regions. This test involved the same procedure as previously described in Section 1.

Degree day (°d) models, used to predict the number of generations produced by radicicolae life stages across the season, were calculated based on soil temperature information. Data loggers, with attached soil probes to 150mm depth were placed at each varietal location, except for the Upton region where a single data logger was placed in the Cabernet Sauvignon block. Soil temperature was recorded at 15-minute intervals throughout the course of the study. Degree day models were calculated based on developmental studies conducted by Raspi et al (1987), where the pre determined developmental zero was 8.7° and the thermal constant was 281.5 °d, for the entire development of phylloxera from egg to mature adult. The developmental zero was subtracted from mean daily temperature (based on hourly daily readings) to create °d units that were plotted against summed ln + 1 emergence totals at each

sampling period. Linear regressions on the cumulative degree day values against ln + 1 phylloxera emergence at (a) the same sampling period and for (b) degree day intervals moved earlier by one sampling period, were carried out using SPSS Version 11.5.

Results

1. Association between different vineyard trapping methods

Insect numbers collected for the three trapping methods are shown in Figure 17. Only the first-instar or crawler life stages were collected in the emergence and trunk traps. The emergence trapping method had by far the highest yields with summed numbers peaking to over 16,000 crawlers on the 17th January 2001 (Figure 23). All traps indicate consistent changes in abundance, involving a steady increase of life stages from November and December, peaks in life stages during January and February (apart from 31st Jan sampling period), and steady declines in all life-stages from March onwards (Figure 17a).

For the trunk trapping method (Figure 17b), higher crawler numbers were counted in the lower trap than the upper trap. Nonetheless, a significant correlation ($r = 0.921$, $N = 17$, $P < 0.001$), between the upper and lower trunk trap numbers was found.

For the root sampling method (Figure 17c), numbers of eggs recovered from the roots peaked before numbers of 1st and then 2nd instars. The 3rd and 4th instar and adult life stages (both alate and apterous) peaked at the same date around the middle of February. The 1st instar stage was by the far the most abundant stage recovered from the roots.

Direct comparisons of the numbers and distribution of crawler life stages collected with the three trapping methods are shown in Figure 18. These indicate similar patterns for all three trapping methods. This was particularly true for numbers recovered with the trunk and root sampling methods, which showed the same signed changes between time intervals for all 8 time occasions ($x^2 = 8$, $df = 1$, $P = 0.005$). For the comparison of root versus emergence catches and emergence versus

emergence catches, changes in abundance followed the same sign in 7 of the 8 comparisons ($x^2 = 4.5$, df = 1, P = 0.034). Phylloxera numbers therefore changed in the same way over a season regardless of the trapping method and despite differences in the absolute numbers of phylloxera caught with the different methods.

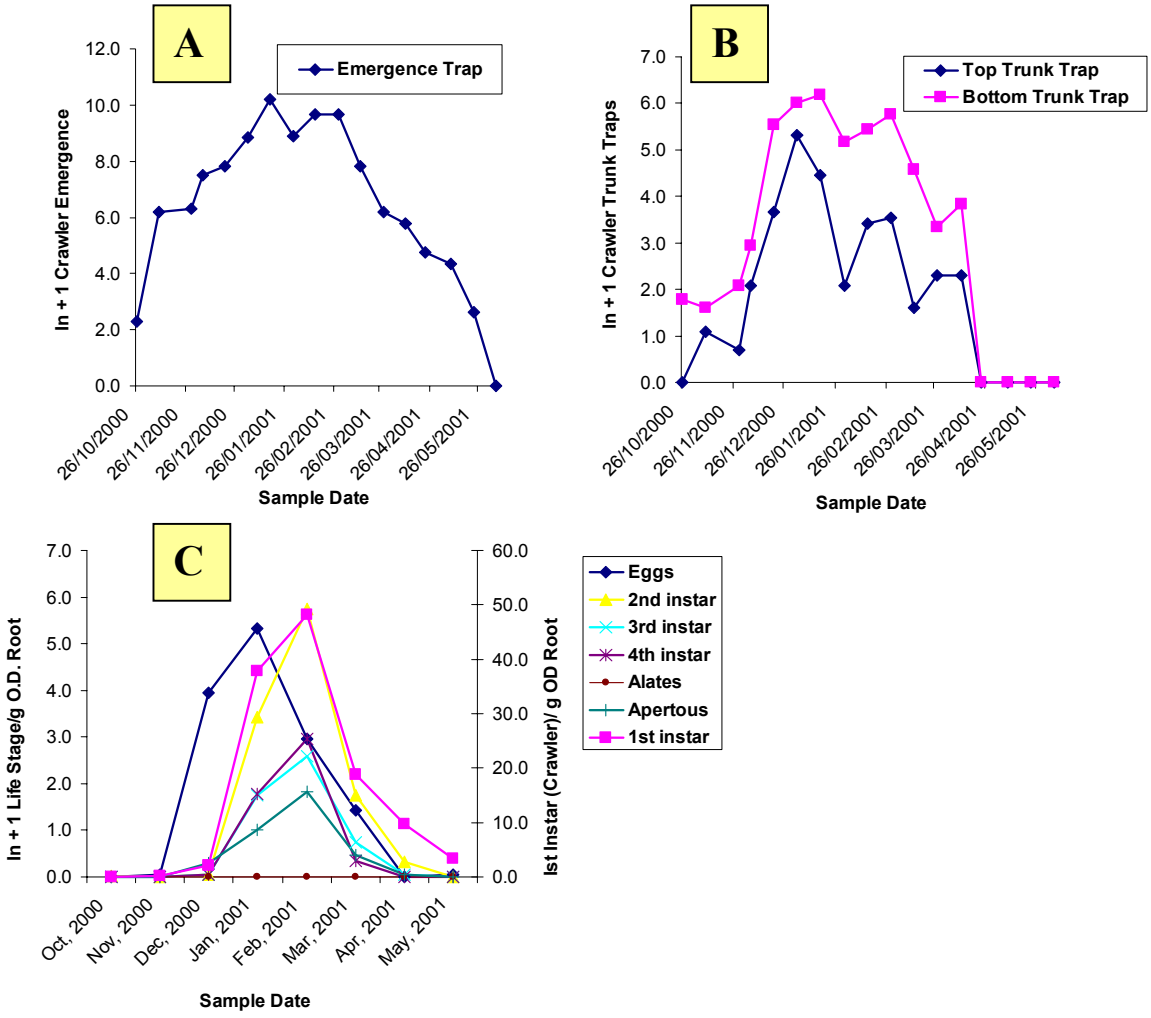


Figure 17. Summed phylloxera (genotype G4) life stage numbers recorded for emergence (A), trunk (B) and oven – dried (O.D.) root (C) sampling methods at a commercial vineyard in the King Valley, NE Victoria for the 2001 vintage.

2. Emergence trapping over three consecutive seasons

Emergence numbers in the 2001, 2002 and 2003 vintages are graphed in Figure 19. Each row contained 3 emergence traps, and for each fortnightly sampling period the insect number per row had been summed. Comparisons of emergence data from

successive vintages indicate a marked reduction in total insect numbers from season to season. The time point at which phylloxera crawler numbers peaked was similar for the 2001 and 2002 vintages, with an earlier peak evident for the 2003 vintage (Figure 19). The 2002 emergence data showed a bimodal pattern with a second peak occurring during March 2002, but this was not evident in the other years.

Univariate ANOVAs were undertaken on data summed by rows and across the entire season to examine differences among rows and among seasons. This analysis indicated that there was an overall significant difference in crawler emergence across the three seasons ($F_{(2,\ 24)}$ = 30.83, P< 0.0001). Individual vine rows were also significantly different from each other ($F_{(3,24)}$ = 3.90, P=0.021), while there was no interaction between these factors ($F_{(6,24)}$ =0.74, P= 0.62) indicating that the same rows showed similar patterns from year to year.

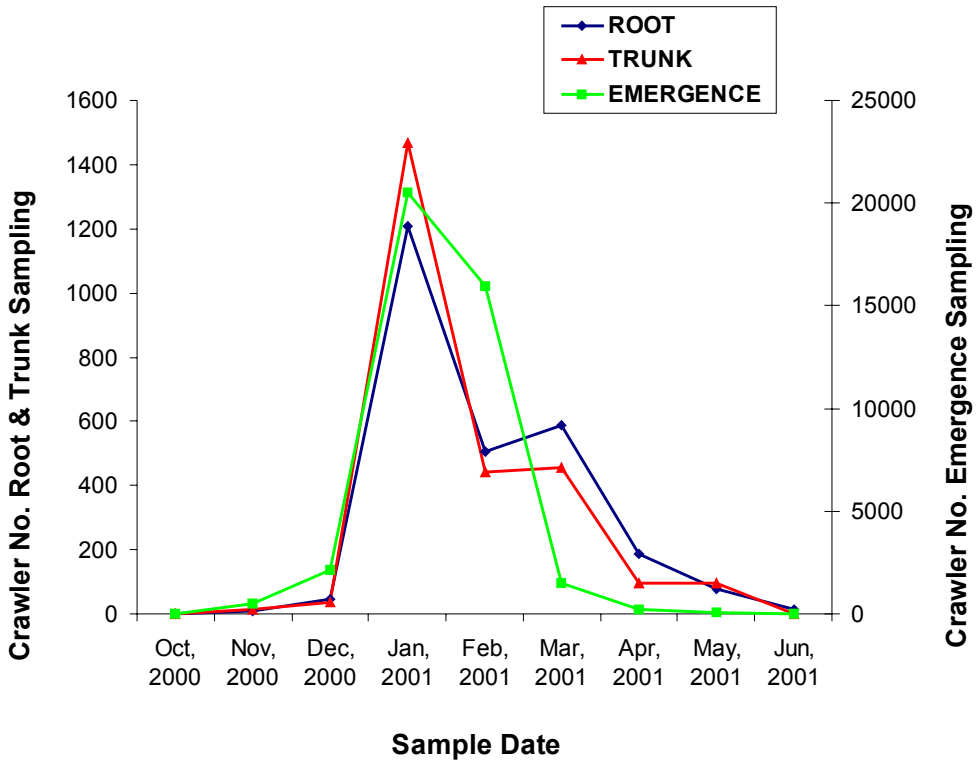


Figure 18. Summed fortnightly crawler numbers for each sampling period collected by three different trapping methods at the King Valley site for the 2001 vintage.

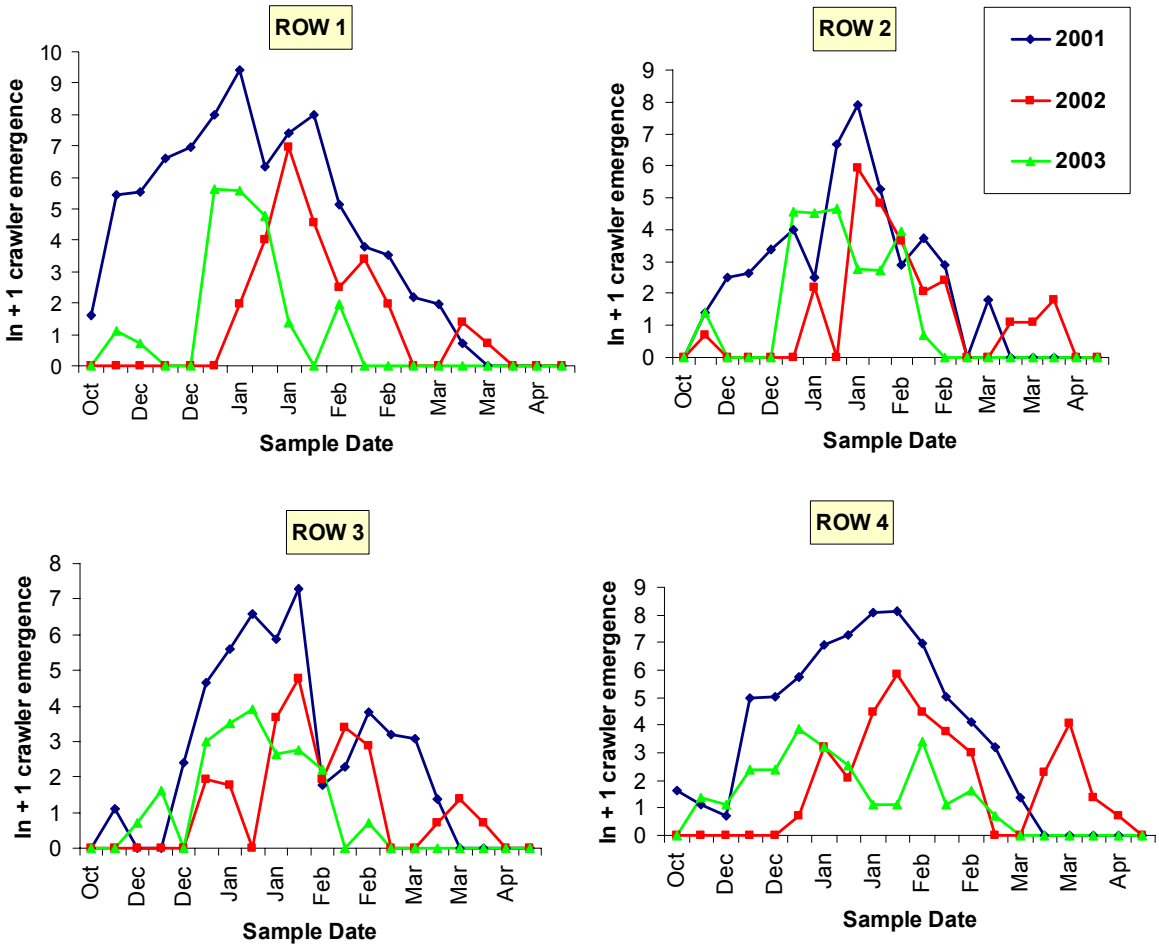


Figure 19. Summed fortnightly ln +1 vine row totals for crawler emergence of G4 phylloxera recorded at a commercial vineyard in the King Valley for three consecutive vintages.

Above-ground symptoms of phylloxera damage were clearly seen across the whole vineyard block throughout the course of this study (as shown in Figure 20). Yield levels for this entire 2.5 hectare block, dropped from 20.5 tonnes in 2001, to 9.6 tonnes in 2002, and in 2003 this variety was not harvested due to uneconomic yields. This suggests that G4 phylloxera had detrimental effects on the grape vines. In the 6 years since G4 phylloxera was detected at this site, grape yields have dropped to an uneconomic level and the grower now intends to remove these vines and replant with phylloxera-resistant rootstocks (R.Carsons, 2003, pers. comm.).



Figure 20. Visual changes associated with phylloxera damage observed on ungrafted Sauvignon Blanc vine at the commencement of the trial in 2000 (left) and at the conclusion of the trial (2003).

3. Variation in phylloxera emergence across different regions.

Crawler emergence numbers across the phylloxera-infested regions are shown in Figure 21. Peaks in crawler emergence differed in the three regions, potentially due to a number of site-related factors such as vine age, temperature, soil-type and differences as well as management practices.

The numbers of crawlers collected across the vineyard regions varied dramatically from peak levels of 14 insects at Rutherglen to over 16,000 crawlers detected on Cabernet Sauvignon vines in the King Valley. The very high numbers of crawlers collected at this site during January 2003 are evident in Figure 27.

Large proportions of winged alates were recorded from Cabernet Sauvignon vines in the King Valley, which closely matched the distribution of apterous life-stages (Figure 28). Where 2 varieties were studied for the same region (Figures 21a and 21c), the pattern of emergence was similar between grape varieties. A significant association between the Cabernet Sauvignon and Cabernet Franc varieties was recorded at Upton (x^2 = 15, df = 1, P= <0.001), and also between the Cabernet Sauvignon and Sauvignon Blanc varieties in the King Valley (x^2 = 5.4, df = 1, P = 0.02).

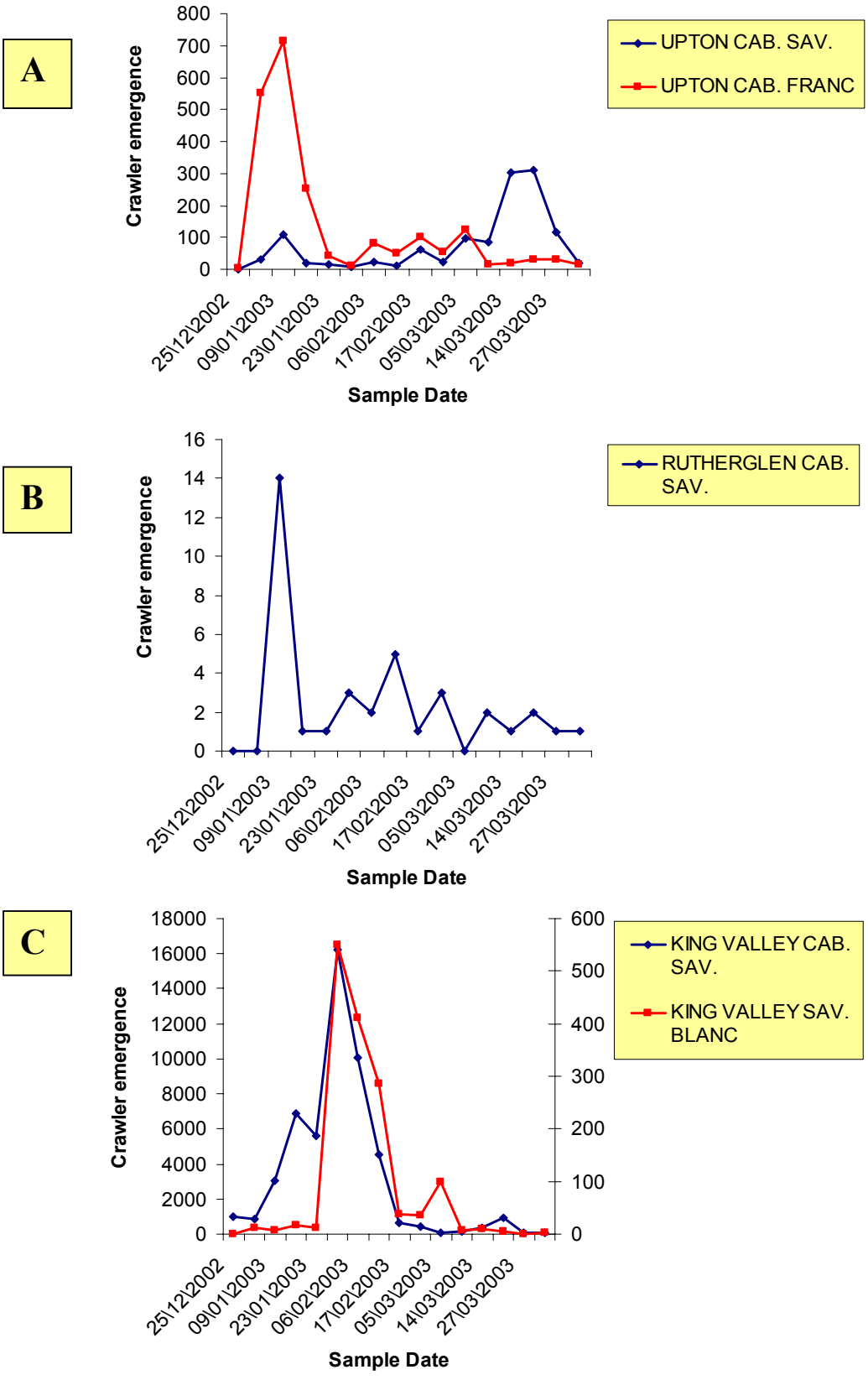


Figure 21. Crawler emergence at selected vineyards across north-east Victoria: (A) Upton region with G1 phylloxera; (B) Rutherglen with a mixed G23 and G29 phylloxera population; and (C) King Valley with G4 phylloxera.

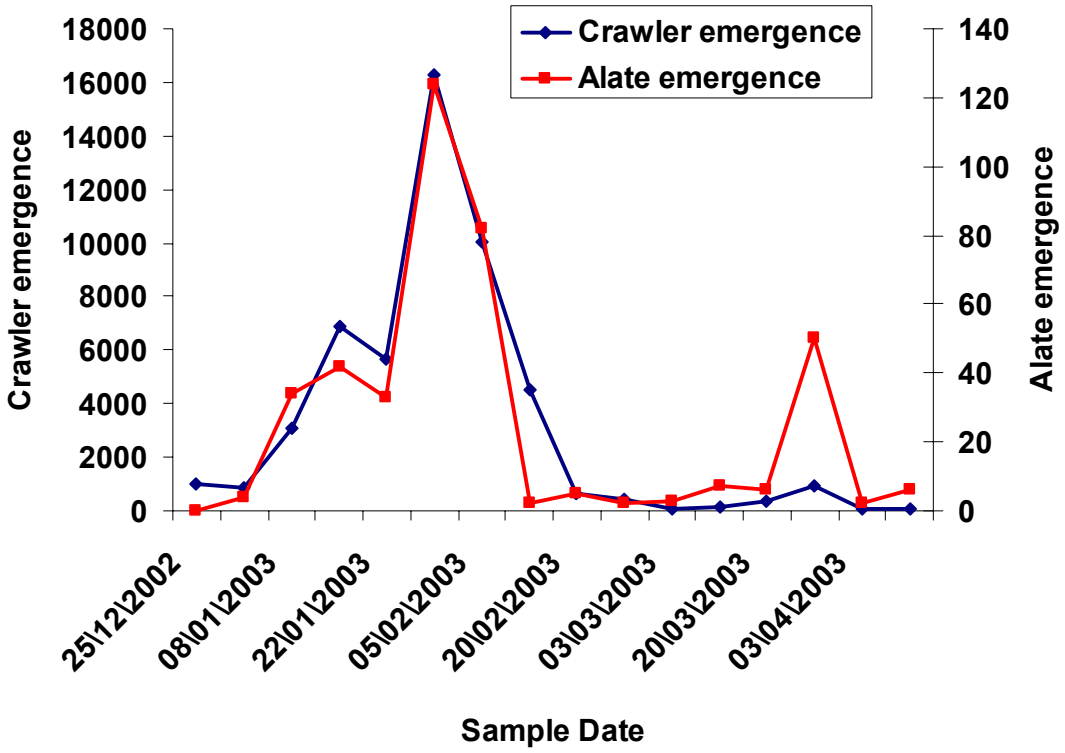


Figure 22. Comparison of G4 crawler and alate crawler numbers at the Cabernet Sauvignon site in the King Valley throughout the 2003 vintage.

Harvest data from the 2003 vintage (refer Table 8), indicates that the highest yielding Cabernet Sauvignon vineyards was located at Rutherglen, followed by the King Valley and Upton regions respectively.

The results from the degree day models (Figure 25), indicate that soil temperature data cannot used alone to consistently predict phylloxera emergence across the 3 regions. As a whole, degree-day values based on soil temperature (Figure 25) indicate that higher emergence rates were possible than were actually observed. Degree –day values offset by one sampling period closely matched emergence in the King Valley region where significant ($P < 0.05$) correlations existed both for the Cabernet Sauvignon ($P = 0.04$) and Sauvignon Blanc varieties (0.02) (refer to Table 9 for statistical summary). A significant correlation was found for the Cabernet Sauvignon variety ($P < 0.01$) but not the Sauvignon Franc variety ($P = 0.09$) located in the Upton region (Table 9). The fact that temperature data for both these varieties

was generated from a single data logger located in the Cabernet Sauvignon block may have affected this result. There was no significance in degree-day values with phylloxera emergence for Cabernet Sauvignon located in the Rutherglen region (Table 9). For this site in particular, phylloxera emergence numbers were well below the minimal threshold (Figure 25). Hence, factors other than temperature must be effecting phylloxera development and controlling the timing of the peaks in 1st instar numbers.

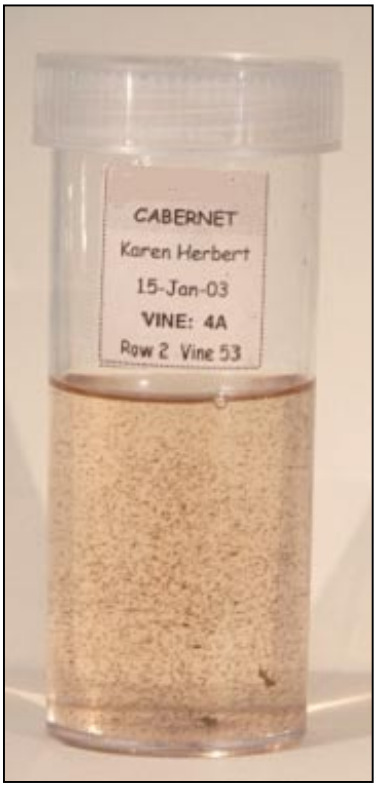


Figure 23. An example of an emergence trap sample collected from a single emergence trap in the King Valley Cabernet Sauvignon block on the 15th January, 2003. The brown flecks visible are 1st instars or crawler life-stages. There were more than 16,000 crawlers in the vial.

REGION & VARIETY	BW (g)	se	BN	se	50 BW (g)	se	BRIX	se	pH	se
Rutherglen Cab. Sav.	7483.7	505.0	126.7	6.6	31.7	1.3	26.3	0.2	3.6	0.0
King Valley Cab. Sav.	7541.7	651.9	97.8	5.6	50.4	2.3	18.5	0.6	3.4	0.0
Upton Cab. Sav.	1523.3	335.9	29.7	5.2	44.1	3.6	24.5	0.2	3.4	0.0
Upton Cab. Franc	835.0	281.7	19.2	5.4	37.9	7.2	22.3	4.5	2.9	0.6
King Valley Sauv. Blanc	548.3	122.3	36.7	6.8	44.5	3.2	23.7	0.4	3.6	0.0

Table 8. Mean harvest variables (and standard errors) from the 2003 vintage from the 3 different grape regions (BW = bunch weight, BN = bunch number, 50 BW = 50 berry weight).

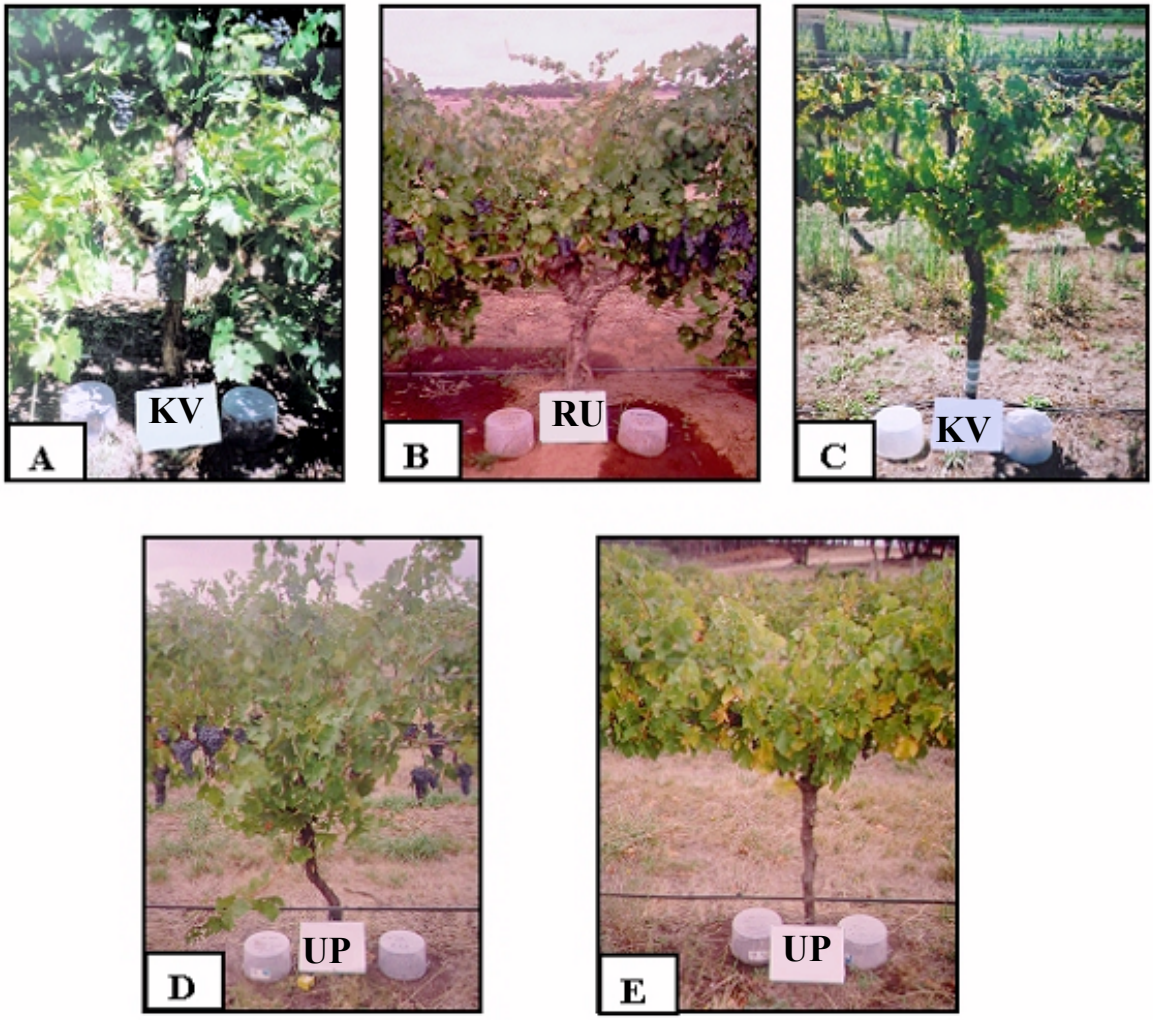


Figure 24. Highest yielding vines from the different sampling regions: A; Cabernet Sauvignon, King Valley (KV); B; Cabernet Sauvignon Rutherglen (RU); C; Sauvignon Blanc, King Valley; D; Cabernet Sauvignon Upton (UP); E; Cabernet Franc, Upton. There is no visual difference in vine vigour between the RU infested vine, which has been infested for the longest time when, compared to vines from the other two regions.

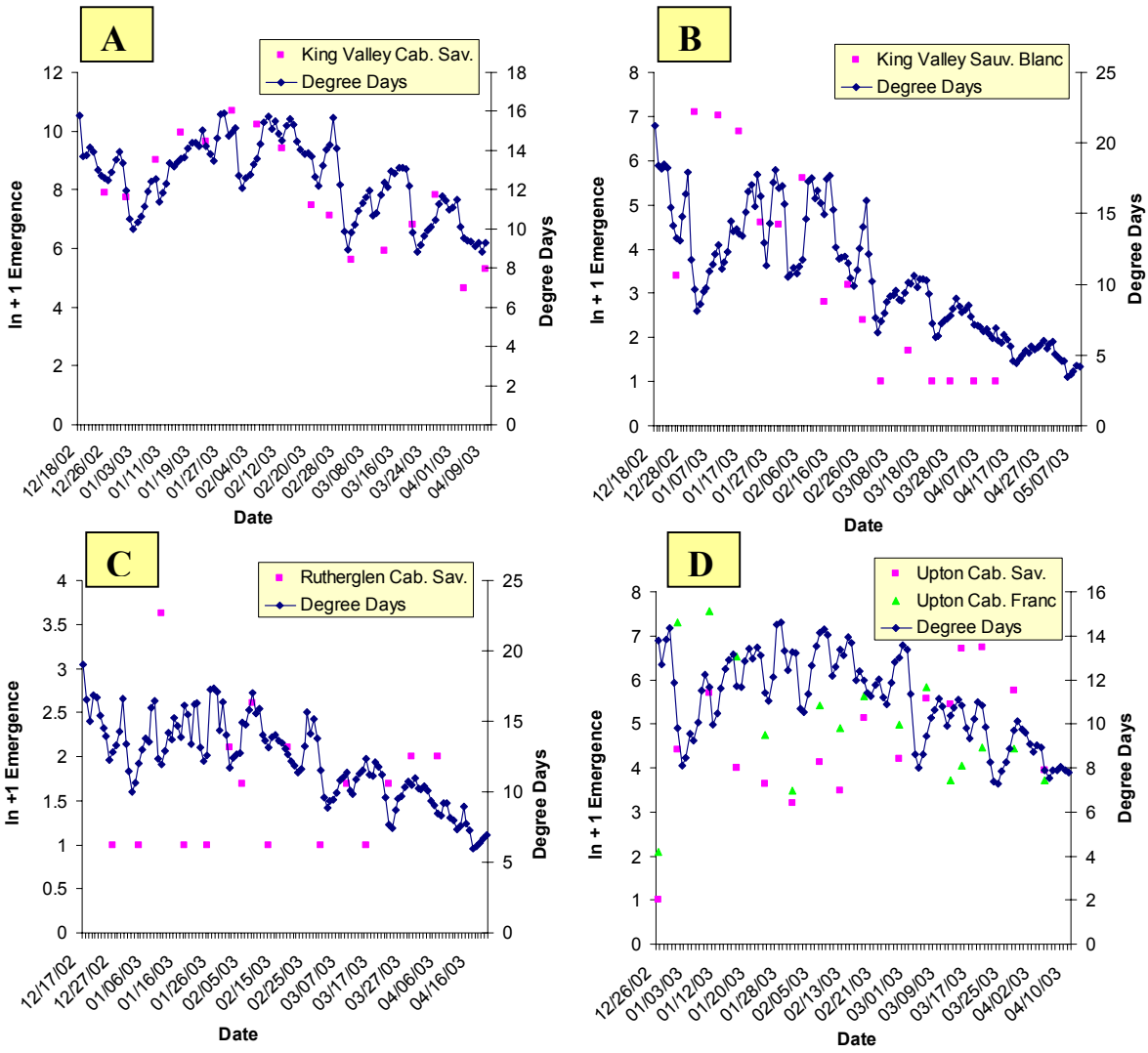


Figure 25. Predicted values of degree days (°d) as computed from Raspi et al (1987) based on soil temperature data (depth 150mm), against summed emergence totals as categorised as: King Valley, Cabernet Sauvignon (A); King Valley, Sauvignon Blanc (B); Rutherglen, Cabernet Sauvignon (C); and Upton, Cabernet Franc and Cabernet Sauvignon (D).

Region and Variey	Effect	df	MS	F	SE	P
King Valley Cab. Sav.	Cumulative degree days (CDD)/sampling interval	1	10.23	3.43	0.024	0.085
	CDD earlier by 1 sampling interval	1	13.36	5.47	0.022	0.036
King Valley Sauv. Blanc	CDD/sampling interval	1	10.38	2.14	0.024	0.165
	CDD earlier by 1 sampling interval	1	24.43	6.65	0.025	0.023
Upton Cab. Sav.	CDD/sampling interval	1	9.20	13.33	0.013	0.003
	CDD earlier by 1 sampling interval	1	3.75	3.30	0.016	0.094
Upton Cab. Franc	CDD/sampling interval	1	1.57	0.97	0.019	0.343
	CDD earlier by 1 sampling interval	1	3.54	2.52	0.018	0.139
Rutherglen Cab. Sav.	CDD/sampling interval	1	0.84	1.53	0.007	0.237
	CDD earlier by 1 sampling interval	1	0.02	0.03	0.010	0.872

Table 9. Summary of linear regression analysis conducted on predicted cumulative degree day (CDD) values with phylloxera emergence for the same sampling interval and also predicted CDD comparing with phylloxera emergence that are earlier than by one sampling interval.

Discussion

The data shows that the different trapping methods vary in their trapping efficiency, with emergence traps capturing more phylloxera than trunk traps and also being a more efficient way of sampling for phylloxera than root sampling. These findings are consistent with Powell et al (2000), who found crawler numbers in the King Valley to be ten times higher in emergence traps compared to trunk traps. Previously Buchanan (1990) had used a form of emergence trap in an ungrafted vineyard in the Nagambie region to show that first-instar crawlers, originating from populations of phylloxera on roots, were the most abundant dispersive stage of phylloxera. Crawler populations at the soil surface appear to be greater than those moving up (or down) the vine trunk. Nonetheless, all three trapping methods showed concordant seasonal changes, suggesting that they are all suitable for assessing changes in phylloxera populations.

For all of the 3 field sites studied, changes in phylloxera numbers followed a similar seasonal trend. Low phylloxera populations occurred in early spring during the vegetative vine growth stage, rising exponentially during January to peak numbers

seen in the mid-ripening stages, and lastly dropped to low levels during February/March before grape harvest. Peaks in all life-stages occurred during the vegetative and mid-ripening growth stages, rather than the post harvest stage. Similar seasonal trends have been found in Canada (Stevenson, 1964), the USA (Granett et al, 1997) and in other Australian studies (Buchanan, 1990, Powell et al, 2000, 2003). However these patterns differ from those found in field population studies in the USA by Omer et al (1997) on *V. vinifera* Chardonnay, who found the highest phylloxera numbers during the postharvest period rather than the vegetative or mid-ripening periods.

When comparing the King Valley emergence data over the three vintages, significant site and seasonal differences were evident. The most visually affected vines in Row 1 (Figure 1) had the highest recorded phylloxera numbers compared to the other 3 rows for the entire 3 years this vineyard was monitored. This finding is in contrast to field studies by Buchanan (1990), who recorded the highest number of phylloxera from emergence traps that were located at relatively vigorous vines at the edge of the phylloxera 'weak spot' that showed little visual evidence of infestation.

Phylloxera development is influenced by temperature (Granett and Timper, 1987; Belcari and Antonelli, 1989; Connelly 1995; Turley et al 1996). Laboratory experiments by Turley et al (1996) showed that the temperature must exceed 18°C for phylloxera to establish feeding sites. However, results of the degree day models based on soil temperature (Figure 12) indicate a decline in phylloxera number where the minimal threshold was being exceeded. Therefore, phylloxera development cannot be attributed to temperature alone. Omer et al (1997) have also suggested that temperature does not account for changes in phylloxera numbers.

Variation in phylloxera abundance is likely to be caused by a number of direct and indirect factors. Buchanan (1990) reported an influence of environmental factors such as temperature on the survival rates of phylloxera. The severity of phylloxera root infestations has been found by Nougaret and Lapham (1928) to be closely related to soil texture. In heavy clay soils with moderate temperatures, vine decline tends to be rapid compared to very hot or cold climates and in sandy soils (Nougaret and

Lapham, 1928; Stevenson, 1963). Omer et al (1997) suggest that a combination of extrinsic factors including temperature, quality and quantity of roots and the activity of soil inhabiting of pathogens of phylloxera are likely to account for variation in population dynamics. Survival studies of grape phylloxera populations by Granett et al (2001a) indicate that fluctuations in population may be largely due to plant physiological changes, mediated by season. Effects of plant physiology on insect demography have been found in other studies on gall-forming insects (Fay et al, 1996, Larson and Witham, 1997).

The emergence trapping technique was the easiest method to use with regards to sample collection in the field and quantification of insect number in the laboratory. Current phylloxera detection methods are based on root assessments for visual evidence of nodosities and tuberosities. Root assessments are very labour intensive and rely on the visual detection of nodosities and tuberosities, which are not always present (K.Herbert, personal observation). Emergence traps may be an alternative to root sampling due to their simple and effective application, as well as lower labour requirement. The optimal time period to sample for phylloxera, independent of the sampling method used, was typically found to occur between mid January and mid February for the 3 field sites studied; insect numbers peaked in all three sites within this 4-5 week period. Predicting the timing of peaks in phylloxera number on individual vines would be difficult, given the site and regional variation due to unknown factors.

Phylloxera populations on the root system showed a synchronised pattern of life-stage development. Even though the phylloxera life cycle is complicated with overlapping generations, peaks in egg numbers were detected during December, followed by peaks in 1st instar number in mid-January, shortly followed by peaks in the 2nd instar life-stage in late January. The numbers of alates were low, or absent at all sites, apart from the King Valley Cabernet Sauvignon vineyard, where numbers where high enough to record a similar pattern of seasonal distribution to that of the apterous life stages. Field studies in Australia in the Nagambie region also indicate alate numbers to be much lower than crawlers, however comparable field studies in New Zealand by King et al (1981), found similar trends in the ratio of these two forms. The physiological mechanism by which the alate form is triggered is not known (Granett

et al, 2001a), however it may be stimulated by crowding (Granett et al 2001a), host plant quality (Dixon, 1973) or high soil moisture (Maillet, 1957). Not surprisingly, 1st instars, were the most abundant life stage detected, as has already been well documented in Australia (Buchanan, 1990; Powell et al, 2000) and overseas (Stevenson, 1964; Granett et al 2001, Omer et al 1997 and 2002). 1st instars have been shown to be the predominant life stage in other field studies. This life-stage therefore poses the greatest threat to quarantine. Due to the very high crawler numbers detected on the canopy floor, this can cause a substantial risk of transfer from infested to uninfested vineyards via machinery, vine material or footwear and clothing of vineyard personnel.

If the G1 and G4 infested study sites are ranked according to the age of the particular infestation, there is a similar ranking in total bunch weight, and total bunch number (refer Table 8). Buchanan (1990) found a significant relationship ($P < 0.001$) between the infestation of vineyards and the ages of the grapevines. A notable exception was the apparent lack of phylloxera-related symptoms and associated yield decline recorded for Cabernet Sauvignon vines located at Rutherglen. As previously mentioned, these vines have been infested with a number of relatively unique genotypes for greater than 25 years, with no obvious impact on either vine damage (Figure 24) or harvest variables (Table 8). In contrast, the remaining grape varieties studied had G1 or G4 infestations that were 6 years or less, and all these appeared to be varying stages of vine decline proportionate to the infestation age (Figure 24). This issue was explored in the previous chapter but reiterates the importance of considering vine damage in relation to variation in aggressiveness of particular phylloxera genotypes.

In summary, it appears that a combination of environmental, physiological and genetic factors seem likely to be responsible for seasonal and regional changes in phylloxera numbers. These factors all appear to impact vine damage intensities, in some cases where the associated symptoms are pronounced, ie in the King Valley, or in other cases absent ie in the Rutherglen region. Phylloxera numbers were found to be highly variable within the same and across seasons, however overall seasonal trends in phylloxera number were consistent between two varieties in same/nearby vineyard(s). Predicting peaks in phylloxera populations for timing of chemical

insecticide application would only be possible after a thorough understanding of the parameters determining variation at particular site. Furthermore, extensive monitoring of phylloxera would also need to be conducted. Fortunately emergence traps would provide an easy empirical means of carrying out such assessments given that they can collect large numbers of phylloxera with relatively little effort and that numbers in these traps reflect activity of phylloxera on roots and moving up the trunk.

Developing an assay for testing the potential of two systemic insecticides for phylloxera suppression on grapevines.

Objective 2:

Evaluate alternative and novel methods of phylloxera control including the use of systemic insecticides (sub-project 2)

Introduction

This study was aimed at designing an *in vitro* glasshouse-based assay to assess the effectiveness of chemicals in suppressing phylloxera populations. The assay was tested with two systemic neonicotinoid insecticides previously not tested for phylloxera control in Australia: the upwardly mobile Confidor™, (active ingredient Imidacloprid) belonging to the Thianicotinyl compound subclass, and the downwardly mobile Actara™ (active ingredient Thiamethoxam), belonging to the chloronicotinyl subclass. Before commencement of chemical screening, a glasshouse trial was conducted to collect quantitative data on phylloxera populations to assess their dynamics for optimising the timing of insecticide applications. To optimise chemical concentration for use against phylloxera, an initial assessment of phylloxera mortality was carried out on phylloxera developing using an excised root bioassay system described in Granett et al 1987. The results of the preliminary glasshouse trial and excised root assay were then used to develop a glasshouse trial to assess the effect of the chemicals to suppress phylloxera populations and to investigate the impact of the chemicals on vine vigour. Findings from this study provide the basis for future field testing.

Materials and Methods

Glasshouse-based population trial

One-year old grapevine (*Vitis vinifera* cv. Sauvignon Blanc clone FVH5V10) rootlings were obtained during April 2001 from Sunraysia Nurseries (Reg. No N627), Gol Gol, NSW, 2738. Rootlings were potted into autoclaved 20cm plastic pots using

a sterilised soil-perlite composite (80% potting mix, 20% perlite) and transferred to a controlled temperature glasshouse prior to phylloxera inoculation. The primary cane was pruned to 15 nodes with secondary and subsequent canes removed so that each vine was standardised for the experiment. The trial was conducted over a 6 month (164 day) period in a temperature controlled glasshouse cycling between 24 °C ± 2 °C (600-1800 h) and 20 °C ± 2 °C (1800-600 h). This temperature range was optimal for both vine and phylloxera development during the course of the trial. Dataloggers monitored temperate at 15 minute intervals. A breakdown in the heating during days 139-140 resulted in mean temperature dropping between 6 and 10 °C. An automatic dripper watering system was established. Vines were watered until the pots just started to drain (equivalent to field capacity) once a day, with adjustments made as water use increased during the season. Growth lights were on for 12 hours each day (600 – 1800 h), with an additional spike from 000 – 100 h to offset dormancy due to changes in daylength.

A randomised design was used to examine the effects of grapevine phylloxera infestation on *Vitis vinifera*. The trial comprised of 2 treatments - infested and uninfested grapevines - randomised over a 3 X 3 grid. The grid comprised 9 upturned wire cages, with 3 vines at random per crate. To prevent contamination of uninfested vines with phylloxera, pots were enclosed in individual 45mm X 35mm polyester/polyamide drawstring bags with an 53µm aperture size (Figure 26). The root system of every vine was encased in this bag which was secured to the vine trunk using a plastic cable tie. Tanglefoot™ was applied to the neck of the bag and around the cable tie as a preventative measure to prevent phylloxera escape.

A single G4 phylloxera genotype was selected for this study as it is one of the two most commonly found phylloxera genotypes present in Australia (Corrie et al 2002). This genotype has been found to cause high level damage to *Vitis vinifera* in the field (refer to page 64). Insects were collected from a commercial vineyard in the King Valley region, from north eastern Victoria and reared *in vitro* on excised root pieces based on the method by Granett et al (1987). One hundred phylloxera eggs were placed on moistened filter paper (10cm diameter) discs. The roots of the vines were exposed and the filter paper placed in contact with the root system. The vines were

then placed back into their respective pot and bagged (note that uninfested vines were bagged prior to the phylloxera infestation of the above mentioned vines to ensure they remained phylloxera free). This point marked the beginning of the time course trial.

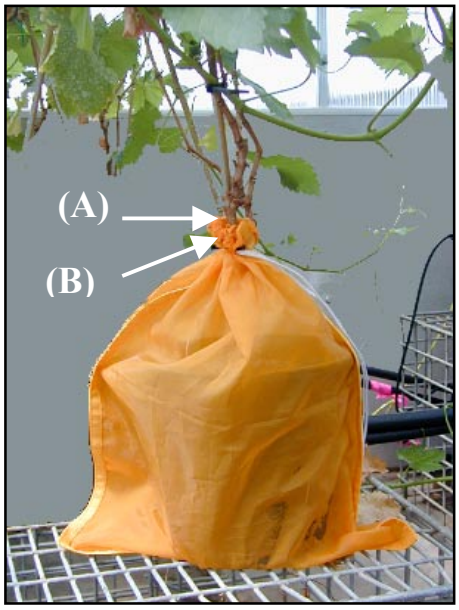


Figure 26. Drawstring polyester/polyamide bags with 53µm mesh size used to contain phylloxera populations to individual pots. (A) Site of Tanglefoot™ application at neck of bag; (B) Plastic cable tie used to secure bag to vine trunk.

Two infested and two uninfested vines were sampled at 40, 58, 79,101,122,144 and 164 days post infestation with phylloxera. At each sample date, infested vines were removed from their pots and the root system scored for the level of phylloxera damage (as per Boubals, 1966). Uninfested vines were also examined to ensure that no contamination had occurred. Root, shoot, leaf mass as well leaf as leaf area (using a Paton™ electric planimeter), were determined for each vine. Three representative sub-samples (approximately 2g wet weight) were taken from the root system of each vine and washed through a 60µm mesh brass sieve with the collected filtrate containing phylloxera washed into a screw-top plastic container containing 70% ethanol. Phylloxera life-stages, categorised as eggs, crawlers or 1st instars or post-

crawler (2^{nd}, 3^{rd}, 4^{th} instars and apterous/alate adults) were determined using a dissector microscope with a X10 objective. To adjust phylloxera numbers for the amount of root tissue sampled, root pieces were oven dried and weighed.

***In vitro* insecticide screening**

In vitro egg trials were undertaken to assess the impact of different insecticide concentrations on phylloxera egg viability and subsequent crawler emergence. Egg viability (or crawler emergence) was used rather than direct assessment of later life stage development due to the fact that phylloxera is extremely sensitive to changes in environment and is known to suffer high mortality rates when handled (K.Herbert, personal observation). It is also difficult to follow the fate of known numbers of insects on whole plants, or to test survival of insects in excised root bioassays because in some instances these can fail to adequately support phylloxera populations as well as healthy root tissue.

Based on unpublished overseas laboratory results (K. Powell, pers comm.), rates tested for both chemicals were 250ppm, 500ppm, 750ppm and 2000ppm active ingredient, as well as a water control. Five hundred G4 phylloxera eggs (1-3 days old) were harvested from excised root bioassays and placed on moistened filter paper discs separated in-groups of 20. Each replicate of 20 eggs were transferred to a Millipore filtration apparatus (glass microanalysis system with stainless steel support), fitted with a 60µm nylon filter and rinsed briefly with distilled water from a wash bottle. Insecticide (or water control) solutions were, for each replicate, prepared independently. Insecticide (or water) was drawn through the filter using a hand vacuum pump (Millipore), immersing the eggs in solution. Eggs were immersed for a period of 3 minutes and the solution was then drained under vacuum. Treated eggs again were rinsed briefly in distilled water and transferred from the nylon filter to new moistened filter paper disc, placing eggs evenly spaced along the midline. Papers discs were placed inside 20cm petri dish containers and sealed with parafilm™ and monitored twice daily over a 7-day period for egg viability and crawler emergence. Crawler mortality was assessed visually using colour and observations of crawler mobility. Mobile crawlers or crawlers observed greater than 2cm from the midline were scored as viable. Crawlers observed less than 2cm from the midline, including

those crawlers not fully emerged from its egg case, or eggs that appeared black in colour (having not hatched) were scored as non-viable.

Glasshouse-based insecticide screening

Sixty grapevine (*Vitis vinifera* cv. Sauvignon Blanc) rootlings were sourced and established and infested with G4 phylloxera as above. Results from the *in vitro* egg emergence trials determined that a concentration of 2000ppm would be the optimal concentration for both insecticides. There were 4 treatments in total, with 3 replicates per treatment per sampling period: (1) one and (2) two Confidor™ applications at a concentration of 2000ppm active ingredient, (3) two applications of Actara™ and (4) no insecticide application (control). The insecticide application times, as determined from the previous population dynamics were at 80 (first application) and 100 (second application) days post infestation with phylloxera. Insecticides were applied at 500ml per vine using a small watering can. Controls were watered with 500ml distilled water. The destructive sampling dates were kept similar (to within 1-2 days) as those dates nominated for the population study. There was one initial vine sampling period after infestation at 79 days (post infestation with phylloxera) followed by the first application of insecticides at 80 days, destructive vine sampling at 98 days, the second application of insecticides at 100 days, followed by vine sampling at 122 and 143 and 164 days post infestation respectively. Note that the duration of the insecticide trial was 42 days less than the duration of the population trial (164 days verses 204 days). Destructive vine sampling and analysis was carried out to collect total vine assessments of leaf square area (cm^2) and O.D. leaf, stem and root weights (g).

Analysis

Life stage and vine growth assessments conducted as part of the glasshouse population trial were compared among treatments using one-way ANOVAs carried out with SPSS Version 11.5. Similarly, one-way ANOVAs were also used to assess crawler mortality and egg hatch values at the different insecticide concentration levels tested for the *in vitro* screening method.

Results

Glasshouse-based population trial

A summary of mean life stage number, taken from sub-samples of individual vine roots is shown in Figure 27. A general increase in numbers of eggs and crawlers is evident in the first 7 sampling periods, apart from the 143-day sampling date. A breakdown in the heating system two days prior to this sample date probably accounted for the low numbers at this point in time. Numbers peaked for egg and crawler life stages at day 164, and then decreased for the 2 remaining sample dates. One way ANOVAs indicated significant ($P<0.05$) effects of infestation time on both egg number and crawler number (F 8.11(8)=7855.79, P=0.002, F 8.12(8)=17801.44, P=0.002 respectively) but not for the combined post-crawler life stage numbers (F 2.92(8)=39.71, P= 0.07).

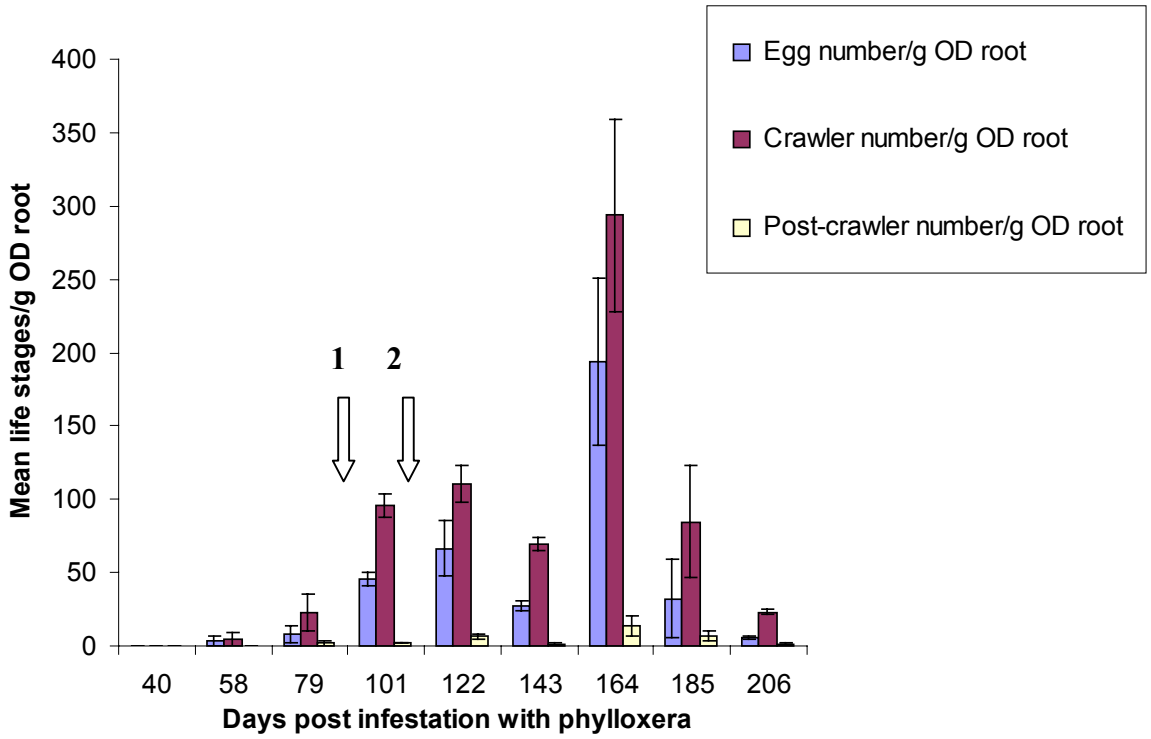


Figure 27. Mean numbers of phylloxera life-stages per oven dried (OD) gram of root sampled. Post-crawler life stages refer to 2nd- 4th instars and adults that were pooled. Arrows indicate optimum timing of insecticides at around day 80 and 100.

There were significant ($P < 0.05$) effects of phylloxera infestation on all measures of vine morphology. For leaf area there was a significant overall effect of time and phylloxera infestation and a marginally significant interaction between these two factors (Table 10). For whole root weight and stem weight, significant time and treatment effects were also recorded, but no significant interaction between these two factors (Table 10). Phylloxera infestation significantly decreased leaf area, root and stem weight (Figure 28). The effects of phylloxera infestation were evident on leaf area and root weight assessments before being evident for stem width; differences between the two treatments were evident for leaf area and root weight assessments at day 79, compared to day 101, when similar differences were seen for mean stem width.

These results were used to determine optimum application timings for tested pesticides. It was evident that any treatment would need to be applied before 164 days, prior to the rapid increase in phylloxera numbers. Therefore, insecticide applications times (as indicated with arrows in Figure 27) were set at day 80 and day 100 (in the case of two applications) after phylloxera infestation.

***In vitro* insecticide screening**

The pesticides did not significantly influence egg mortality (ANOVA, $F\ 1.24(4) = 1452.04$, $P = 0.29$). In contrast, there was a significant ($F\ 11.76(4) = 943.75$, $P = <0.001$) effect on crawler mortality. For both chemicals, treatments increased crawler mortality particularly at higher concentrations of the chemical treatments (Figure 29). At 0 ppm, crawler mortalities of 16% (Actara™) and 13% (Confidor™) were observed, indicating that some crawlers died even when water was applied. It is possible that the combined egg transfer and treatment procedure in the filtration apparatus may have affected egg viability, however phylloxera is known to exhibit high crawler mortalities (Granett et al 1997). Because both chemicals produced relatively higher mortalities at the highest concentration, 2000 ppm was selected for the glasshouse *in planta* trial. This concentration was within the manufacturers recommended concentrations; higher levels were not tested to minimise toxicity and residual levels.

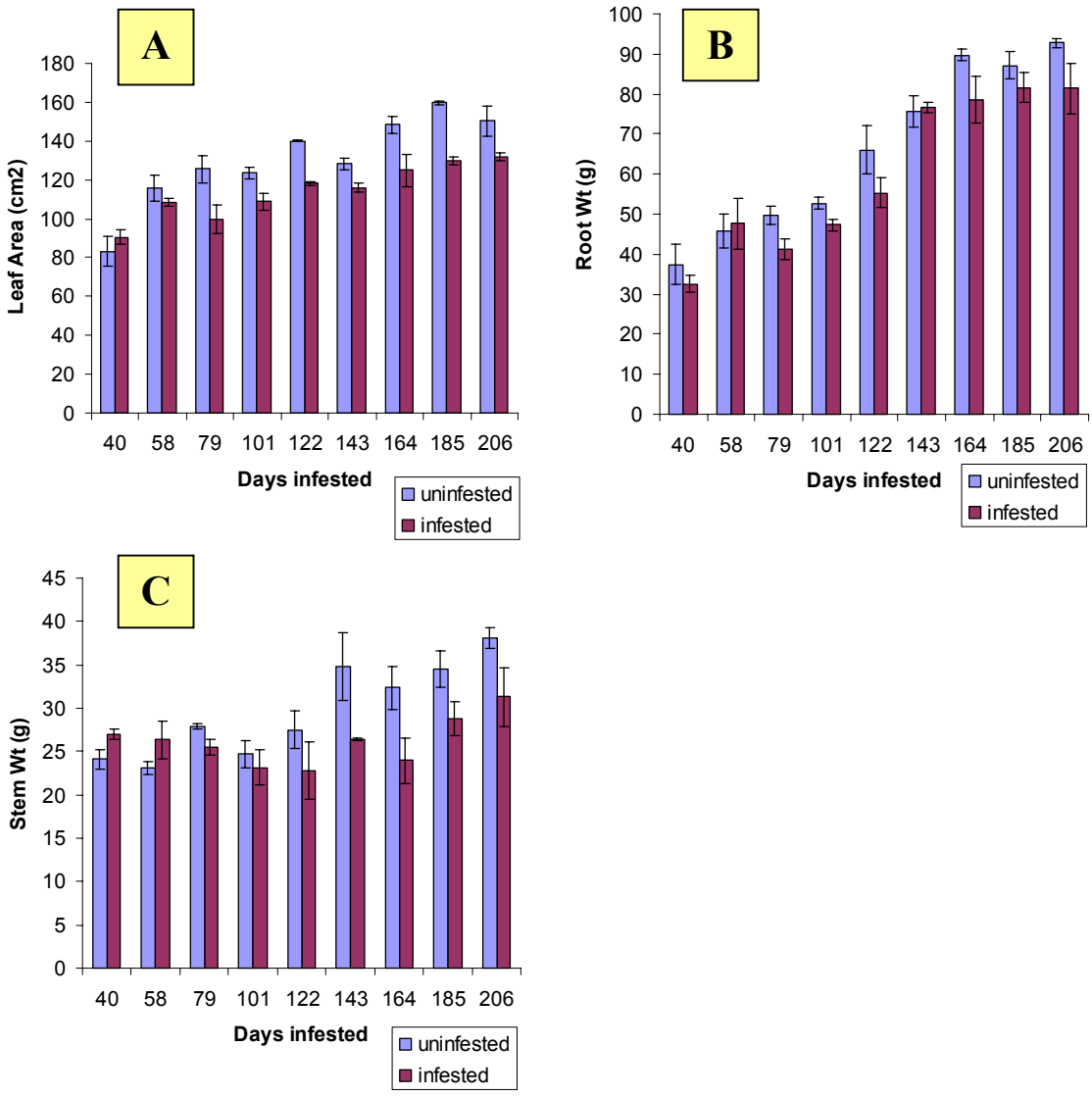


Figure 28. Differences in (A) mean leaf area (cm^2); (B) mean root weight; and (C) mean stem weight (g) of phylloxera-infested grapevines recorded at nine sampling periods.

Vine Measurement	Effect	df	MS	F	P
Mean Leaf Area (cm^2)	Time	8	1302.065	26.970	<0.001
	Treatment	1	2422.772	50.184	<0.001
	Treatment by time	8	127.048	2.632	0.042
	Error	18	48.278		
Mean Stem Wt (g)	Time	8	54.096	6.164	<0.001
	Treatment	1	112.148	12.779	0.002
	Treatment by time	8	19.516	2.224	0.076
	Error	18	8.776		
Mean Root Wt (g)	Time	8	1781.489	49.210	<0.001
	Treatment	1	691.865	19.112	<0.001
	Treatment by time	8	32.560	0.899	0.537
	Error	18	36.201		

Table 10. Results of ANOVAs on vine morphology variables in the pilot experiment.

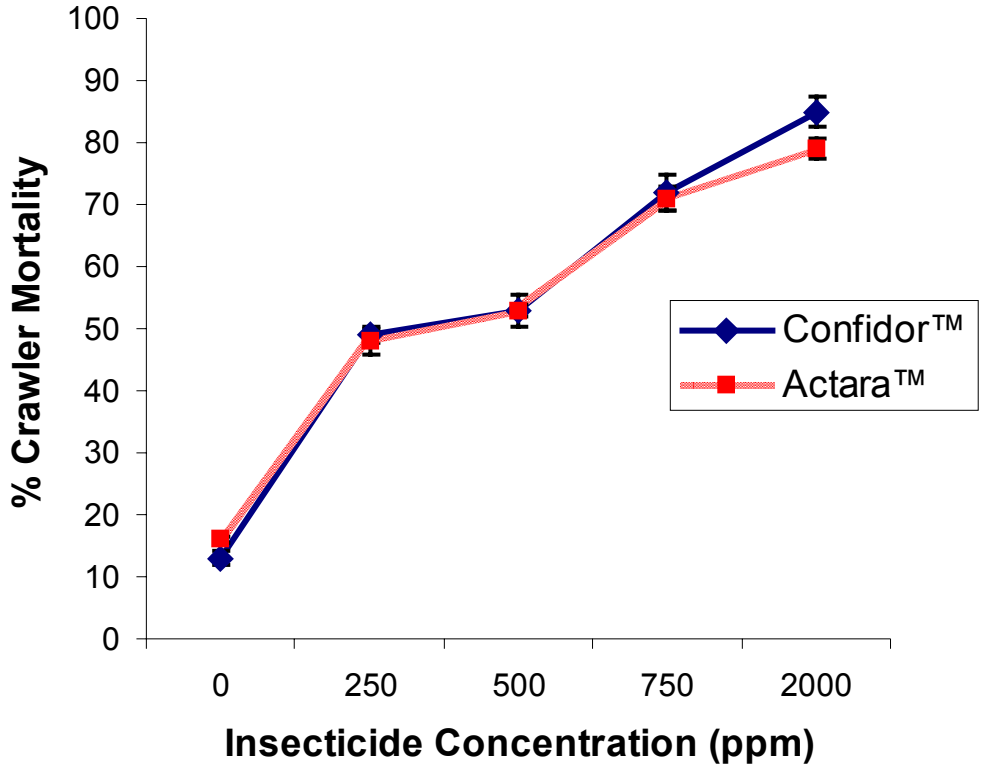


Figure 29. Mean percentage crawler mortalities following Actara™ and Confidor™ application after a 7-day period. For the control treatment (0 ppm), eggs were treated with distilled water.

Glasshouse-based insecticide screening

The mean numbers of each life stage shown in Figure 30 were significantly lower than in the initial experiment to determine the timing of pesticides (Figure 27). The reason for this difference is not clear, but might reflect minor changes in root development as a consequence of different vine planting material sourced from the nursery at different years. Nevertheless, the efficacy of the chemical treatments was clear-cut. There was no significant ($P < 0.05$) effects of the insecticide treatments on the egg life stage (Table 11). However, all insecticide treatments influenced the number of crawler and post-crawler stages ($P < 0.001$), with interaction between these factors (Table 11).

Impacts of the insecticide treatments on the distribution of different life stages on grapevine roots are compared in Figure 30. Reductions in the numbers of crawlers and post-crawler life stages were seen in the Confidor™ treatments verses the control. With no pesticide application, the proportions of egg and crawler life stages are fairly uniform across the sampling periods. This ratio changes with pesticide application; for the Confidor™ treatment, the egg proportion almost doubled with crawler number reduced more than 50% reduced from 122 days.

High levels of variation were observed in vine morphology across the treatments (Figure 32). There were overall significant ($P < 0.05$) time and treatment effects of insecticides on leaf area and root mass but no interaction between these factors (Table 12). There was no overall significant effect of both time and treatment on stem weight. Visual differences of the effects of insecticide on root mass (Figure 33) show clear differences in the root volumes with and without insecticide application. The amounts of fibrous root growth for the Confidor™ treatment are much more abundant than compared to vine roots that received no insecticide application.

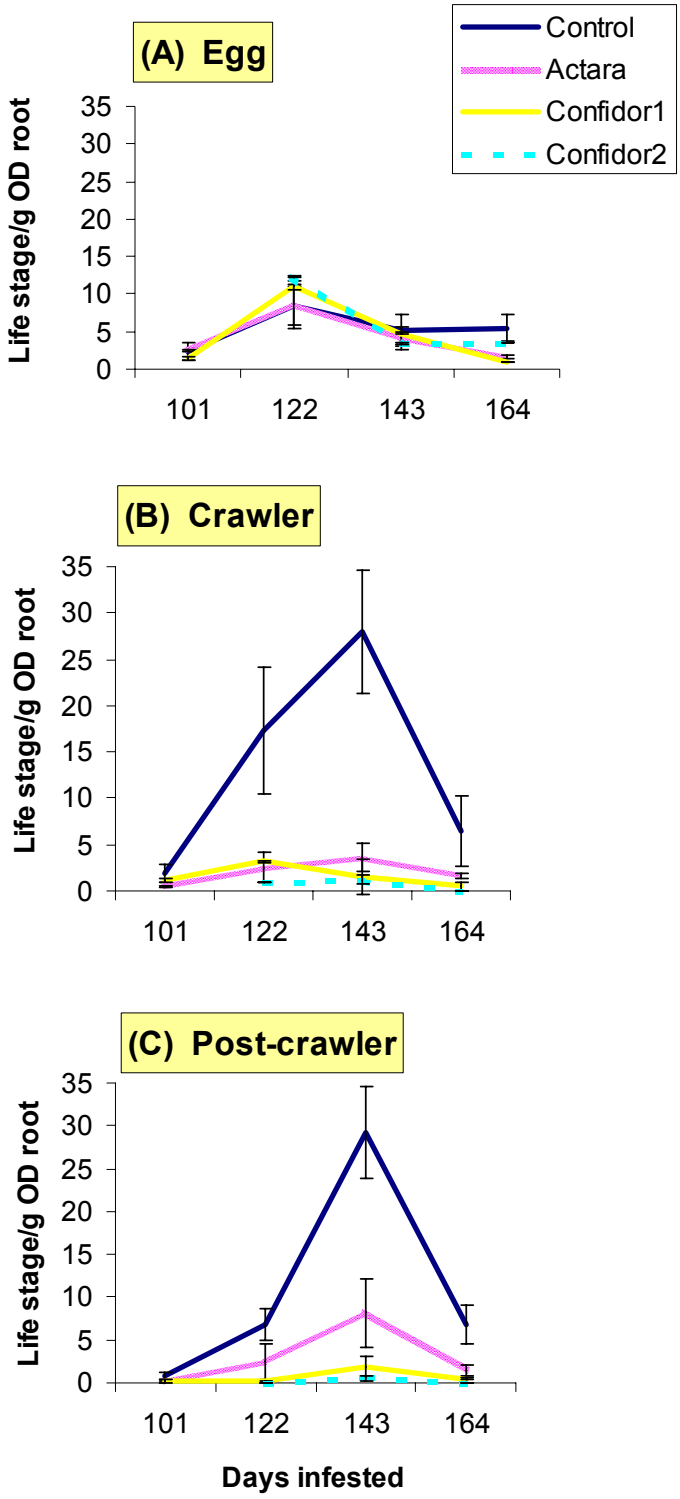


Figure 30. Mean counts of different life stages per gram OD root for Actara™ and Confidor™ glasshouse *in planta* trial (Control= water treatment, Actara™= 2000 ppm application, Confidor™ 1 = single application at 2000 ppm, Confidor™ 2= double application at 2000 ppm). (a) eggs, (b) crawler, (c) post-crawler (=2nd, 3rd, 4th instars and adults).

Insect Life Stage	Effect	df	MS	F	P
Egg	Time	3.00	263.80	9.94	<0.001
	Treatment	3.00	65.39	2.46	0.082
	Treatment by time	8.00	13.19	0.50	0.849
	Error	30.00	26.54		
Crawler	Time	3	164.88	7.07	<0.001
	Treatment	3	450.20	19.30	0.000
	Treatment by time	8	95.35	4.09	0.002
	Error	30	23.33		
Post-Crawler	Time	3	239.37	20.15	<0.001
	Treatment	3	314.38	26.46	<0.001
	Treatment by time	8	101.99	8.58	<0.001
	Error	30	11.88		

Table 11. Results of one-way ANOVAs comparing the effects of pesticide treatment and time since phylloxera establishment on numbers of life stages per gram of OD root.

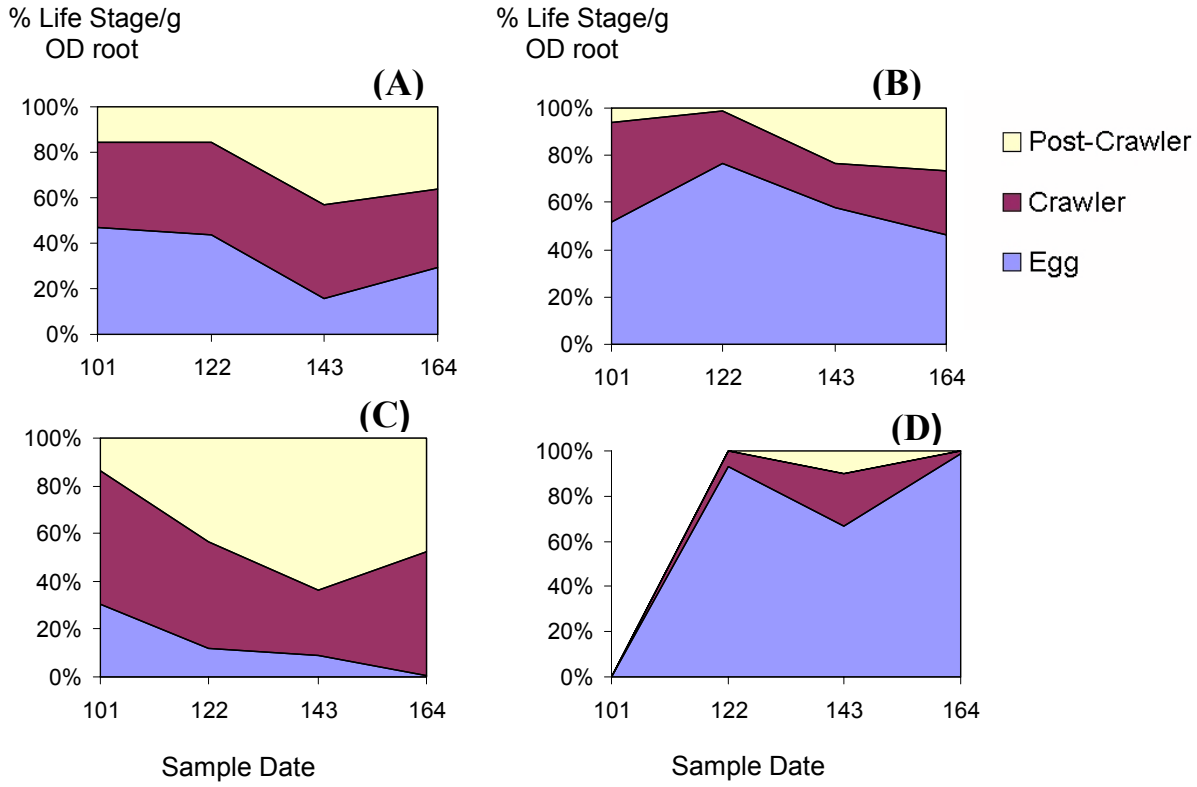


Figure 31. Stacked area graphs of the various life stages/g OD root for following insecticide treatments: Control (A), Confidor™, single application (B), Actara™ (C) and Confidor™, double application (D). Note that phylloxera were not recovered from (D) until 122 days.

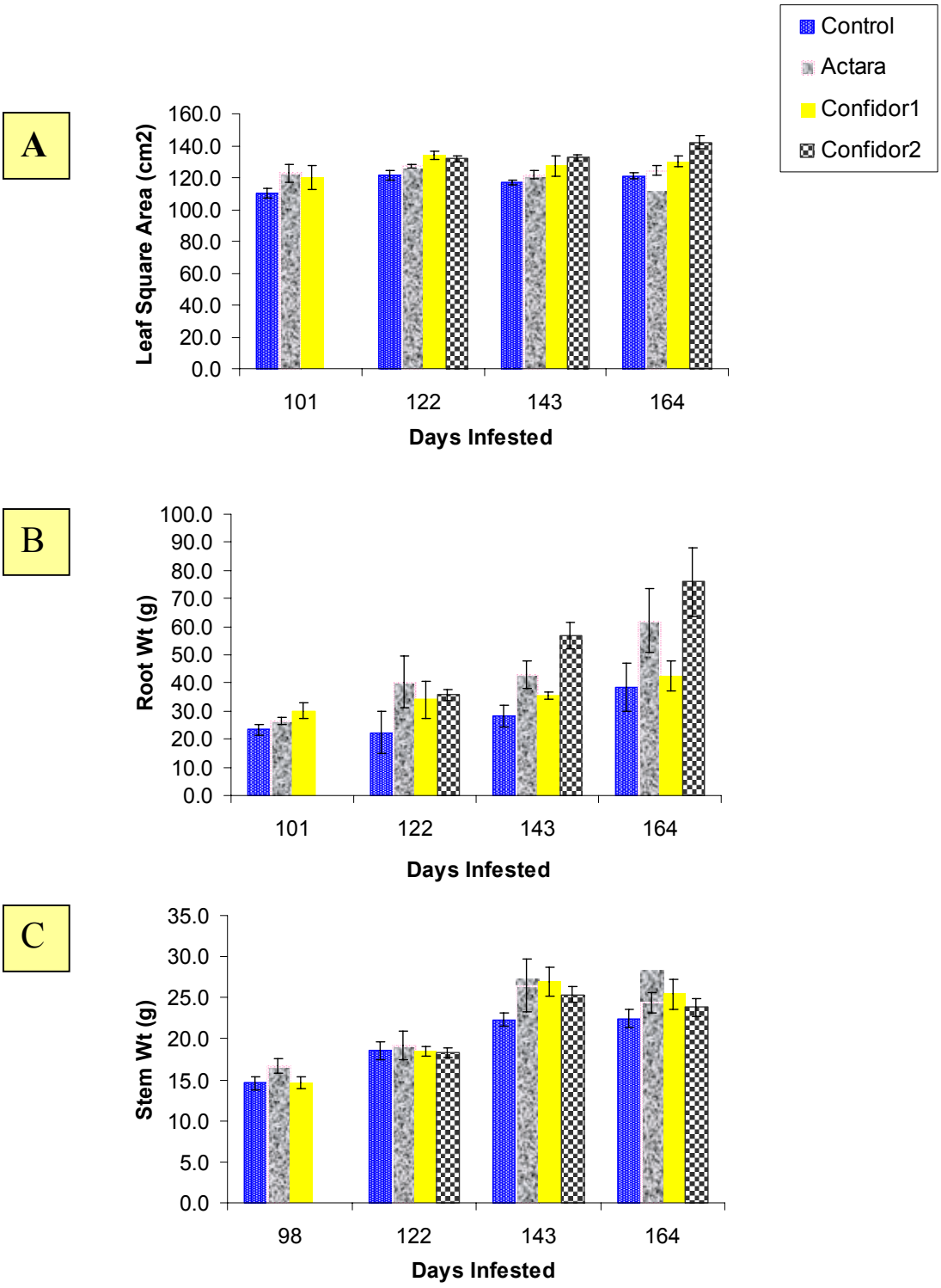


Figure 32. Vine morphology comparisons of (A) mean leaf square area (mm^2), (B) mean root wt (g) and (C) mean stem wt (g), for the insecticide treatments. Control = no insecticide application, Actara™ = 2000ppm double application, Confidor™ 1 = 2000ppm single application, Confidor™ 2 = 2000ppm double application.



Figure 33. Comparisons of the infested root systems with (A), no insecticide application and (B), two applications of Confidor™ at 2000ppm.

Vine Measurement	Effect	df	MS	F	P
Mean Leaf Area (cm^2)	Time	4	274.05	5.52	<0.001
	Treatment	3	472.55	9.51	<0.001
	Treatment by time	8	36.33	0.73	0.66
	Error	32	49.68		
Mean Stem Wt (g)	Time	4	184.41	29.92	<0.001
	Treatment	3	15.81	2.57	0.07
	Treatment by time	8	4.91	0.80	0.61
	Error	32	6.16		
Mean Root Wt (g)	Time	3	1141.62	6.05	<0.001
	Treatment	3	1272.32	6.74	<0.001
	Treatment by time	8	155.98	0.83	0.59
	Error	32	49.68		

Table 12. Results of ANOVAS on vine morphology variables for the insecticide pot trial.

Discussion

This study represents one of the few attempts to investigate chemical effects on phylloxera under controlled conditions. The egg hatching bioassay was similar to one outlined by Granett et al (1997), however in their assay; crawler mortality and life-stage counts were assessed via an excised root bioassay method. Results from the egg bioassay and glasshouse trials indicate that the chemicals studied do not appear to affect eggs. It has been well documented that eggs are often most resistant life stage to insecticides (Sutter et al, 1990; Gubran et al, 1992; Devine et al, 1999). In the current study, hatched crawlers where killed after contact with the egg case. For effective phylloxera population control, it is imperative that the insecticide dramatically reduces at least one or more life-stages directly or indirectly as the above example illustrates.

Using a pot-based glasshouse trial was an effective means of evaluating both insecticide efficacy as well as assessing vine health. Similar published assays of this nature that have been used successfully for other pest problems (Barratt et al 1995, Ninkovic et al 2001, Lígia et al 2004). The best physiological monitors of phylloxera infestation in this study were changes in leaf area between infested and non-infested treatments and also between insecticide-treated vines. This is suggested to be related to leaf changes, such as premature yellowing, which are usually the first signs above-ground symptoms of phylloxera infestation (Granett et al 2001). Significant changes ($P < 0.05$) in leaf area are seen 79 days after commencement of the potted trial and may represent the best indicator of vine health. Omer et al (2002) found a relationship between the plant growth stage on the overall performance and proportion of phylloxera life stages. They report higher levels of crawlers and eggs in the vegetative and mid-ripening vine stages (approx 60 days post-budburst) than compared to post-harvest stages (greater than 125 days). It is difficult to compare the findings of Omer et al (2002) with those found in the present study because, for the duration of the glasshouse trial, vines remained in a vegetative growth stage. Future pot trials over a longer time course including harvest periods and with a greater range of phylloxera infestation could be undertaken to further test the utility of this measure.

Furthermore, once fully optimised in the glasshouse, field studies are required to access the ability of the given insecticides to penetrate different soil types to determine how effectively they can access root systems several metres deep in the soil.

Phylloxera has a high potential for rapid population growth from comparatively low population reservoirs. Generation time may be less than a month, giving vineyards three to ten generations per year (Granett et al 2001). Hence a high level of mortality is necessary to negate the high reproductive potential of phylloxera. Two applications of Confidor™ had the highest mortality: 85% of total crawlers in the bioassay trial were killed, and there was also a significant reduction in crawler and post-crawler life-stages for the pot trial. Since 100% effectiveness was not achieved, these results suggest that multiple applications of insecticide would be required in order to achieve continued suppression to avoid phylloxera resurgence from resistant egg life stages and other life-stages not killed by the initial insecticide application.

A 20% reduction in crawler number was observed following two applications of Confidor™, and this has important implications for effective quarantine of recently infested vineyards. First instar phylloxera, or crawlers represent the most active and dispersive stage of the insect and have been found in relatively high numbers both below and above-ground during the vine-growing season (Powell et al. 2000). A reduction in the number of this life stage would reduce the substantial risk of transfer from infested to uninfested vines, hence strengthening quarantine measures within an existing vineyard and also between neighbouring vineyards.

Systemic insecticides containing Imidacloprid have been used successfully to manage whiteflies on many crops. Bethke and Redka (1997) showed that applications of Imidacloprid at rates of 20, 40 and 90mg active ingredient (AI) per litre pot volume to poinsettias caused over 94% adult mortality of the silverleaf whitefly, *Bemesia argentifolii* after 48 hours exposure to the plants. These authors found that adults exposed to poinsettias that had been treated 150 days earlier at the same rates had 79% greater mortality than the adults exposed to untreated plants (Bethke and Redak, 1997). Results by Bi et al (2002) on the efficacy of six novel insecticides against the

greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood), on strawberries, indicated that Imidacloprid applied at rates of 10,20 and 40mg AI per pot cause adult mortalities ranging from 82% to 96%. Applications of Imidacloprid under field conditions were also found to significantly suppress adult populations and have no adverse effects on beneficial organisms (Bi et al, 2002). The same authors also found that applications of chemicals containing the AI Thiamethaxam (the same AI of Actara™) at 0.5, 1.0 times its label concentration, caused 67% and 90% nymph mortality (respectively) with no apparent effect on densities of 3rd or 4th instars of greenhouse whiteflies on strawberries (Bi et al, 2002).

The residual activity of Imidacloprid (the AI of Confidor™) on other economically important crops has been reported to control both thrips and aphids on tobacco for at least 14 days (Crow et al 1996), and on corn crops against the corn leaf aphid for up to 28 days (Harvey et al 1996, Goumet et al, 1996). In California, where Confidor™ is registered for phylloxera suppression, two applications of between 16 to 32 fl oz/A, between budbreak and the pea-berry stage are recommended to reduce phylloxera populations and allow for an increase in root growth. Actara™ is reported to be more mobile in soil than Confidor™, and has a residual activity of between 4-21 days for aphid control on lettuce (ag.arizona.edu/crops/vegetables/insects/general/ reviewinsect.htm), and 7-10 days residual for aphid pests of cotton (Novartis™, release notes, 1999) but is currently not currently registered in any country for phylloxera suppression. In the U.S., its recommended rate for grape and variegated leafhopper control is between 12.5-16.5 fl. oz. A (Novartis™, release notes, 1999).

In summary, these results suggest that Confidor™ and Actara™ cause a reduction of crawlers and egg life stages that leads to some significant improvements in vine vigour. Field trials of Confidor™ need to be undertaken to assess the overall economic benefit of using this insecticide as cost-effective interim management strategy in a phylloxera-infested vineyard. To achieve success, a continued suppression of the phylloxera population would need to occur, leading to a long-term effect on vine viability and maintenance of productivity of the vines.

Outcomes and Conclusions

Implications for early detection:

Two approaches for the early detection of grapevine phylloxera were developed and assessed within the course of this project. The three-fold aim of the detection systems were to (i) produce a new detection system that can detect presence of phylloxera more rapidly than current methods used (ii) improve the specificity of the new detection systems and (iii) develop simpler less labour intensive routine screening approaches. In this project one detection approach examined used a leaf sampling protocol and the second used a soil sampling protocol.

The first approach to be evaluated was the potential identification of pathogenesis-related (PR) proteins induced as a stress response to phylloxera infestation. Four PR proteins were screened from the leaves of phylloxera infested grapevines under controlled glasshouse conditions. Based on our results, two PR proteins, VvTL1 and VvTL2, were not specifically up-regulated by phylloxera infestation and are deemed unsuitable as leaf-expressed markers in an early detection system. The PR proteins, VvGlub and VvChi3, showed promise as more specific leaf markers particularly after 150 days of phylloxera infestation. However the results were inconsistent.

In order to determine if the PR approach might be successful in the future as an improved phylloxera detection system several questions would need to be addressed. Firstly are the PR proteins phylloxera-specific or can the same proteins be induced by other abiotic or biotic stresses. Secondly a wider pool of PR proteins would need to be screened for phylloxera-specificity before this detection approach could be discounted. Thirdly if a phylloxera-specific PR protein were to be found it would need to be tested against a range of vine types infested with a range of phylloxera genotypes to ensure its robustness.

The second approach researched was towards the development of a DNA probe with the potential to detect phylloxera DNA in soil samples from phylloxera infested vineyards. Phylloxera-specific primers were developed and evaluated against a range

of organisms including (i) phylloxera (ii) other aphid genera and (iii) commonly found vineyard organisms. When tested against a range of phylloxera genotypes the primers were able to 'detect' or positively amplify product for all ten genotypes screened. When insects (bees, wasps, fruit fly, springtails, thrips, and caterpillars), mites and nematodes commonly found in vineyards were screened the primers did not positively amplify product. However, when aphids were screened, the primers positively amplified DNA product three and four aphid genera using elongation factor and 28S D3 rDNA primers respectively. Before industry could use a DNA probe to detect phylloxera the primer specificity needs further refinement to ensure that aphid DNA does not produce 'false positive' results. Overall the phylloxera-specific probe approach by its very nature, because it relies on expression of the target organisms DNA, is more reliable than the PR approach as a potential detection system. Because it targets the insects DNA, sampling protocols could be modified depending to examine not only detection in soil samples but also in trap samples.

In addition the influence of phylloxera genotype on the rate of vine decline and population dynamics was also examined because any detection system needs to take into account these factors. This approach showed that genotype can influence the rate of vine decline and therefore the potential for detection using standard visual assessment protocols. It was also shown that population dynamics is influenced by phylloxera genotype and the implications of this for industry are that any phylloxera specific detection system would need to be extremely sensitive to ensure that less 'virulent' genotypes can be detected.

Implications for interim management:

There are several approaches that have potential for interim management of phylloxera. However, very few studies have been conducted under controlled conditions to evaluate the options systematically. Within this project one approach chemical control was assessed with a view to using this option in combination with an improved early detection system. The longer it takes to detect phylloxera the more difficult it is to control or suppress phylloxera populations in the vineyard.

In this project two relatively new chemical insecticides, the upwardly mobile Imidacloprid and the downwardly mobile Thiamethoxam, were evaluated using two newly developed rapid screening protocols involving glasshouse and laboratory trials. In addition population studies in the field and glasshouse highlighted the importance of optimising timing to significantly maximise the impact of the insecticide on reduction of phylloxera crawlers. Crawlers are the most abundant life stage on the grapevine roots and also the main active dispersive stage thereby representing a significant quarantine risk. Both insecticides significantly reduced egg, crawler and post-crawler levels. In addition vine vigour, as assessed by enhanced root development and leaf area was improved when insecticides were applied.

Although field trials, using insecticides, were not conducted in this study the population studies did highlight optimum timing dates for application whist the controlled environment studies highlighted optimum application rates. The next logical step in evaluation would be field trials. However, other factors on vineyard sites, such as soil type, climate and phylloxera genotype could potentially affect the efficacy of the insecticide treatments and careful evaluation is required before specific recommendations can be made to industry.

Chemical insecticides have a considerable advantage over other 'alternative 'approaches to phylloxera control in that they (i) could be used to suppress phylloxera populations more rapidly thereby reducing the impact of the pest on vine health and associated yield decline and (ii) reduce the risk of spread of crawlers by reducing the population levels and (iii) allow growers more time to develop and implement a

rootstock replanting program. By using an integrated approach of an early detection system combined with a more efficient control option phylloxera populations may be suppressed and the potential for spread delayed and the quarantine risks minimised.

Recommendations

The Australian Viticulture Industry recognises that grapevine phylloxera is the major insect pest constraint to long-term sustainable production of grapevines. A critical factor in the management of phylloxera in Australia is therefore early detection of new infestations and ensuring rapid management options are implemented before irreversible damage to grapevines occurs and to ensure that quarantine breakdown is minimised. There is an urgent need to develop a phylloxera-specific detection system, which is able to recognise either specific chemical markers induced in the host plant when attacked by phylloxera or molecular markers which identify the insect itself. There is also a need to evaluate interim management strategies, which could be used in specific instances to reduce phylloxera levels and allow development and phased implementation of sustainable rootstock replanting strategies.

The following recommendations can be made based on the outcomes of this research project:

(i) Whilst the PR protein leaf marker approach in our study was limited to only four potential approach further studies may look at screening other candidate proteins for phylloxera specificity.

(ii) The 'phylloxera specific' primers developed within the course of this project are specific to phylloxera but also detect some aphid genera. Refinement of the primer system is required before a truly phylloxera-specific DNA probe can be developed. Once this is achieved extensive controlled environment and field validation is required to ensure its effectiveness over a range of phylloxera genotypes and soil environments. A standardised simple sampling protocol will also need to be developed so that industry can utilise the detection system to its fullest advantage.

(iii) A comparison of the sensitivity and accuracy levels of the phylloxera-specific DNA probe to conventional detection methods should be undertaken in the future by testing detection systems within known phylloxera-infested vineyards.

(iv) Whilst the options for interim management of phylloxera-infested grapevines using chemical insecticides may be limited by restrictions on residue levels and potential environmental issues there is clearly a need to develop interim approaches for phylloxera management. Field evaluation of chemical insecticides would be required before specific recommendations on their use can be made to industry.

(v) Evidence form this study has highlighted in both field and controlled environment studies that the rate of grapevine decline and population dynamics of phylloxera is likely to be influenced by several factors including phylloxera genotype and site-related factors. Further studies on phylloxera genotype and grapevine interactions are required to ensure the robustness of any early detection system and interim management approach.

Appendix 1: Communication and Publications

The outcomes and progress of the project was relayed to industry through a variety of communications. A selected list is shown below:

Posters:

K. Herbert, K. Powell, A. Hoffmann, Y. Parsons, K. Ophel- Keller and R. van Heeswijck (2004) A DNA-based diagnostic test for the early detection of phylloxera in ungrafted vineyards. 4th Australasian Soil Diseases Symposium, Rowland Flat, SA, 8-11 February.

K. Herbert, K. Powell, A. Hoffmann, Y. Parsons, K. Ophel- Keller and R. van Heeswijck : (2004)"A DNA-based diagnostic test for the early detection of phylloxera in ungrafted vineyards" Twelfth Australian Wine Industry Technical Conference, 24-29 July, Melbourne, 2004.

Workshops:

Herbert, K. (2001) Early detection and alternative management of grapevine phylloxera in ungrafted vineyards. Oral presentation at three National Phylloxera Identification Workshops in Nagambie and Rutherglen, January-February 2001.

Herbert, K. (2002, 2003) Early detection and alternative management of grapevine phylloxera in ungrafted vineyards. Oral presentation at four National Phylloxera Identification Workshops in Nagambie and Rutherglen, November 2002-January 2003.

Herbert, K. (2004) Early detection and alternative management of grapevine phylloxera in ungrafted vineyards. Oral presentation at one National Phylloxera Identification Workshops in Rutherglen, January 2004.

Oral Presentations:

Herbert, K. (2004). Rutherglen Research Institute Seminar Series 2004– presentation on Industry Placement at Orlando-Wyndham.

Herbert, K. (2004)"A DNA based diagnostic test for the early detection of phylloxera in ungrafted vineyards" at the Twelfth Australian Wine Industry Technical Conference, 24-29 July, Melbourne, 2004.

Herbert, K. (2002) Annual Phylloxera Technical Meeting – 11th November 2002, Melbourne

Herbert, K. (2000) Early detection and alternative management of grapevine phylloxera on ungrafted vines. Oral presentation at University of Adelaide for Annual Student assessment, November 2000.

Herbert, K. (2000) Early detection and alternative management of grapevine phylloxera on ungrafted vines. Oral presentation at CRCV Annual Review Meeting, University of Adelaide, October 2000.

Herbert, K. & Powell, K.S. (2000) Early detection and alternative management of grapevine phylloxera in ungrafted vineyards. Oral presentation at the National Phylloxera Technical Reference Group Meeting, Adelaide, May 2000.

Herbert, K. (2000) Early detection and alternative management of grapevine phylloxera on ungrafted vines. Oral presentation as part of Rutherglen Research Institute Seminar Series, December 2000.

Herbert, K. (2000) Early detection and alternative management of grapevine phylloxera on ungrafted vines – research overview. Oral Presentation at Phylloxera Annual General Meeting, University of Adelaide, August 2000.

Van Heeswijck (2000) Phylloxera research: New knowledge on an old problem. Oral presentation at Industry Outreach Seminar. Department of Horticulture, Viticulture & Oenology, Adelaide University, November 2000.

Conference proceedings:

Herbert et al., (2005). "A DNA based diagnostic test for the early detection of phylloxera in ungrafted vineyards" (2005). Proceedings Twelfth Australian Wine Industry Technical Conference, 24-29 July, Melbourne, 2004.

Industry Articles:

Herbert, K.S., Powell, K.S., Hoffmann, A. A., Parsons, Y. M., Ophel-Keller, K. and van Heeswijck, R. (2003), Early Detection of Phylloxera – Present and Future Directions). Australian and New Zealand Grapegrower and Winemaker – Annual Technical Issue.

Media reports:

Herbert, K. (2001) Phylloxera season is a busy time for researchers. CRCV Newsletter Autumn, Vol. 7. p3.

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Appendix 2: Intellectual Property

The Corporation and the Research Organisation agree that the Corporation's share of title to all intellectual property and project income will be allocated on a percentage basis according to the proportion of funding for the project provided by the Corporation in relation to the funding provided by the Research Organisation.

Appendix 3: References

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Appendix 4: Staff

Department of Primary Industries, Victoria

Dr Kevin Powell (Project Leader & co-supervisor)

CESAR (La Trobe University)

Karen Herbert (PhD student)
Professor Ary Hoffmann (co-supervisor post June 2003)
Dr Yvonne Parsons (co-supervisor post June 2003).

University of Adelaide

Dr Robyn Van Heeswijck (co-supervisor until June 2003).

SARDI

Dr Kathy Opel Keller

Appendix 5: Sequence Alignments

(A) EF alignments:

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     5         15         25         35         45         55

D_VIT1          AGCAACAAAA CCACGCCGCA ATTCTTTAAC TGATACATTC TTTACATTGA ATCCAACATT
D_VIT2          AGCAACAAAA CCACGACGCA ATTCTTTAAC TGATACATTC TTTACATTGA ATCCAACATT
D_VIT3          AGCAACAAAA CCACGACGCA ATTCTTTAAC TGATACATTC TTTACATTGA ATCCAACATT
D_VIT4          AGCAACAAAA CCACGACGCA ATTCTTTAAC TGATACATTC TTTACATTGA ATCCAACATT
D_VIT5          AGCAACAAAA CCACGACGCA ATTCTTTAAC TGATACATTC TTTACATTGA ATCCAACATT
Sitobion        AGCAACAAAA CCACGACGCA ATTCTTTAAC TGATACATTC TTTACATTGA ATCCAACATT
RHOPALASIP      AGCAACAAAA CCACGACGCA ATTCTTTAAC TGATACATTC TTTACATTGA ATCCAACATT
HYADAPHIS       AGCAACAAAA CCACGACGCA ATTCTTTAAAC TGATACATTC TTTACATTGA ATCCAACATT
Trama rara      AGCTACAAAA CCACGTCTCA ATTCTTTGAC TGAAACATTC TTTACATTGA AACCAACATT
Stomaphis       AGCAACAAAA CCACGTCTCA ATTCTTTGAC TGAAACATTC TTTACATTGA AACCAACATT
Platypalpu      AGCTACATAA CCACGACGCA ATTCTTTAAC TGAAACGTTC TTTACGTTGA AACCAACATT
Pherocera       AGCAACATAA CCACGACGTA ATTCTTTAAC TGATACATTC TTAACATTGA AACCAACATT
Stenomphra      AGCTACATAA CCACGACGTA ATTCCTTAAC TGACACGTTC TTAACGTTGA AACCAACATT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115

D_VIT1          GTCTCCAGGA ACAGCTTCAG TAAGGGCTTC ATGGTGCATT TCCACAGATT TTACTTCAGT
D_VIT2          GTCTCCAGGA ACAGCTTCAG TAAGGGCTTC ATGGTGCATT TCCACAGATT TTACTTCAGT
D_VIT3          GTCTCCAGGA ACAGCTTCAG TAAGGGCTTC ATGGTGCATT TCCACAGATT TTACTTCAGT
D_VIT4          GTCTCCAGGA ACAGCTTCAG TAAGGGCTTC ATGGTGCATT TCCACAGATT TTACTTCAGT
D_VIT5          GTCTCCAGGA ACAGCTTCAG TAAGGGCTTC ATGGTGCATT TCCACAGATT TTACTTCAGT
Sitobion        GTCTCCAGGA ACAGCTTCAG TAAGGGCTTC ATGGTGCATT TCCACAGATT ATACTTCAGT
RHOPALASIP      GTCTCCAGGA ACAGCTTCAG TAAGGGCTTC ATGGTGCATT TCCACAGATT TTACTTCAGT
HYADAPHIS       GTCTCCAGGA ACAGCTTCAG TAAGGGCTTC ATGGTGCATT TCCACAGATT TTACTTCAGT
Trama rara      ATCTCCAGGA ACAGCTTCCG TCAAAGCTTC ATGGTGCATC TCCACAGACT TGACTTCAGT
Stomaphis       GTCTCCAGGA ACAGCTTCTG TCAAAGCTTC GTGGTGCATC TCTACAGATT TAACTTCAGT
Platypalpu      ATCACCGGGT ACAGCTTCGG GTAAAGCTTC ATGATGCATT TCAACGGATT TTACTTCAGT
Pherocera       ATCACCAGGT ACAGCTTCAG TTAAAGCTTC GTGATGCATT TCAACTGATT TAACTTCAGT
Stenomphra      ATCACCAGGT ACAGCTTCAG GAAGAGCTTC ATGATGCATT TCAACTGATT TAACTTCAGT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175

D_VIT1          AGTAATGTTG GCAGGTGCAA AGACAACAAC CATACCAGGT TTCAATACAC CAGTTTCAAC
D_VIT2          AGTAATGTTG GCAGGTGCAA AGACAACAAC CATACCAGGT TTCAATACAC CAGTTTCAAC
D_VIT3          AGTAATGTTG GCAGGTGCAA AGACAACAAC CATACCAGGT TTCAATACAC CAGTTTCAAC
D_VIT4          AGTAATGTTG GCAGGTGCAA AGACAACAAC CATACCAGGT TTCAATACAC CAGTTTCAAC
D_VIT5          AGTAATGTTG GCAGGTGCAA AGACAACAAC CATACCAGGT TTCAATACAC CAGTTTCAAC
Sitobion        AGTAATGTTG GCAGGTGCAA AGACAACAAC CATACCAGGT TTCAATACAC CAGTTTCAAC
RHOPALASIP      AGTAATGTTG GCAGGTGCAA AGACAACAAC CATACCAGGT TTCAATACAC CAGTTTCAAC
HYADAPHIS       AGTAATGTTG GCAGGTGCAA AGACAACAAC CATACCAGGT TTCAATACAC CAGTTTCAAC
Trama rara      GGTGATGTTG GCAGGAGCGA ACACAACAAC CATACCAGGT TTCAACAAAC CAGTTTCCAC
Stomaphis       GGTGATGTTG GCAGGAGCAA ACACAACAAC CATACCAGGT TTCAACAGAC CAGTTTCTAC
Platypalpu      AGTAATATTA GCTGGAGCAA ATACGACAAC ACAACCTGGT TTCAAAACAC CAGTTTCAAC
Pherocera       TGTTATATTA GCTGGTGCAA ATACAACAAC GGTACCAGGT TTTAAAACAC CAGTTTCAAC
Stenomphra      GGTAATATTG GCTGGAGCGA AAACAACAAC CATACCGGGT TTTAAGACAC CAGTTTCAAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
D_VIT1          ACGACCAACA GGGACTGTTC CAATACCTCC TCTCTTGTAC ACGTCTTGAA GTGGAAGACG
D_VIT2          ACGACCAACA GGGACTGTTC CAATACCTCC TATCTTGTAC ACGTCTTGAA GTGGAAGACG
D_VIT3          ACGACCAACA GGGACTGTTC CAATACCTCC TATCTTGTAC ACGTCTTGAA GTGGAAGACG
D_VIT4          ACGACCAACA GGGACTGTTC CAATACCTCC TATCTTGTAC ACGTCTTGAA GTGGAAGACG
D_VIT5          ACGACCAACA GGGACTGTTC CAATACCTCC TATCTTGTAC ACGTCTTGAA GTGGAAGACG
Sitobion        ACGACCAACA GGGACTGTTC CAATACCTCC TATCTTGTAC ACGTCTTGAA GTGGAAGACG
RHOPALASIP      ACGACCAACA GGGACTGTTC CAATACCTCC TATCTTGTAC ACGTCNNGAA GTGGAAGACG
HYADAPHIS       ACGACCAACA GGGACTGTTC CAATACCTCC TATCTTGTAC ACGTCNNGAA GTGGTAGACG
Trama rara      ACGACCAACG GGGACTGTTC CAATACCTCC TATCTTGTAT ACGTCTTGAA GTGGGAGACG
Stomaphis       ACGACCAACA GGAACTGTTC CAATACCTCC TATCTTGTAT ACATCCTGAA GTGGAAGACG
Platypalpu      ACGACCTACT GGTACTGTTC CAATACCGCC TATTTTGTAA ACATCTTGAA GTGGAAGACG
Pherocera       ACGACCAACT GGTACTGTTC CAATACCACC AATTTTATAA ACATCTTGTA ATGGAAGACG
Stenomphra      ACGACCTACT GGTACAGTTC CAATACCACC TATTTTGTAT ACGTCTTGGA GTGGAAGACG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
D_VIT1          AAGAGCCTTG TCAGTTGGAC GACTTGGTGG TAGGATAGCA TCTAAAGCTT CAATTAAACA
D_VIT2          AAGAGCCTTG TCAGTTGGAC GACTTGGTGG TAGGATAGCA TCTAAAGCTT CAATTAAACA
D_VIT3          AAGAGCCTTG TCAGTTGGAC GACTTGGTGG TAGGATAGCA TCTAAAGCTT CAATTAAACA
D_VIT4          AAGAGCTTTG TCAGTTGGAC GACTTGGTGG TAGGATAGCA TCTAAAGCTT CAATTAAACA
D_VIT5          AAGAGCTTTG TCAGTTGGAC GACTTGGTGG TAGGATAGCA TCTAAAGCTT CAATTAAACA
Sitobion        AAGAGCCATG TCAGTTGGAC GACTTGGTGG TAGGATAGCA TCTAAAGCTT CAATTAAACA
RHOPALASIP      AAGAGCTTTG TCAGTTGGAC GACTTGGTGG TAGGATAGCA TCTAAAGCTT CAATTAAACA
HYADAPHIS       AAGAGCCTTG TCAGTTGGAC GACTTGGTGG TAGGATAGCA TCTAAAGCTT CAATTAAACA
Trama rara      AAGAGCCTTG TCAGTTGGAC GACTGGGTGG CAGGATAGCA TCTAAAGCTT CAATCAAACA
Stomaphis       AAGAGCTTTG TCAGTTGGGC GACTTGGTGG CAGGATAGCA TCTAAAGCTT CAATCAAACA
Platypalpu      AAGTGGTTTT TCAGTTGGAC GACTTGGTGG TAAGATAGCA TCCAAAGCTT CAATCAAAGT
Pherocera       AAGTGGTTTA TCAGTTGGAC GACTTGGTGG TAAAATGGCA TCAAGAGCAT CAATTAAAGT
Stenomphra      GAGAGGTTTA TCAGTTGGGC GAGATGGTGG AAGGATAGCA TCCAAAGCAT CAATTAAAGT
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                ....|....| ....|...
                   305        315
D_VIT1          TTTACCGTCA GCCTTTTC
D_VIT2          TTTACCGTCA GCCTTTCC
D_VIT3          TTTACCGTCA GCCTTTTC
D_VIT4          TTTACCGTCA GCCTTTCC
D_VIT5          TTTACCGTCA GCCTTTCC
Sitobion        TTTACCGTCA GCCTTTCC
RHOPALASIP      TTTACCGTCA GCCTTTCC
HYADAPHIS       TTTACCGTCA GCCTTTCC
Trama rara      TTTACCATCG GCCTTTCC
Stomaphis       TTTACCGTCA GCCTTTCC
Platypalpu      TTTACCGTCA ACCTTTCC
Pherocera       TTTACCTTCA GCATTTCC
Stenomphra      TTTTCCTTCA GCATTTCC
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(B) 28S D3 alignments:

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   5          15         25         35         45         55
DVIT1           CCGAACCCGC TGGCGTTCAA AGTCGCGCCG GTCCGCAGTC CGGGGGCGTC TCCGCCGGGT
DVIT2           CCGAACCCGC AGGCGTTCAA AGTCGCGCCG GTCCGCAGTC CGGGGGCGTC TCCGCCGGGT
MYZUS           CCGAACCCGC CGGCGTTCAA AGTCGCGCTG GTCCGCAGTC CGGGGGCGTC TCCGCCAGGT
MICROSIP        CCGAACCCGC CGGCGTTCAA AGTCGCGCTG GTCCGCAGTC CGGGGGCGTC TCCGCCAGGT
SITOBION        CCGAACCCGC CGGCGTTCAA AGTCGCGCTG GTCCGCAGTC CGGGGGCGTC TCCGCCAGGT
PEMPHIGUS       CCGAACCCGC CGGCGTTCAA AGTCGCGCTG GTCCGCAGTC CGGGGGCGTC TCCGCCAGGT
Mantis          CCGGACCCCC CGGCGTTCAA AGCCGCGCCG GCCCGCAATC CGGGGGCGTC CCCGCCGGGA
Tenodera        CCGGACCCCC CGGCGTTCAA AGCCGCGCCG GCCCGCAATC CGTGGGCGTC CCCGCCGGGA

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                   65         75         85         95        105        115
DVIT1           CGGCTTCGGC CGTCCGGGGG CGCACCCTTA GCGCACACGT TGGTACCCGA AAGATGGTGA
DVIT2           CGGCTTCGGC CGTCCGGGGG CGCACCCTTA GCGCACACGT TGGTACCCGA AAGATGGTGA
MYZUS           CGGCTTCGGC CGTCCGGGGG CGCACCCTTA GCGCACACGT TGGTACCCGA AAGATGGTGA
MICROSIP        CGGCTTCGGC CGTCCGGGGG CGCACCCTTA GCGCACACGT TGGTACCCGA AAGATGGTGA
SITOBION        CGGCTTCGGC CGTCCGGGGG CGCACCCTTA GCGCACACGT TGGTACCCGA AAGATGGTGA
PEMPHIGUS       CGGCTTCGGC CGTCCGGGGG CGCACCCTTA GCGCACACGT TGGTACCCGA AAGATGGTGA
Mantis          CAGCTTTGGC CGTCCGTGGG CGCACTCTGA GCGTACACGT TGGGACCCGA AAGATGGTGA
Tenodera        CAGCTTTGGC CTTCCGTGGG CGCACTCTGA GCGTACACGT TGGGACCCGA AAGATGGTGA

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                  125        135        145        155        165        175
DVIT1           ACCTATGCCT GGCCAGGATG AAGTCAGGGG AAAACCCTGA TGGAGGTCCG CAGGCGATTC
DVIT2           ACCTATGCCT GGCCAGGATG AAGTCAGGGG AAAACCCTGA TGGAGGTCCG CAGGCGATTC
MYZUS           ACCTATGCCT GGCCAGGATG AAGTCAGGGG AAAACCCTGA TGGAGGTCCG CAGGCGATTC
MICROSIP        ACCTATGCCT GGCCAGGATG AAGTCAGGGG AAAACCCTGA TGGAGGTCCG CAGGCGATTC
SITOBION        ACCTATGCCT GGCCAGGATG AAGTCAGGGG AAAACCCTGA TGGAGGTCCG CAGGCGATTC
PEMPHIGUS       ACCTATGCCT GGCCAGGATG AAGTCAGGGG AAAACCCTGA TGGAGGTCCG CAGGCGATTC
Mantis          ACCTATGCCT GGCCAGGACG AAGTCAGGGG AAANCCCTGA TGGAGGTCCG CAGNCGATTC
Tenodera        ACCTATGCCT GGTCAGGACG AAGTCAGGGG AAANCCCTGA TGGAGGTCCG CAGNCGATTC

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                  185        195        205        215        225        235
DVIT1           TGACCGTGCA AATCGATCGT CTGAGCTGGG TATAAGGGCG AAAGACTAAT CGAACCATCT
DVIT2           TGACCGTGCA AATCGATCGT CTGAGCTGGG TATAAGGGCG AAAGACTAAT CGAACCATCT
MYZUS           TGACCGTGCA AATCGATCGT CTGAGCTGGG TATAGGGGCG AAAGACTAAT CGAACCATCT
MICROSIP        TGACCGTGCA AATCGATCGT CTGAGCTGGG TATAGGGGCG AAAGACTAAT CGAACCATCT
SITOBION        TGACCGTGCA AATCGATCGT CTGAGCTGGG TATAGGGGCG AAAGACTAAT CGAACCATCT
PEMPHIGUS       TGACCGTGCA AATCGATCGT CTGAGCTGGG TATAGGGGCG AAAGACTAAT CGAACCATCT
Mantis          TGACNGTGCA AATCGATCGT CAGAGCTGGG TATAGGGGCG AAAGACTAAT CGAACCATCT
Tenodera        TGACNGTGCA AATCGATCGT CAGAGCTGGG TATAGGGGCG AAAGACTAAT CGAACCATCT

                ....|....| ....|....| ....|.
                  245        255        265
DVIT1           AGTAGCTGGT TCCCCNNNNN NNTTCC
DVIT2           AGTAGCTGGT TCCCCNNNNN NNTTCC
MYZUS           AGTAGCTGTT TCCCCNNNNN NNTTCC
MICROSIP        AGTAGCTGTT TCCCCNNNNN NNTTCC
SITOBION        AGTAGCTGTT TCCCCNNNNN NNTTCC
PEMPHIGUS       AGTAGCTGTT TCCCCNNNNN NNTTCC
Mantis          AGTAGCTGGT TCCCTCCGAA GTTTCC
Tenodera        AGTAGCTGGT TCCCTCCGAA GTTTCC
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