# DIGESTIVE AND FEEDING PHYSIOLOGY OF GRAPE PHYLLOXERA (*DAKTULOSPHAIRA VITIFOLIAE* FITCH)

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**A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE AUSTRALIAN NATIONAL UNIVERSITY**

SCHOOL OF BOTANY AND ZOOLOGY

FACULTY OF SCIENCE

THE AUSTRALIAN NATIONAL UNIVERSITY

CANBERRA, ACT

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**FEBRUARY 2007**

This thesis does not contain any material that has been accepted for the award of any other degree or diploma at this or any other University. To the best of my knowledge, this thesis does not contain any material that has been published previously, except where due reference is made in the text. The research described in this thesis is my own original work, except where due acknowledgement has been made in the text.

Part of the content of Chapters 2 and 5 are currently *in press* for publication for *Proceedings of the Third International Phylloxera Symposium* in *Acta Horticulturae*. For both articles I am the principle author and contributor to the work.

Kim Bernadette Kingston

01 February 2007

# ACKNOWLEDGEMENTS

I would like to thank my supervisors for their continued support, guidance, and encouragement throughout my PhD studies. Dr Kevin Powell (Department of Primary Industries; DPI) originally offered me this fantastic opportunity, and I must thank him for allowing a 'soil microbiologist' to endeavour into the world of entomology and host-plant interactions. Dr Paul Cooper (Australian National University; ANU) has provided continual strength to me both personally and academically, I always felt refreshed and revitalised after our conversations. Both supervisors brought different attributes to my PhD journey, and I knew from early on in the project that I was in a fortunate situation.

Going back to the start of this endeavour, I also wish to thank Angela Avery (DPI) for initially planting the seed of thought in my mind that perhaps it was time to 'think outside the square' for the future direction of my career. Without that late night chat, I would never have gone down this path. I also need to thank the Department of Primary Industries for allowing me four years leave without pay to complete my PhD studies. My scholarship and the funds for the project were generously provided by the Grape and Wine Research Corporation.

During the course of the PhD I have been a part of *'team phylloxera!'* My workmates have made my task very enjoyable, and I have benefited from the professional relationship and social developments that have come from being part of this team. Therefore acknowledgment must be made of the assistance and support provided to me by past and present colleagues of the grape phylloxera research team at DPI-Rutherglen, particularly: Anna Burns, Annette Blanchfield, Carolyn Trethowan, Ginger Korosi, Karen Herbert, Rob Bederian and Sarah Brown.

I would also like to acknowledge the psychological assistance that has been provided to me by my workmates at DPI-Rutherglen. It is true that the "people make the workplace", and the friendships that I have established here have been critical the completion of this research. A general thank you for all of the chats, both in and outside of the tea room. The pub lunches were pretty good too! A special thank you to Kyra-Jane Huhn for offering assistance with the formatting of this thesis.

Now to the first of a list of professional thankyous …

The statistical analysis presented throughout the thesis has been performed in consultation with Dr Alvin Milner and Sorn Norng (DPI). Thank you to for your direction, and most importantly, your patience. I may not share your passion for biometrics, but I certainly appreciate your support. Special thanks to Sorn for reviewing the final chapters.

The microscopy research presented in chapter 2 could not have been performed without the direction and assistance of Neda Plovanic (ANU). Neda, a 'master' at sectioning, provided technical support and training for the light microscopy sectioning. A number of sections presented in this thesis were cut by her steady hand. The electron microscopy images presented were captured with the use of the facilities at the ANU Electron Microscopy Unit under the direction and experience of Dr Roger Heady. All scanning electron microscopy images in this thesis were taken by Dr Roger Heady and Dr Paul Cooper.

The molecular symbiont research presented in chapter 3 was assisted by a number of people. An initial refresher course in the skills of molecular biology was provided by Dr Lyn Cook (ANU); I was appreciative of the opportunity to regain some confidence in this area. Further assistance in field of molecular biology was provided by Dr Daryl Nelson, Dr Andrew Oxley and Annette Blanchfield (DPI). I especially need to thank

Andrew and Annette for assistance in the laboratory and great discussions about the best approach to take. Finally, I need to thank Dr Trudy Wharton (ANU) who provided me with the Monterey pine aphids used as a positive PCR control. The DNA sequencing presented in this thesis was completed by Saturn Biotech, Murdoch University.

The EPG analysis of grape phylloxera feeding behaviour would not have been possible without the training and assistance provided by Dr Freddy Tjallingii (Wageningen University, The Netherlands). Freddy is considered in many circles as the 'godfather' of EPG, and the insight that he brought to the analysis of the grape phylloxera waveforms was critical for the development of my knowledge in this area. I have also been rewarded by meeting a number of researchers undertaking EPG based studies. Various members of the EPG community have provided support and guidance to me for the development of an EPG system for a tiny, root-feeding insect, and inspired me along the way into the belief that everything was possible.

A professional thank you must also be extended to the providers of the grapevine root material that is essential for maintaining the grape phylloxera populations in the laboratory, and was also utilised for the EPG studies. In particular, thank you to Peter Clingleffer (CSIRO, Merbein) for continuously allowing the phylloxera research team to 'harvest' the root system of the vineyard planted grapevines. And a special thank you to the vineyard manager in the King Valley, Victoria, who has managed to keep grape phylloxera out of his vineyard, but still lets the phylloxera research team enter to collect root material. The Börner rootstock material was used in agreement with Professor Ernst Ruhl (Geisenheim Research Institute, Germany).

Back to a personal level …

I would sincerely like to acknowledge my parents, Rex and Joy Andrews, who have always supported me in my endeavours and ambitions. They mightn't understand the 'nut and bolts' of what I do, but they have provided encouragement ever since I decided (half a lifetime ago) to pursue a career in science. Coming from a small country town, not everyone thought that "girls did science". I share in the pride that mum and dad display for all of their children, and for that I am grateful.

And finally my eternal gratitude to my husband and best friend, Merrick Kingston. Without Merrick's continual belief in me, and support for my periods of absence from our family home, I would not have been able to complete this journey. I don't think that either of us really knew what we were getting ourselves into to, but I thank Merrick for his patience and exceptional personal qualities. Merrick's perceptiveness always brings reality to my life, and for that I am extremely grateful.

This thesis has been completed in loving memory of Anya.

# ABSTRACT

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) is a worldwide pest of the viticulture industry due to damage caused to the root system of the European grapevine, *Vitis vinifera* L. (Vitaceae). The main management option for radicicolae grape phylloxera is the use of resistant rootstocks, however due to the success of this management option a range of questions relating to the biology, nutrition and feeding behaviour of the pest insect remain unanswered. This thesis addressed a number of these questions in order to extend the current knowledge of the biological characteristics of grape phylloxera and the possible influences on host-plant responses.

The internal morphology of grape phylloxera was dominated by a large, non-coiled midgut, and developing eggs in adults. The midgut was compartmentalised by an atypical junction with the hindgut. The hindgut extended to the posterior end of the insect, and although an anus was present, no waste excretion was observed in live insects. Parthenogenetic production of relatively large eggs may compress the digestive system, increasing pressure in both the midgut and hindgut chambers. The evolution of the compartmentalised midgut is suggested to permit the storage of food and ensure the continual supply of food to grape phylloxera during periods of egg production and during dispersal to new *Vitis* plants.

Molecular and microbiological evidence showed that radicicolae grape phylloxera has a transient association with an undefined bacteria species. The association appeared to be related to the health of the grapevine root and the insect population, and may influence the variable survival rates of insects observed in laboratory controlled experiments. The bacteria species differed from a bacterial association identified in

gallicolae grape phylloxera, and unlike the endosymbiotic relationship of Aphididae, was not essential.

The development of an artificial feeding system for radicicolae grape phylloxera was complicated by the size of the insect, the extended survival of 'control' insects, and a lack of detail regarding the composition of the natural food source. Due to the sedentary feeding behaviour of grape phylloxera, the design of the diet chamber was modified to allow renewal of the artificial diet solution with minimal disturbance to the insect. The survival time of grape phylloxera was significantly extended by two diet formulations, however neither result was reproducible. Insect probing activity was generally stimulated by acidic diet formulations containing sucrose and amino acids, indicating that grape phylloxera were attracted to the artificial feeding system although the diet formulation was not optimised.

Electrical penetration graph (EPG) studies identified that the apterous life stages of radicicolae grape phylloxera exhibit differential feeding behaviour on *V. vinifera* root material. The first application of EPG to grape phylloxera research identified 14 waveforms associated with probe penetration and continual feeding activity, with the two most common waveforms anticipated to represent salivation and ingestion. The restrictions of working with a root-feeding insect were overcome by the use of excised root pieces and tissue culture grapevines. Both root systems produced the same EPG waveforms.

The content of this thesis lays the ground work for further research into grape phylloxera host-plant interactions with susceptible and resistant *Vitis* species. The long-term aim of this research is the development of alternative management options for the pest insect, and to ensure the sustainability of resistant rootstocks as the main management option for the viticulture industry.

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# CHAPTER 1

# INTRODUCTION

# 1 HISTORICAL BACKGROUND

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) cause economic loss to vineyards planted with the susceptible European grapevine host-plant, *Vitis vinifera* L. (Vitaceae). The grafting of American *Vitis* species as resistant rootstocks to *V. vinifera* is the main management option for grape phylloxera infested vineyards; however there are reports in America and Europe of adaptation by grape phylloxera leading to the breakdown of this resistance (Granett *et al.*, 1987; Boubals, 1994; Porten *et al.*, 2000). Most studies on the interaction between grape phylloxera and *Vitis* species have focused on population dynamics (Powell *et al.*, 2000; Powell *et al.*, 2003; Herbert *et al.*, 2006) and the genetic variability of the insect (Corrie *et al.*, 2002; Corrie *et al.*, 2003; Vorwerk and Forneck, 2006). There is limited understanding on the general biology and nutritional requirements of grape phylloxera, and the feeding behaviour of the insect on susceptible and resistant varieties of the *Vitis* host-plant. Understanding the biology and interactions of grape phylloxera on the host-plant is fundamentally important for ensuring the long-term management of the pest insect for the viticulture industry.

## *1.1 TAXONOMIC CLASSIFICATION*

Grape phylloxera are a plant-sucking insect that feed solely on species of *Vitis*, causing characteristic galls to form on the roots and leaves of grapevines (Figure 1). Since being described in 1854, over 32 synonyms have been recorded for the scientific name of the species (Ponsen, 1997). Grape phylloxera taxonomically are classified within the superfamily Aphidoidea, which contains three sister family groups: Phylloxeridae, Adelgidae and Aphididae (Carver *et al.*, 1991). Phylloxeridae are monoecious, and the family incorporates around 70 species, including: the pear phylloxera (*Aphanostigma piri* Cholodkovsky), pecan phylloxera (*Phylloxera devastatrix* Pergande), oak phylloxera

(*Phylloxera coccinea* Heyden) and southern pecan leaf phylloxera (*Phylloxera russellae* Stoetzel).

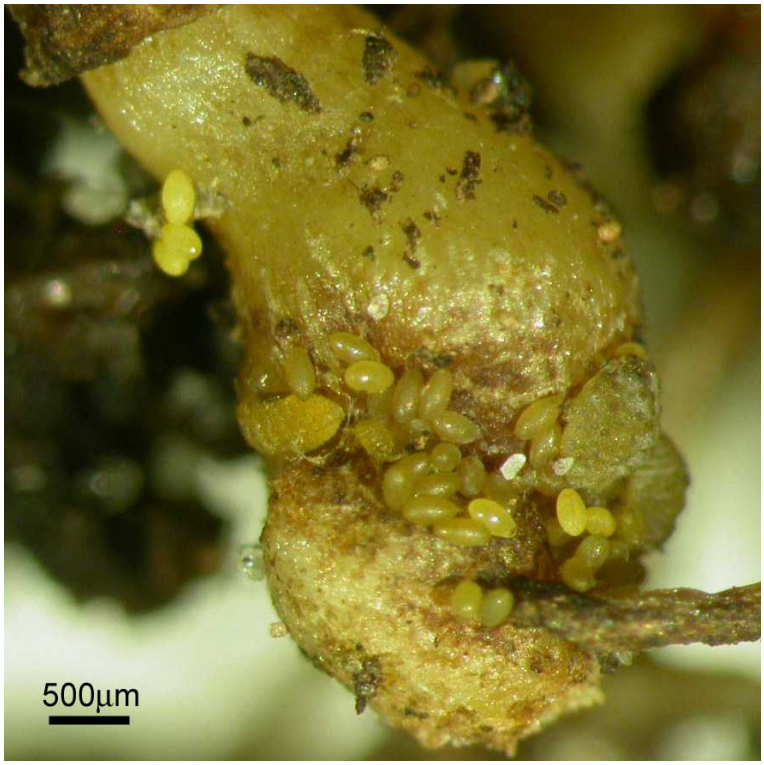


**Figure 1.** The development of a nodosity gall induced by radicicolae grape phylloxera feeding on the fibrous root of a *V. vinifera* potted grapevine; apterous adults and eggs are present. Scale bar 500 µm.

Feeding by Phylloxeridae leads to the development of galls, swellings or necrotic tissue at the root, shoot or stem feeding sites on woody deciduous plants (Ponsen, 1997). The taxonomic classification of grape phylloxera within the aphid (Aphidoidea) superfamily results in the insect being described as “aphid-like”, which may lead to false assumptions regarding the similarity of grape phylloxera to members of the Aphididae. The sister families Aphididae and Phylloxeridae are taxonomically separated by external morphological features, however there are also differences between the two families based on internal morphology and nutritional requirements (Ponsen, 1997) which limit the level of extrapolation from biological studies between the two families.

### *1.2 GEOGRAPHICAL DISTRIBUTION*

Grape phylloxera was first recognised as a pest for the viticulture industry following detection in France during the late 1860s. Feeding on *V. vinifera*, the insect rapidly spread through vineyards causing damage and death of grapevines and resulting in considerable economic impact (Ordish, 1987). By the end of the 19$^{th}$ Century grape phylloxera was present in most of the viticulture regions throughout Europe, North America, South America, South Africa, New Zealand and Australia. Grape phylloxera is currently found all major viticulture regions of the world, with the exception of Chile, China and parts of Australia (Granett *et al.*, 2001b). In Australia, the distribution of the insect has been quarantined to defined regions of infestation.

Grape phylloxera was first detected in Australia in 1877 in a commercial vineyard at Geelong, Victoria (Boehm, 1996). Despite the implementation of eradication programs involving the uprooting of infested vineyards, grape phylloxera was later identified in other important Victorian viticultural regions, including Bendigo (1893) and Rutherglen (1899). Grape phylloxera was also identified within regions of New South Wales (1884) and Queensland (1910). The pest insect has not been detected in any of the

remaining states and territories of Australia, including Tasmania, South Australia, Western Australia, Northern Territory and the Australian Capital Territory (Boehm, 1996).

Victorian state-based legislation was implemented to restrict the movement of grapevine material from designated grape phylloxera quarantine zones, termed Vine Disease Districts (VDD). Initial districts had broad boundaries, and were inclusive of both infested and non-infested vineyards; however the VDD were later separated into discrete regions following new surveys on the distribution of the pest insect (Buchanan, 1987; Boehm, 1996). With the implementation of the National Phylloxera Management Protocols in 2000, the VDD terminology was changed to Phylloxera Infested Zones (PIZ), which in Victoria included the viticulture regions of King Valley, North-East, Mooroopna, Upton and Nagambie (National Phylloxera Management Protocol, 2003). However the spread of grape phylloxera into non-infested regions remains a constant risk to the Victorian viticulture industry. Despite the quarantine regulations, a number of PIZ regions have been extended or proclaimed in recent years in response to the identification of grape phylloxera in vineyards previously considered 'free' of the pest insect (Powell *et al.*, 2006; Phylloxera Update, 2007; Kennedy, 2007).

## 2 INTERACTIONS OF THE HOST-PLANT MODEL

There are around 60 species of *Vitis* around the world, each differing in the degree of resistance or susceptibility to grape phylloxera infestation. Grape phylloxera are native to the temperate-subtropical regions of eastern and south western North America, including Mexico, where the insect feeds on wild grapes (Vitaceae: *Vitis* species). Within the native habitat, grape phylloxera cause limited damage to the host-plant, (Gullan and Cranston, 2005). American *Vitis* species have developed tolerance and resistance mechanisms to the impact of grape phylloxera feeding through coevolution.

However the European grapevine *V. vinifera* is highly susceptible to grape phylloxera feeding, and the damage caused by the development of galls on the fibrous and lignified root system, together with a secondary fungal infection, lead to an economic decline in grapevine growth and production (Granett *et al.*, 1998; Omer *et al.*, 1999a). The impact of grape phylloxera on *V. vinifera* production varies across infested viticulture regions due to genetic diversity of the insect in the population both within and between countries (King and Rilling, 1985;1991). Environmental factors including temperature, soil type, organic matter and soil moisture are also likely to influence the level of damage caused by grape phylloxera (Granett *et al.*, 1998). The life cycle of grape phylloxera is complex, with distinct differences occurring on the native American *Vitis* species and on the European *V. vinifera* (Granett *et al.*, 2001b; Corrie *et al.*, 2002; Vorwerk and Forneck, 2006).

## *2.1 THE INSECT MODEL – GRAPE PHYLLOXERA*

Within Australian vineyards, the genetic variation of grape phylloxera has been investigated (Corrie *et al.*, 1997; Corrie *et al.*, 2002), and was found to be associated with variable damage caused by grape phylloxera infestation (Corrie *et al.*, 2003). New genotypic classes of grape phylloxera continue to be identified, with the most recent publication identifying 83 genetically different clones from Australian vineyards (Umina *et al.*, 2007). However no common genotypes have been identified between the Australian and European populations of grape phylloxera (Vorwerk and Forneck, 2006). In Australian vineyards, the presence of two genotypic classes (G1 and G4) dominate the grape phylloxera profile across most infested regions (Corrie *et al.*, 2002). Variation in the population genetics of grape phylloxera represents a risk for the breakdown of current management options, and highlights the requirement for understanding the biology of the insect for the benefit of maintaining existing, and developing alternative, management options in infested vineyards.

### 2.1.1 GRAPE PHYLLOXERA LIFE CYCLE ON AMERICAN VITIS

Grape phylloxera are oviparous, and in the North America native habitat complete the full life cycle on American *Vitis* species. Holocyclic reproduction (incorporating both parthenogenetic and sexual phases) begins in spring with the hatching of a sexual over-wintering egg (Figure 2). The hatching fundatrix (or stem mother) represents the first parthenogenetic generation following the sexual cycle. The fundatrix feeds on the upper surface of American *Vitis* leaves causing the formation of a pocket-like gall on the underside of the leaf. After developing into an apterous adult, the fundatrix parthenogenetically reproduces several hundred (up to 500) eggs, and dies. The eggs hatch and develop through four moults into gallicolae apterous adults. Gallicolae grape phylloxera feed on the leaves of *Vitis* resulting in the formation of additional pocket-like galls, and continue the parthenogenetic reproduction of up to 200 eggs for four to seven generations. The first instar searches the grapevine for alternative feeding sites, and once feeding begins typically remains at the same feeding location throughout the developmental life stages (Coombe, 1963).

During summer, first instars migrate either onto new leaves (continuing the gallicolae parthenogenetic cycle), or onto the roots of the grapevine. Root feeding apterous females, termed radicicolae, produce galls at the root feeding site. Hook-shaped galls, termed nodosities, develop on fibrous roots; feeding on lignified roots does not appear to impact on American *Vitis* species. Radicicolae grape phylloxera parthenogenetically produce, on average, three to five (and up to 10) generations of eggs throughout spring and summer, and during late summer and autumn may emerge from the soil surface and develop into alate females. The alate initiates the beginning of sexual reproduction, laying several male and female eggs. The sexual eggs hatch, mature without feeding and mate leading to the development of a single over-wintering egg in the sexual female. The over-winter egg is deposited in the bark of the grapevine stem and the sexual female dies, completing the holocyclic life cycle (Coombe, 1963). First

instar radicicolae grape phylloxera may hibernate on the roots of the grapevine over winter, continuing the parthenogenetic life cycle independent of the sexual life cycle.

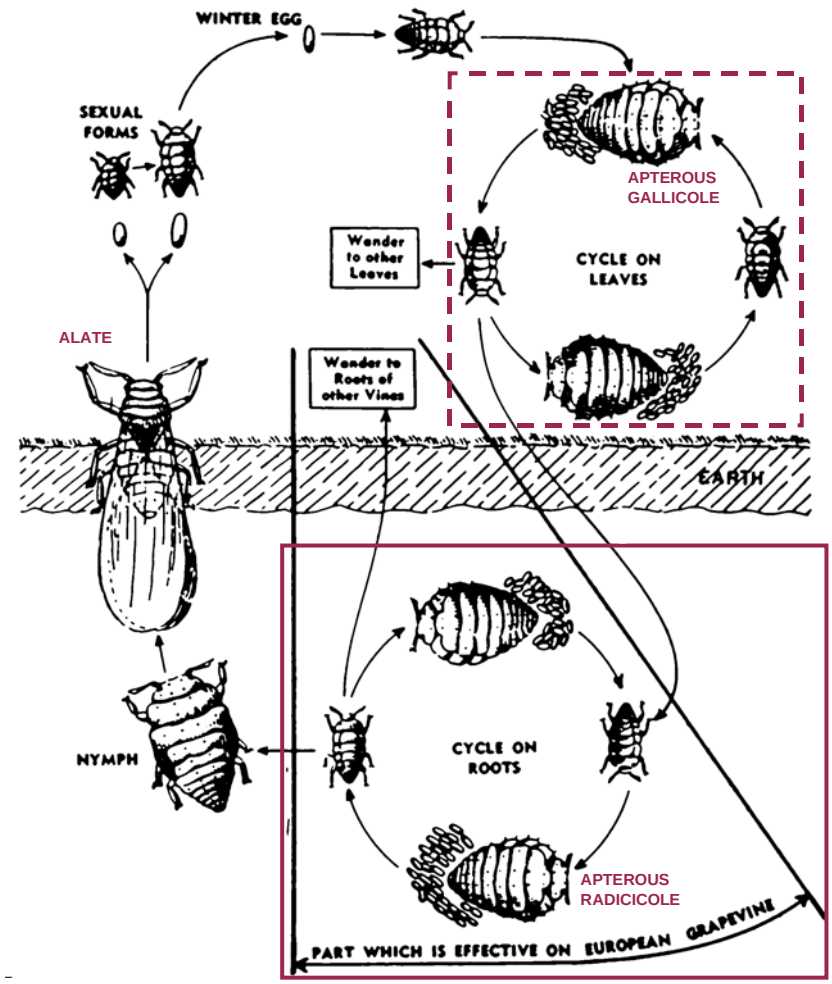


**Figure 2.** The holocyclic life cycle of grape phylloxera begins with the hatching of the over-wintering egg. Several parthenogenetic generations of apterous gallicolae (dashed box) and radicicolae (solid box) occur during the summer months. In autumn, alate insects emerge from the soil surface to complete the sexual cycle. Anholocyclic reproduction of radicicolae grape phylloxera occurs on the European grapevine, *V. vinifera* (adapted from Coombe, 1963).

### 2.1.2 *GRAPE PHYLLOXERA LIFE CYCLE ON* V. VINIFERA

Radicicolae are the principal apterous female on *V. vinifera*, with gallicolae generally failing to establish leaf galls. Although the development of the alate life stage is observed, sexual maturity is not achieved and sexual reproduction does not occur on *V. vinifera*. Radicicolae grape phylloxera feed on both fibrous and lignified roots of *V. vinifera*, resulting in the development of galls on both systems. Additional to the formation of nodosities on the fibrous root system, gall swellings, termed tuberosities, form on lignified roots giving the root a wart-like appearance (Coombe, 1963). The anholocyclic life cycle of grape phylloxera on *V. vinifera* continues with the parthenogenetic production of eggs by radicicolae, and the winter hibernation of the insect as first instars. On *V. vinifera*, up to 15 generations of radicicolae grape phylloxera may occur per season. Early researchers considered sexual reproduction essential for grape phylloxera survival (Despeissis, 1895), however the continued existence of the pest insect on *V. vinifera* proves that the anholocyclic life cycle is sufficient for the maintenance of a grape phylloxera population within an infested vineyard.

Although *V. vinifera* is unable to support the sexual life cycle of grape phylloxera due to absence of suitable leaf material, viable sexual reproduction may occur in Australian vineyards due to the presence of American *Vitis* species as rootstocks. The development of suckers on American *Vitis* provides the leaf material required for gallicolae development, and completion of the grape phylloxera holocyclic life cycle (Buchanan and Hardie, 1978). However a study into the genetic structure of grape phylloxera by Corrie *et al.* (2002) identified parthenogenetic reproduction to be predominant under Australian conditions, with sexual reproduction considered a rare event. In the study, radicicolae grape phylloxera were more common than gallicolae, with limited evidence for sexual recombination between the two populations to indicate a holocyclic life cycle.

### 2.1.3 AUSTRALIAN POPULATION DYNAMICS

Population studies conducted in Australian *V. vinifera* vineyards showed that radicicolae grape phylloxera numbers peak during early summer (January-February), and decline through autumn (April-May). Although sexual reproduction is rare, the alate and first instar life stages are active above ground during the summer months (Powell *et al.*, 2000; Herbert *et al.*, 2006). The presence of first instar grape phylloxera on the soil and above-ground regions of the grapevine allows for wind- and human-assisted dispersal of the insect. First instars have been detected up to 20 m from the nearest infested grapevine, contributing to within vineyard migration rate of between 15-100 m per year (King and Buchanan, 1986; Powell *et al.*, 2000). Due to limited evidence of sexual recombination in radicicolae grape phylloxera under Australian conditions, the alate life stage (and potential sexual reproduction) is considered a low risk for the spread of the insect (Corrie *et al.*, 2002).

## 2.2 THE PLANT MODEL – GRAPEVINES

The performance and productivity of grapevines in a grape phylloxera infested vineyard is dependent upon a number of factors, including: grape phylloxera genetics; population density; environmental conditions (including climate and water availability); soil characteristics (classification, temperature, structure, chemistry and microbiology); and vineyard location (Granett *et al.*, 1983; Herbert *et al.*, 2006; Reisenzein *et al.*, 2007). The impact of grape phylloxera in susceptible *V. vinifera* vineyards is variable, with some vineyards requiring replanting within 5 years of detection due to economic levels of grape phylloxera damage, and some vineyards remaining economically viable for decades (Viduka *et al.*, 2003).

### 2.2.1 HOST-PLANT RESPONSE

The nodosities and tuberosities induced by radicicolae grape phylloxera feeding act as a nutrient sink, supplying photosynthates, starch, amino acids and amides for insect

survival and development (Kellow *et al.*, 2004). Although grapevine root damage in the form of growth abnormalities and metabolism disturbance are primarily due to grape phylloxera feeding activity (Rilling, 1975), the presence of the nutrient sink is not sufficient to be the singular cause of decline in grapevine health. It is speculated that microorganisms act as secondary parasites, enabling a three-way interaction between grape phylloxera, grapevines and fungi to cause root decay (Vannacci *et al.*, 1984). Laboratory, glasshouse and field studies have shown that the detrimental impacts of grape phylloxera infestation are intensified in the presence of specific species of fungi, in particular *Fusarium* (Omer *et al.*, 1995; Omer *et al.*, 1999b; Omer and Granett, 2000; Edwards *et al.*, 2007). This soil microbiology relationship, together with grape phylloxera and grapevine genetics, grapevine health, vineyard management, soil type and environmental conditions, complicates the impact of grape phylloxera infestation on the grapevine (Granett, 2000).

### *2.2.2 GALL FORMATION*

Radicicolae grape phylloxera feed from the cortex of the root, where the stylet penetrates the parenchyma cells on an intracellular pathway. The stylet typically penetrates 4-5 cell layers below the epidermis of the root, and the phloem tissue of the stele is not penetrated (Kellow *et al.*, 2004). The intracellular stylet penetration of the feeding site initiates the development of root galls (Pollard, 1973), and the degree of gall formation depends upon the sensitivity of the plant tissue to grape phylloxera feeding. Radicicolae feeding activity produces gall formations due to a depression in the growth rate of cells at the feeding site, and the stimulation of cell growth rate on the opposite side of the root (Miles, 1972). Gall formation is thought to be stimulated by amino acids, including glutamine, asparagine, serine, alanine and aspartic acid (Hori, 1992). The gall acts as a nutrient sink for grape phylloxera due to the accumulation of starch, amino acids and amides, and although *V. vinifera* recognises the presence of the pest insect, the susceptible grapevine produces limited defensive responses

(Kellow *et al.*, 2004). The amino acid compounds within the saliva of grape phylloxera may be detoxified in the tissue of resistant *Vitis*, therefore inhibiting gall formation (Miles, 1990). Other compounds possibly associated with gall formation are: plant hormones and auxins, including indole acetic acid (IAA); or other growth stimulators, including IAA synergists, phenolic compounds; or phenol oxidases (Hori, 1992).

Resistant *Vitis* commonly display two forms of genetic resistance to grape phylloxera feeding: (1) antixenosis, involving the repulsion of the insect by the root; and (2) antibiosis, the formation of a corky layer around the feeding site which varies in thickness according to the level of resistance (Boubals, 1966a; Hardie and Cirami, 1988). Sealing off root swellings with localised necrosis may also reduce the impact of grape phylloxera feeding. El-Nady and Schroder (2003) identified two types of necrosis as a response to radicicolae grape phylloxera feeding activity: Type I developed with 12-14 hours, and Type II within 2-5 days. Both resulted in the death of the plant tissue and prevention of insect feeding. A build up of allelochemical phenolics causes the necrosis, destroying the food source for grape phylloxera and preventing the secondary rot that leads to grapevine decline (Miles, 1990). In *V. vinifera*, these defensive phenolics accumulate diffusely throughout the root tissue and have limited impact on reducing grape phylloxera damage or grapevine decline.

### *2.2.3 RESISTANT ROOTSTOCK SELECTION*

Variability in American *Vitis* species resistance to grape phylloxera feeding has been investigated by a number of researchers (Boubals, 1966a; King and Rilling, 1985; Kellow, 2000; Forneck *et al.*, 2002; Viduka *et al.*, 2003). Several genes are expected to control resistance, with dominance displayed in *V. rotundifolia*, *V. berlandieri*, *V. cinerea* and *V. rubra* (Boubals, 1966b). Resistant *Vitis* species have been utilised in rootstock breeding programs, although the mechanism of resistance differs among species resulting in varying levels of resistance to grape phylloxera feeding. Kellow *et*

*al.* (2002) examined five resistant rootstocks, in comparison with susceptible *V. vinifera*, and classified the level of resistance to grape phylloxera feeding as 'resistant', 'highly resistant' and 'immune' (Table 1). Resistance ratings were based on grape phylloxera survival rates and the level of root nodosity formation on micropropagated tissue culture grapevines. Variation in the levels of nutrition provided by the host-plant to the pest insect may be a possible explanation for the reduced levels of grape phylloxera survival and fecundity on resistant rootstocks (Granett *et al.*, 1983).

The resistant rootstocks are grafted to the productive, although susceptible, *V. vinifera*, and due to the success of resistant rootstocks as a management tool, there has been limited research into the biology, nutrition and feeding behaviour of grape phylloxera. However there are examples of rootstock failure, especially if *V. vinifera* was included in the parentage of the resistant rootstock breeding program, and expression of *V. vinifera* susceptibility has resulted in insufficient levels of grape phylloxera resistance (Boubals, 1994). Rootstock AXR#1 (*V. vinifera* x *V. rupestris*) demonstrated a failure of resistance to grape phylloxera after several years of establishment in California vineyards in the United States of America (Granett *et al.*, 2001b). There are also reports in Europe of adaptation by grape phylloxera leading to the breakdown of resistance in resistant rootstocks which do not contain *V. vinifera* parentage (Boubals, 1994; Porten *et al.*, 2000).

Resistant rootstocks generally display tolerance to the damage caused by grape phylloxera, but do not necessarily deter feeding or display complete resistance to the insect. Some rootstocks reduce egg production, first instar development and survival rates in comparison with *V. vinifera* (Granett *et al.*, 1983). The level of resistance displayed by the rootstock is also influenced by grape phylloxera genetics (Powell *et al.*, 2006). Resistant rootstocks also vary in levels of tolerance to a number of other viticultural management factors, including nematodes, salt, drought and lime (Coombe,

1963; Granett *et al.*, 2001b); therefore management issues beyond grape phylloxera influence vineyard rootstock selection.

**Table 1.** Resistance ratings for grape phylloxera resistant rootstocks (adapted from Kellow *et al.*, 2002)

| *Vitis* species (cv.) | resistance rating | criteria for resistance status/comments |
|---|---|---|
| *V. vinifera* (Shiraz) | susceptible | Grape phylloxera established nodosities, reached reproductive maturity within the time of the assay[1] and had relatively high rates of survival. No browning or necrosis was visible on any of the nodosities formed. |
| *V. champini* (Ramsey) | resistant | Grape phylloxera initiated nodosities, but only a small number of insects reached reproductive age within the time of the assay[1] and survival rates were relatively low. Nodosities were relatively small with brown, necrotic regions. |
| *V. riparia x V. rupestris* (Schwarzmann) | highly resistant | Grape phylloxera were unable to initiate nodosity formation. No survival of insects. |
| *V. riparia* | highly resistant | Grape phylloxera attempted to feed and a small number of nodosities were initiated but failed to develop. Feeding sites became brown and necrotic. No survival of insects. |
| *V. cinerea* x *V. riparia* (Börner) | highly resistant | Grape phylloxera attempted to feed but no nodosities were initiated. Feeding sites became brown and necrotic. No survival of insects. |
| *V.rotundifolia* | immune | No evidence that grape phylloxera attempted to feed and no nodosities initiated. No insects, dead or alive, observed on the roots. |

[1] time period for assay was 30 days

# 3 GRAPE PHYLLOXERA MANAGEMENT OPTIONS

Methods to eradicate grape phylloxera from vineyards, or at least reduce the damage to *V. vinifera* caused by feeding, have been investigated since the insect was first recognised in France in the late 1860s. Innovative and eccentric remedies were trialled, including the use of tobacco leaves, oil, sulphur, seawater, phenol, incense and manures (Ordish, 1987). The use of resistant rootstocks is currently the main management strategy used by the viticulture industry to combat the damaged caused by grape phylloxera. Quarantine, chemical, cultural and biological control measures, outlined below, may also be implemented to reduce the impact of grape phylloxera infestation within a vineyard production. There is currently no management strategy for the eradication of grape phylloxera from the viticulture industry.

## *3.1 QUARANTINE MANAGEMENT*

In Australia, the implementation of quarantine protocols during the late 1900s has limited the spread of grape phylloxera into new vineyards, although new infestations continue to occur (Powell *et al.*, 2006; Phylloxera Update, 2007; Kennedy, 2007). The National Phylloxera Management Protocols place restrictions on the movement of grapevine material, including cuttings, roots and fruit, out of grape phylloxera infested regions (National Phylloxera Management Protocol, 2003). Grape phylloxera dispersal may be human assisted by the transport of soil and grapevine material attached to machinery, equipment and footwear (King and Buchanan, 1986), and cleaning procedures for all viticulture equipment is incorporated into the management protocols. An assessment of grape phylloxera survival during a range of wine production post-harvest processes, including transportation, crushing and pressing, highlighted the requirement for the quarantine protocols in order to restrict the accidental movement of the pest insect (Deretic *et al.*, 2003).

### *3.2 CHEMICAL CONTROL*

The most extensively used chemical for grape phylloxera control in the 1800s was carbon bisulphide. The toxic fumigant was injected into the soil at rates of up to 300 g per grapevine, killing grape phylloxera, all other soil biota and in some cases the grapevine itself. Occasionally the grapevine regrew, however carbon bisulphide provided no long-term success due to the constant reinfestation of the vineyard (Ordish, 1987). There has been limited success in the development of a chemical control for radicicolae grape phylloxera due to the distribution of the insect on the root system below-ground.

For success in a soil environment, a downwardly mobile systemic insecticide is required that provides an even distribution of the chemical to all feeding locations, and at sufficient toxicity levels (Coombe, 1963). The value of carbofuran as a possible chemical control agent for grape phylloxera was investigated (Granett *et al.*, 1986; Kim *et al.*, 2002), although the long-term control of grape phylloxera remains an issue (Buchanan, 1990). In a number of viticulture counties of the United State of America, carbofuran is banned due to adverse impacts on bird populations (Weber *et al.*, 1996). More recent studies have found systemic neonicotinoid insecticides to suppress grape phylloxera and improve grapevine vigour under controlled glasshouse conditions, although the effectiveness of the insecticide under field conditions has not been validated (Herbert, 2005).

### *3.3 CULTURAL CONTROL*

Cultural management options generally attempt to manage grapevine nutrition and water requirements for short-term improvements in grapevine vigour, and a general reduction in the stress levels caused by radicicolae grape phylloxera feeding on the root system. The organic management of vineyards infested with grape phylloxera in Germany induced changes in a range of soil biotic and abiotic factors, having a

beneficial impact on grapevine vigour and a reduction in the impact of grape phylloxera (Huber *et al.*, 2003). Field and laboratory trials have also shown a negative correlation between the rate of nitrogen fertiliser application and the development of grape phylloxera induced root nodosities (Kopf *et al.*, 2000). A strong negative correlation was determined from the application of organic matter to grape phylloxera survival rates (Porten *et al.*, 2000), however under Australian conditions the response of grape phylloxera populations to the application of organic matter as composts has been variable. The application of green waste compost increased the number of insects collected in the vineyard during above-ground dispersal periods (Powell *et al.*, 2007a), while composted grape-marc waste material reduced the number of dispersal insects (Powell *et al.*, 2007b).

Vineyard location and soil type can also have an impact of grape phylloxera population dynamics. Grape phylloxera infested vineyards growing on fertile, aerated soils generally outperform infested vineyards on compact, stony soils (Vega, 1956), however grape phylloxera establishment is disrupted if the vineyard is planted on sandy soil (Boehm, 1996). Therefore soil texture, and specifically clay and humus content, is expected to affect grape phylloxera infestation levels (Reisenzein *et al.*, 2007). In flat vineyards, the radicicolae grape phylloxera population may be reduced by flooding the grapevines over the winter months with 0.1 m of water for 40 days (Boehm, 1996).

### *3.4 BIOLOGICAL CONTROL*

Effective biological control measures for grape phylloxera are currently limited to the use of resistant rootstocks, although natural pathogens of the insect are being investigated (Granett, 2000). A range of insect biological control agents exist for gallicolae grape phylloxera (Gorkavenko, 1972; Rack and Rilling, 1978; Wheeler and Henry, 1978; Wheeler and Jubb, 1979), however they have limited impact on the radicicolae population due to the soil distribution of the insect.

Biological control measures currently being examined for radicicolae grape phylloxera include: entomopathogenic nematodes (English-Loeb *et al.*, 1999); mites (Forneck *et al.*, 1998); entomopathogenic fungal species (Goral *et al.*, 1977; Kirchmair *et al.*, 2004); and plasmodiophorid parasites (Huber *et al.*, 2004). The field assessment of the biological control agents has been hindered by the rapid mineralisation of the relatively small insects, and post-infection by other soil bacteria and fungi (Huber and Kirchmair, 2007). To date there has been limited success in validating the application of any individual biological control method to radicicolae grape phylloxera under field conditions.

# 4 GRAPE PHYLLOXERA DIGESTION

Grape phylloxera feed solely on *Vitis* species, therefore understanding the digestion of the monoecious food source is an important consideration for interpreting host-plant interactions. Despite being recognised as a major pest insect to the viticulture industry since the late 1800s, few studies on the processes of digestion, food absorption and waste excretion in grape phylloxera have been published.

## *4.1 STRUCTURE OF THE DIGESTIVE SYSTEM*

The common structure of the digestive system within the Aphidoidea superfamily is based on sucking of liquid food from a plant, through stylet mouthparts into the pharyngeal duct, where the pharyngeal pump forces the food into the foregut, and the food is then vented into the midgut via the oesophageal valve. The midgut is the longest part of the digestive system, and passes waste products into the hindgut and rectum, prior to excretion via the anal opening. A filter chamber absorbs excess fluid from the midgut prior to absorption, although Malpighian tubules for urinary excretion are lacking. The anal opening is generally in a ventral position at the posterior end of Aphididae, although the opening is in a dorsal position in Adelgidae (Ponsen, 1987).

The basic structure of the Phylloxeridae digestive system is similar to other members of the Aphidoidea superfamily, although a number of exceptions exist. The length of the midgut is reduced and no filter chamber is present. However, a major physiological difference that raises questions over the method of waste excretion in Phylloxeridae is the lack of anal dorsal muscles and the absence of visible honeydew droplets (Ponsen, 1997). Grape phylloxera is often reported as not having a complete digestive system due to the lack of anal excretion. Sobetskiy and Derzhavina (1973) reported the loss of the anal opening due to the degeneration of a large section of the digestive system. The intestinal tract has been reported as closed in all developmental stages of grape phylloxera, with the digestive system ending blindly (Federov, 1959); however Schaller (1960) stated that the anal opening closed and inhibited excretion in adults only. The absence of honeydew excretion (Riley, 1870), existing in most gall-inhabiting aphids, is also stated as evidence that anal excretion is lacking in grape phylloxera (Ponsen, 1997).

In the absence of anal excretion, well-developed salivary glands in grape phylloxera have supported proposals for alternative methods for waste disposal. Federov (1959) concluded that excretory organs were not required due to the extra-intestinal digestion of the *Vitis* food source prior to ingestion. The periodic discharge of waste products back into the plant gall via the salivary glands has also been proposed (Sobetskiy and Derzhavina, 1973). Both methods of waste excretion would have an impact on grape phylloxera host-plant interactions. Alternatively, grape phylloxera have been reported to discharge excess waste during oviposition (Rilling, as cited in Forneck *et al.*, 2001), although no evidence of a connection between the digestive and reproductive system was provided.

In contradiction to evidence that grape phylloxera have an incomplete digestive system, Ponsen (1997) reported an anal opening in a similar position to Adelgidae, in a

dorsal orientation at the posterior end of the insect. Ponsen (1997) however questioned the ability of grape phylloxera to excrete waste through the anal opening due to the absence of anal dorsal muscles.

The structure of the digestive system of grape phylloxera remains unclear; however an understanding of the processes of digestion, food absorption and waste excretion is important for the interpretation of host-plant interactions. The structure of the digestive system of grape phylloxera can influence insect feeding behaviour, survival, developmental rate and fecundity levels, and therefore contribute to the success of the insect on *Vitis* species.

### *4.2 ENDOSYMBIONTS*

Endosymbiont organisms include gram-negative bacteria and yeasts which live either intracellularly or free in the haemolymph of a large number of insects (Houk, 1987). Endosymbionts provide essential elements to the diet of the insect not obtained directly from feeding, including the biosynthesis of amino acids, sterols, vitamins or the enzyme polysaccharase for carbohydrate digestion (Houk, 1987).

All Aphidoidea feeding on phloem sap have an obligatory relationship with endosymbionts; the *Buchnera* bacterial endosymbiont is present in Aphididae, although the Adelgidae and Phylloxeridae are excluded from this association (Houk, 1987; Douglas, 1998). Evolutionary divergence of the Phylloxeridae family from the Aphididae occurred prior to the infection of Aphididae with *Buchnera* (Martinez-Torres *et al.*, 2001). Additionally, symbionts are not expected to be required by grape phylloxera due to the insect feeding on parenchyma cells, which have a different nutrient profile to the phloem sap of the Aphididae diet (Buchner, 1965).

Early microscopy studies identified endosymbiont structures in members of the Phylloxeridae, and bacterial symbionts were cultured from grape phylloxera (Buchner,

1965; Ponsen, 1997). However the detection of endosymbionts in Phylloxeridae was transient, and grape phylloxera was concluded not to contain bacterial endosymbionts. However, a more recent study utilising molecular and morphological techniques identified the presence of bacterial DNA in gallicolae grape phylloxera (Vorwerk *et al.*, 2007).

The status of bacterial endosymbionts within radicicolae grape phylloxera has not been determined with the use of molecular technology. Obligatory endosymbiont relationships in other insects may be exploited for the benefit of pest management (Douglas, 1998), therefore the confirmation of endosymbiont status for radicicolae grape phylloxera may provide an opportunity for the development of an alternative management strategy. Interpreting the relationship between grape phylloxera and the bacterial association would also provide insight into the nutritional requirements of the insect.

## 5 GRAPE PHYLLOXERA NUTRITIONAL REQUIREMENTS

The reliance of grape phylloxera on *Vitis* species for survival suggests a unique evolutionary relationship, including specialised sensory recognition of the host-plant. However the nutritional basis of this relationship is poorly understood. Unlike members of the Aphididae family, grape phylloxera feed on parenchyma cells and settle on a feeding location prior to reaching phloem (Kellow *et al.*, 2004). Although the exact chemistry of the parenchyma cell food source is unknown, the nutritional requirements of grape phylloxera can be inferred by investigating the general chemistry of the grapevine root, and changes in chemistry with gall development.

### *5.1 GRAPEVINE CHEMISTRY*

The nutritional physiology of grapevine roots change with seasonal development and vineyard management, affecting abscisic acid, sugar and amino acid profiles (Stoll *et al.*, 2000). These changes are related to the growth stage of the grapevine and are known to influence radicicolae grape phylloxera development, with higher rates of survival and fecundity observed post-harvest during periods of high sucrose loading in the root parenchyma cells (Omer *et al.*, 2002). Additionally, the impact of cultural management on the observed level of grape phylloxera damage also indicates that vineyard management and grapevine physiology influences the pest insect population (Huber *et al.*, 2003). Generally however the effect of changes in the host-plant chemistry on grape phylloxera feeding, survival and fecundity is poorly understood.

Localised changes in sugar concentrations, particularly an increase in sucrose, have been identified by investigating the changes in grapevine physiology surrounding grape phylloxera feeding sites (Ryan *et al.*, 2000). Starch, amide and amino acids have also been shown to accumulate at the grape phylloxera feeding site during gall development (Forneck *et al.*, 2002; Kellow *et al.*, 2004), allowing the root nodosity to function as a nutrient sink for insect growth and development.

### *5.2 ARTIFICIAL DIET DEVELOPMENT*

The limited knowledge of the exact diet chemistry of radicicolae grape phylloxera increases the complexity of developing an artificial diet formulation for the insect. Artificial feeding systems have been developed for a range of Aphididae species since the late 1960s, with some species able to be maintained on near permanent cultures maintaining 18-20 generations (Dadd and Mittler, 1966; Akey and Beck, 1971). However, nearly all published Aphididae diets are designed for vascular tissue leaf-feeders, with little detail on root-feeding insects. Sobetskiy and Derzhavina (1973) studied the chemistry of gallicolae grape phylloxera leaf galls and found higher levels of

nitrogen and reduced levels of carbohydrate substances in comparison with the diet of phloem feeding Aphididae. Nevertheless there are similarities in the essential dietary components included in the artificial diet formulations for a wide range of insect species, including amino acids, lipids, vitamins and minerals (Cohen, 2003).

Wöhrle (1999) investigated the development of an artificial feeding system for gallicolae grape phylloxera, and formulated an artificial diet solution capable of maintaining fourth instars for a maximum of 15 days. The artificial diet formulations of '5% sucrose' and '5% sucrose plus an amino acid mix' were equally successful, and gallicolae grape phylloxera survival was significantly longer on these diet formulations than on '2% sucrose', '2.5% glucose plus 2.5% fructose' and the control of 'water only'. The successful sucrose concentration of 5% was comparatively low compared with optimal concentrations for the pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae), of 20-25% sucrose (Srivastava and Auclair, 1971). However due to differences in grapevine root and leaf chemistry, the artificial diet formulation developed for gallicolae grape phylloxera may not be suitable for radicicolae grape phylloxera. Rilling *et al.* (1975) found that free amino acid profiles differed between grapevine leaf and root galls induced by grape phylloxera feeding. Previously, Rilling and Steffan (1972) had identified differences in sugar utilisation between the leaf and root feeding insects, and found that radicicolae grape phylloxera ingested 1.5 times the volume of sucrose in comparison with gallicolae grape phylloxera.

The development of an artificial feeding system for radicicolae grape phylloxera would have multiple applications, including the application as a bioassay for the rapid testing of new control agents, the investigation of essential nutritional requirements, and predicting the impact of vineyard management practices on grape phylloxera population dynamics.

# 6 GRAPE PHYLLOXERA FEEDING BEHAVIOUR

Current rootstock resistance trials infer resistance to radicicolae grape phylloxera feeding activity based on insect survival rates and root gall formation (Kellow *et al.*, 2002; Powell *et al.*, 2006). However these experiments infest the grapevine roots with eggs, and all future host-plant interactions are dependent upon the establishment and survival of first instars. These trials therefore deliver no outcomes based on the interactions of later life stages of grape phylloxera with the *Vitis* species, and do not directly provide information about the feeding behaviour of the insect during the establishment of a feeding site and gall indication. As a consequence, the feeding behaviour of radicicolae grape phylloxera is poorly understood.

## *6.1 FEEDING LOCATION*

Grape phylloxera penetrate the cortex of the root on an intracellular pathway to a parenchyma cell food source (Pollard, 1973). The stylet of the insect progresses along on a row of adjacent cells toward the stele, although vascular tissue is not penetrated. Penetrated parenchyma cells accumulate tannins and are collapsed in appearance, and although surrounding cells appear granular in content they are generally not damaged (Niklowitz, 1954; Kellow *et al.*, 2004). The chemistry of the radicicolae grape phylloxera feeding site is unknown as previous light microscopy studies were unable to differentiate the exact position of the stylet tip within the parenchyma cell, or the mechanism of solute transport (Kellow *et al.*, 2004).

Fluorescent light microscopy studies have displayed only one stylet track at each feeding site on susceptible *V. vinifera*. As grape phylloxera is a sedentary feeder, it is speculated that the insect is able to mobilise nutrients to maintain sufficient food intake to support survival and reproductive demands (Kellow *et al.*, 2004). The saliva injected into the plant tissue by the stylet of the insect may induce this plant response. Based on the Aphididae model, grape phylloxera is expected to develop a stylet sheath when

feeding and inject two types of saliva into the plant. Viscous saliva hardens to form the stylet sheath, and the watery saliva contains compounds which assist stylet entry into the plant, extracellular feeding and food intake into the stylet food canal (Chapman, 1969).

### *6.2 PROBING ACTIVITY*

The maintenance of radicicolae grape phylloxera populations on some resistant *Vitis* varieties highlights the potential for insect adaptation leading to rootstock resistance breakdown. Grape phylloxera feeding behaviour on both susceptible and resistant *Vitis* species is an essential component of host-plant interactions, however there is limited information available relating to this area of research. Grape phylloxera feeding behaviour can be interpreted by examining probing activity with the application of the electrical penetration graph (EPG). EPG amplifies the electrical signal that is created when the stylet of a fluid feeding insect penetrates a conductive food source during a feeding event (Tjallingii, 1988). The pattern of the electrical signal generated is dependent upon the feeding activity of the insect, and correlation of the EPG waveforms with insect activity allows for the interpretation of host-plant interactions and insect feeding behaviour.

First developed for Aphididae, EPG has been applied to a range of piercing arthropods (Reese *et al.*, 2000). EPG analysis is able to differentiate the feeding behaviour of an insect based on the processes of stylet penetration, stylet pathway to the food source, salivation and ingestion. EPG waveforms provide information on the feeding location of the insect within the plant food source, and EPG technology can be applied to investigate the differential feeding behaviour for an insect on a range of host-plants, including susceptible and resistant varieties (van Helden and Tjallingii, 2000). The detail obtained on insect feeding behaviour from EPG studies may assist with the

determination of host-plant resistance mechanisms and virus transmission characteristics.

The application of EPG technology to grape phylloxera has not been reported, however examining the probing behaviour and feeding activity of radicicolae grape phylloxera is important for assessing variable survival and fecundity rates on susceptible and resistant *Vitis* species. Interpretations of the resistance mechanisms for rootstocks resistant to grape phylloxera feeding will assist in ensuring the continued long-term success of resistant rootstocks as a management option for the pest insect.

## 7 OUTLINE OF EXPERIMENTAL RESEARCH

Grape phylloxera is a major pest of the viticulture industry worldwide, however there is currently no means of eradicating the pest insect once a vineyard becomes infested. Resistant rootstocks are at present the only long-term management option; however due to the success of resistant rootstocks a number of questions relating to the biology, nutritional requirements and feeding behaviour of the radicicolae grape phylloxera have not been examined. The investigation of these areas remains an important consideration for grape phylloxera research in order to develop alternative management strategies that directly target the biological characteristics of the insect. Investigating the insect relationship to host-plant interactions with resistant rootstocks is also important to ensure the continued success of the viticulture industries main management strategy. The research described in this thesis makes a contribution to the current understanding of the fundamental interactions between radicicolae grape phylloxera and *Vitis* species.

The thesis has been divided into several chapters to address a range of questions relating to the biology, nutritional requirements and feeding behaviour of the radicicolae grape phylloxera. The content of each self-contained chapter, including four data chapters and a final conclusion, is briefly outlined below.

The internal and external morphology of radicicolae grape phylloxera was examined with the application of scanning electron microscopy and light microscopy techniques (Chapter 2). A number of biological features relating to host-plant interactions were analysed, including specific morphological changes linked to the reproduction of parthenogenetic eggs.

Bacterial culturing and morphological techniques, together with molecular identification, was used to determine the status and importance of bacterial symbiotic associations with radicicolae grape phylloxera (Chapter 3).

The development of an artificial feeding system for radicicolae grape phylloxera was trialled (Chapter 4). The impact of diet chamber design and the importance of a number of chemical components on grape phylloxera survival were identified.

Grape phylloxera feeding behaviour on *V. vinifera* was examined with the application of EPG (Chapter 5). Differences in feeding behaviour based on grape phylloxera life stage were identified, and initial 'proof of concept' trials involving feeding behaviour of resistant rootstocks were undertaken.

In the concluding chapter, the interrelated conclusions extending from the collective areas of grape phylloxera research were identified, and future directions highlighted (Chapter 6).

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# CHAPTER 2

# STRUCTURE OF THE DIGESTIVE SYSTEM OF GRAPE PHYLLOXERA

Sections of this chapter are currently *in press* for publication as part of the *Proceedings of the Third International Phylloxera Symposium*:

Kingston, K. B., Cooper, P. D. and Powell, K. S. (2007). Grape phylloxera external morphology observations under scanning electron microscopy. *Acta Horticulturae*.

# 1 SUMMARY

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) is a major pest of the viticulture industry, however limited research is published relating to the structure of the digestive and reproductive biology of the insect. The current study re-examined the structure of the digestive system of radicicolae grape phylloxera to clarify a number of features important for host plant interactions. Light microscopy sections of grape phylloxera highlighted a midgut compartmentalised into two sections, an anterior and a posterior region, separated by the hindgut connection. The division and dorsal connection to the hindgut that had not previously been documented. The midgut may be compressed during the development of parthenogenetically produced eggs in adult insects; eggs are approximately 40% of the adult length and in volume internally occupy around 5% of the body cavity. The midgut storage chamber was hypothesised to be essential for the continual supply of energy during periods of reduced food intake. The presence of the hindgut and an anal opening indicated that waste excretion occurred through an anus, although excretion was not observed. In the absence of anal dorsal muscles, longitudinal muscles were proposed to provide sufficient pressure in the hindgut chamber to ensure the expulsion of low levels of fluid waste. The structure of the digestive system of radicicolae grape phylloxera was atypical, containing adaptations that possibly assist with insect dispersal to a new *Vitis* food source.

## 2 INTRODUCTION

The common structure of the digestive system within the Aphidoidea superfamily is based on the sucking of liquid plant food through stylet mouthparts into the pharyngeal duct, where the pharyngeal pump forces the food into the foregut, and the food is then vented into the midgut via the oesophageal valve. The midgut is the longest part of the digestive system, and passes waste products into the hindgut and rectum, prior to excretion via the anal opening. A filter chamber passes excess fluid directly from the foregut, and therefore prior to absorption in the midgut, to the hindgut for excretion. Malpighian tubules for urinary excretion are lacking. The anal opening is generally in a ventral position at the posterior end of the aphid, although the opening is located dorsally in the Adelgidae sister group (Ponsen, 1987).

The digestive system of grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) has previously been investigated by a number of researchers since first being recognised as an economically significant pest to the viticulture industry in the late 1800s. Most studies concluded that the insect does not have a complete digestive system, and therefore lacks the capacity for anal waste excretion. Grape phylloxera do not provide any visible clues for anal waste excretion due to the absence of sugary honeydew production (Riley, 1870). Evidence supporting the theory that grape phylloxera lacks anal waste excretion included the degeneration of a large portion of the gut, the loss of the anal opening (Sobetskiy and Derzhavina, 1973), and a closed intestinal tract, or midgut, that resulted in a blind-ending digestive system (Federov, 1959). Extra-intestinal digestion was cited as the reason for the lack of excretory organs in grape phylloxera. Studies have also implied that the anal opening of grape phylloxera closes during life stage development, and anal excretion was only inhibited in adults (Schaller, 1960). Alternative methods of waste excretion proposed for grape phylloxera included the periodic elimination of waste back into the grapevine gall via

the salivary glands (Schaller, 1960; Sobetskiy and Derzhavina, 1973) and waste excretion during oviposition (Rilling, as cited in Forneck *et al.*, 2001). The excretion of waste products into the host-plant has been associated with cell division and gall development (Hori, 1992), therefore determination of the mode of waste excretion in radicicolae grape phylloxera has implications of investigating host-plant interactions.

Limited studies have provided evidence for a complete digestive system in grape phylloxera. Diagrams presented by Breider (1952) indicated an anal opening dorsal to the gonopore in gallicolae grape phylloxera. Ponsen (1997) provided a major contribution to the understanding of the digestive system of grape phylloxera during a histological description of the alimentary tract of members of the Phylloxeridae. Ponsen's review (1997) included both gallicolae and radicicolae grape phylloxera.

The internal digestive system of grape phylloxera was concluded to contain a short foregut, stomach, crenated intestine, caecal intestine, rectum and an epidermal invagination terminating at the anal opening (Ponsen, 1997). A sharp loop in the digestive system marked the transition of the stomach to the intestine. The caecal intestine, which had no cuticular lining, was defined as an open connection between the crenated intestine and posterior region of the alimentary tract between the rectum and the anal opening, where cuticular lining was present. Ponsen did describe two species of Phylloxeridae as having an incomplete digestive system with a blind-ending intestine, however neither of these were grape phylloxera.

Characteristic features of Phylloxeridae (in comparison with Aphididae) included an enlarged salivary pump, a short crenated intestine, the absence of a filter chamber, the absence of anal dorsal muscles and a lack of honeydew production (Ponsen, 1997). A complete digestive system was identified in grape phylloxera, but the lack of anal dorsal muscles (typically used to open an anal opening for waste excretion) provided an explanation for the unobserved levels of honeydew production and waste excretion.

Grape phylloxera reproduce by parthenogenesis, and have a high reproductive capacity which results in elevated population levels of the insect in vineyards during the summer months of the growing season. Adult grape phylloxera lay 3-6 eggs per day during a reproductive period of 1-2 months (Granett *et al.*, 1983). Following the establishment of a feeding site, the sedentary feeders remain attached to the same feeding location during life stage development, and accommodate for the continual production of eggs by repositioning the posterior end during oviposition (De Klerk, 1974). The energy requirements for the continual egg production would be high, and the rate of food intake would need to equal the rate of energy and water loss during oviposition. Grape phylloxera may feed on non-vascular cells that are higher in protein and contain less fluid and carbohydrate when compared with the Aphididae diet of phloem sap (Ponsen, 1997). However it remains unclear what digestive and reproductive features of grape phylloxera accommodate for the high rate energy and water intake necessary for of egg production.

Despite previous research into the digestive system of grape phylloxera, several questions remain to be addressed. The degree of contradiction in previous publications regarding the completeness of the grape phylloxera digestive system, and method of waste excretion require further investigation. Relationships between the digestive and reproductive systems of grape phylloxera have also not been addressed. Few publications exist on the external morphology of grape phylloxera, and these investigations were limited to light microscopy technology (Davidson and Nougaret, 1921; De Klerk, 1974; Buchanan, 1990). Therefore the current study utilised advanced techniques over previous research, including scanning electron microscopy and thin sectioning, to review the external and internal morphology of grape phylloxera. Light microscopy measurements of the external features of radicicolae grape phylloxera were obtained to validate the application of the more advanced techniques.

# 3 MATERIAL AND METHODS

## *3.1 INSECT MATERIAL*

Radicicolae grape phylloxera were originally collected from infested vineyards in Victoria, Australia. Populations were maintained at the Department of Primary Industries – Rutherglen Centre on excised *Vitis vinifera* L. (Vitaceae) root pieces (approximately 1 cm width x 10 cm length), prepared using a protocol slightly modified from Granett *et al.* (1985). The modifications were: (1) roots washed clean of attached soil with a soft brush under running water; (2) roots soaked for 5 minutes in Ridomil® Gold Plus systemic fungicide solution (2.3 g/L); (3) roots triple rinsed with sterile distilled water prior to air drying; (4) both ends of roots wrapped in moist cotton wool; and (5) the size of the petri dish was increased to 15 cm, with the increase in area reducing the level of water condensation within the chamber. Grape phylloxera populations were incubated in the dark at a constant temperature of 25 ± 3°C.

Only apterous radicicolae grape phylloxera were used for the current study, and developmental life stages were determined by comparative increases in size. The presence of nearby eggs indicated adult reproduction. If present, neighbouring moulted cuticles were counted to confirm the insect life stage. For collection, insects were handled using a moist sable-haired paintbrush to avoid damage.

## *3.2 OVIPOSITION DURATION*

Apterous adult grape phylloxera were observed *in situ* on the excised root piece to determine the time required to complete oviposition. Insect activity was viewed with a stereo microscope and JVC camera (TK-C1381), and simultaneously video captured by a VHS recorder (LG-FC930W). Video tapes were reviewed to observe the time required to completely deposit an egg, and the time period between oviposition. The beginning of oviposition was defined by the extension of the posterior end of the insect

and visualisation of the egg surface. Oviposition was considered complete when the egg was fully emerged and detached from the posterior end of the insect.

## *3.3 EXTERNAL MORPHOLOGY*

Observations of the external body features of grape phylloxera were made using both light and electron microscopy techniques. Light microscopy enabled measurements to 10 µm degree of accuracy, while higher levels of resolution were achieved with electron microscopy and measurements made to 1 µm degree of accuracy. Comparison of external body measurements from live insects via light microscopy and fixed insects via electron microscopy assisted in validating the use of the electron microscopy technique, and to determine the level of artefact introduced by preparative fixation techniques. All apterous life stages were observed.

### *3.3.1 LIGHT MICROSCOPY*

Insects were collected from excised root pieces and placed onto moist filter paper in a 3.5 cm petri dish. Measurements (±10 µm) were immediately taken of the length, width and depth of each live insect using an Olympus BH2 microscope and a graduated eyepiece.

### *3.3.2 SCANNING ELECTRON MICROSCOPY*

Several preparation techniques were trialled for viewing grape phylloxera with scanning electron microscopy (SEM). SEM was performed in a Cambridge Instruments Stereoscan 360 SEM. The external morphology of the insects were examined using a 15kV electron beam, with images captured digitally.

#### *i. AIR DRYING*

Insects were collected from excised root pieces and stored in 100% ethanol. Insects were mounted onto a SEM stub with clear nail polish and air-dried overnight, prior to 20 nm gold plate coating.

#### *ii. CRITICAL POINT DRYING*

Insects were collected from excised root pieces and fixed either with 2.5% glutaraldehyde or 100% ethanol. Insects were post-fixed with 1% osmium tetroxide, placed in a porous vial and immersed in 100% ethanol overnight before undergoing critical point drying using the dry ice technique. Insects were mounted onto the SEM stub with double-sided tape and gold coated (20 nm).

#### *iii. COLD STAGE*

Insects were collected from excised root pieces into cryotubes, snap frozen in liquid nitrogen and stored at -80°C until required. Insects were fixed onto cold stage stubs with a mounting paste of 50:50 graphite and cryo-protectant, and then frozen in liquid nitrogen. Insect stubs were coated with 10 nm of gold and immediately transferred to the cold stage SEM. Measurements of external body features (±1 µm) were made from the SEM images using ImageJ public domain software (Rasband, 2005). Stylet and labium length were measured as an indicator of possible feeding locations within the root structure, and examination was made of the posterior end of the insect for evidence of an anal opening. Antennae length was also measured. Sample numbers used for each life stage measurement and observation were variable due to the loss of some insects during preparation. The small size of grape phylloxera and the speed of preparation required for cold stage SEM prevented the correct orientation of all insects prior to gold coating. Incorrect orientation prevented the measurement of all morphological features on some individuals. Some insects were physically damaged during preparation and not used for morphological descriptions.

### *3.4 INTERNAL MORPHOLOGY*

Observations of the internal structure of the digestive system of grape phylloxera were made using both light and electron microscopy techniques. Longitudinal and transverse light microscopy thin sectioning provided a morphological characterisation

of the structure of the midgut, hindgut and reproductive organs of the insect. Cold stage SEM provided an *in situ* representation of these features. Apterous fourth instar and adult life stages were used for both studies to compare changes as the insect developed into the reproductive life stage. Adult insects were defined by the internal presence of developing eggs.

### *3.4.1 LIGHT MICROSCOPY*

Insects were collected from excised root pieces and rinsed with sterile distilled water. Samples were immersed in 2.5% glutaraldehyde solution and fixed either overnight at 4°C or for a few hours on ice to allow penetration of the insect body parts. The glutaraldehyde solution was removed and the insects rinsed several times with buffer solution (38.5 ml 4.52% $NaH_2PO_4.6H_2O$, 6.5 ml 5.04% NaOH, pH 7.2). Insects were stored in 70% ethanol until required, then prepared for sectioning with a series of ethanol washes (1 x 95%, 2 x 100%) prior to embedding in LR White Resin. A series of ethanol:resin washes (2:1, 1:2, 100% resin) occurred on ice under vacuum for 30 minutes. Samples were set in a resin cap at 60°C under nitrogen gas overnight. Longitudinal and transverse sections (1 µm) were cut using a glass knife on a Reichert microtome (Austria OmU2). Sections were heat-dried onto glass slides, stained with 0.05% toluidine blue solution, and mounted under a cover slip with Permount® solution.

Insect sections (4 fourth instar and 7 adult) were viewed using an Olympus AX70 Provis microscope and digital images captured with a SPOT RT™ (Diagnostic Instruments) camera and software. Measurements (length and width) were made of the maximum dimensions of the insects external body, digestive system (midgut and hindgut) and developing eggs (adults only). Light microscopy measurements (±10 µm) were made using an Olympus BH2 microscope and a graduated eyepiece. Additional measurements were made from the digital images using ImageJ public domain software (Rasband, 2005).

### 3.4.2 *Scanning Electron Microscopy*

Insects were collected from excised root pieces into cryotubes, snap frozen in liquid nitrogen and stored at -80°C until required. Insects (7 fourth instar and 8 adult) were fixed onto cold stage stubs with a mounting paste of 50:50 graphite and cryo-protectant, and re-frozen in liquid nitrogen. Individual insects were fractured using a triangular glass knife cryomicrotome (Reichert FC4) prior to placing the stub into the cold stage SEM. The SEM chamber was held at -196°C using liquid nitrogen. The specimens were initially etched with the electron beam to improve visualisation, prior to coating with 20 nm of gold. Measurements (±1 µm) were made from the SEM images using ImageJ public domain software (Rasband, 2005).

## 3.5 *Volume Calculations*

The volume of selected grape phylloxera morphology features was calculated for an approximate comparison of cavity size. Of interest was variation in the cavity size of internal features (midgut, hindgut and developing egg) of fourth instar and adult insects; changes in size of internally developing eggs in comparison with externally oviposited eggs; and the volume of externally deposited droplets of fluid. Due to the cylindrical-rounded shape of these features, the ellipsoid volume formula was used: $^1/_6\pi$(length x width x height). For morphology features where only the length and width measurements were known, the width value was assumed to also equal height.

## 3.6 *Statistical Analysis*

GenStat-Eighth Edition© (Lawes Agricultural Trust) was used to calculate the mean and standard deviation of all measurements. Statistical differences in external body measurements between life stages, from both the light and electron microscopy techniques, was determined by general analysis of variance (ANOVA). The unbalanced sample number for SEM external body measurements was resolved by the inclusion of missing values to ensure a standard sample number (n = 7); the light

microscopy measurements were balanced (n = 10). ANOVA was also used for comparison of the within life stage statistical relatedness between the light microscopy and SEM external body measurements; all sample numbers were adjusted with missing values to ensure an even sample number (n = 10). Statistical significance for all ANOVA tests was based at $p < 0.05$. The sample number for other measurements (internal dimensions and volume calculations via light microscopy and SEM) were too low for statistical analysis.

# 4 RESULTS

Video observation of adult grape phylloxera activity on excised root feeding material determined that the time required for egg oviposition, where an egg passed through the gonopore and separated from the posterior end of the insect, was 43 ± 34 minutes (mean ± standard deviation, n = 6). Subsequent oviposition activity, with the expansion of the posterior end and visualisation of the egg surface, was observed for an individual insect within 180 minutes (n = 1). Although the egg was moist at the time of oviposition, honeydew excretion was not observed in live insects.

## *4.1 EXTERNAL MORPHOLOGY*

Measurements of the external body dimensions of grape phylloxera were determined by both light microscopy and SEM techniques. The use of SEM for external body measurements was opportunistic following the use of the technique to view the fine external morphology features of the insect. Comparisons of external body dimensions from SEM images with live light microscopy measurements assisted in validating the use of the SEM technique to observe the external morphology of grape phylloxera, and provided a comparison for possible artefacts induced by SEM preparation.

### *4.1.1 EXTERNAL MEASUREMENTS BY LIGHT MICROSCOPY*

Light microscopy measurements of live grape phylloxera indicated an approximate 20% increase in both length (anterior-posterior) and width (lateral) between each life stage (Table 1). There was a significant increase in size with life stage for all external body measurements; except for the width expansion between eggs and first instars, and the depth expansion between first and second instars. Adult grape phylloxera measured 690 µm in length and 387 µm in width. Fertile eggs were 42% of the reproductive adult length.

**Table 1.** Live grape phylloxera external body measurements of all apterous life stages, from light microscopy observation. Sample number n = 10 for all life stages.

| life stage | length[1] (µm) | width[2] (µm) | depth[3] (µm) |
|---|---|---|---|
| adult | 690 ± 61[a] | 387 ± 23[a] | 297 ± 55[a] |
| fourth instar | 591 ± 42[b] | 310 ± 22[b] | 218 ± 20[b] |
| third instar | 491 ± 20[c] | 267 ± 19[c] | 178 ± 16[c] |
| second instar | 394 ± 30[d] | 199 ± 17[d] | 131 ± 18[d] |
| first instar | 326 ± 31[e] | 160 ± 13[e] | 107 ± 11[d] |
| egg | 288 ± 9[f] | 150 ± 13[e] | NA |
| ANOVA p | < 0.001 | < 0.001 | < 0.001 |
| l.s.d. | 34.29 | 14.78 | 27.16 |

Mean values ± standard deviation, n = 10; measurements of egg depth not applicable (NA).
Insect orientations: [1] anterior – posterior measurement, [2] lateral measurement, [3] dorsal – ventral
[abcdef] denotes significant difference groupings of mean values, ANOVA p and l.s.d. values indicated

### *4.1.2 SEM TECHNIQUE COMPARISONS*

Air drying and critical point drying preparation for SEM resulted in the compression and shrinkage of the external cuticle of grape phylloxera (Figures 1a and 1b). The splitting of the stylet was an induced artefact of these techniques (Figure 1c). Cold stage SEM provided the optimum preparation for grape phylloxera (Figure 1d), with no evidence of cuticle compression or other artefacts induced by the liquid nitrogen snap freezing preparation of the insects.

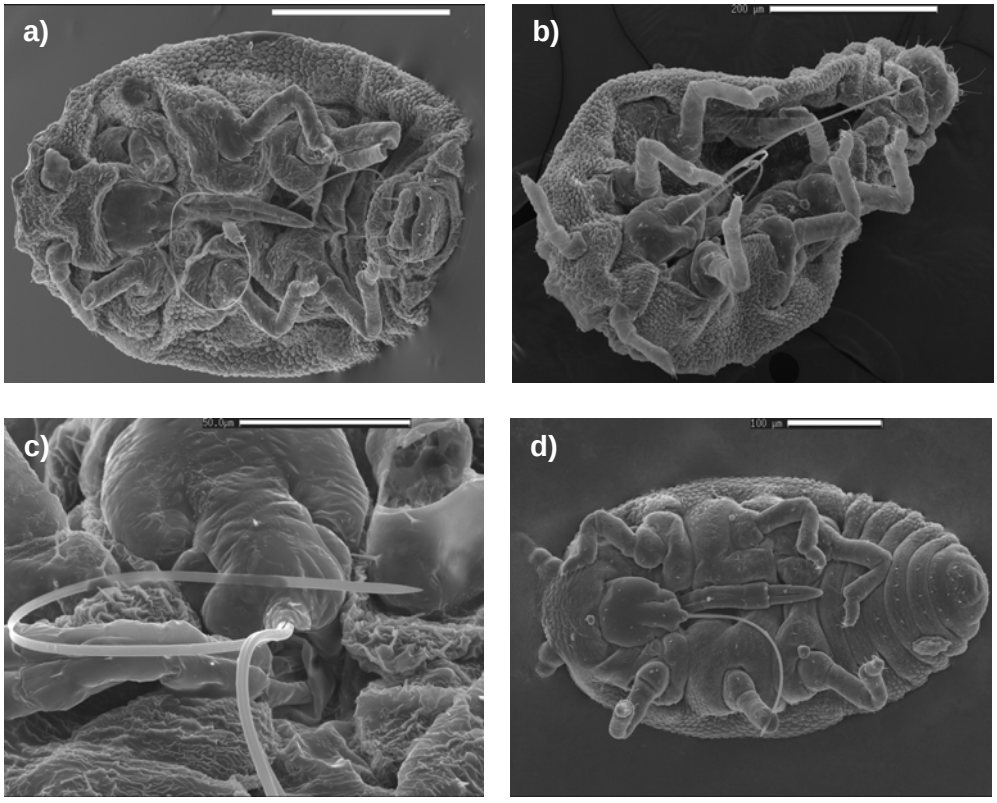


**Figure 1.** Grape phylloxera comparative structural damage caused by SEM sample preparation: **a)** cuticle compression observed with air drying, scale bar 200 µm; **b)** cuticle compression induced by critical point drying, scale bar 200 µm; **c)** stylet splitting experienced with both air and critical point drying procedures, scale bar 50 µm; **d)** no cuticle compression was observed with cold stage SEM preparation, scale bar 100 µm.

### *4.1.3 EXTERNAL MEASUREMENTS BY SEM*

Cold stage SEM measurements of frozen grape phylloxera indicated a continual increase in both length and width with each developmental life stage (Table 2), although the increase in size was not always statistically significant. The adult life stage recorded the largest external body measurement, length 701 µm and width 426 µm, and was significantly 42% larger (length and width) than the fourth instar. The fourth instar was significantly 20% larger (length and width) than the third instar, however the length of the third instar was not significantly more than the second or first instars where there was 10-15% growth with each life stage. Eggs were the same size as first instars, but smaller than the second instar developmental life stage. Oviposited eggs were 41% of the reproductive adult length (Table 2).

ANOVA comparison of grape phylloxera external body measurements (length and width) by the light microscopy and SEM techniques indicated that the two methods provided comparable results. The light microscopy length measurements were generally larger than the SEM measurements, however there was no significant difference between the two techniques for the egg, first instar, second instar and adult life stages; the third and fourth instar SEM measurements were significantly smaller ($p = 0.010$). The light microscopy width measurements were also generally larger than the SEM measurements, however there was no significant difference between the egg, first, second, third or fourth instar values; the adult SEM measurement was significantly larger than the light microscopy measurement ($p = 0.037$). The comparable external body measurements presented by the two techniques indicated that cold stage SEM sample preparation did not induce cuticle shrinkage or distortion, and therefore cold stage SEM was a suitable method for viewing the fine external morphology features on grape phylloxera.

**Table 2.** External morphology measurements of all grape phylloxera apterous life stages from cold stage SEM images. Some body features are not applicable (NA) to developed eggs.

| life stage | external body (µm) | | mouthparts (µm) | | posterior opening | antennae (µm) | |
|---|---|---|---|---|---|---|---|
| | length[1] | width[2] | labium | stylet | (µm) | right | left |
| adult | 701 ± 155$^{a}$ (6) | 426 ± 122$^{a}$ (7) | 187 ± 54 (4) | 282 ± 50$^{a}$ (4) | 29 ± 5.0$^{a}$ (2) | 124 ± 20$^{a}$ (3) | 114 ± 4.6$^{a}$ (3) |
| fourth instar | 495 ± 33$^{b}$ (7) | 300 ± 33$^{b}$ (7) | 136 ± 16 (5) | 211 ± 40$^{b}$ (6) | 24 ± 12$^{ab}$ (5) | 95 ± 12$^{b}$ (3) | 95 ± 9.0$^{b}$ (3) |
| third instar | 414 ± 37$^{c}$ (4) | 242 ± 2.1$^{c}$ (4) | 133 ± 26 (3) | 162 ± 28$^{bc}$ (4) | 15 ± 5.0$^{c}$ (2) | 100 ± 2.9$^{bc}$ (3) | 87 ± 14$^{b}$ (3) |
| second instar | 361 ± 33$^{c}$ (5) | 183 ± 8.6$^{cd}$ (5) | 164 ± 57 (2) | 149 ± 19$^{c}$ (3) | 18 ± 2.5$^{b}$ (3) | 90 ± 2.5$^{c}$ (3) | 91 ± 6.4$^{b}$ (2) |
| first instar | 324 ± 42$^{cd}$ (6) | 154 ± 19$^{de}$ (6) | 158 ± 27 (6) | 124 ± 0$^{d}$ (1) | 22 ± 2.6$^{ab}$ (6) | 84 ± 16$^{c}$ (6) | 91 ± 4.2$^{b}$ (5) |
| egg | 284 ± 21$^{d}$ (2) | 126 ± 22$^{e}$ (2) | NA | NA | NA | NA | NA |
| ANOVA p | < 0.001 | < 0.001 | 0.232 | < 0.001 | 0.007 | 0.003 | 0.002 |

Mean values ± standard deviation; sample number in parenthesis (n) was variable for each morphology feature

External body insect orientations: [1] anterior – posterior measurement, [2] lateral measurement

$^{abcde}$ denotes significant difference groupings of mean values, ANOVA p-value indicated

### *4.1.4 EXTERNAL MORPHOLOGY OBSERVATIONS BY SEM*

The external body size of grape phylloxera increased with developmental life stage (Table 2), but there was limited change in the external morphology of grape phylloxera between instars (Figure 2). The largest increase in size (42%) was between the fourth instar and adult life stages. Adult grape phylloxera displayed a visual increase in size, and an expansion or swelling of the posterior region (Figure 2e), as evidenced in the SEM images. This expansion was due to the development and oviposition of relatively large eggs (Figure 2f).

The stylet and labium of grape phylloxera was positioned in a medial anterior-posterior orientation, although displacement of the stylet from the labium grove was evident in some SEM images (Figure 2). Stylet length increased with life stage and was significantly longer in adults compared with fourth instars; fourth instar stylet length was the same as third instars and third instar stylet length was the same as second instars (Table 2). There was only one stylet length measurement for first instar insects as the insects were collected prior to feeding and the stylet was still enclosed within the labium. The first instar stylet length measurement was significantly the shortest, and although the stylet length of adults (282 µm) was 227% the length of first instars (124 µm), there was no change in the ratio of stylet length to external body length. There was no significant difference in the length of the labium with life stage, and overall mean length for all life stages was 201 µm.

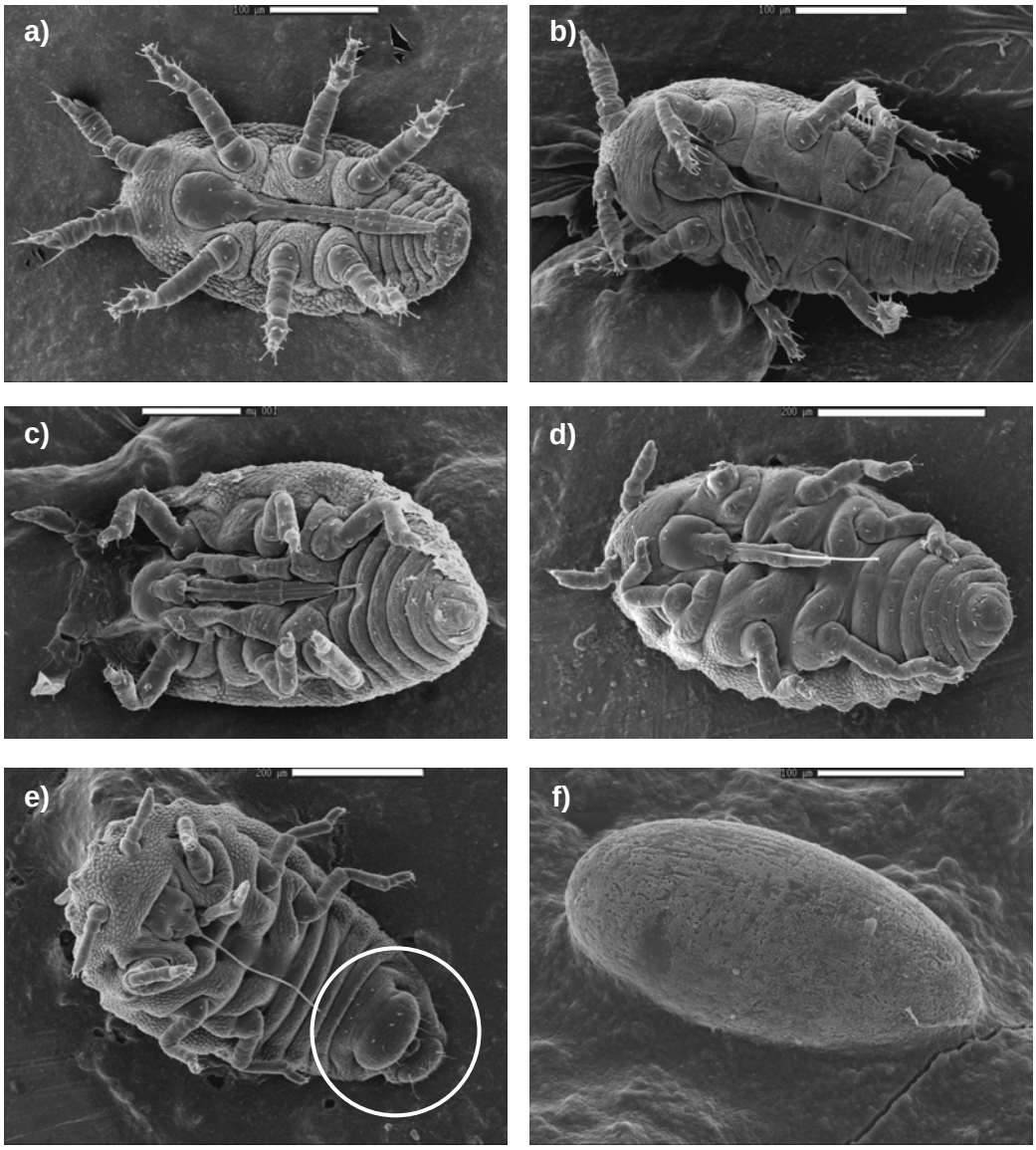


**Figure 2.** Cold stage SEM images of all apterous life stages of grape phylloxera: **a)** first instar, scale bar 100 µm; **b)** second instar, scale bar 100 µm; **c)** third instar, scale bar 100 µm; **d)** fourth instar, scale bar 200 µm; **e)** adult; the circle highlights the swelling of the posterior region, scale bar 200 µm; **f)** egg; note that the base is sunken into the stub mounting paste, scale bar 100 µm. In images a)-e) the stylet was positioned in a medial anterior – posterior orientation, in the first instar (a) only the labium was visible; the stylet was positioned within the labium in images c) and d), however the labium was displaced in images b) and e).

The gonopore was ventrally positioned at the posterior end of grape phylloxera, and an additional posterior opening was identified as an indentation with a lateral orientation in a dorsal position to the gonopore (Figure 3a). The size of the putative anal opening did not correlate with life stage development (Table 2) as third instars significantly had the smallest posterior opening, and there was no significant difference in size among the first instar, fourth instar or adult life stages.

Limited measurements were recorded for the posterior opening of the adult life stage due to the presence of the gonopore and insect orientation on the SEM stub inhibiting the view. A droplet of fluid present at the posterior end of two individual adult grape phylloxera (Figure 3b) also inhibited observation of the posterior opening.

There were slight variations in the measurement of left and right antennae length; however there was a general increase in the length of the antennae with life stage development (Table 2). The antennae of adult grape phylloxera were significantly larger than all instars, although the ratio of antennae length to insect length decreased as life stage increased and was highest for first instars. Sensory pits were observed at the end of the antennae of all life stages (Figure 4), but measurements were not taken for size comparison due to limitations in visibility caused by SEM image orientation.

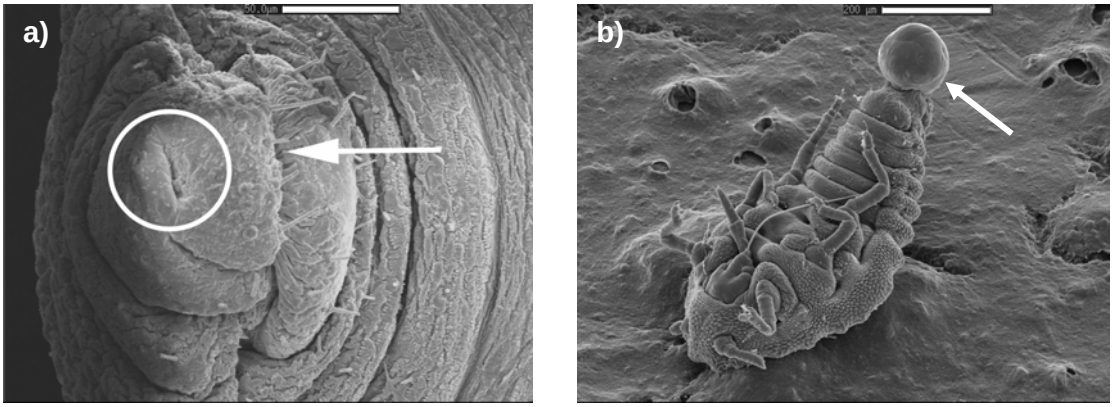


**Figure 3.** Cold stage SEM images of adult grape phylloxera: **a)** the posterior opening measured as potential evidence for waste excretion through an anal opening; the posterior opening shown by the circle, the gonopore indicated by the arrow. Scale bar 50 µm; **b)** droplet of fluid (indicated by the arrow) at the posterior end of the insect. Scale bar 200 µm.

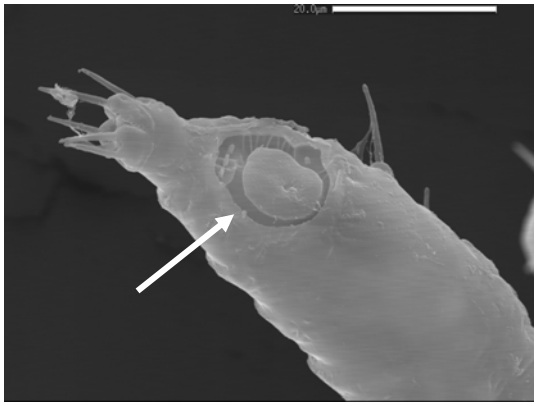

**Figure 4.** Cold stage SEM image of the sensory pit (indicated by the arrow) at the end of the antennae of the apterous first instar – adult life stages of grape phylloxera. Scale bar 20 µm.

### *4.1.5 EXTERNAL MORPHOLOGY VOLUME CALCULATIONS*

From SEM images, the volume capacity of a single developed egg was 3.1 x $10^6$ $\mu m^3$ (Table 3). The volume of a single developed egg occupied approximately 6% of the adult body cavity (55 x $10^6$ $\mu m^3$) as a fully developed egg just prior to oviposition. In comparison with volume measurements calculated from live grape phylloxera measured with light microscopy (Table 1), the developed egg (3.4 x $10^6$ $\mu m^3$) was approximately 8% of the adult body cavity volume (42 x $10^6$ $\mu m^3$).

The dimensions of the fluid droplet at the posterior end of grape phylloxera identified in Figure 3b were 111 µm x 143 µm (Table 3). The volume capacity of the fluid droplet was 1.2 x $10^6$ $\mu m^3$.

**Table 3.** Adult grape phylloxera cold stage SEM external morphology feature dimension and volume measurements.

| measurement | | external body[1] (n = 5) | developed egg[2] (n = 1) | posterior fluid droplet (n = 2) |
|---|---|---|---|---|
| dimension (µm) | l | 651 ± 107 | 298 | 112 ± 18 |
| | w | 377 ± 108 | 141 | 143 ± 14 |
| volume ($\mu m^3$) | | 55x$10^6$ ± 44x$10^6$ | 3.1x$10^6$ | 1.2x$10^6$ ± 0.046x$10^6$ |

Value of maximum dimensions, n value in parenthesis; external body and posterior fluid droplet mean ± standard deviation, absolute value for developed egg; length (l) and width (w) dimensions of each feature indicated

[1] external body values differ to Table 2 as volume calculations were only made from insects with both length and width measurements

[2] developed egg values differ to Table 2 as only included the egg measurement with a low percentage of the base sunken into the stub mounting paste

## 4.2 INTERNAL MORPHOLOGY

Grape phylloxera internal morphology characteristics were determined by longitudinal and transverse sectioning (1 µm) of fourth instar and adult insects. Fracturing whole insects and viewing the internal morphology of grape phylloxera with cold stage SEM complemented the sectioning observations.

### 4.2.1 INTERNAL MORPHOLOGY BY LIGHT MICROSCOPY

Longitudinal sectioning of grape phylloxera highlighted a specialised digestive system. Food passed from the stylet food canals into a short foregut before entering a compartmentalised midgut. The dimensions of the midgut were largest at the medial position of the insects (Figure 5a). The midgut ended blindly in the posterior region and the hindgut was positioned dorsal to the midgut. The midgut connected to the hindgut in a dorsal position forming a separation in the midgut (Figure 5b). The ovaries were also dorsal to the midgut, and the hindgut passed between the ovaries and dorsal to the oviduct toward the posterior end of the insect. The position of the oviduct inhibited a posterior connection between the midgut and hindgut (Figure 5c). The hindgut extended to the posterior end of the insect in a dorsal position to the gonopore; however the presence of an anal opening was not confirmed. No filter chamber was present. The midgut was asymmetrical and reduced in capacity on the side of the insect where the ovaries were located, allowing for the development of eggs in the adult life stage (Figure 5d).

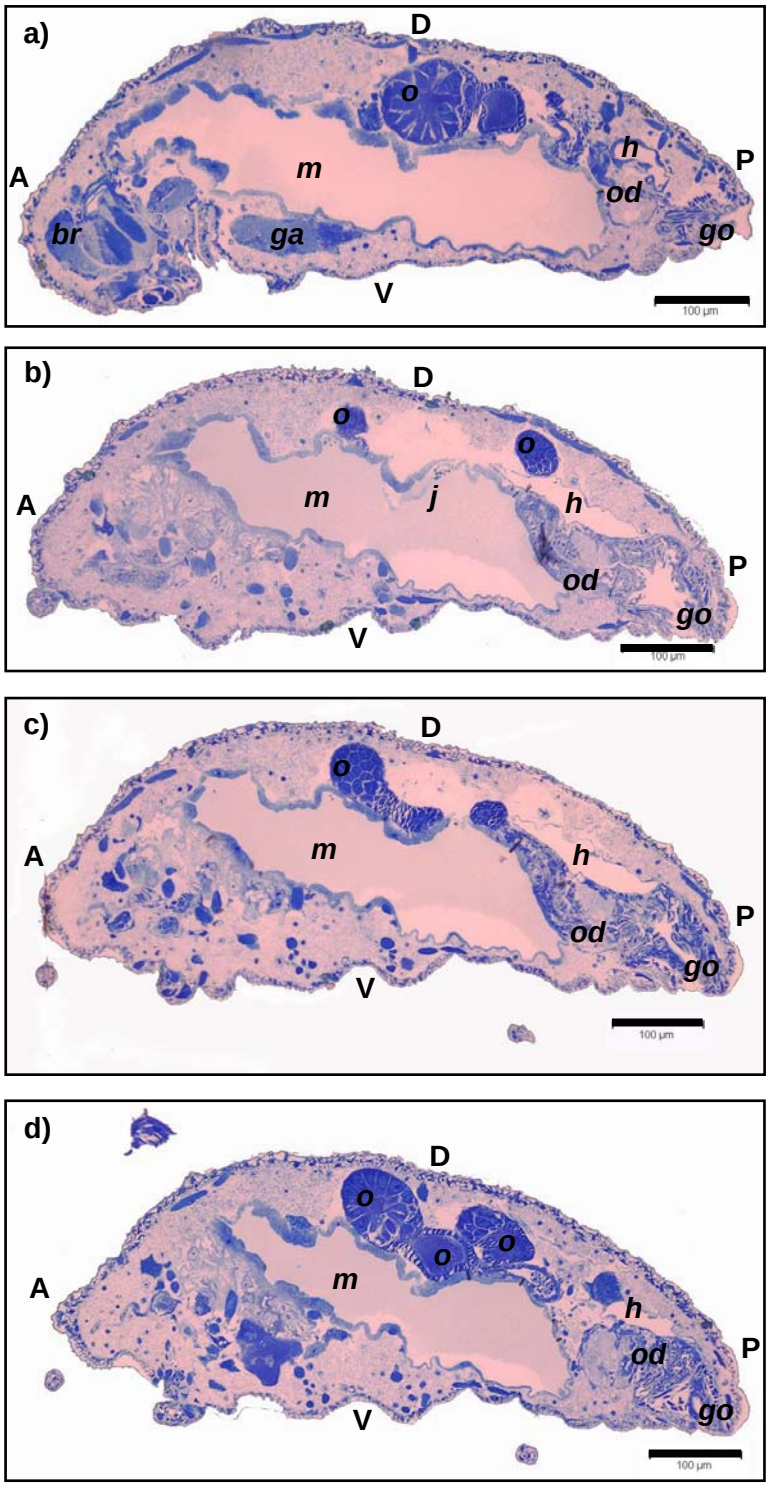


**Figure 5.** Caption on the following page.

**Figure 5 caption.** Series of longitudinal sections of fourth instar grape phylloxera: **a)** the medial position of the insect with the head visible in the anterior region, the midgut was the main component of the body cavity; **b)** the hindgut, oviduct and gonopore are more evident, the hindgut connected with the midgut in a dorsal position at the midgut-hindgut junction; **c)** the hindgut remained dorsal to the midgut in all sections, the position of the oviduct inhibited a posterior connection between the gut cavities; **d)** the midgut was reduced in size, the ovaries are positioned dorsal to the digestive system. Scale bar 100 µm.

Abbreviations: ***br*** brain, ***ga*** ganglion, ***m*** midgut, ***o*** ovaries, ***h*** hindgut, ***od*** oviduct, ***go*** gonopore, ***j*** junction between midgut and hindgut

Insect orientation indicated: **A** anterior, **P** posterior, **D** dorsal, **V** ventral

Adult grape phylloxera displayed multiple egg development which resulted in the compression of the midgut (Figure 6a). The hindgut remained positioned in the posterior region of the insect and in a dorsal orientation to the oviduct (Figure 6b). There was evidence for the simultaneous development of eggs in both a dorsal and ventral position which lead to the compression of the midgut and an asymmetrical structure.

Transverse sectioning of adult grape phylloxera confirmed the interpretation of the longitudinal sections. In a medial position of the adult insect, the body cavity was dominated by the midgut and developing eggs, which were positioned in both dorsal and ventral positions (Figure 7a). The hindgut appeared in close proximity to the midgut in a dorsal position. Egg size increased toward the posterior region of the insect, and caused the compression of the posterior region of the midgut where extensive folding was present (Figures 7b-7d). The hindgut was constantly located in a dorsal position to the midgut, and the oviduct blocked a posterior connection between the two gut cavities (Figure 7c). At the posterior end of the midgut (Figure 7e), the midgut and hindgut did not connect. The hindgut passed between developing eggs (Figure 7d), and remained in a dorsal position to the gonopore (Figure 7e). The hindgut was tracked to the posterior end of the insect, beyond the termination point of the gonopore (Figure 7f), although an external opening in the cuticle of the insect was not observed in light microscopy sections.

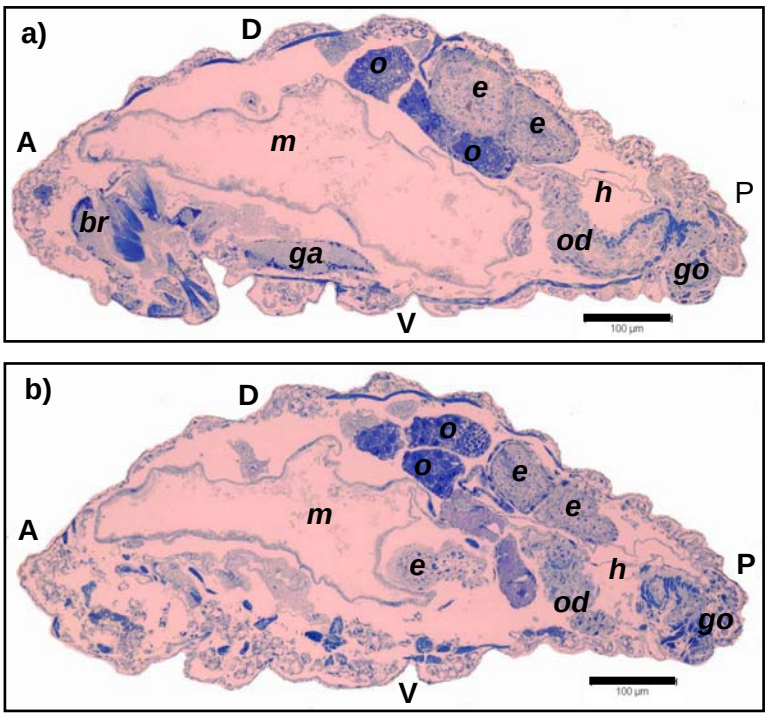


**Figure 6.** Longitudinal sections of adult grape phylloxera: **a)** the medial position of the insect with the head visible in the anterior region, multiple eggs were developing simultaneously dorsal to the midgut, the hindgut passed between the oviduct and the development of multiple eggs compressed the size of the midgut compartment and may have blocked the hindgut, which was intertwined with the oviduct; **b)** the midgut was reduced in size, the hindgut intertwined through the ovaries and developing eggs and was evident at the posterior region of the insect, egg development was present in both a dorsal and ventral position causing the structure of the midgut to be asymmetrical. Scale bar 100 µm.

Abbreviations: ***br*** brain, ***ga*** ganglion, ***m*** midgut, ***o*** ovaries, ***e*** egg, ***h*** hindgut, ***od*** oviduct, ***go*** gonopore
Insect orientation indicated: **A** anterior, **P** posterior, **D** dorsal, **V** ventral

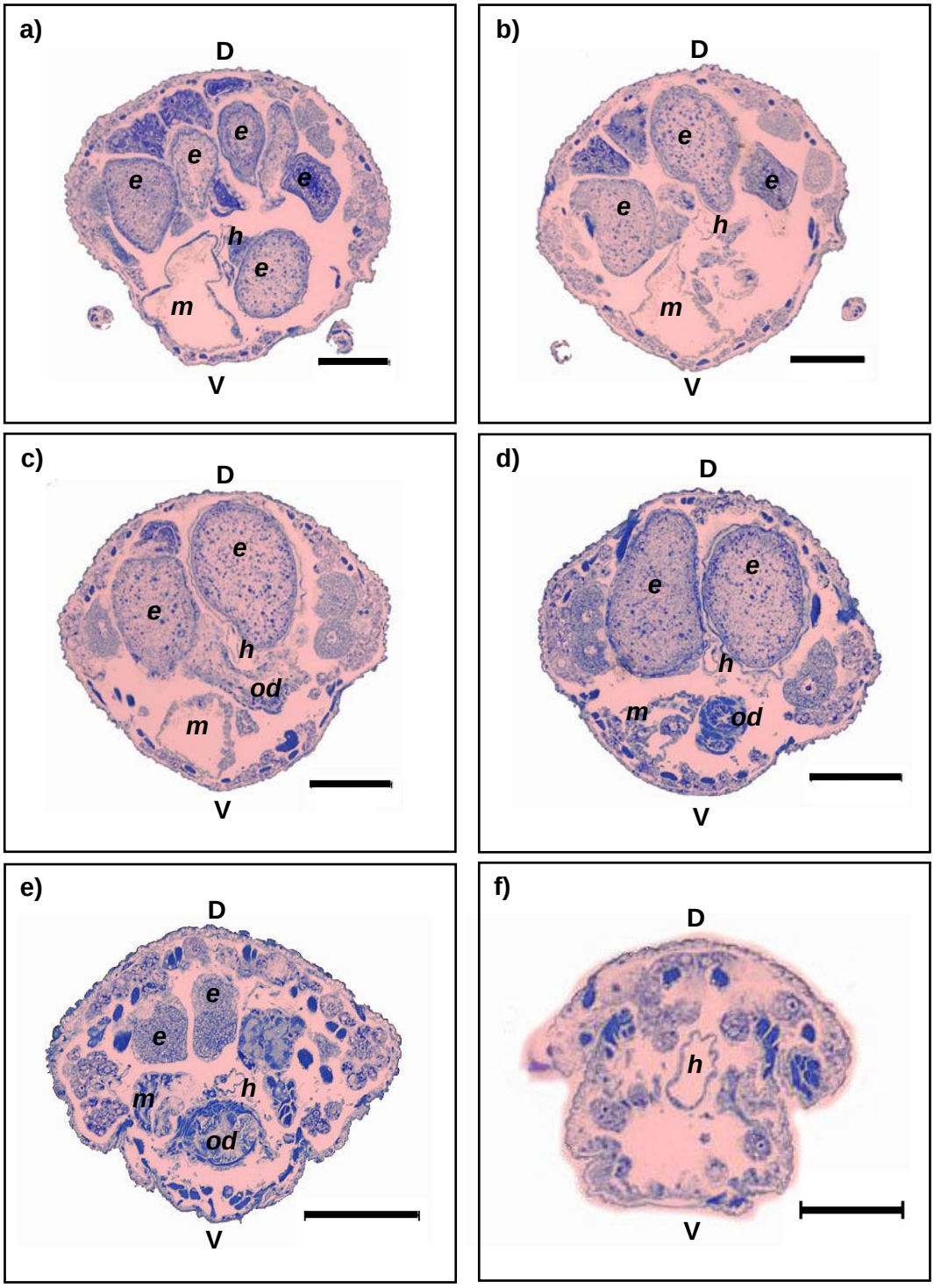


**Figure 7.** Caption on the following page.

**Figure 7 caption.** Series of transverse sections of adult grape phylloxera highlighted the progressive changes in the digestive and reproductive system from the medial position of the insect (a) toward the posterior end (f): **a)** the body cavity was dominated by the midgut and developing eggs, eggs are positioned in dorsal and ventral positions, the hindgut was dorsal to the midgut; **b)** the hindgut remained dorsal to the midgut, the two gut cavities are more distinct; **c)** the oviduct was present between the midgut and the hindgut, inhibiting a posterior connection between the gut cavities; **d)** the midgut was reduced in size, the hindgut was positioned between two large, developing eggs; **e)** the hindgut and midgut remained separated at the posterior end of the midgut, the oviduct increased in size; **f)** the hindgut was present at the posterior end of the insect. Scale bar a) – e) 100 µm, scale bar f) 50 µm.

Abbreviations: ***m*** midgut, ***e*** egg, ***h*** hindgut, ***od*** oviduct
Insect orientation indicated: **D** dorsal, **V** ventral

### *4.2.2 INTERNAL MORPHOLOGY BY SEM*

Developing eggs, an open lattice digestive system and tubules were identified by fracturing adult grape phylloxera and viewing via cold stage SEM (Figure 8). The positioning of the open lattice structure corresponded with the location of the midgut and hindgut chambers from light microscopy sectioning. The tubule opening located ventral to the developing egg, and adjacent to the midgut in a dorsal position (Figure 8 insert), was in the approximate location of the midgut-hindgut junction. The indentation in the egg, in line with the midgut-hindgut junction, may have been caused by the hindgut chamber en route to the posterior end of the insect.

In another individual, the ovaries of adult grape phylloxera were visible (Figure 9). The developing egg was displaced during sample preparation and the area utilised by the egg was represented by the empty cavity. Positioned dorsal to the empty egg cavity were three circular structures that corresponded to the ovaries of the insect. The midgut was present in a ventral position.

The hindgut open lattice structure was observed in the posterior region of fourth instar grape phylloxera (Figure 10). The gonopore was ventral to the hindgut position, and aligned with the light microscopy sections.

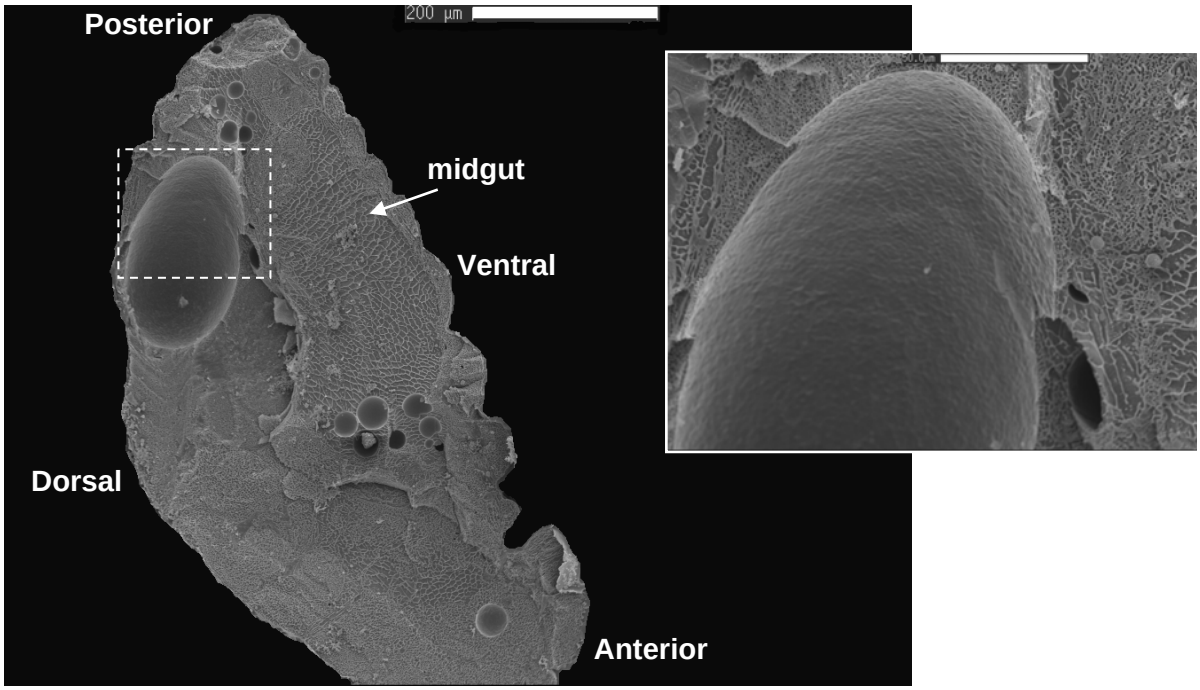


**Figure 8.** Cold stage SEM images of the internal structure of adult grape phylloxera, insect orientations labelled. Insert box (indicated on main image) is a close up view of a developing egg, with a tubular opening present in a ventral position. The open lattice structure ventral to the egg (indicated by the arrow) was the midgut of the insect. Scale bar on main picture 200 µm, on insert picture 50 µm.

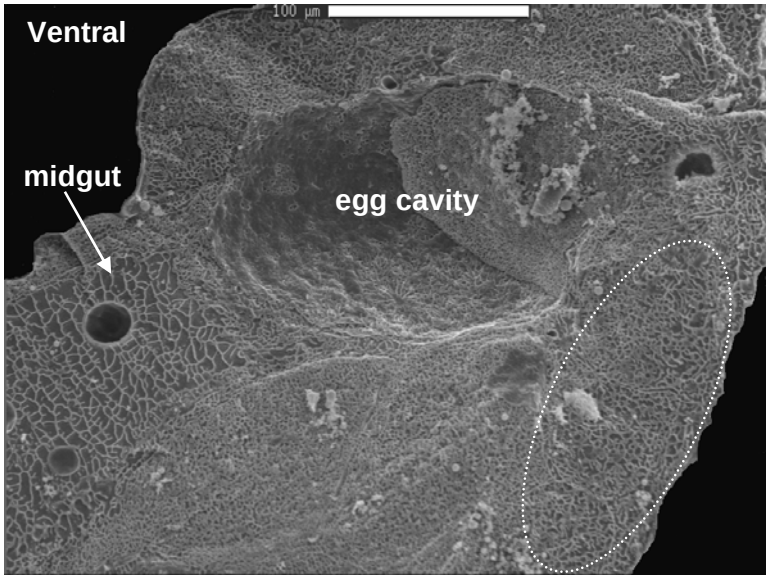


**Figure 9.** Cold stage SEM image of the internal structure of adult grape phylloxera, medial position with the insect ventral orientation labelled. The open lattice structure of the midgut (indicated by the arrow) was situated in a ventral position, three circular ovaries were visible in a dorsal position (indicated by the dotted oval), and an empty egg cavity was present due to the dislodgment of a developing egg during preparation. Scale bar 100 µm.

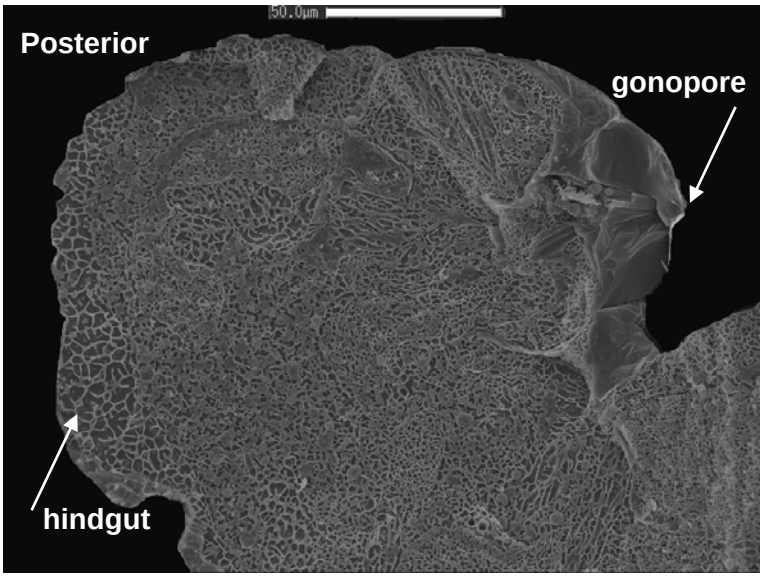


**Figure 10.** Cold stage SEM image of the internal structure of fourth instar grape phylloxera, insect posterior orientation labelled. The gonopore was situated in a ventral position; the open lattice structure of the hindgut (indicated by the arrow) was in a dorsal position. Scale bar 50 µm.

### *4.2.3 INTERNAL MORPHOLOGY MEASUREMENTS*

Measurements of grape phylloxera internal features from limited light microscopy longitudinal sections indicated adult insects were larger than fourth instar insects in maximum dimensions of all features, except the width of the midgut (Table 4). The hindgut widths were the same dimension in both life stages. The ratio of the length and width of the digestive system to the external body dimension were however reduced in reproductive adults. Developing eggs were 29% of the adult external body length.

The external body dimensions of adult grape phylloxera viewed with internal cold stage SEM images (Table 5) were similar to the light microscopy sections, and developing eggs were also 29% of the adult body length. The midgut-hindgut junction observed in Figure 9 was 26 x 10 µm in size. A measurement of a similar midgut-hindgut junction in a light microscopy transverse section was 42 x 9 µm.

**Table 4.** Grape phylloxera fourth instar and adult external body dimensions and internal (digestive system and developing egg) measurements from longitudinal sections.

| life stage | | external | digestive system | | developing |
|---|---|---|---|---|---|
| | | body (µm) | midgut (µm) | hindgut (µm)[1] | egg (µm) |
| adult | l | 803 ± 46 | 488 ± 92 | 233 ± 117 | 233 ± 12 |
| (n = 4) | w | 308 ± 61 | 79 ± 35 | 28 ± 3.5 | 134 ± 14 |
| fourth instar | l | 680 ± 170 | 463 ± 124 | 180 ± 0 | NA |
| (n = 2) | w | 225 ± 78 | 98 ± 3.6 | 28 ± 3.5 | NA |

Mean values of maximum dimensions ± standard deviation, n value in parenthesis; developing egg measurements not applicable (NA) for fourth instar; length (l) and width (w) of each feature indicated

[1] adult n value = 2

**Table 5.** Adult grape phylloxera internal cold stage SEM dimension and volume measurements of the external body cavity and internal features.

| measurement | | external body (n = 2) | developing egg (n = 2) | midgut-hindgut junction (n = 1) |
|---|---|---|---|---|
| dimensions (µm) | l | 718 ± 86 | 210 ± 7.8 | 26 |
| | w | 367 ± 28 | 126 ± 0.7 | 10 |
| volume ($\mu m^3$) | | $51x10^6 \pm 14x10^6$ | $1.7x10^6 \pm 0.045x10^6$ | $1.4x10^3$ |

Value of maximum dimensions, n value in parenthesis; external body and developing egg mean ± standard deviation, absolute value for midgut-hindgut junction; length (l) and width (w) dimensions of each feature indicated

### *4.2.4 INTERNAL MORPHOLOGY VOLUME CALCULATIONS*

Grape phylloxera volume calculations of external and internal features from light microscopy sections indicated an increase in the external body cavity of adults compared with fourth instar insects (Table 6). The increase in volume of the external body cavity did not fully translate to the internal measurements of the digestive system; the hindgut volume was increased in the adult, although the volume of the midgut was reduced.

Developing eggs had a maximum volume of 2.2 x $10^6$ $\mu m^3$ (Table 6), which represented approximately 5% of the adult body cavity. The volume of an internally developing egg, viewed with cold stage SEM, was 1.7 x $10^6$ $\mu m^3$, which represented approximately 3% of the adult body cavity (Table 5).

The midgut-hindgut junction observed in Figure 9 had a volume of approximately 1,361 $\mu m^3$ (Table 5), which was similar to the volume capacity of a midgut-hindgut junction observed in a light microscopy transverse section (1,781 $\mu m^3$).

**Table 6.** Grape phylloxera fourth instar and adult external body cavity and internal (digestive system and developing egg) volume measurements from longitudinal sections.

| life stage | external | digestive system | | developing |
|---|---|---|---|---|
| | body ($\mu m^3$) | midgut ($\mu m^3$) | hindgut ($\mu m^3$)[1] | egg ($\mu m^3$) |
| adult (n = 4) | $41x10^6 \pm 16x10^6$ | $1.9x10^6 \pm 1.5x10^6$ | $8.7x10^4 \pm 2.3x10^4$ | $2.2x10^6 \pm 0.43x10^6$ |
| fourth instar (n = 1) | $21x10^6 \pm 17x10^6$ | $2.3x10^6 \pm 0.78x10^6$ | $7.2x10^4 \pm 1.8x10^4$ | NA |

Mean values of maximum dimensions ± standard deviation, n value in parenthesis; developing egg measurements not applicable (NA) for fourth instar

[1] adult n value for hindgut area measurement = 2

### *4.2.5 SUMMARY OF INTERNAL MORPHOLOGY*

The main digestive and reproductive features of grape phylloxera are represented schematically in Figure 11. The main features of interest were the location and size of the midgut, hindgut, ovaries (and developing eggs) and the oviduct. More inclusive terminology was used in comparison with Ponsen (1997). The midgut compartment was defined as a complete unit as the individual sections, including the stomach, crenated intestine and caecal intestine, were unable to be differentiated. The term hindgut included the anal opening, rectum and the ileum chamber connecting with the midgut. From the light microscopy sectioning it was unable to be determined if the ileum had cuticular lining (and therefore was technically 'hindgut'), but the chamber was included within this classification to make a clear definition in function from the midgut chamber.

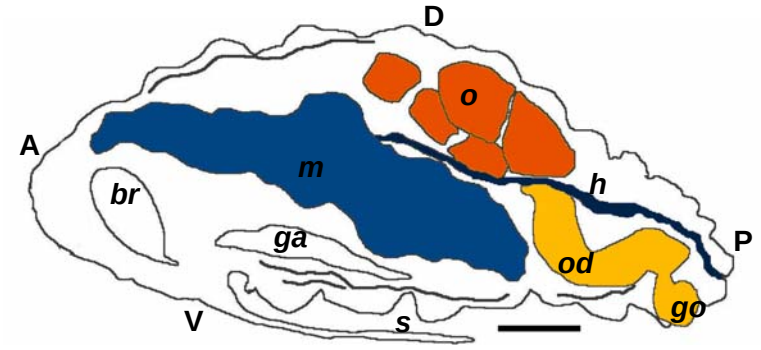


**Figure 11.** Schematic diagram of the digestive and reproductive features of grape phylloxera. Scale bar 100 µm.

Abbreviations: ***s*** stylet, ***br*** brain, ***ga*** ganglion, ***m*** midgut, ***o*** ovaries, ***h*** hindgut, ***od*** oviduct, ***go*** gonopore
Insect orientation indicated: **A** anterior, **P** posterior, **D** dorsal, **V** ventral

# 5 DISCUSSION

The similarity in external body measurements for insects via light microscopy and SEM techniques validated the use of SEM to characterise the fine external morphology features of radicicolae grape phylloxera. The development of grape phylloxera from egg to adult involves four nymphal moults, resulting in four instar life stages prior to the development of the reproductive adult. SEM external body measurements of grape phylloxera displayed a continual increase in size with each life stage development; however the transition from fourth instar into adult resulted in the largest size increase (42%). This increase in size may be representative of an observation by Davidson and Nougaret (1921) of a growth phase during the fourth instar life stage. Between the third and the fourth moult, the fourth instar was observed to increase in size by 25%. Adult insects used for measurement in the current study were typically the largest insects within the population; smaller insects were observed that could be classified as adults, but these were not collected. Selecting the larger adult insects, along with the possibility of collecting early moult fourth instars, would exacerbate the variation in size between the fourth instar and adult life stage.

In comparison with previous research using light microscopy technology (Davidson and Nougaret, 1921; Buchanan, 1990), most of the life stage external body measurements recorded by light microscopy and SEM were smaller. This variation may be due to the differences in quality of the grape phylloxera food source. Davidson and Nougaret (1921) recorded adult insects feeding on fleshy and succulent roots at 1000 µm in length and 550 µm in width, while insects feeding on other regions of the grapevine root were smaller in size. The insects used for the current study were collected from populations feeding on excised root pieces in the laboratory. These insects were smaller in size to insects collected from potted vine populations (data not shown), and are not expected to grow to a size comparable with field collected insects due to the

limiting environmental and nutritional factors exhibited by excised roots. Despite the smaller size recorded for adults in the current study, the egg measurements are similar to values previously reported by De Klerk (1974).

### *5.1 FEEDING SITE LOCATION*

The feeding location of radicicolae grape phylloxera has been reported to be a single parenchyma cell within the cortex tissue of the grapevine root, with the stylet of the insect penetrating the root on an intracellular path (Kellow *et al.*, 2004). However the stylets of gallicolae grape phylloxera have been reported to reach the phloem (Rosen, cited in Pollard, 1973) Stylet length was measured as an indicator of the depth of feeding by grape phylloxera within the grapevine root system. Stylets were difficult to measure accurately due to possible distortion of the SEM image, and uncertainty if the stylet was fully extended at the time of insect collection. However the values presented in the current study are similar to existing data (Davidson and Nougaret, 1921).

Following examination of sections of several *Vitis* species, the thickness of the cortex tissue of grapevine roots was determined, and compared with the depth of stylet penetration required for phloem feeding. The thickness of the cortex in *V. vinifera* ranged from 200-300 µm, or 7-12 cell layers deep (Kellow, 2000). Stylet penetration from the plant surface to the food source in Hemipteran insects is generally indirect, involving trial-and-error sampling of potential food sources before settling in a satisfactory location (Tjallingii and Hogen Esch, 1993). However, even if the stylet of grape phylloxera did penetrate the root surface and travel through the cortex on a straight intracellular path, the stylet measurements recorded indicated that it was unlikely radicicolae grape phylloxera can reach phloem cells. Only fourth instar and adult life stages recorded a stylet length in excess of 200 µm. Alternatively to species of the closely related Aphididae that feed predominately on phloem tissue, these

measurements support the expectation that grape phylloxera feed primarily on the parenchyma tissue of grapevine roots.

### *5.2 WASTE EXCRETION*

The location of a posterior opening was investigated by SEM as evidence for the presence of anal waste excretion. All instar life stages displayed one posterior opening, which was variable in size. Adult grape phylloxera have an enlarged gonopore posterior opening for oviposition, however in limited SEM images (n = 2) the presence of a second posterior opening, dorsal to the gonopore, was visible. The presence of the additional posterior opening provided evidence for the existence of an anus in grape phylloxera for waste excretion. The location of the second posterior opening aligned with the expected termination point of the hindgut from longitudinal and transverse light microscopy sections, and was also supported by the internal SEM data. Although the anal opening observed by SEM was not captured in sections observed using light microscopy, other light microscopy studies support the presence of an anal opening in grape phylloxera (Breider, 1952; Ponsen, 1997; Kemper, 2004). The posterior opening was therefore interpreted as the exit point of an anus.

An absence of honeydew production (Riley, 1870) has been cited as evidence that grape phylloxera does not have a system of anal excretion for waste (Ponsen, 1997). Honeydew was not visible when observing insects actively feeding on grapevine roots, however the possible presence of honeydew was observed by SEM as a droplet of fluid at the posterior end of two individual adult grape phylloxera insects. The expulsion of the droplet of fluid was probably the result of pressure applied to the abdomen during preparation, although the exact exit point could not to be determined. The volume of the hindgut ($8.7 \times 10^4$ $\mu m^3$) was less than the volume of the fluid droplet ($1.2 \times 10^6$ $\mu m^3$); however the dimensions (and therefore volume measurements) for the hindgut cavity are expected to be an underestimation due to inaccuracies caused by

not viewing the entire hindgut structure within the one light microscopy section for measurement. The actual volume capacity of the hindgut was not determined.

A preliminary ion analysis of the posterior droplet of fluid with cold stage SEM, using energy dispersive X-ray microanalysis (EDAX), indicated that the ion concentration was at non-detectable levels (data not shown), and therefore did not provide an indication of a feeding location. However the gold coating of the sample as preparation for the SEM would interfere with the detection of low level ion concentrations and therefore decrease detection limits. It is also possible that the droplet of fluid was not associated with waste excretion and was alternatively fluid from the gonopore passage assisting oviposition. Further investigation is required to resolve the origin of the posterior droplet of fluid.

Similar fluid droplets have been observed in the oak phylloxera, *Phylloxera coccinea* Heyden (Hemiptera: Phylloxeridae). *P. coccinea* do not display visible levels of honeydew production, however a fluid droplet of 'honeydew' was been observed with the application of pressure to the abdomen (Ponsen, 1997). Ponsen inferred that Phylloxeridae feeding on parenchyma cell contents have reduced fluid intake in comparison to phloem feeding, and honeydew generating, Aphididae. The waste excretion levels generated by Phylloxeridae (including grape phylloxera) may therefore be too low for visible detection under standard conditions. Adelgidae however also feed on parenchyma tissue, and do excrete waste (Ponsen, 2006). The variation in waste excretion between Phylloxeridae and Adelgidae has been explained by the absence of anal dorsal muscles in Phylloxeridae, which are important for opening the anal aperture in both Adelgidae and Aphididae (Ponsen, 2006). The low levels of honeydew excretion observed in Phylloxeridae with the application of pressure to the abdomen of the insect was proposed to be due to the contraction of anal lateral

muscles; the lateral muscles may have the capacity to open the anal opening, although to a lesser degree than dorsal muscles (Ponsen, 1997).

The location of grape phylloxera muscles was not a focus of the current study, however longitudinal muscle fibres were observed in most light microscopy sections. Anal waste excretion of low volumes of honeydew are proposed to occur in grape phylloxera, in the absence of anal dorsal muscles, by the contraction of these longitudinal muscles to compress the posterior end of the insect. Compression of the posterior end of grape phylloxera was regularly observed in the adult life stage during oviposition. By compressing the posterior end, the volume of the hindgut would be decreased, therefore increasing the pressure of fluid in the chamber to allow for the excretion of waste material. The passing of eggs through the oviduct during oviposition in adults would also increase the pressure in the hindgut, and potentially 'push' the fluid waste material out the anal opening with oviposition. Freshly laid eggs were observed to be moist immediately following oviposition; this fluid may be from the oviduct or represent waste fluid excretion from the dorsally positioned anal opening. The low, non-detectable, volumes of anal waste excretion by grape phylloxera indicated that food utilisation was very high.

The alternative methods of waste excretion proposed for grape phylloxera, including the periodic elimination of waste back into the grapevine gall via the salivary glands for (Schaller, 1960; Sobetskiy and Derzhavina, 1973) and waste excretion during oviposition (Rilling, as cited in Forneck *et al.*, 2001), although not directly addressed by the current study may still be theoretically discussed. Ponsen (1997) identified a large salivary pump in Phylloxeridae, with three pairs of muscles. These were more developed than in Aphididae, where it was expected less muscular power was required to pump saliva into phloem tissue than into individual parenchyma cells. The modifications observed in the salivary glands of grape phylloxera have previously

provided evidence for salivary waste excretion (Schaller, 1960). Grape phylloxera are sedentary feeders on individual parenchyma cells, and therefore by definition have a limited food source; the excretion of waste products back into this food source appears an unlikely mechanism for waste excretion. No physiological connection between the oviduct and the midgut or hindgut was observed in adult grape phylloxera light microscopy sections to support the possibility of waste excretion during oviposition. The only possible link identified between oviposition and waste excretion was the pressure placed on the hindgut during the movement of the egg through the oviduct which may result in the excretion of waste material at the same time as oviposition, although through a different passage.

The morphology and function of the salivary glands in grape phylloxera were not addressed in the current study, but may require further investigation. Enzyme histochemistry studies identified dark staining by alkaline phosphatase in the salivary glands of grape phylloxera, indicating that the cells were involved in the active transport of material across the salivary gland membrane (Kemper, 2004). Radioactively labelled $^{14}C$ sucrose studies with grape phylloxera also strongly marked the salivary glands (Rilling and Steffan, 1972), although the midgut was only weakly labelled indicating that the sucrose passed through the midgut almost unchanged. Extra-intestinal digestion by grape phylloxera has been suggested as evidence for a closed digestive system (Federov, 1959); however extra-intestinal ingestion does not exclude the necessity for waste excretion. Although the current study has found the digestive system of grape phylloxera to be complete, enlarged salivary glands and the associated alkaline phosphatase and $^{14}C$ sucrose activity suggested that extra-intestinal digestion, as a requirement to liquefy and pre-digest the parenchyma cell contents, may still occur. All of the amino acids Schaller (1960) identified in the saliva of grape phylloxera as evidence for waste excretion via the salivary glands increase in concentration during the development of the 'nutrient-sink' gall in *V. vinifera* (Kellow *et*

*al.*, 2004), and therefore may represent residual pre-digested cell contents as apposed to excreted waste products.

### *5.3 INSECT DISPERSAL ADAPTATIONS*

The ratio of grape phylloxera antennae length to external body length was greatest for the first instar active life stage. The function of the sensory pore located at the end of each antenna was unknown, but may be important during dispersal, host-plant displacement and the establishment of new feeding sites. Hemipteran antennae detect olfactory cues from plants to assist in long-distance orientation and plant surface exploration during host plant settling (Backus, 1988). Grape phylloxera feeds exclusively on *Vitis* species (Granett *et al.*, 2001b); therefore a highly specific sensory system would be important for the location of a new host-plant. The antennae measurements made by SEM were similar to those presented by Davidson and Nougaret (1921) from light microscopy studies.

The internal structure of the grape phylloxera digestive system would assist in providing sustained energy for the insect during periods of low food intake due to dispersal to a new *Vitis* food source and/or the compression of the midgut cavity during egg development. The midgut was compartmentalised by the junction of the hindgut in a dorsal position; the impact of the structure of the digestive system on grape phylloxera biology was implied by the survival of intermediate instars for up to nine days in an artificial feeding system without nutritional intake (Chapter 4). Enzyme histochemistry studies (Kemper, 2004) confirmed the separation in midgut function, with the anterior region of the midgut staining heavily for alkaline phosphatase and minimal staining in the posterior region of the midgut. This implied that the anterior midgut was actively involved in the transport of nutrients across the membrane surface, and the posterior midgut was a storage system to sustain the insects survival when active feeding on grapevines was not possible. The compartmentalisation of the grape

phylloxera midgut, together with extra-intestinal digestion, may assist with the slow digestion of the complex high protein and low carbohydrate diet of parenchyma cell contents.

### *5.4 REPRODUCTIVE IMPACTS ON FOOD INTAKE*

The internal features of grape phylloxera were dominated in the ventral orientation by the midgut chamber and in the dorsal position by the ovaries and developing eggs (in adults). There were no loops or coils in the alimentary tract, which was in contrast with members of the Aphididae where 3-4, and up to 11, loops have been observed (Ponsen, 2006). Due to the absence of loops, the alimentary tract of gallicolae grape phylloxera was measured to be four times shorter than the digestive system of the Aphididae *Myzus persicae* (Ponsen, 1997).

Adult grape phylloxera experience multiple egg development, with an oviposited egg measuring approximately 40% of the insect length, and equivalent to 6-8% of the internal body cavity. The internal development of eggs resulted in the compression of the digestive system. This compression was highlighted by the reduction in volume of the midgut chamber in adults compared with fourth instar insects. The length of a developing egg was approximately 30% of the adult length, and occupied between 3-5% of the internal body cavity. However this is the size of a single egg, and does not represent the entire area utilised by egg development in the ovaries. Egg development in both dorsal and ventral positions compressed the midgut chamber, forcing food from the posterior storage system into the anterior midgut for absorption, providing the energy to support the development of egg production.

The positioning of the hindgut between the oviduct and the developing eggs may result in the blocking of the hindgut passage during oviposition. The internal SEM image of the indentation in the developing egg provided evidence for the compression of the hindgut during the movement of the egg through the oviduct. The increase in hindgut

internal pressure as a result of this blockage may assist with the excretion of waste material during oviposition and the contraction of the longitudinal muscles. The hindgut blockage and limitation in midgut size (due to egg development) may also periodically inhibit adult grape phylloxera feeding, with the insect remaining attached to the grapevine root food source but experiencing no active ingestion of parenchyma cell contents. The occlusion of the digestive system has also been reported in fluid feeding larval Neuroptera where little solid waste was excreted (Chapman, 1969).

The midgut-hindgut junction observed in longitudinal and transverse light microscopy sections, and in internal cold stage SEM images, would be an important adaptation for sustaining energy supplies in an insect experiencing periodic feeding. The midgut-hindgut junction had not previously been reported for grape phylloxera, but the resulting compartmentalisation of the midgut chamber would allow for food absorption during periods of restricted intake. Grape phylloxera lay 3-6 eggs per day for a period of 1-2 months (Granett *et al.*, 1983), or between 90-360 eggs during the lifetime of a reproductive adult. Oviposition takes 43 minutes from the beginning of expulsion from the gonopore to detachment from the insect; therefore egg development must be rapid as the internal development of 6 simultaneous eggs would equal 30% of the internal body cavity volume. Periodic feeding events would be short periods of time separated by episodes of hindgut blockage with oviposition, and may be essential to supply the energy requirements necessary to produce up to 360 eggs. Studies investigating the feeding behaviour of radicicolae grape phylloxera (Chapter 5) are required to identify the potential periodic feeding events occurring in the adult life stage.

### *5.5 COMPARISON WITH PREVIOUS RESEARCH*

The structure of the digestive system reported in the current study was similar to previous studies that concluded grape phylloxera to have a complete digestive system (Breider, 1952; Ponsen, 1997). However both Breider and Ponsen interpreted the

midgut-hindgut junction to be a twist in the cavity of the digestive system, with the midgut connected to the hindgut in the posterior end. Breider (1952) identified the gonopore position to be in a similar posterior-ventral position, with the anal opening positioned dorsal to the gonopore. Both studies concluded grape phylloxera to have a complete digestive system. Federov (1959) concluded grape phylloxera to have a blind-ending midgut, and used this to support a lack of anal excretion. The current study did show the midgut of grape phylloxera to be 'blind-ending' in the posterior region, although the complete digestive system was not blind-ending due to the dorsal midgut-hindgut junction. The hindgut was tracked to the extreme posterior of the insect, although an anal opening observed by SEM was not identified through light microscopy sectioning.

The method of waste excretion in grape phylloxera remains undefined, although evidence of an external posterior opening in all apterous life stages, and the internal tracking of the hindgut to the posterior region of the insect, was indicative of the potential for anal waste excretion. The incidence of a droplet of fluid at the posterior end of two adult insects (viewed with cold stage SEM) provided evidence for the presence of fluid within the chamber. In comparison with phloem sap, the food source of grape phylloxera is high in protein (nutrition) and low in sugar and water (waste products) and would therefore require reduced levels of waste excretion. The slow digestion of this food source in the compartmentalised midgut may be an important adaptation for providing the energy requirements to sustain continual egg production during the adult life stage, and may also be an important evolutionary adaptation during periods of dispersal to a new *Vitis* food source.

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# CHAPTER 3

# INVESTIGATING BACTERIAL SYMBIOTIC RELATIONSHIPS IN GRAPE PHYLLOXERA

# 1 SUMMARY

Bacterial symbiotic relationships with radicicolae grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) have not been extensively investigated using molecular techniques, although a bacterial association with gallicolae grape phylloxera has recently been reported. An undefined bacteria species was cultured from radicicolae grape phylloxera; however PCR and sequence analysis indicated that the taxonomy of the bacteria differed to the *Pantoea* spp previously identified in association with gallicolae grape phylloxera. The 91% identity of the radicicolae grape phylloxera bacterial DNA sequence with uncultured bacterium confirmed the successful amplification of the 16S rRNA gene, however the level of identify was too low to classify the species. The identification of the bacterial DNA in radicicolae grape phylloxera was transient, and potentially associated with grapevine health and insect feeding location. The proposed bacterial symbiotic association in radicicolae grape phylloxera would be classified as non-essential; however the association may still impact on insect survival rates and host-plant interactions.

## 2 INTRODUCTION

Symbiotic relationships occur when two different species live in close association with each other on either a permanent or long-term basis. The symbiotic relationship may benefit one species (commensalism), both species (mutualism), or result in harm to one of the species (parasitism). An organism inhabiting another species in a symbiotic relationship is termed an endosymbiont (Raven and Johnson, 1989). Several insect groups, including cockroaches, beetles, whiteflies, scale insects, psyllids, leafhoppers, cicadas and aphids, have endosymbiotic associations with intracellular microorganisms (Gullan and Cranston, 2005). Typically, the symbiotic relationship develops due to the insect diet being deficient in essential nutrients, which is biosynthesised by the microorganism. All members of the Hemipteran superfamily Aphidoidea contain the bacterial endosymbiont *Buchnera*, except for members of the Adelgidae and Phylloxeridae families (Douglas, 1998). *Buchnera* provide Aphididae with up to 90% of their essential amino acids which are not naturally obtained from the insects diet of phloem sap (Douglas, 2006); the endosymbiont has lost the ability to produce non-essential amino acids, and these are supplied to the bacteria by the host (Baumann, 2005). The maternally inherited endosymbiotic relationship is therefore considered essential and mutualistic. The bacterial endosymbiont cannot be cultured outside of the host insect, and the growth of the aphid is inhibited in the absence of the endosymbiont (Martinez-Torres *et al.*, 2001).

Members of the Aphididae family may contain bacterial symbiotic relationships additional to the primary symbiont *Buchnera*. Aphididae primary symbionts are spherical in shape and located in specialised cells (termed mycetocytes) within the haemocoel of the insect (Baumann *et al.*, 1995). Aphididae secondary symbionts are rod-shaped bacteria located in the tissue surrounding the mycetocytes, and tertiary symbionts are rod-shaped bacteria located free within the haemolymph (Chen *et al.*,

1996). By definition, secondary (and tertiary) symbionts coexist with the more ancient primary symbiont infection (Moran *et al.*, 2005), and the presence of secondary (and tertiary) symbionts relies upon repeated infection by the bacteria (Sandstrom *et al.*, 2001). Therefore, unlike primary symbionts, secondary (and tertiary) symbionts are transient and are generally not considered to offer nutritional advantage to the aphid (Douglas, 1998). However secondary symbionts have been associated with beneficial impacts on insect biology, including improved reproduction, resistance to parasitoids and tolerance to heat stress (Moran *et al.*, 2005). The horizontal transfer of Aphidoidea secondary symbionts has resulted in the independent acquisition of bacteria by unrelated insect species relatively recently in the evolutionary timescale (Russell *et al.*, 2003).

Evolutionary divergence between the Aphididae and the Phylloxeridae families occurred prior to infection of the Aphididae with *Buchnera* around 100-250 mya (Martinez-Torres *et al.*, 2001). Insects feeding on plant parenchyma cell contents, like the Phylloxeridae, may not require bacterial endosymbiotic relationships to supply the essential nutritional requirements of the insect (Buchner, 1965); although members of the parenchyma-feeding Adelgidae family have evolved endosymbiotic relationships (Ponsen, 2006). In a review of previous research, Buchner (1965) included claims of bacterial endosymbionts being cultured from *Phylloxera vastatrix* Pl. (a synonym of *Daktulosphaira vitifoliae* Fitch), and of rod-shaped bacteria being identified in the closely related oak phylloxera, *Phylloxera quercus* Boyer de Fonscolombe (Hemiptera: Phylloxeridae). The identification of the bacteria proved transient, and it was concluded that grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) did not contain endosymbionts (Buchner, 1965). However, until the recent identification of bacterial DNA in gallicolae grape phylloxera (Vorwerk *et al.*, 2007), there had been limited re-examination of grape phylloxera bacterial symbiotic relationships utilising molecular technology. There are currently a range of molecular

PCR primers available that specifically amplify DNA from the bacterial 16S small ribosomal subunit. The 16S rDNA primers identify bacterial DNA within a homogenised insect DNA sample. Specific insect nuclear small subunit (SSU) rDNA primers are also available to pre-test the insect DNA sample for PCR amplification inhibitors.

The horizontal transfer of secondary symbionts in the Aphididae highlighted the theoretical possibility that members of the Phylloxeridae (including grape phylloxera) have been infected with a non-essential, transient bacterial species post evolutionary divergence. Therefore the current study utilised culturing and molecular techniques to determine the status of bacterial associations within radicicolae grape phylloxera. Clarification of the endosymbiont status of radicicolae grape phylloxera was considered important for understanding host-plant interactions because the presence of endosymbionts may play an important role in the nutritional physiology and development of grape phylloxera. An understanding of the influences of bacterial associations on insect biology would also be important for the future development of an artificial feeding system (Chapter 4) as insects with a symbiotic relationship are capable of synthesising amino acids missing from an artificial diet formulation, while aposymbiotic (symbiont-free) insects require the inclusion of all essential amino acids for survival (Douglas, 2006). Therefore an endosymbiotic relationship in radicicolae grape phylloxera could be targeted as a possible control option for the pest insect.

The current study utilised microbiological and molecular techniques to re-examine the status of bacterial symbiotic associations with radicicolae grape phylloxera. Specific bacterial primers (16S rDNA) provided an initial screening for bacterial DNA prior to sequencing. *Pantoea* specific primers developed to align with the gallicolae grape phylloxera associated bacteria (Vorwerk *et al.*, 2007) were also examined.

# 3 MATERIAL AND METHODS

## *3.1 INSECT MATERIAL*

Radicicolae grape phylloxera were originally collected from infested vineyards in the King Valley, Victoria, Australia. Grape phylloxera from this region have been identified as a single genotypic class, G4 (Corrie *et al.*, 2002). Populations were maintained at the Department of Primary Industries – Rutherglen Centre on excised *Vitis vinifera* L. (Vitaceae) cv. Shiraz root pieces (approximately 1 cm width x 10 cm length), prepared using a protocol slightly modified from Granett *et al.* (1985). The modifications were: (1) roots washed clean of attached soil with a soft brush under running water; (2) roots soaked for 5 minutes in Ridomil® Gold Plus systemic fungicide solution (2.3 g/L); (3) roots triple rinsed with sterile distilled water prior to air drying; (4) both ends of roots wrapped in moist cotton wool; and (5) the size of the petri dish was increased to 15 cm, with the increase in area reducing the impact of water condensation. Grape phylloxera populations were incubated in the dark at a constant temperature of 25 ± 3°C.

Only fourth instar and adult apterous radicicolae grape phylloxera were used for this study. Insects used for bacterial endosymbiont screening were collected directly from the excised root pieces, or from populations of grape phylloxera that had been transferred to *V. vinifera* cv. Shiraz tissue culture or potted grapevines. Micropropagated grapevines were prepared as outlined by Kellow *et al.* (2002). Slight modifications included: (1) agar medium was not supplemented with benzyl aminopurine or napthaleneacetic acid; and (2) tissue culture plantlets were not transferred to a second agar medium prior to the perlite-based medium. Potted grapevines were maintained under glasshouse conditions with an alternating 12 hour temperature range of 18-26°C and 18-22°C. Natural lighting was supplemented with artificial growth lights for a 14 hour day period, and an additional 1 hour exposure at night (Korosi *et al.*, 2007).

### *3.2 CULTURING OF INTERNAL BACTERIA*

In preparation for culturing, apterous radicicolae grape phylloxera (n = 5-10) were collected from the potted grapevine root source into a sterile 2 ml tube for sterilisation. The insects were mixed with 70% ethanol for 30 seconds or 5 minutes, and then rinsed twice with sterile water. The insects were aseptically transferred to a new sterile 2 ml tube containing 200 µl of nutrient broth and homogenised with a microtube pestle. The inoculant was incubated at 25°C for 48-96 hours prior to spreading (10 µl) in triplicate onto nutrient agar plates (13 g nutrient broth, 10 g agar bacteriological per L). Agar plates were incubated at 25°C for 1-2 days. Non-inoculated nutrient agar plates were also incubated as a negative control. Culturing reagents were sourced from OXOID™.

Single bacteria colonies were viewed with a stereo microscope and characterised by a range of morphological features including diameter, colour, form, elevation, and margin. Gram smears were prepared as described by Black (1999) and examined using an Olympus BH2 light microscope. Digital images were captured with a Nikon Coolpix 4500 camera using a microscope eyepiece adaptor.

### *3.3 DNA EXTRACTION*

In preparation for DNA extraction, apterous radicicolae grape phylloxera were collected into a sterile 2 ml tube for sterilisation. Groups (n = 10) of the insects were mixed with 70% ethanol for 30 seconds or 5 minutes, and then rinsed with sterile water. The insects were aseptically transferred to a new sterile 2 ml tube for DNA extraction.

Grape phylloxera DNA extractions were performed using a QIAGEN® DNeasy® Tissue DNA purification kit. Specifically, the QIAGEN® DNeasy® DNA purification procedure for the isolation of genomic DNA from insects with microtube pestles was used. DNA extractions were also performed on a group (n = 5) of Monterey pine aphids (*Essigella californica*, Hemiptera: Aphidoidea: Lachnidae: Cinarinae) as a positive control (insects

supplied by Dr Trudy Wharton, Australian National University, Canberra). The endosymbiont status of the Monterey pine aphid had not previously been examined, however the insect was expected to contain *Buchnera* as closely related species (Aphidoidea: Lachnidae: Cinarinae) have been confirmed to contain the primary symbiont (Martinez-Torres *et al.*, 2001). Monterey pine aphids were stored in 100% ethanol at -20°C until required. An additional DNA extraction was performed with no insects as a negative control to confirm the sterility of the procedure.

### *3.4 PCR CONDITIONS AND PRIMER COMBINATIONS*

All DNA extractions were amplified with insect nuclear SSU rDNA, bacterial 16S rDNA and *Pantoea* primers in a BioRad® iCycler® Thermal Cycler. Non-lysed bacteria cells cultured from grape phylloxera samples were also amplified and provided a negative control for the SSU rDNA primers and a positive control for the 16S rDNA primers. PCR mixtures contained 1.5 µl 50 mM $MgCl_2$, 2.5 µl 10x reaction buffer (BIOLINE™), 0.5 µl 20 mM dNTP, 0.5 µl 10 µM each forward and reverse primer, 1 µl 1U BIOTAQ™ RED DNA polymerase, 2 µl of DNA template and nuclease free sterile water to final volume of 25 µl. All PCR mixtures were prepared with negative (sterile water) and positive (Monterey pine aphid DNA) control. PCR reagents were sourced from BIOLINE™ and primers from GeneWorks™.

PCR products (10 µl) were detected by electrophoresis on a 1% agarose gel stained with ethidium bromide. DNA samples were run with a 100 bp (0.5 µg) DNA ladder and either a 1 kb (0.5 µg) or a 2-Log: 0.1-10 kb (1.0 µg) DNA ladder (New England Biolabs®). DNA bands were visualised with a BioRad® Molecular Imager Gel Doc™ System. Images were captured digitally using BioRad® Quantity One® 1-D Analysis Software.

### *3.4.1 SSU rDNA PRIMERS*

Amplification with nuclear SSU rDNA primers 2880 (5'-CTGGTTGATCCTGCCAGTAG-3') and B- (5'-CCGCGGCTGCTGGCACCAGA-3') (Cook *et al.*, 2002) confirmed that DNA extractions contained no PCR inhibiting compounds. The SSU rDNA primers amplify insect DNA but do not align with bacteria DNA for amplification. PCR mixtures were incubated in a preheated (94°C) thermal cycler at 94°C for 3 minutes (initial denaturation); followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds (annealing) and 72°C for 1 minute, the time for each step increased by 0.02 seconds per cycle; followed by a final extension period of 7 minutes at 72°C. The SSU rDNA primers produced an amplification product approximately 650 bp in length.

### *3.4.2 16S rDNA PRIMERS*

Bacterial specific 16S rDNA forward primer 27F (5'-AGATTTGATCMTGGCTCAG-3') and universal reverse primer 1492R (5'-GGYTACCTTGTTACGACTT-3') were used to investigate the presence of the bacterial rRNA gene within grape phylloxera DNA extractions. PCR mixtures were incubated in a preheated (94°C) thermal cycler at 94°C for 3 minutes (initial denaturation); followed by 30 cycles at 94°C for 30 seconds, 50°C for 45 seconds (annealing) and 72°C for 90 seconds; followed by a final extension period of 7 minutes at 72°C (modified from Dojka *et al.*, 2000). The 16S rDNA primers produced an amplification product approximately 1.5 kb in length.

### *3.4.3* PANTOEA *PRIMERS*

Gram-negative bacteria closely related to *Pantoea agglomerans* had previously been identified in an association with gallicolae grape phylloxera populations (sequence alignment 99-100%), and specific 16S rDNA primers for the amplification of *P. agglomerans* were supplied by Sonja Vorwerk, University of Hohenheim (Stuttgart, Germany). *Pantoea* specific forward primer *pagF* (5'-CACTGGAAACGGTGGCTAAT-3') and reverse primer *pagR* (5'-CGGCAGTCTCCTTTGAGTTC-3') were used to

determine the status of the *P. agglomerans* association with radicicolae grape phylloxera. PCR mixtures were incubated in a preheated (94°C) thermal cycler at 94°C for 3 minutes (initial denaturation); followed by 40 cycles at 94°C for 30 seconds, 48°C for 45 seconds (annealing) and 72°C for 45 seconds; followed by a final extension period of 7 minutes at 72°C. The *Pantoea* primers produced an amplification product approximately 1 kb in length (Vorwerk *et al.*, 2007). The *Pantoea* primers amplify a region of the 16S rRNA gene within the amplification region of the 16S rDNA primers.

### *3.5 CLONING AND SEQUENCING*

The 16S rDNA PCR amplification product of radicicolae grape phylloxera DNA collected from the potted grapevine root source was cloned and sequenced. Two DNA samples (30 second and 5 minute 70% ethanol sterilisation time) were supplied in dehydrated format to Saturn Biotech, Murdoch University (Perth, Western Australia) for PCR, cloning, sequencing and sequence editing.

PCR was performed with the 27F and 1492R 16S rDNA primers, and each reaction contained 3 µl 25 mM $MgCl_2$, 2.5 µl 10x reaction buffer (Applied Biosystems™), 1.2 µl 20 mM dNTP, 1.0 µl 10 µM each forward and reverse primer, 0.25 µl 5U Applied Biosystems™ Taq polymerase, 1.5 or 1.0 µl of DNA template (30 second and 5 minute DNA extractions respectively) and nuclease free sterile water to final volume of 25 µl. PCR mixtures were incubated in a thermal cycler at 94°C for 3 minutes (initial denaturation); followed by 45 cycles at 94°C for 50 seconds, 50°C for 30 seconds (annealing) and 72°C for 90 seconds; followed by a final extension period of 7 minutes at 72°C. The amplified products were separated on a 1.2% agarose gel, fragments excised and purified with a QIAGEN® QIAquick® Gel Extraction Kit. DNA fragments were cloned (20 clones for the 30-second and 16 clones for the 5-minute PCR amplification products) into the Promega™ pGEM®-T Easy Vector. The cloned DNA

fragments were bidirectionally sequenced with the Promega™ pGEM®-T Easy Vector T7 and SP6 RNA polymerase promoter primers in an Applied Biosystems™ 3730 DNA analyser. The final sequence was edited and assembled with Applied Biosystems™ SeqEd software (version 1.0.3).

The GenBank Basic Local Alignment Search Tool (BLAST) was used to identify sequence alignment between bacteria species in the GenBank database and the 16S rDNA sequence associated with radicicolae grape phylloxera.

# 4 RESULTS

## *4.1 BACTERIA CULTURE*

Samples of homogenised grape phylloxera grew uniform bacterial colonies on nutrient agar; insect sterilisation time (30 seconds or 5 minutes) did not impact on colony growth. The colonies were generally 1-2 mm in diameter, opaque yellow-cream in colour, circular in form, with an entire margin and a convex elevation (Figure 1). The bacteria cells stained as gram-positive rod-shaped bacilli with rounded ends and parallel sides (Figure 2). No bacteria colonies grew on the non-inoculated nutrient agar negative control plates.

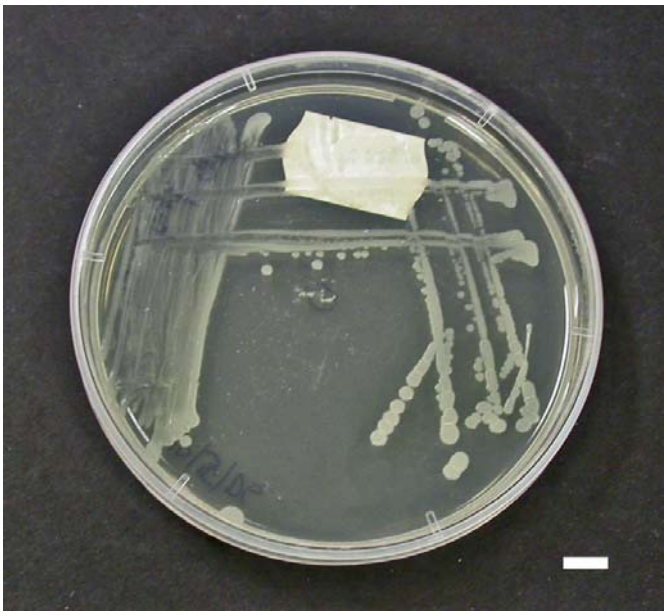

**Figure 1.** Morphology of bacteria colonies cultured from radicicolae grape phylloxera growing on nutrient agar. Scale bar 10 mm.

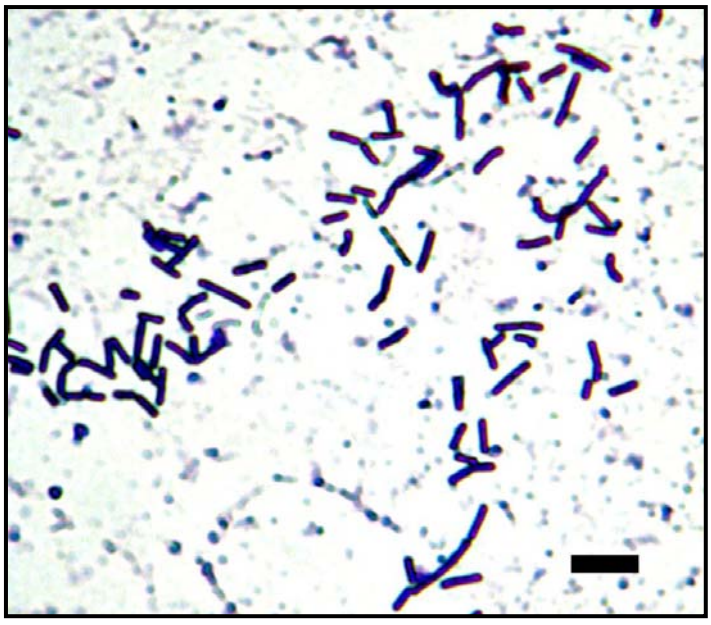

**Figure 2.** Gram-positive (purple) rod-shaped bacteria cultured from radicicolae grape phylloxera. Scale bar 10 µm.

### *4.2 PCR AMPLIFICATION*

DNA extractions were performed on grape phylloxera collected from three different root sources (tissue culture, excised root and potted grapevine) and at two different sterilisation times (30 second and 5 minute). All insect DNA extractions amplified with the SSU rDNA primers (Table 1), confirming that the DNA was not contaminated with PCR inhibiting compounds. Cultured bacteria cells from grape phylloxera, the DNA extraction negative control, and the PCR negative control samples did not amplify the SSU rDNA primers (Figure 3).

Inconsistent product amplification of grape phylloxera DNA extractions was obtained with the 16S rDNA primers (Table 1). All grape phylloxera DNA extractions from the potted grapevine root source amplified a 1.5 kb product, independent of sterilisation time. There was no amplification of grape phylloxera DNA extractions from the excised root or tissue culture sources. All negative amplification results were confirmed with (at least) a second PCR attempt. The Monterey pine aphid DNA amplified with the 16S rDNA primers, although only genomic DNA (>10 kb) was present for the grape phylloxera cultured bacteria cells, lane 5 (Figure 4). The DNA extraction negative control and the PCR negative control samples did not amplify the 16S rDNA primers.

The *Pantoea* primers produced variable amplification of grape phylloxera DNA (Table 1). The 1 kb product was observed in the DNA extraction of grape phylloxera collected from the tissue culture source, the grape phylloxera cultured bacteria cells and the Monterey pine aphid. Multiple non-specific bands between 300-800 bp were also amplified, and were observed in all other grape phylloxera DNA extractions (Figure 5). The PCR negative control did not amplify the *Pantoea* primers.

**Table 1.** The result of PCR amplification with SSU rDNA, 16S rDNA and *Pantoea* primers for grape phylloxera DNA extractions. Grape phylloxera DNA extractions identified by the root collection source and the 70% ethanol sterilisation time; the DNA extraction negative control and PCR positive (Monterey pine aphid) and negative (water) controls are also indicated.

| phylloxera DNA extraction | root collection source | ethanol sterilisation | primer amplification result | | |
|---|---|---|---|---|---|
| | | | SSU rDNA | 16S rDNA | Pantoea |
| 1 | potted vine | 30 seconds | ✓ | ✓ | * |
| 2 | potted vine | 5 minutes | ✓ | ✓ | * |
| 3 | bacteria cells[1] | 5 minutes[2] | × | ✓ | ✓ |
| 4 | excised root | 30 seconds | ✓ | × | * |
| 5 | excised root | 5 minutes | ✓ | × | * |
| 6 | tissue culture | 5 minutes | ✓ | × | ✓ |
| DNA extraction negative control | | NA | × | × | × |
| Monterey pine aphid | | 5 minutes | ✓ | ✓ | ✓ |
| PCR negative control (water) | | NA | × | × | × |

Primer amplification result codes: ✓ = amplification product; × = no amplification product; * = amplification product represented non-specific bands

[1] non-lysed cells from a grape phylloxera cultured bacteria colony used for PCR DNA template

[2] 5 minute ethanol sterilisation of grape phylloxera collected from the potted grapevine root source prior to culturing on nutrient agar; there was no additional ethanol sterilisation step for the bacteria cells prior to PCR

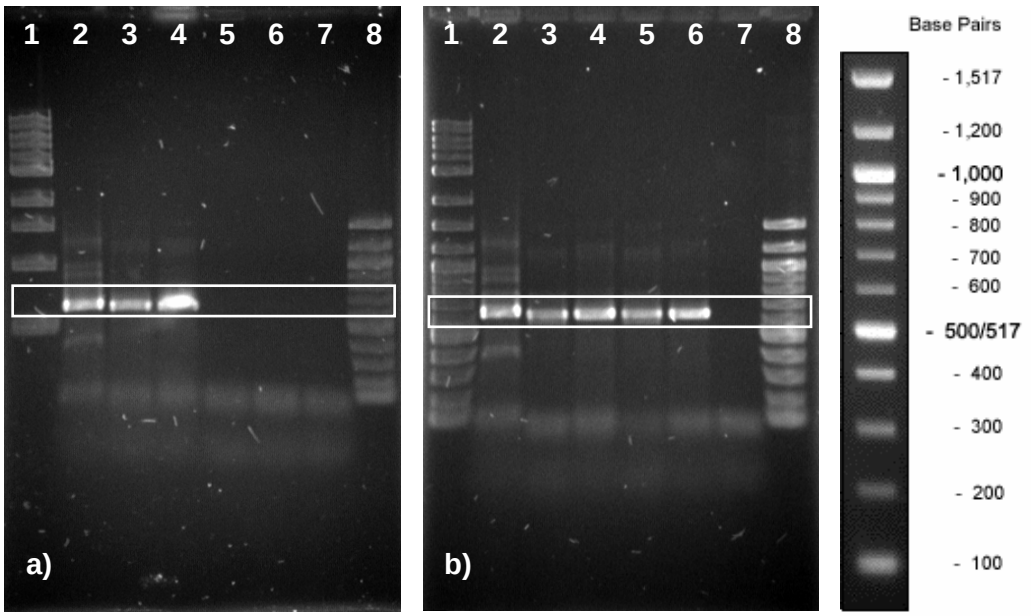


**Figure 3.** PCR amplification products with SSU rDNA primers; the 650 bp products are boxed (the 100 bp DNA ladder is shown for product size alignment). Lane numbers 1-8 are indicated, grape phylloxera DNA extractions are denoted by root collection source, and ethanol sterilisation time in parenthesis.

Agarose gel **3a)** lane 1: 1 kb DNA ladder, lane 2: Monterey pine aphid, lane 3: potted grapevine (30 sec), lane 4: potted grapevine (5 min), lane 5: bacteria cells, lane 6: DNA extraction negative control, lane 7: PCR negative control, lane 8: 100 bp DNA ladder.

Agarose gel **3b)** lane 1: 2-Log DNA ladder, lane 2: Monterey pine aphid, lane 3: excised root (30 sec), lane 4: potted grapevine (30sec), lane 5: excised root (5 min), lane 6: tissue culture (5 min), lane 7: PCR negative control, lane 8: 100 bp DNA ladder.

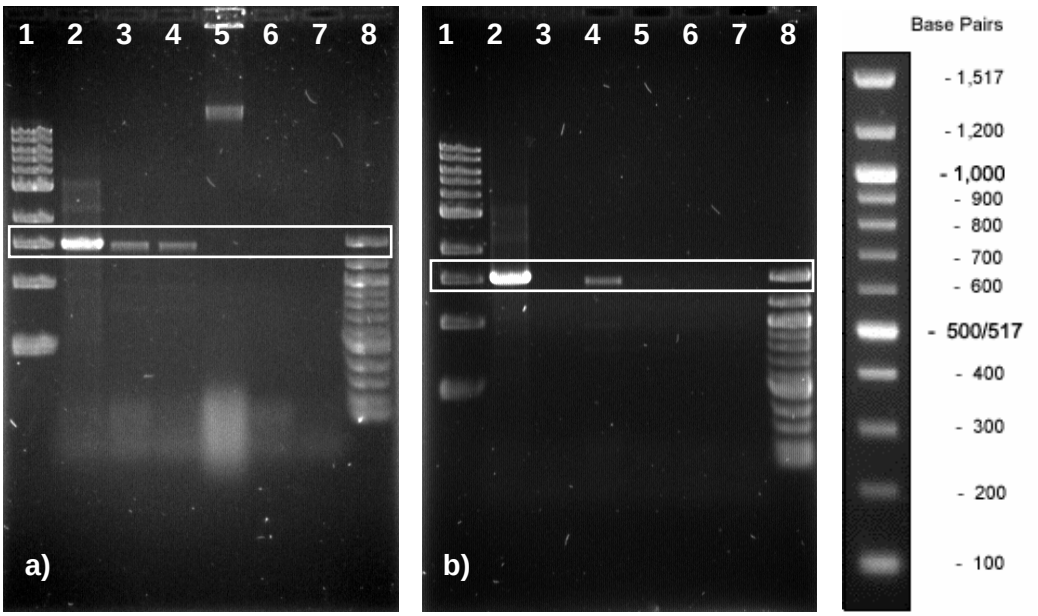


**Figure 4.** PCR amplification products with 16S rDNA primers; the 1.5 kb products are boxed (the 100 bp DNA ladder is shown for product size alignment). Lane numbers 1-8 are indicated, grape phylloxera DNA extractions denoted by root collection source, and ethanol sterilisation time in parenthesis.

Agarose gel **4a)** lane 1: 1 kb DNA ladder, lane 2: Monterey pine aphid, lane 3: potted grapevine (30 sec), lane 4: potted grapevine (5 min), lane 5: bacteria cells, lane 6: DNA extraction negative control, lane 7: PCR negative control, lane 8: 100 bp DNA ladder.

Agarose gel **4b)** lane 1: 1 kb DNA ladder, lane 2: Monterey pine aphid, lane 3: excised root (30 sec), lane 4: potted grapevine (30 sec), lane 5: excised root (5 min), lane 6: tissue culture (5 min), lane 7: PCR negative control, lane 8: 100 bp DNA ladder.

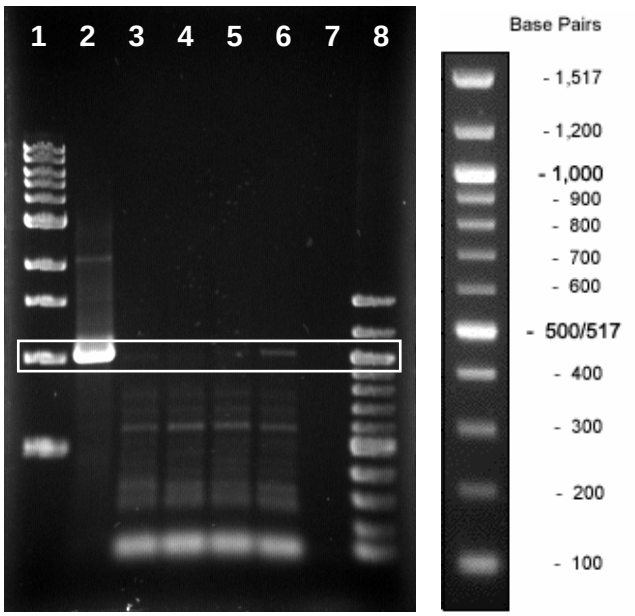


**Figure 5.** PCR amplification products with *Pantoea* primers; the 1 kb products are boxed and non-specific bands unboxed (the 100 bp DNA ladder is shown for product size alignment). Lane numbers 1-8 are indicated, grape phylloxera DNA extractions denoted by root collection source, and ethanol sterilisation time in parenthesis.

Agarose gel lane 1: 1 kb DNA ladder, lane 2: Monterey pine aphid, lane 3: excised root (30 sec), lane 4: potted grapevine (30 sec), lane 5: excised root (5 min), lane 6: tissue culture (5 min), lane 7: PCR negative control, lane 8: 100 bp DNA ladder.

### *4.3 BACTERIAL DNA SEQUENCE*

There was limited variation in the DNA sequence of the individually cloned PCR products from the radicicolae grape phylloxera DNA extractions (30 second and 5 minute ethanol sterilisation times); therefore a composite DNA sequence from all cloned PCR products was produced. Sequencing of 16S rDNA amplified with the 27F and 1492R primers produced a 1.49 kb fragment (Figure 6). The DNA sequence was not identified following comparison with the GenBank database using megaBLAST; the maximum identity was 91% to an environmental uncultured bacterium 16S rRNA gene (GenBank accession number DQ354732). There was 85% identity with the bacteria sequence identified in gallicolae grape phylloxera (GenBank accession number AM050138) by two sequence alignment (GenBank bl2seq).

```
1    GGCTACCTTG TTACGACTTC ACCCCAGTCA TCGGCCACAC CGTGGTAAGC GCCCTCCTTA
61   CGGTTAGGCT ACCTACTTCT GGTGCAACAA ACTCCCATGG TGTGACGGGC GGTGTGTACA
121  AGGCCCGGGA ACGTATTCAC CGCGACATTC TGATCCGCGA TTACTAGCGA TTCCGACTTC
181  ATGCACTCGA GTTGCAGACT GCAATCCGGA CTACGATCGG CTTTTTGAGA TTAGCTCGCC
241  CTCGCGGGTT GGCAACCCTC TGTACCGACC ATTGTATGAC GTGTGTAGCC CTGCCCATAA
301  GGGCCATGAT GACTTGACGT CATCCCCACC TTCCTCCGGT TTGTCACTGG CAGTATCCTT
361  AGAGTGCCCA TGCTAAATGC TGGCAAATAA TGAAAAGGGT TGCGCTCGTT GCGGGACTTA
421  ACCCAACATC TCACGACACG AGCTGACGAC AGCCATGCAG CACCTGTGTT AAGATTCCCT
481  AACAAGCACT CTCCTATCTC TCCAGAATTC CAGACATGTC AAGGGTAGGT AAGGTTCTTC
541  GCGTTGCATC GAATTAAACC ACATGCTCCA CCGCTTGTGC GGGTCCCCGT CAATTCCTTT
601  GAGTTTTAAC CTTGCGACCG TACTCCCCAG GCGGTCAACT TATCGCGTTA GCTGCGCCAC
661  TGAAGACTCA AGCCCCCCAA CAGCTAGTTG ACATCGTTTA CGGCGTGGAC TACCAGGGTA
721  TCTAATCCTG TTTGCTCCCC ACGCTTTCGC GCATCAGCGT CAGTAATAGC CCAGAAGGTT
781  GCCTTCGCCA TCGGTGTTCC TCCAGATATC TACGCATTTC ACCGCTACAC TCGGAATTCC
841  ACCCTCCTCT TCCATACTCT AGCCTTGCCA GTCTCGAATG CAATTCCCAA GTTAAGCCCG
901  GGGATTTCAC ATCTGACTTG CCAAGCCGCC TGCGCGCGCT TTACGCCCAG TAATTCCGAT
961  TAACGCTTGC ACCCTACGTA TTACCGCGGC TGCTGGCACG GAGTTAGCCG GTGCTTATTC
1021 TTCGGGTACC GTCATCATTC GCGGGTATTT GTCCCAGGGG CCTTCTTCCC CGCCAAAAGC
1081 ACTTTACAAC CAAAAGGCCT TCTTCACGCA CGCGGCATGG CTGGATCAGG CTTTCGCCCA
1141 TTGTCCAAAA TTCCCCACTG CTGCCTCCCG TAGGAGTCTG GGCCGTGTCT CAGTTCCAGT
1201 GTGGCGGATC ATCCTCTCAG ACCAGCTACA GATCGTCGCC TTGGTGAGCC TTTACCTCAC
1261 CAACTAGCTA ATCTGACGTG GGCTCATCTG TTAGCGAGAG CAATAAAGCC CCCTTTCTCC
1321 CGAAGGGCTG ATGCGGTATT AGCGCAAGTT TCCGAGAGTT ATTCCCCACC ATCGGGCAGG
1381 TTCCCAGGCA TTACTCACCC GTTCGCCGCT CTATATCCCG ATAGCAACCT AACTAAACCG
1441 TTCGACTTGC ATGTGTTAGG CCTGCCGCCA GCGTTCAATC TGAGCCATGA TCAAACTCT
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**Figure 6.** DNA sequence (1.49 kb) amplified by bacterial 16S rDNA primers in radicicolae grape phylloxera.

# 5 DISCUSSION

Bacterial morphology and molecular techniques identified a bacterial association in radicicolae grape phylloxera. The ability to culture the bacteria species indicated that the association was not a mutually dependent endosymbiotic relationship, and differed from the essential and mutualistic association that Aphididae have with their primary symbiont *Buchnera*. However in the absence of alternative symbiotic relationships, the bacterial association identified for radicicolae grape phylloxera would signify a primary symbiont relationship.

## *5.1 CULTURAL IDENTIFICATION OF GRAPE PHYLLOXERA BACTERIA*

The gram-positive rod-shaped bacteria associated with radicicolae grape phylloxera differed morphologically from the spherical-shaped cocci of the Aphididae primary symbiont *Buchnera* (Baumann et al., 1995). The radicicolae grape phylloxera bacteria also differed to the gram-negative short bacilli associated with gallicolae grape phylloxera; the gallicolae bacteria was morphologically characterised to the family *Enterobacteriaceae* (Vorwerk *et al.*, 2007). Aphididae secondary symbionts are also typically rod-shaped *Enterobacteriaceae*, although few of these symbionts have been cultured outside of the host (Moran *et al.*, 2005). Therefore the bacteria cultured from radicicolae grape phylloxera did not align with the gram-negative symbiotic relationships previously recognised in Aphididae or Phylloxeridae. Rod-shaped endosymbionts have been identified within the parenchyma feeding Adelgidae (Ponsen, 2006), although further morphological description was not provided for comparison with the radicicolae grape phylloxera rod-shaped bacteria.

Aphididae primary and secondary symbionts are located with the haemocoel of the insect, however bacterial associations have also been identified in the gut of some species (Grenier *et al.*, 1994). Microscopy studies located gram-positive cocci and gram-negative bacilli inside the intestinal tract of the pea aphid, *Acyrthosiphon pisum*

Harris (Hemiptera: Aphididae). The undefined bacteria were not observed in all life stages of *A. pisum*, and were suspected to be accidental contaminants from host-plants or vascular tissue. The bacteria were capable of reproducing in the digestive system of the insect, but were not considered to perform a vital function for *A. pisum*, and in certain circumstances may have represented a parasitic association (Grenier *et al.*, 1994).

Rod-shaped gram-positive bacteria have also been identified in the intestinal tract of a range of soil-dwelling insects (Margulis *et al.*, 1998). The bacteria were ingested from soil particles or faeces and attached to the wall of the intestine as filamentous, spore-forming structures. Cells released by the filaments were passed during waste excretion by the insects to complete the cycle of infection. The symbiont was identified as the intestinal stage of the soil bacterium *Bacillus cereus*, although no direct evidence was provided for the mutualistic, parasitic or commensalistic basis of the symbiotic association (Margulis *et al.*, 1998).

The identification of bacterial cells in the digestive system of grape phylloxera was not a focus of the digestive physiology research presented in this thesis (Chapter 2). Although the presence of bacterial cells within the digestive system was not evident, more detailed analysis, including transmission electron microscopy studies, may be required to locate isolated bacterial cells within the intestinal tract. The association of bacteria with the digestive system of *A. pisum* and soil-dwelling insects indicated that a similar association may be present in radicicolae grape phylloxera, explaining the transient detection of a bacterial association. The morphology of the bacteria cultured from radicicolae grape phylloxera appeared similar to *Bacillus* spp. (*Bergey's Manual of Systematic Bacteriology*, 1986), however further morphological and enzymological studies are required to fully identify the bacteria species.

## *5.2 MOLECULAR IDENTIFICATION OF GRAPE PHYLLOXERA BACTERIA*

The presence of bacterial DNA associated with radicicolae grape phylloxera was confirmed with molecular analysis, although the amplification of the 16S rDNA primers was variable. Grape phylloxera DNA extractions from the potted grapevine source amplified the 1.5 kb product; however there was no amplification of DNA extractions from the excised root or tissue culture collection sources. The transient detection of bacterial DNA in grape phylloxera samples was further highlighted by the previous amplification of the 16S rDNA primers by DNA extractions (obtained from a non-QIAGEN extraction method) from both the excised root and the tissue culture sources (data not shown). The amplification of bacteria cells cultured from grape phylloxera was initially inhibited by the overloading of the PCR with bacteria cells, as indicated by the large (>10 kb) genomic DNA band on the agarose gel. The positive amplification of the bacteria cells cultured from grape phylloxera with the 16S rDNA primers was confirmed in an additional PCR (data not shown).

Bacterial DNA was also identified in the grape phylloxera DNA extractions with the amplification of the *Pantoea* primers; however the amplification results did not correlate with the 16S rDNA primers. Grape phylloxera DNA extractions from the potted grapevine amplified the 16S rDNA primers; however the *Pantoea* primers produced non-specific bands. Non-specific bands were also amplified from grape phylloxera excised root sources, and positive amplification was only observed in the grape phylloxera tissue culture and bacteria cell sources. Due to the internal alignment of the *Pantoea* primers within the 16S rDNA sequence, there was no explanation for the amplification of the grape phylloxera tissue culture DNA with the *Pantoea* primers but not the 16S rDNA primers. DNA from the Monterey pine aphid unexpectedly amplified the *Pantoea* specific primers; however Vorwerk *et al.* (2007) did not present the degree of specificity of the *Pantoea* primer combination beyond gallicolae grape phylloxera DNA, and it was undisclosed if Aphididae species were tested. Therefore it was

unknown if other Aphididae species would also amplify the *Pantoea* primers or if this amplification represented a specific relationship with the Monterey pine aphid.

The morphological characteristics of the bacteria cultured from radicicolae grape phylloxera indicated that the taxonomy of the species differed from the bacterial association identified in gallicolae grape phylloxera. The non-specific binding of the *Pantoea* primers to the radicicolae grape phylloxera DNA and the low level sequence identity (85%) supported the identification of a new bacterial association in radicicolae grape phylloxera. In an attempt to identify the 16S rDNA sequence amplified from radicicolae grape phylloxera DNA, the DNA sequence was also aligned with the 16S rRNA gene of Aphididae secondary symbionts (Russell *et al.*, 2003) and *Bacillus* spp (Margulis *et al.*, 1998). The 85-86% identity with Aphididae secondary symbionts (GenBank accession numbers AY136167, AY136146 and AY136162) confirmed the previously stated non-alignment of the morphological characteristics of radicicolae grape phylloxera cultured bacteria with Aphididae secondary symbionts. The same level of genetic identity (85-86%) was also identified in comparison with *Bacillus* spp (GenBank accession numbers Y15466, X55060 and D16266) identified as intestinal symbionts of soil-dwelling insects (Margulis *et al.*, 1998).

The 16S rDNA gene is highly conserved; therefore the 85-86% identity of the radicicolae grape phylloxera bacteria DNA sequence with a range of insect symbiont 16S rRNA genes, and the variable amplification (including non-specific bands) of the *Pantoea* primers, was due to the genetic conservation of the DNA sequence and not species identification. The 91% identity to any sequence in the GenBank database indicated that the bacterial sequence from radicicolae grape phylloxera may represent a novel species, and further investigations are required in order to resolve the identity of the organism.

### *5.3 MOLECULAR CONTROLS*

Due to the variable and transient amplification of the radicicolae grape phylloxera DNA extractions during PCR with both 16S rDNA and *Pantoea* primers, multiple controls were incorporated into the molecular experiments to confirm the results: (1) DNA quality was not the cause of the amplification variation as all grape phylloxera DNA extractions amplified with the insect SSU rDNA primers to confirm the absence of PCR inhibiting compounds; (2) The possibility of external contamination during the DNA extraction procedure (causing the transient positive amplification results) was eliminated by the non-amplification of the DNA extraction negative control in all PCR. The non-amplification of all PCR negative controls eliminated the possibility of contamination during the PCR set up; (3) The Monterey pine aphid DNA extraction was a positive control for all PCR, with amplification of the correct product size occurring in all reactions. The successful amplification of the Monterey pine aphid confirmed that all primer combinations were working; (4) Insect sterilisation time was initially considered a possible factor for the non-amplification of the 16S rDNA primers, with the 5 minute 70% ethanol sterilisation time potentially having a negative impact on bacterial DNA detection. However the positive amplification of grape phylloxera DNA extractions from the potted grapevine source exposed to 70% ethanol for both 30 seconds and 5 minutes confirmed that sterilisation time was not a factor.

### *5.4 IMPACTS OF FEEDING LOCATION*

The transient amplification of bacterial 16S rDNA primers in grape phylloxera DNA extractions may have been related to the root collection source. The potted grapevine provided the most 'natural' environment for radicicolae grape phylloxera, with the insect surrounded by soil and feeding on a relatively mature whole-plant system. Excised roots, although initially collected from a soil environment, are removed from a soil environment and surface treated with fungicide prior to insect infestation. The excised roots are restricted from a whole-plant response to feeding by grape phylloxera; the

tissue culture grapevines provide a whole-plant response, although the root system is relatively immature and in a sterile, soil-less environment.

If the bacteria detected in the potted grapevine DNA extractions 'infected' the grape phylloxera from the external environment, then bacteria would not be present in the sterile tissue culture system, explaining the non-amplification result for the tissue culture DNA extraction. The previous amplification of the 16S rDNA primers with grape phylloxera tissue culture DNA extractions (data not shown), and the amplification of the *Pantoea* primers, may have represented the transient retention of the bacterial association from a previous infection. Vorwerk *et al.* (2007) identified bacterial DNA in field collected adult gallicolae grape phylloxera, and in their parthenogenetically produced eggs. The presence of bacterial DNA in the eggs of the insect suggested the maternal inheritance of the microorganism association. Investigation of the presence of bacterial DNA in the eggs of radicicolae grape phylloxera was not attempted, leaving the issue of internal transfer unresolved; however the absence of bacterial DNA in the excised root samples did not support the maternal transmission of the bacteria.

The excised roots used for collection of grape phylloxera for DNA extraction were nutritionally poor, with the grape phylloxera population observed to have a reduced fecundity and growth rate compared with 'typical' excised roots described in earlier studies (Powell *et al.*, 2006). The causes for this poor population growth were unknown, but interestingly, previous DNA extractions of grape phylloxera collected from 'typical' excised roots were amplified with the 16S rDNA primers (data not shown). The absence of the bacterial association with grape phylloxera on poor quality excised root pieces highlighted the possibility that there may be a correlation between grapevine health, insect development and the presence of bacteria. The field location of excised root collection, and the soils natural biota, may also be a factor. Secondary symbionts in Aphididae have been associated with beneficial impacts on insect biology

for reproduction and changes in host-plant range (Moran *et al.*, 2005), and nutritional contributions have been identified as the possible role of symbionts in Adelgidae (Havill and Foottit, 2007). A similar bacterial association in grape phylloxera would provide an additional cause, beyond grapevine health, for the variable insect survival rates observed in laboratory controlled experiments.

### *5.5 COMPARISON WITH PREVIOUS RESEARCH*

The transient detection of bacterial DNA in grape phylloxera had similarities to the observation of rod-shaped organisms in *P. quercus*. These organisms were concluded to be of low significance with only occasional occurrence (Buchner, 1965). Grape phylloxera do not have an association with the Aphididae primary symbiont *Buchnera* (Vorwerk *et al.*, 2007), and previous molecular studies have failed to locate the presence of secondary or tertiary Aphididae symbionts (Chen *et al.*, 1996; Chen and Purcell, 1997). Speculatively however, there remains the possibility that a non-essential, transient bacterial relationship developed in grape phylloxera after evolutionary divergence from the Aphididae family. Transient bacteria may be ingested with food, and either lost with the passing of waste, or during life stage moulting (Douglas, 1998). The mechanism of waste disposal in grape phylloxera is not clearly defined, and may occur either as anal waste excretion, or via the salivary glands into the feeding cell of the grapevine root (Chapter 2). The regurgitation of waste products back into the feeding location may allow for the re-infection of the insect with the bacterial symbiont. Equally, the close distribution of radicicolae grape phylloxera on a grapevine gall would allow for the infection of first instar insects with the waste products of later life stages prior to the establishment of feeding. This method of infection parallels the cycle outlined for *B. cereus* in soil-dwelling insects (Margulis *et al.*, 1998)

The transient detection of the bacterial DNA in radicicolae grape phylloxera indicated that the association was not essential for the survival of the insect, although an insect

bacterial infection may have some physiological and ecological impact on the insect (Sandstrom *et al.*, 2001). Aphididae secondary symbionts are facultative, however they are still considered to have influenced aphid ecology and evolution (Russell *et al.*, 2003). The bacterial association identified in radicicolae grape phylloxera was distinct from the Aphididae secondary symbiont and the gallicolae grape phylloxera bacterial association; however the bacteria species may still have an impact on the biology of the insect. There are similarities between the bacteria isolated from radicicolae grape phylloxera and intestinal symbionts identified in *A. pisum* (Grenier *et al.*, 1994) and soil-dwelling insects (Margulis *et al.*, 1998); however the identity of the bacteria species has not been resolved.

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# CHAPTER 4

# DEVELOPMENT OF AN ARTIFICIAL FEEDING SYSTEM FOR GRAPE PHYLLOXERA

# 1 SUMMARY

Factors important for the development of an artificial feeding system for radicicolae grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) included chamber design, diet formulation (sugar and amino acid composition), solution pH and insect life stage. Two artificial diet chambers were trialled, and although the filter paper chamber provided a suitable environment for the survival of grape phylloxera, the humidity chamber was considered an improved design for application to a grape phylloxera feeding system. The humidity chamber simplified the observation of the insects during the diet experiments, reduced the impact of condensed water droplets within the insect chambers and enabled the renewal of the diet solution without disturbing insect feeding behaviour. From 15 diet experiments with grape phylloxera, only two artificial diet formulations resulted in significant increases in survival time; however these results were not reproducible. Insect probing activity did not relate to increased insect survival time, and a significantly higher proportion of probing insects was only observed on one of these diet formulations: *5% sucrose pH 4.5 plus amino acid*. Probing activity inferred insect attraction to the artificial diet formulation, but did not imply ingestion of the diet solution. Factors considered critical for the successful development of a radicicolae grape phylloxera artificial diet formulation included sugar in the form of sucrose, the inclusion of a number of essential amino acids and an acidic solution, although grape phylloxera may be able to feed at a range of pH values.

## 2 INTRODUCTION

An artificial diet is a mix of synthesised ingredients that provide food and nutrition to a living organism. An important application of artificial diets is to provide a method for maintaining insect colonies in a laboratory environment, in the absence of a natural (plant) food source. Artificial diets are also used as a model system to undertake biological studies, including: the impact of specific dietary compounds (sugars, amino acids, vitamins) on insect survival and food uptake (Srivastava and Auclair, 1971; Febvay *et al.*, 1988); examination of the nutritional contribution from bacterial symbiotic relationships (Sasaki *et al.*, 1991); and the investigation of the impacts of possible control agents, including lectins, protease inhibitors and enzymes (Powell *et al.*, 1993). The artificial diet composition may be defined as holidic, and contain a fully defined list of chemicals, as meridic, containing a mix of defined and non-defined compounds or as an oligidic diet, and contain a mix of non-defined and/or non-purified compounds (Cohen, 2003).

Artificial diets have been formulated for a number of plant-sucking insect species within the orders of Thysanoptera and Hemiptera. The success of artificial diets has varied, with experiments reporting insect development through the nymphal life stages, but failing to support adult development (Jancovich *et al.*, 1997); the maintenance of an insect population for several weeks and through several generations (Auclair, 1965); or the continuation of the same insect colony for extended periods of time, and in exceptional cases for up to 26 years (van Emden, 2003). Insects grown on artificial diet systems typically experience extended developmental rates, and reduced weight gains in comparison with plant-fed insects. However there are some exceptions within artificial diet systems where insect developmental time has been similar to the developmental time on a natural food source, with increased survival time and adult insects ovipositing more eggs (Debolt, 1982).

Many variations in diet formulation and chamber design exist, with specific modifications often required to reflect the natural food source of the insect. The nutritional requirements of plant-sucking insects are essentially the same as other insects, and include amino acids, water-soluble vitamins, salts and sucrose (Auclair, 1969). The success of fluid artificial diets for plant-sucking insects has been assisted by the utilisation of a double layer of Parafilm® to encase the liquid artificial diet. Since the first application of Parafilm® to the artificial diet system by Mittler and Dadd (1962), Parafilm® has become a common part of the artificial diet chamber design for plant-sucking insects. The transparent nature of Parafilm®, and the self-sealing characteristics, allow observation of the insect in the diet chamber and prevent the leakage of the diet solution with insect stylet penetration (van Emden, 2003).

A number of artificial diet systems have been established for the plant-sucking Aphididae. The nutritional requirements of agriculturally significant pests with the Aphididae have been studied with the application of artificial diet systems, including the pea aphid (*Acyrthosiphon pisum* Harris), the bean aphid (*Aphis fabae* Scopoli), the cotton aphid (*Aphis gossypii* Glover), the potato aphid (*Macrosphum euphorbiae* Thomas) and the green peach aphid (*Myzus persicae* Sulzer) (reviewed in Auclair, 1969).

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) has been a significant agricultural pest insect since the late 1800s, however only one research group has published details of an artificial diet system for Phylloxeridae (Wöhrle, 1999; Forneck and Wöhrle, 2003). Using an artificial diet formulation of '5% sucrose' or '5% sucrose plus a 10 amino acid mix', fourth instar gallicolae grape phylloxera survived on the artificial diet a maximum of 14 days (Wöhrle, 1999). However, in Australian vineyards radicicolae grape phylloxera pose the greatest threat to grapevine health and

production, and no artificial diet studies have previously been reported on radicicolae grape phylloxera.

The natural diet of most Aphididae with an established artificial feeding system is leaf phloem sap, however radicicolae grape phylloxera reportedly feed on parenchyma cells within the root cortex (Kellow *et al.*, 2004). This differential feeding site complicates the development of an artificial feeding system for grape phylloxera as the chemistry of the grapevine parenchyma cell is poorly understood. Changes in grapevine root chemistry (sugar and amino acid concentrations) induced by grape phylloxera feeding and gall development have been studied, including variation in amino acid concentrations (Rilling *et al.*, 1975; Kellow *et al.*, 2004) and sugars (Ryan *et al.*, 2000). There is also a general understanding of the pH of grapevines, and how the values are affected by vineyard irrigation management (Stoll *et al.*, 2000).

With this knowledge, and assisted by previous artificial diet formulations for Aphididae, the development of an artificial diet system for radicicolae grape phylloxera was investigated. The initial requirement of a 'successful' artificial feeding system was the maintenance of the grape phylloxera population for an extended period of time in comparison with control treatments. The development of the artificial diet was aimed at examining the essential nutritional requirements of grape phylloxera, and enabling the long-term capability of developing a model system for the testing of novel control agents. Two diet chamber designs were trialled for radicicolae grape phylloxera during examination of diet formulations varying in the composition of sugar, amino acids and solution pH. The success of the diet formulations was determined by relative increases in survival time in comparison with control treatments. The attraction of radicicolae grape phylloxera to the diet formulation was determined by the level of probing activity observed.

# 3 MATERIAL AND METHODS

## *3.1 INSECT COLLECTION*

Radicicolae grape phylloxera were originally collected from infested vineyards in Victoria, Australia. Populations were maintained at the Department of Primary Industries – Rutherglen Centre on excised *Vitis vinifera* L. (Vitaceae) cv. Sultana root pieces (approximately 1 cm width x 10 cm length), prepared using a protocol slightly modified from Granett *et al.* (1985). The modifications were: (1) roots washed clean of attached soil with a soft brush under running water; (2) roots soaked for 5 minutes in Ridomil® Gold Plus systemic fungicide solution (2.3 g/L); (3) roots triple rinsed with sterile distilled water prior to air drying; (4) both ends of roots wrapped in moist cotton wool; and (5) the size of the petri dish was increased to 15 cm, with the increase in area reducing the level of water condensation within the chamber. Grape phylloxera populations were incubated in the dark at a constant temperature of 25 ± 3°C.

Egg hatcheries were established to enable the simultaneous collection of a large number of first instar insects. Eggs from excised root pieces were placed onto moist filter paper in a 35 mm petri dish. The petri dish was sealed with Parafilm® M membrane and incubated in the dark at a constant temperature of 25 ± 3°C. The hatcheries were monitored daily until sufficient first instars were available for the artificial diet experiment (2-3 days post establishment). Active insects were then transferred directly from the hatchery into the artificial diet chamber without prior feeding on grapevine root material.

Only apterous radicicolae grape phylloxera were used for this study. For collection from excised root pieces, developmental life stages were determined by comparative increases in size. If present, neighbouring moulted cuticles were counted to confirm the insect life stage. Insects were handled using a moist sable-haired paintbrush to

avoid damage. Two apterous life stages were used in the artificial diet experiments: 'first instar' and an 'intermediate instar' group that included second – fourth instars. First instars were collected while actively moving on the excised root piece or directly from egg hatcheries. Intermediate instars were gently removed from the excised root surface with a moist paintbrush.

## *3.2 ARTIFICIAL DIET CHAMBERS*

Two artificial diet chamber designs were trialled for grape phylloxera: the 'filter paper' and the 'humidity chamber'. Experiments involving both designs were set up in a randomised complete block design and replicated typically 3-5, although the application (and renewal) of the diet solution was performed in treatment groups to avoid cross-contamination of solutions. Replicated blocks represented one chamber per artificial diet formulation (including control diets); treatment groups represented all chambers containing the same artificial diet formulation. Artificial diet chambers were incubated in the dark at a constant temperature of 25 ± 3°C. Artificial diet solutions were renewed every two days to avoid deterioration of the chemical components and reduce microbial contamination. All artificial diet preparation and renewal procedures were performed in an air-filtered cabinet or on a clean bench laboratory to limit microbial contamination.

### *3.2.1 FILTER PAPER CHAMBER DESIGN*

Based on Powell *et al.* (1993), the filter paper chamber consisted of a sterile 35 mm diameter petri dish, in the base of which was a sterile 32 mm diameter filter paper moistened with 50 µl sterile ultra-pure water (Figure 1a). Insects were placed into the chamber with a moist sable-haired paintbrush and the chamber covered immediately with a layer of stretched Parafilm® M membrane. Insects were 'starved' inside the chambers, in the dark, for a period of 1-2 hours to reduce the impacts of prior feeding. First instar insects collected from the egg hatcheries were not starved as 'hatchery first

instars' were prevented from having food in their digestive system due to a lack of exposure to grapevine root material.

Following the starvation period, the artificial diet solution (250 µl) was placed in the middle of the diet membrane surface, and covered with a second layer of stretched Parafilm® M. A water weight, enclosed in a plastic container and sealed with Parafilm®, of 30-50 g was placed above the Parafilm® M membrane surface to increase the pressurisation of the diet solution, except for diet experiments 1 and 2, Table 2. Diet experiment 3 provided a comparison between the survival rate of grape phylloxera under non-pressurised and pressurised chamber conditions.

The filter paper chamber artificial diet solution was renewed by removing the double Parafilm® membrane from the diet chamber. Insects in contact with the diet membrane at the time of artificial diet renewal were carefully repositioned onto the moist filter paper with a moist sable-haired paintbrush. The diet chamber was recovered with a new layer of stretched Parafilm®, and fresh diet solution enclosed with a second layer of new stretched Parafilm®.

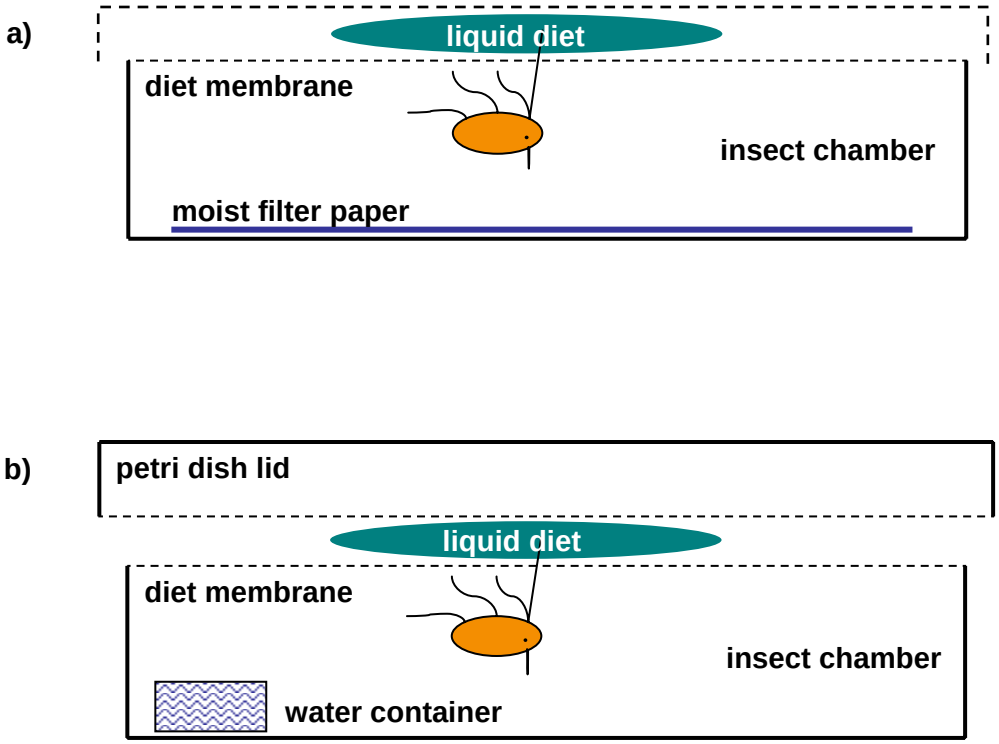


**Figure 1.** Diagrammatic representation of the two chamber designs trialled for grape phylloxera artificial diet experiments. The solid line represents the 35 mm diameter petri dish used for the diet chamber, the dashed line represents a layer of Parafilm® M. The insect is depicted on the diet membrane, inserting its stylet into the liquid diet.

**a)** filter paper chamber with moist filter paper in the base of the petri dish and the diet enclosed within a double layer of Parafilm® M;

**b)** humidity chamber with the lid of a 0.5 ml tube providing a water container, the chamber was covered with a layer of Parafilm® M and the diet enclosed with a second layer of Parafilm® M attached to the lid of the petri dish.

### *3.2.2 HUMIDITY CHAMBER DESIGN*

The humidity chamber was simplified from the chamber model previously used to develop an artificial diet feeding system for gallicolae grape phylloxera (Forneck and Wöhrle, 2003). The humidity chamber consisted of a sterile 35 mm diameter petri dish with the sterile lid of a 0.5 ml tube in the base (dimensions 5 mm x 5 mm). The tube lid was a water container filled with 50-70 µl of sterile water and covered with a pre-moistened circle of fine mesh (Figure 1b). The mesh (60 µm pore) provided a cover over the water container to allow insects to move across the water surface and reduced the level of accidental drowning. The original chamber design (Forneck and Wöhrle, 2003) used a saturated solution of $K_2SO_4$ to fill the container. Water was used for these experiments following initial trials which provided evidence that contact with the $K_2SO_4$ solution had a negative impact on grape phylloxera survival rates (data not shown).

Insects were placed into the chamber with a moist sable-haired paintbrush and the chamber was covered immediately with a layer of stretched Parafilm® M membrane. The lid of the petri dish was also covered with a layer of stretched Parafilm® and placed directly above the diet membrane Parafilm® layer. The two membrane surfaces were kept aligned by placing a 30 g water weight on the petri dish lid (Figure 2). Insects were 'starved' inside the chambers, in the dark, for a period of 1-4 hours. First instar insects collected from the egg hatcheries were not starved as 'hatchery first instars' were prevented from having food in their digestive system due to a lack of exposure to grapevine root material.

Following the starvation period, the two membrane surfaces were separated and the artificial diet solution (50-70 µl) was placed in the middle of the diet membrane surface. The lid membrane was replaced back on top of the diet membrane to enclose the diet solution, and the water weight placed back onto the petri dish lid to keep the two

membrane surfaces aligned, and to increase the pressurisation of the diet solution. The volume of diet solution used for the humidity chamber was reduced from the filter paper chamber due to the alterations in the method of diet enclosure; higher volumes of diet solution were unable to be contained on the diet membrane during the application of the lid membrane.

The artificial diet solution in the humidity chamber design was renewed without disturbing insect activity. The water weight and lid membrane were removed, and the diet solution gently poured off the diet membrane surface. Both Parafilm® membranes were rinsed with 1 ml of sterile water and blotted dry with a clean tissue. Fresh diet solution was placed onto the diet membrane and the surface was recovered with the lid membrane and water weight.

**Figure 2.** The humidity chamber with a 30 g water weight positioned to keep the diet and petri dish lid membrane surfaces aligned, and to increase the pressurisation of the diet solution.

## *3.3 ARTIFICIAL DIET FORMULATION*

A range of artificial diet formulations were trialled for grape phylloxera (Table 1). Holidic diet formulations varied in sugar type, sugar concentration, amino acid profile and pH. Oligidic and meridic diet formulations were also trialled. In the text, diet formulations are written in *italic* to clarify interpretation. Controls for all artificial diet experiments were ultra-pure water adjusted to the same pH as the experimental diet solution, with the exceptions of diet experiments 3 and 5 (Table 2). An additional control treatment of 'no-diet', where the two layers of Parafilm® membrane enclosed no liquid solution, was occasionally used to observe insect survival time in the absence of any fluid diet solution.

### *3.3.1 CHEMICALS AND MATERIALS*

All sugar and amino acid chemical reagents used for artificial diet formulations (Table 1) were obtained from the chemical company Sigma-Aldrich. Grape phylloxera artificial diet solutions were prepared using acid-washed glassware, sterile ultra-pure water and high purity chemicals. Diet solutions were pH adjusted with 0.5M KOH and 0.5M HCl to a range of pH 4.5-7.5. Diet solutions were filter sterilised with a Millipore™ 25 mm Millex® filter unit (0.22 µm) and stored in sterile plastic vials at -20°C. During diet experiments, stock diet solutions were stored at 4°C to reduce the rate of deterioration.

### *3.3.2 SUGAR TYPE AND CONCENTRATION*

The majority of artificial diet formulations contained sugar in the form of sucrose. Variation in the sucrose concentration was based on successful aphid diets with a range of 10-20% (Mittler, 1967). Generally, 5% sugar (sucrose, glucose or fructose) was incorporated into the diet formulation. This was based on chemical analysis of grape phylloxera feeding sites that identified sucrose to have a concentration range 13-55 mg/g dry weight (Ryan *et al.*, 2000). Sucrose, glucose and fructose were identified as the main sugars at the feeding sites.

**Table 1.** Formulations for 15 holidic artificial diet solutions trialled to increase the survival time of grape phylloxera within an artificial diet system. Dietary components consisted of a range of sugars and amino acids (AA); values represent g/100ml ultra pure water, solution pH range indicated.

| | 5% sucrose | 10% sucrose | 20% sucrose | 5% glucose | 5% fructose | 5% sucrose + 5 AA | 5% sucrose + AA | 5% glucose + AA | 5% fructose + AA | 10 AA |
|---|---|---|---|---|---|---|---|---|---|---|
| **sugar** | | | | | | | | | | |
| D (+) sucrose | 5 | 10 | 20 | | | 5 | 5 | | | |
| D (+) glucose | | | | 5 | | | | | 5 | |
| D (-) fructose | | | | | 5 | | | 5 | | |
| **amino acid** | | | | | | | | | | |
| L-arginine-HCl | | | | | | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| L-glutamine | | | | | | 0.6 | | | | |
| L-histidine-HCl | | | | | | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| L-isoleucine | | | | | | | 0.2 | 0.2 | 0.2 | 0.2 |
| L-leucine | | | | | | | 0.2 | 0.2 | 0.2 | 0.2 |
| L-lysine-HCl | | | | | | | 0.2 | 0.2 | 0.2 | 0.2 |
| L-methionine | | | | | | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| L-phenylalanine | | | | | | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| L-threonine | | | | | | | 0.2 | 0.2 | 0.2 | 0.2 |
| L-tryptophan | | | | | | | 0.1 | 0.1 | 0.1 | 0.1 |
| L-valine | | | | | | | 0.2 | 0.2 | 0.2 | 0.2 |

**Table 1 cont.**

| | 5% sucrose | 10% sucrose | 20% sucrose | 5% glucose | 5% fructose | 5% sucrose + 5 AA | 5% sucrose + AA | 5% glucose + AA | 5% fructose + AA | 10 AA |
|---|---|---|---|---|---|---|---|---|---|---|
| **pH values** | | | | | | | | | | |
| | 4.5 | 7.0 | 7.0 | 7.5 | 7.5 | 7.0 | 4.5 | 4.5 | 6.5 | 6.5 |
| | 6.0 | | | | | | 6.5 | 6.5 | | |
| | 7.0 | | | | | | | | | |
| | 7.5 | | | | | | | | | |

### *3.3.3 AMINO ACIDS*

Amino acids were added to grape phylloxera artificial diet formulations due to evidence of increased amino acid concentrations at the feeding site of radicicolae grape phylloxera (Kellow *et al.*, 2004). Two different amino acid profiles were trialled in artificial diet formulations.

The amino acid profile containing five amino acids was based on changes identified in the grapevine root amino acid profile with gall formation following grape phylloxera feeding (Kellow *et al.*, 2004). Histidine, arginine and phenylalanine recorded the largest increase in concentration with gall formation, and glutamine occurred at the highest concentration of all amino acids detected. Methionine recorded a mid-range increase in concentration, but was included in the five amino acid profile (instead of other amino acids with a higher increase in concentration) due to evidence that the addition of methionine, in association with sucrose, enhanced the uptake of artificial diet solutions by aphids (Mittler, 1988). The total amino acid concentration was 1.4%. In diet formulations, the five amino acid profile was denoted as '*plus five amino acids*' or '+ 5AA'.

The amino acid profile containing 10 amino acids was defined by the amino acids considered 'essential' for insect nutrition (Cohen, 2003). These amino acids were methionine, threonine, tryptophan, valine, isoleucine, leucine, phenylalanine, lysine, arginine and histidine. The total amino acid concentration was 1.9%. In diet formulations, the 10 amino acid profile was denoted as '*plus amino acids*' or '+ AA'.

In the absence of previous artificial diet formulations for radicicolae grape phylloxera, the individual amino acid component concentrations in both amino acid profiles were based on a holidic diet developed for *A. pisum*, the pea aphid (Akey and Beck, 1971).

### *3.3.4 pH Level*

Artificial diet formulations were initially adjusted to a neutral or slightly alkaline pH (7.0-7.5), based on the aphid model. Aphids obtain optimal growth and reproduction in artificial diet systems with slightly alkaline pH (7.4-7.8), reflecting the pH of the phloem sap of their preferred host-plants (Mittler, 1988). Cohen (2003) reported that artificial diets were generally formulated to be slightly acidic to improve palatability and microbial control. Therefore grape phylloxera artificial diet formulations were made slightly acidic (pH 6.0-6.5). However the pH of the xylem sap in canes of *V. vinifera* was reported to be in the acidic range, pH 4.2-4.8 (Stoll *et al.*, 2000). To reflect the pH of the food source of grape phylloxera, acidic (pH 4.5) artificial diet formulations were also trialled.

Although the specific pH of parenchyma cell contents was not directly measured, the pH of the grapevine root food source of grape phylloxera was confirmed by measurements from micropropagated *V. vinifera* cv. Shiraz (Kellow *et al.*, 2002). Leaf, stem and root material from the micropropagated grapevine were ground with a small amount of water in a mortar and pestle. The ground mix was pH tested with indicator tape, pH range 2-9 (Merck). Each sample was measured in triplicate.

### *3.3.5 Grapevine Root Extract*

Grapevine root extracts were added to diet formulations to provide a 'natural' feeding stimulus. The root extracts contained ground root material from the natural grapevine diet of grape phylloxera, and provided a more complex diet formulation in comparison with the chemically defined, holidic, artificial diets. Two grapevine root extract formulations were trialled in grape phylloxera artificial diet experiments. The root extracts were prepared from different micropropagated grapevines.

In the non-defined, oligidic, artificial diet, micropropagated *V. vinifera* cv. Shiraz roots were prepared using a protocol slightly modified from the root pH measurement

preparation outlined previously. Modifications included: (1) grinding three complete root systems; and (2) the addition of 2.5 ml sterile water pH 7.5 to the mortar and pestle solution. The root solution was left to diffuse for 10 minutes, and then filter sterilised with a Millipore™ 25 mm Millex® filter unit (0.22 µm) and stored in sterile plastic vials at 4°C. The root extract was pH tested with indicator tape, pH range 2-9 (Merck). The root extract diet solution was prepared immediately prior to the start of the artificial diet experiment, and stored at 4°C during the experimental period.

In the meridic artificial diet that consisted of both chemically non-defined and defined components, micropropagated *V. vinifera* cv. Shiraz roots were prepared using an alternative method. The root material from a micropropagated grapevine was ground in liquid nitrogen with a mortar and pestle, and added in liquid form (200 µl) to a *5% sucrose pH 4.5 plus amino acids* diet solution (600 µl). The grapevine root extract was not filter sterilised prior to addition to the holidic diet solution. The final pH of the diet formulation was not tested. The meridic diet was stored at 4°C during the diet experiment to preserve the contents for diet renewal.

### *3.4 ARTIFICIAL DIET MONITORING*

Grape phylloxera survival, location and feeding status in the artificial diet experiments was monitored daily (either once or twice) in each of the replicated blocks. For monitoring, artificial diet chambers were viewed with a stereo microscope to count the number of insects alive and dead. The filter paper chamber required inversion of the chamber to view insects below the filter paper; inversion was not necessary to view all insects in the water humidity chamber. Insects in a stationary position on the diet membrane during observation for insect survival were recorded as 'probing' the diet solution. For some probing insects, the stylet was in a vertical position and appeared to penetrate the diet membrane. Monitoring of artificial diets continued until all insects in all chambers were dead.

Images of grape phylloxera on the diet membrane of the artificial diets were digitally captured with a SPOT RT™ (Diagnostic Instruments) camera mounted onto an Olympus AX70 Provis microscope. External cold-source lighting was provided to increase the contrast of the images. Approximate scale bars (based on the average size of the grape phylloxera life stage, Chapter 2) were digitally applied to the images during post analysis with ImageJ software (Rasband, 2005)

## *3.5 STATISTICAL ANALYSIS*

GenStat-Eighth Edition© (Lawes Agricultural Trust) was used to calculate summary statistics for survival rate (minimum-maximum survival time) and probing activity (mean and total probing number) data. Artificial diet formulation survival rates were further analysed with survival analysis, and probing activity via logistic regression. Statistical significance for both survival analysis and logistic regression was based at $p < 0.05$.

### *3.5.1 SURVIVAL ANALYSIS*

Survival analysis was performed with R statistical software (R Development Core Team, 2006). Survival analysis was used to determine the fraction of grape phylloxera within each diet experiment that survived for each monitoring period, and the rate of death for that time interval. For all diet formulations within each artificial diet experiment, data was formatted to represent individual insect time until death (or the survival time of the insect).

Insects within artificial diet experiments which died due to causes other than the diet formulation, or were removed from the experiment due to external damage to the diet chamber, were excluded (censored) from the survival analysis. Missing insects were identified when the total insect number per chamber did not equal the total sample number (n). The live/dead status of missing insects was calculated by using prior and post insect counts. Non-recovered missing insects were censored; all other missing insect data points were adjusted to represent the most probable live/dead status of the

insect. Artificial diets involving censored data were diet experiments 1, 2, 4, 5, 6 and 11 (Tables 2 and 3). Insects recorded in the location of 'below the filter paper' in the filter paper chamber design, or 'on the water container' in the humidity chamber design were not censored from the data set as the two locations were possible for all insects within the same diet experiment and therefore did not represent a disadvantage to any particular artificial diet formulation.

Survival analysis was performed using the Kaplan-Meier estimate to determine and plot survival distribution. The log-rank test was performed to verify statistical difference between the survival distributions of the artificial diet formulations. Artificial diet experiments with a significant log-rank test p-value were further analysed as a pair-wise comparison with the control. This was to determine which individual diet formulation was significantly different from the control. Experiments containing multiple diet formulations were also run as pair-wise comparisons with the control to investigate individual relationships. If two controls (no-diet and water) were included in the diet experiment, both were run individually in a pair-wise comparison with each diet formulation. Diet formulations were only statistically compared within a single artificial diet experiment.

### *3.5.2 LOGISTIC REGRESSION MODEL*

GenStat-Eighth Edition© logistic regression (log*it*) models were run for each diet experiment to compare the proportion of insects in a probing position on the diet membrane to the total number of insects alive for each monitoring period. As probing was not possible if all insects were dead, the data set was restricted for each replicate chamber to the last monitoring period for recording alive insects. The total and mean number of probing insects was calculated for the same time period, and was based on the number of monitoring events and not necessarily the total time in days (monitoring occurred once or twice daily). As the survival time of grape phylloxera varied between

diet formulations, the total number of monitoring events also varied for both the log*it* and mean calculations. Microsoft® Excel line-scatter plots were generated to depict the total number of probing events per monitoring period for the survival time of the artificial diet experiment.

Proportion of probing insect comparisons for diet experiments containing multiple diet formulations were also run as a pair-wise logistic regression model in comparison with the control to determine individual relationships. If two controls (no-diet and water) were included in the diet experiment, both were run individually in a pair-wise comparison with each diet formulation. Grape phylloxera probing activity was only statistically compared within a single artificial diet experiment.

# 4 RESULTS

Grape phylloxera artificial diet experiments were analysed to determine the influence of chamber design, life stage and diet formulation on insect survival time and the level of probing activity on the diet membrane. In total, 15 artificial diet experiments are presented, involving 17 different artificial diet formulations.

## *4.1 ARTIFICIAL DIET CHAMBER DESIGN*

First and intermediate instar grape phylloxera were used in both artificial diet chamber designs. Of the fifteen artificial diet experiments reported, the filter paper chamber was used for six artificial diet experiments, and the humidity chamber for nine artificial diet experiments. Not all 17 artificial diet formulations were used in the two chamber designs; the filter paper chamber experiments involved 10 artificial diet formulations, while the humidity chamber experiments examined 11 artificial diet formulations.

### 4.1.1 *FILTER PAPER CHAMBER DESIGN*

The filter paper chamber design produced variable humidity and condensation levels within the chamber, confounding grape phylloxera survival on the artificial diet due to restricted insect mobility and access to the diet solution. As individual insects moved across the surface of the diet membrane they became immobilised in (relatively) large condensed droplets of water. Insects also became trapped in water droplets below the filter paper surface, and 30% (range 0-81%) of insects across all filter paper diet experiments died below the filter paper. The condensed droplets of water on the diet membrane and below the filter paper both resulted in restricted insect movement, and therefore inhibited interaction with the artificial diet solution. The restricted insect movement resulted in a negative impact on the aim of the experiment to compare insect survival on variable artificial diet formulations.

The solid filter paper in the base of the diet chamber restricted observation of the entire diet chamber, and it was necessary to invert the chamber in order to investigate the location of insects on the underside of the filter paper surface. Additional disturbance to the sedentary feeding grape phylloxera was caused by the repeated removal of the diet membrane during artificial diet renewal. The continual contact caused by relocating the insects to the filter paper surface appeared to have a negative impact on insect fitness, and therefore the ability of grape phylloxera to feed on the diet formulation.

### 4.1.2 *HUMIDITY CHAMBER DESIGN*

In comparison with the filter paper chamber, the humidity chamber design produced more even condensation levels within the chamber. Droplets of water did still occur (Figure 3), however they were smaller than the water droplets that occurred in the filter paper chamber and had less impact on the movement of the insect across the diet membrane. In some humidity chambers the water droplets would adhere to the insect

as they crossed the membrane, but (unlike the filter paper chamber) they did not completely immobilise the insects and the insects continued to interact with the diet membrane and, if stimulated, probe the diet solution. The absence of the filter paper prevented insects from becoming trapped below the filter paper surface, and also improved the visibility of the diet chamber for monitoring. The design modification allowed for renewal of the artificial diet solution with reduced disruption to the insect as the diet membrane was not removed. Individual grape phylloxera were observed to remain in a stationary feeding position on the diet membrane during and following diet renewal.

A proportion of insects in every humidity chamber would interact with the water container, and across all humidity chamber diet experiments 22% (range 10-42%) of insects died in contact with the water container. However the addition of fine mesh to cover the top of the water container did allow some insects to successfully move over the surface of the water and re-interact with the artificial diet membrane for potential probing activity. Although there was no ability to track the activity and movement of individual insects, reductions in the number of insects recorded on the water container surface between monitoring times provided evidence for the re-location of some insects from the water container surface (data not shown).

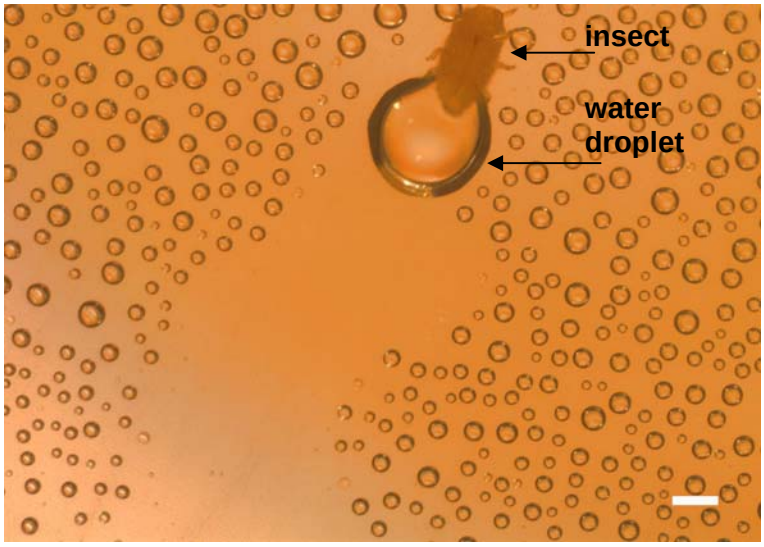


**Figure 3.** An intermediate grape phylloxera on the underside of the humidity chamber diet membrane; condensed droplets of water adhered to the insect walking across the membrane surface, leaving a dry membrane trail. As the water droplet near the posterior end of the insect increased in size, mobility decreased. Insect mobility was further affected in the filter paper chamber where larger droplets of condensed water were observed on the diet membrane. Scale bar 200 µm.

### *4.2 ARTIFICIAL DIET SURVIVAL TIME*

In total, 17 artificial diet formulations were trialled on grape phylloxera (Table 1, plus the oligidic and meridic diet formulations) across 15 artificial diet experiments. Grape phylloxera on control diets of no-diet (no liquid solution) or water survived several days without exposure to an artificial diet formulation or nutritional intake. The maximum survival time of first instar insects on the water control diet ranged from 4-7 days across all artificial diet experiments; the maximum survival time for the intermediate instars was 6.5-9 days (Tables 2 and 3). The maximum survival time was reduced on the no-diet control to 4 days for first instars and 7 days for intermediate instars.

Across all artificial diet experiments and formulations (including control diets), there was an increase in the mean grape phylloxera survival rate of 1.4 days when the artificial diet chamber design was changed from the filter paper to the humidity chamber (Tables 2 and 3). This increase in survival rate was also associated with a change in the grape phylloxera life stage used for the artificial diet experiments from predominantly first instars to intermediate instars. Life stage development was indicated by the presence of moulted cuticles in the diet chambers. Moulted cuticles were observed in intermediate instar diet chambers in the early stages of the diet experiments, however no moulting was observed in the first instar diet chambers.

**Table 2.** Grape phylloxera survival rate in artificial diet experiments conducted using the filter paper chamber; life stage, diet formulation and survival rate (days) indicated. Number of insects per diet formulation indicated by the life stage (n) value.

| diet number | life stage (n) | | artificial diet formulation and survival rate | | | | | log-rank test p-value |
|---|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | e | |
| 1 | hatchery first instar | formulation | **no-diet** | **water pH 7.0** | 5% sucrose pH 7.0 | 10% sucrose pH 7.0 | 20% sucrose pH 7.0 | |
| | (25) | mean (s.e.) | 2.96* (0.13) | 3.00 (0.16) | 3.00 (0.30) | 3.28 (0.16) | 3.22* (0.26) | 0.72 |
| | | range | 2-4 | 1-5 | 1-8 | 1-5 | 1-6 | |
| 2 | first and intermediate instar | formulation | *first instar* **water pH 7.0** | *first instar* 10% sucrose pH 7.0 | *intermediate* **water pH 7.0** | *intermediate* 10% sucrose pH 7.0 | | |
| | (15) | mean (s.e.) | 3.27 (0.15) | 3.18* (0.25) | 3.38* (0.33) | 3.17* (0.36) | | 0.73 |
| | | range | 2-4 | 1-4 | 1-4 | 1-4 | | |
| 3 | first instar | formulation | *no pressure* **10% sucrose pH 7.0** | *water pressure* 10% sucrose pH 7.0 | | | | |
| | (15) | mean (s.e.) | 4.20 (0.25) | 4.07 (0.24) | | | | 0.63 |
| | | range | 3-6 | 3-6 | | | | |
| 4 | first instar | formulation | **no-diet** | ***5% sucrose pH 7.0*** | | | | |
| | (15) | mean (s.e.) | 2.35* (0.25) | ***3.13 (0.19)*** | | | | ***0.036*** |
| | | range | 1-4 | ***2-4*** | | | | |

**Table 2 cont.**

| diet number | life stage (n) | | artificial diet formulation and survival rate | | | | | log-rank test p-value |
|---|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | e | |
| 5 | hatchery first instar | formulation | **water pH 7.0** | 5% sucrose pH 7.0 | 5% sucrose pH 7.0 + 5AA[1] | 5% sucrose pH 6.0 | 5% sucrose pH 4.5 | |
| | (16) | mean (s.e.) | 3.86* (0.54) | 3.07 (0.48) | 3.69 (0.58) | 3.44 (0.48) | 3.28 (0.38) | 0.76 |
| | | range | 1-7 | 1-7 | 1-10 | 1-7 | 1-7 | |
| 6 | hatchery first instar | formulation | **water pH 7.5** | root extract[2] pH 5.0 | 5% sucrose pH 7.5 | 5% glucose pH 7.5 | 5% fructose pH 7.5 | |
| | (20) | mean (s.e.) | 2.75* (0.23) | 3.05 (0.11) | 2.75 (0.17) | 2.93* (0.22) | 2.95* (0.17) | 0.90 |
| | | range | 1-4 | 2-4 | 1-4 | 2-4 | 2-4 | |

Control diets for each experiment are highlighted in **bold**, diet formulation characteristics in *italic* indicate experiment variances not based on the artificial diet solution. Survival analysis mean and standard error of mean (s.e.) values, and maximum-minimum range of grape phylloxera survival rates are presented for each diet formulation. Significant log-ranked test p-values, and the diet formulations significantly different from the control diet are highlighted in ***bold italic***.

[1] diet formulation containing the five amino acid profile denoted as '+ 5AA'

[2] oligidic grapevine root extract diet formulation

* data set contains censored values

**Table 3.** Grape phylloxera survival rate in artificial diet experiments conducted using the humidity chamber; life stage, diet formulation and survival rate (days) indicated. Number of insects per diet formulation indicated by the life stage (n) value.

| diet number | life stage (n) | | artificial diet formulation and survival rate | | | | log-rank test p-value |
|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | |
| 7 | intermediate instar | formulation | **water pH 7** | 5% sucrose pH 7.0 + 5AA[1] | | | |
| | (15) | mean (s.e.) | 4.37 (0.43) | 5.03 (0.57) | | | 0.22 |
| | | range | 1.5-6.5 | 0.5-9 | | | |
| 8 | intermediate instar | formulation | **water pH 7.5** | 5% sucrose pH 7.5 | 5% fructose pH 7.5 | 5% glucose pH 7.5 | |
| | (10) | mean (s.e.) | 5.20 (0.55) | 3.95 (0.39) | 5.25 (0.49) | 4.55 (0.60) | 0.15 |
| | | range | 2-9 | 0.5-4.5 | 3-8 | 1-7 | |
| 9 | intermediate instar | formulation | **water pH 7** | 5% fructose pH 6.5 + AA[2] | 5% sucrose pH 6.5 + AA[2] | | |
| | (20) | mean (s.e.) | 4.75 (0.50) | 4.83 (0.48) | 5.33 (0.40) | | 0.27 |
| | | range | 2-13[4] | 2-13[4] | 0.5-8 | | |
| 10 | intermediate instar | formulation | **water pH 7** | 5% glucose pH 6.5 + AA[2] | 10 AA pH 6.5 | | |
| | (30) | mean (s.e.) | 3.80 (0.22) | 3.73 (0.25) | 3.28 (0.17) | | 0.21 |
| | | range | 2-7 | 2-7 | 0.5-4.5 | | |

**Table 3 cont.**

| diet number | life stage (n) | | artificial diet formulation and survival rate | | | | log-rank test p-value |
|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | |
| 11 | intermediate instar | formulation | **water pH 4.5** | 5% sucrose pH 4.5 + $AA^2$ | | | |
| | (30) | mean (s.e.) | 4.79* (0.45) | 4.18 (0.25) | | | 0.18 |
| | | range | 1.5-9 | 1.5-7 | | | |
| 12 | intermediate instar | formulation | **no-diet** | **water pH 4.5** | ***5% sucrose pH 4.5 + $AA^2$*** | 5% glucose pH 4.5 + $AA^2$ | |
| | (40) | mean (s.e.) | 4.80 (0.27) | 4.80 (0.35) | ***6.54 (0.38)*** | 5.05 (0.36) | ***< 0.001*** |
| | | range | 1-7 | 1.5-8 | ***0.5-11*** | 1.5-10 | |
| 13 | intermediate instar | formulation | **water pH 4.5** | 5% sucrose pH 4.5 + $AA^2$ | | | |
| | (40) | mean (s.e.) | 4.00 (0.20) | 4.11 (0.25) | | | 0.53 |
| | | range | 2.5-8 | 1-8 | | | |
| 14 | hatchery first instar | formulation | **water pH 4.5** | 5% sucrose pH 4.5 + $AA^2$ | | | |
| | (30) | mean (s.e.) | 2.30 (0.19) | 2.67 (0.21) | | | 0.32 |
| | | range | 0.5-4 | 0.5-5 | | | |

**Table 3 cont.**

| diet number | life stage (n) | | artificial diet formulation and survival rate | | | | log-rank test p-value |
|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | |
| 15 | first instar | formulation | **water pH 4.5** | 5% sucrose pH 4.5 + AA[2] | 5% sucrose pH 4.5 + AA[2] + root extract[3] | | |
| | (40) | mean (s.e.) | 2.77 (0.25) | 3.36 (0.27) | 3.15 (0.27) | | 0.33 |
| | | range | 0.5-7 | 0.5-7 | 1-7 | | |

Control diets for each experiment are highlighted in **bold**. Survival analysis mean and standard error of mean (s.e.) values, and maximum-minimum range of grape phylloxera survival rates are presented for each diet formulation. Significant log-ranked test p-values, and the diet formulations significantly different from the control diet are highlighted in ***bold italic***.

[1] diet formulation containing the five amino acid profile denoted as '+ 5AA'

[2] diet formulation containing the ten amino acid profile denoted as '+ AA'

[3] meridic grapevine root extract diet formulation

[4] diet experiment not monitored between day 8-13, therefore the maximum survival time stated may not be accurate

* data set contains censored values

### *4.2.1 FILTER PAPER CHAMBER SURVIVAL TIME*

The filter paper chamber was used to investigate grape phylloxera survival rates for six artificial diet experiments (Table 2). All experiments, except for artificial diet experiment 2, used only first instar insects. Diet experiment 2 compared the survival of the first and intermediate instars. Across the six filter paper chamber experiments, diet formulations compared sucrose at a range of concentration levels (5, 10 and 20%), sucrose at a range of pH levels (4.5, 6.0, 7.0, and 7.5), a range of sugars (sucrose, glucose and fructose), sucrose plus five amino acids, and an oligidic grapevine root extract.

The only filter paper chamber artificial diet to have a significant impact on grape phylloxera survival time was trialled in diet experiment 4 (Table 2). In diet experiment 4, the first instar mean survival time on the artificial diet formulation *5% sucrose pH 7.0* was significantly extended (0.78 mean days), in comparison with the no-diet control. Although all insects were recorded dead in both systems at day four (Table 4), the mortality rate was reduced during the first two days of the diet experiment when grape phylloxera were exposed to the *5% sucrose pH 7.0* artificial diet formulation (Figure 4).

There were no further significant impacts on grape phylloxera survival rates determined by pair-wise survival analysis of artificial diet formulations in comparison with the control diets.

**Table 4.** Survival analysis for grape phylloxera artificial diet experiment 4; first instar life stage in the filter paper chamber feeding on diet formulations 'no-diet' or '5% sucrose pH 7.0'.

| diet formulation | time (days) | insects at risk | insects dead | proportion surviving | standard error |
|---|---|---|---|---|---|
| no-diet | 0 | 15 | 0 | 1.000 | 0 |
| | 1 | 15 | 2 | 0.867 | 0.088 |
| | 2 | 12 | 8 | 0.289 | 0.12 |
| | 3 | 3 | 1 | 0.193 | 0.11 |
| | 4 | 2 | 2 | 0.000 | |
| 5% sucrose | 0 | 15 | 0 | 1.000 | 0 |
| pH 7.0 | 2 | 15 | 3 | 0.800 | 0.10 |
| | 3 | 12 | 7 | 0.333 | 0.12 |
| | 4 | 5 | 5 | 0.000 | |

'Insects at risk' was the number of insects alive within the diet chamber prior to observation, 'insects dead' was the number of insects observed dead for each time interval. Gaps in 'insects at risk' and 'insects dead' values are due to censored insects. Time intervals without an observed death of an insect are not presented as the survival distribution value remains the same as the previous time interval.

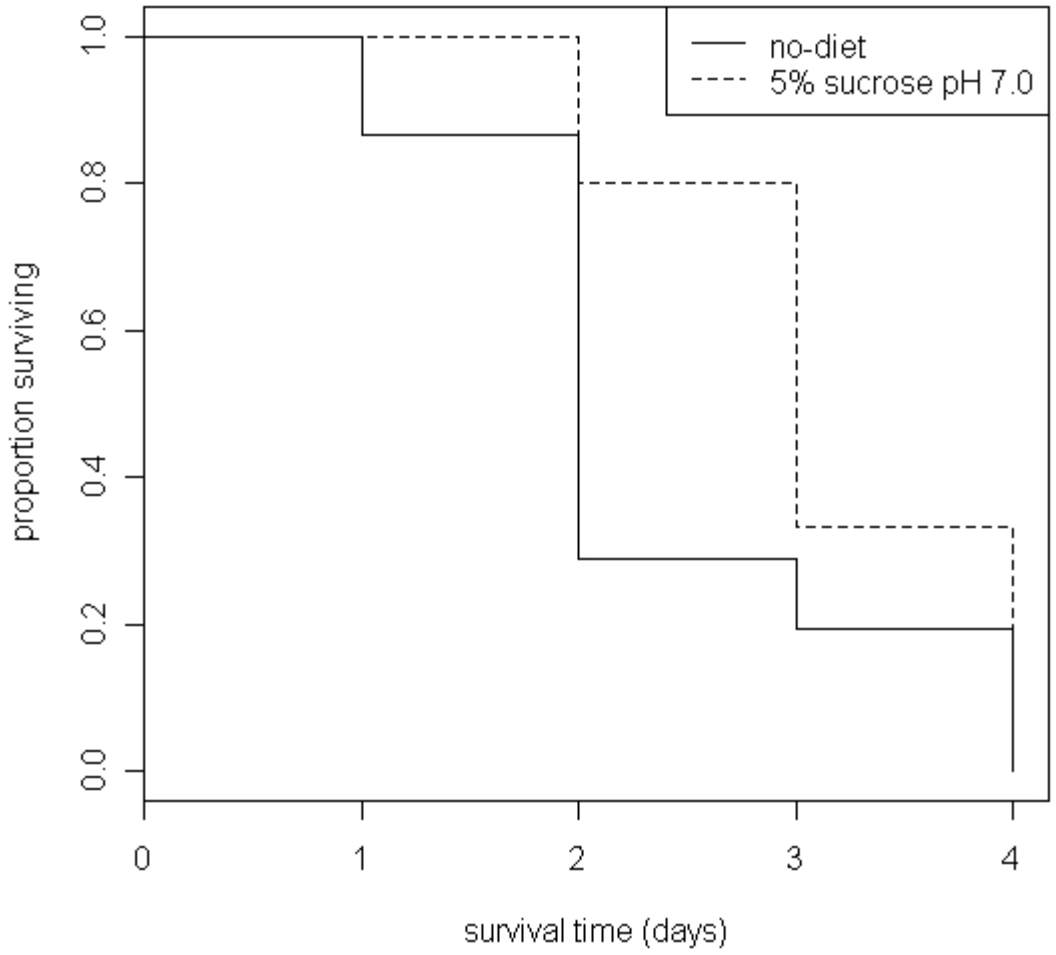


**Figure 4.** Grape phylloxera survival rate in artificial diet experiment 4; survival analysis plot of proportion of insects surviving over time (days). First instar life stage in the filter paper chamber feeding on 'no-diet' or '5% sucrose pH 7.0'.

The artificial diet formulation *5% sucrose pH 7.0* was also trialled in diet experiments 1 and 5 (Table 2). There was no significant difference in survival rates in comparison with either the no-diet or water controls, and the mean survival time for grape phylloxera on the diet formulation *5% sucrose pH 7.0* was either similar to the control diets (diet experiment 1) or reduced (diet experiment 5). The mean survival times for the artificial diet formulation *5% sucrose pH 7.0* were similar across all diet experiments; however the maximum survival times in diet experiments 1 and 5 were longer than in diet experiment 4. The significant result produced in diet experiment 4 was not reproducible.

Diet experiment 1 trialled artificial diet formulations with varying sucrose concentrations (5, 10 and 20%) at pH 7.0; however there was no increase in grape phylloxera survival time. There was also no variation in insect survival time, in comparison with a water control, with the *10% sucrose pH 7.0* artificial diet formulation trialled in diet experiment 2 on both first and intermediate instar grape phylloxera (Table 2). The survival time was not extended by using intermediate instars in comparison with the first instars.

Diet experiment 3 did not include a no-diet or water control as there was a direct comparison between grape phylloxera survival time in the absence or presence of a water weight pressure. The application of the water weight on the diet membrane did not impact on insect survival time; however there was an approximate one day increase in mean survival time between the *10% sucrose pH 7.0* diet formulation in diet experiment 3 in comparison with diet experiment 1. With the absence of a water control in diet experiment 3, no comparison in the natural variance of population survival times could be made between diet experiments 1 and 3.

The 5% sucrose artificial diet formulation was trialled at a range of pH values (4.5, 6.0 and 7.0) in diet experiment 5 (Table 2). There was no significant difference in the mean survival time between the diet formulations, although the maximum survival time

was extended by 3 days with the artificial diet formulation *5% sucrose pH 7.0 plus five amino acids*. Varying the form of sugar used in the diet formulation (pH 7.5) also did not have a significant impact of grape phylloxera survival time (diet experiment 6). In diet experiment 6, the mean survival time was highest with the oligidic *grapevine root extract* artificial diet formulation. The pH of the grapevine root extract was 5.0, which was the same as the measured pH of the root and stem of the micropropagated grapevine, and slightly less acidic than the leaves (Table 5).

**Table 5.** Micropropagated grapevine pH values obtained from ground leaf, stem and root material.

| sample | pH value |
|---|---|
| grapevine leaf | 4.0 |
| grapevine stem | 5.0 |
| grapevine root | 5.0 |

### 4.2.2 HUMIDITY CHAMBER SURVIVAL TIME

The humidity chamber was used to investigate grape phylloxera survival for nine artificial diet experiments (Table 3). These experiments were mainly performed on intermediate instars, although diet experiments 14 and 15 were performed on first instars. Across the nine experiments, diet formulations compared sugars (sucrose, glucose and fructose) with the addition of amino acids (5 and 10 mix) at a range of pH values (4.5, 6.5, 7.0 and 7.5), and a meridic grapevine root extract.

The only humidity chamber artificial diet to have a significant impact on grape phylloxera survival time was diet experiment 12 (Table 3). By pair-wise survival analysis, the *5% sucrose pH 4.5 plus amino acids* artificial diet formulation was confirmed to be significantly different from the control diets. Across all humidity chamber diet experiments, no other artificial diet formulations were significantly different from the control diets by the pair-wise statistical analysis. However individual artificial diet formulations within diet experiments 8 and 10 were marginally different ($p < 0.1$) from the water controls.

In experiment 12, intermediate instar grape phylloxera exposed to an artificial diet formulation of *5% sucrose pH 4.5 plus amino acids* significantly survived an additional 1.7 mean days over both the no-diet ($p < 0.001$) and water ($p = 0.0027$) controls. The maximum survival time of the *5% sucrose pH 4.5 plus amino acids* was 11 days (Table 3). The artificial diet formulation of *5% glucose pH 4.5 plus amino acids* had a maximum survival time of 10 days, but did not significantly extend the survival time of grape phylloxera in comparison with control diets (no-diet $p = 0.17$, water $p = 0.87$). The survival analysis plot illustrated that the proportion of insects surviving was higher in the *5% sucrose pH 4.5 plus amino acids* artificial diet formulation throughout the diet experiment (Figure 5). The mortality was highest for the control diets, where 50% of insects had died between 5.0-5.5 days (Table 6). In comparison, the *5% glucose pH*

*4.5 plus amino acids* artificial diet formulation recorded 50% mortality between 5.5-6.0 days, and the *5% sucrose pH 4.5 plus amino acids* between 6.5-7.0 days.

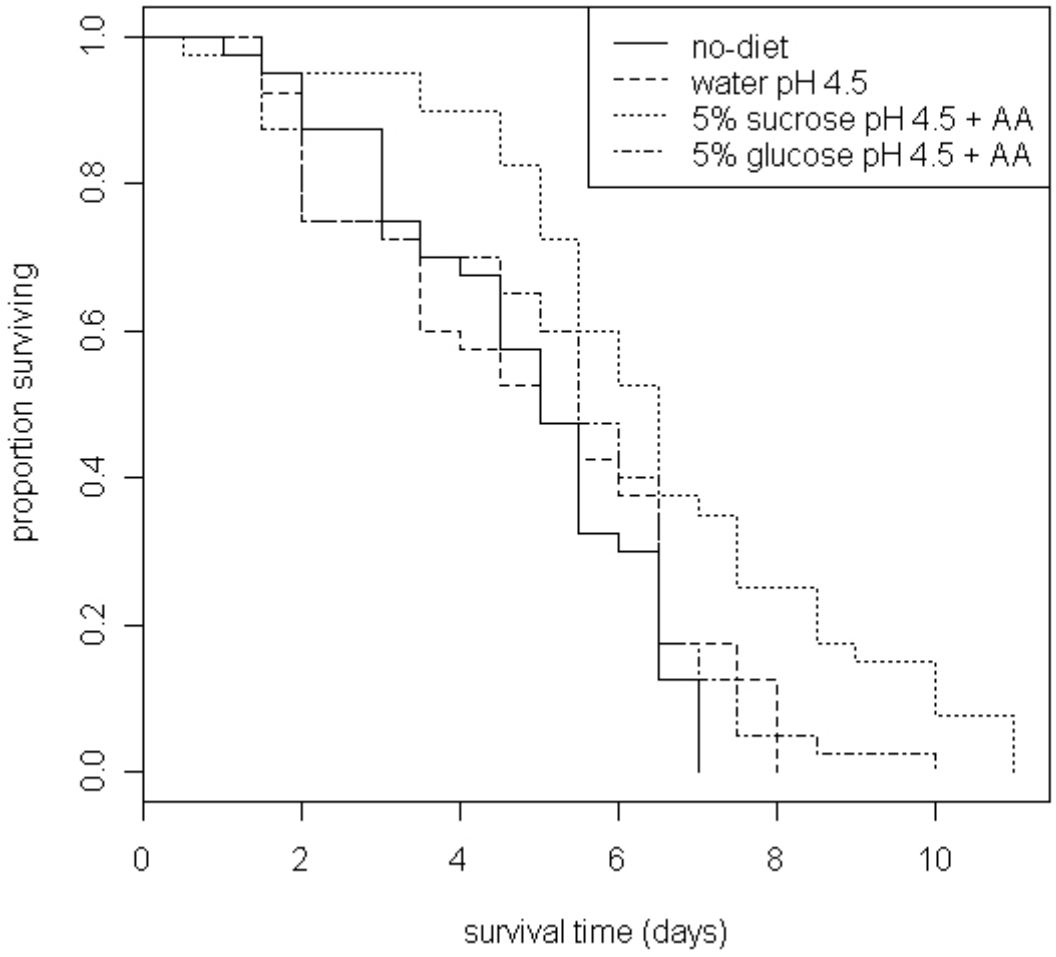


**Figure 5.** Grape phylloxera survival rate in artificial diet experiment 12; survival analysis plot of proportion of insects surviving over time (days). Intermediate life stage in the humidity chamber feeding on 'no-diet', 'water pH 4.5', '5% sucrose pH 4.5 plus amino acids (+ AA)' or '5% glucose pH 4.5 plus amino acids (+ AA)'.

**Table 6.** Survival analysis for grape phylloxera artificial diet experiment 12; intermediate life stage in the humidity chamber feeding on diet formulation 'no-diet', 'water pH 4.5', '5% sucrose pH 4.5 plus amino acids' or '5% glucose pH 4.5 plus amino acids'.

| diet formulation | time (days) | insects at risk | insects dead | proportion surviving | standard error |
|---|---|---|---|---|---|
| no-diet | 0 | 40 | 0 | 1.000 | 0 |
| | 1.0 | 40 | 1 | 0.975 | 0.025 |
| | 1.5 | 39 | 1 | 0.950 | 0.035 |
| | 2.0 | 38 | 3 | 0.875 | 0.052 |
| | 3.0 | 35 | 5 | 0.750 | 0.069 |
| | 3.5 | 30 | 2 | 0.700 | 0.073 |
| | 4.0 | 28 | 1 | 0.675 | 0.074 |
| | 4.5 | 27 | 4 | 0.575 | 0.078 |
| | 5.0 | 23 | 4 | 0.475 | 0.079 |
| | 5.5 | 19 | 6 | 0.325 | 0.074 |
| | 6.0 | 13 | 1 | 0.300 | 0.073 |
| | 6.5 | 12 | 7 | 0.125 | 0.052 |
| | 7.0 | 5 | 5 | 0.000 | |
| water | 0 | 40 | 0 | 1.000 | 0 |
| pH 4.5 | 1.5 | 40 | 3 | 0.925 | 0.042 |
| | 2.0 | 37 | 7 | 0.750 | 0.069 |
| | 3.0 | 30 | 1 | 0.725 | 0.071 |
| | 3.5 | 29 | 5 | 0.600 | 0.078 |
| | 4.0 | 24 | 1 | 0.575 | 0.078 |
| | 4.5 | 23 | 2 | 0.525 | 0.079 |
| | 5.0 | 21 | 2 | 0.475 | 0.079 |
| | 5.5 | 19 | 2 | 0.425 | 0.078 |
| | 6.0 | 17 | 2 | 0.375 | 0.077 |
| | 6.5 | 15 | 8 | 0.175 | 0.060 |
| | 7.5 | 7 | 2 | 0.125 | 0.052 |
| | 8.0 | 5 | 5 | 0.000 | |

**Table 6 continued.**

| diet formulation | time (days) | insects at risk | insects dead | proportion surviving | standard error |
|---|---|---|---|---|---|
| 5% sucrose | 0 | 40 | 0 | 1.000 | 0 |
| pH 4.5 plus | 0.5 | 40 | 1 | 0.975 | 0.025 |
| amino acids | 1.5 | 39 | 1 | 0.950 | 0.035 |
| | 3.5 | 38 | 2 | 0.900 | 0.047 |
| | 4.5 | 36 | 3 | 0.825 | 0.060 |
| | 5.0 | 33 | 4 | 0.725 | 0.071 |
| | 5.5 | 29 | 5 | 0.600 | 0.078 |
| | 6.0 | 24 | 3 | 0.525 | 0.079 |
| | 6.5 | 21 | 6 | 0.375 | 0.077 |
| | 7.0 | 15 | 1 | 0.350 | 0.076 |
| | 7.5 | 14 | 4 | 0.250 | 0.069 |
| | 8.5 | 10 | 3 | 0.175 | 0.060 |
| | 9.0 | 7 | 1 | 0.150 | 0.057 |
| | 10.0 | 6 | 3 | 0.075 | 0.042 |
| | 11.0 | 3 | 3 | 0.000 | |
| 5% glucose | 0 | 40 | 0 | 1.000 | 0 |
| pH 4.5 plus | 1.5 | 40 | 5 | 0.875 | 0.052 |
| amino acids | 2.0 | 35 | 5 | 0.750 | 0.069 |
| | 3.0 | 30 | 1 | 0.725 | 0.071 |
| | 3.5 | 29 | 1 | 0.700 | 0.073 |
| | 4.5 | 28 | 2 | 0.650 | 0.075 |
| | 5.0 | 26 | 2 | 0.600 | 0.078 |
| | 5.5 | 24 | 5 | 0.475 | 0.079 |
| | 6.0 | 19 | 3 | 0.400 | 0.078 |
| | 6.5 | 16 | 9 | 0.175 | 0.060 |
| | 7.0 | 7 | 2 | 0.125 | 0.052 |
| | 7.5 | 5 | 3 | 0.050 | 0.035 |
| | 8.5 | 2 | 1 | 0.025 | 0.025 |
| | 10.0 | 1 | 1 | 0.000 | |

'Insects at risk' was the number of insects alive within the diet chamber prior to observation, 'insects dead' was the number of insects observed dead for each time interval. Gaps in 'insects at risk' and 'insects dead' values are due to censored insects. Time intervals without an observed death of an insect are not presented as the survival distribution value remains the same as the previous time interval.

The artificial diet formulation of *5% sucrose pH 4.5 plus amino acids* was also trialled in diet experiments 11, 13 (intermediate instars), 14 and 15 (first instars), however none of these diet experiments produced similar mean and maximum survival times to those observed in diet experiment 12 (Table 3). The artificial diet formulation *5% sucrose pH 4.5 plus amino acids* was not recorded as significantly different in mean survival time (in comparison with the control diets) in any of the additional diet experiments, although the exposure to the diet formulation did increase the mean survival times (except for diet experiment 11). The mean and maximum survival times observed in diet experiment 11 and 13 (intermediate instars) were similar, as were the values for diet experiments 14 and 15 (first instars).

Artificial diet formulations trialled in diet experiments 7 and 8 included *5% sucrose pH 7.0 plus five amino acids*, and *5% sucrose, fructose* or *glucose pH 7.5*. None of the artificial diet formulations increased grape phylloxera mean survival time in comparison with the water controls (Table 3). Diet experiments 7 and 8 using intermediate instars included the same artificial diet formulations used in first instar diet experiments 5 and 6 (Table 2). Across all diet formulations, the mean and maximum survival times for intermediate instars were higher in comparison with first instars; however no statistical analysis was possible between diet experiments to confirm the trend.

In diet experiment 8, the artificial diet formulation *5% sucrose pH 7.5* had a reduced mean survival time in comparison with the water control. The reduction in survival time was not significant, although as a pair-wise comparison the p-value was approaching the level of significance ($p = 0.054$). A similar reduction in mean survival time was observed in diet experiment 10 with the artificial diet formulation of *10 amino acids pH 6.5* (pair-wise $p = 0.098$). This artificial diet formulation was part of a group of formulations that included a range of sugars at pH 6.5 plus amino acids (diet experiments 9 and 10). In these diet experiments, the artificial diet formulation of *10*

*amino acids pH 6.5* recorded the shortest mean grape phylloxera survival time; the artificial diet formulation *5% sucrose pH 6.5 plus amino acids* the longest (Table 3). The acidity of the sucrose and glucose plus amino acids diet formulations in diet experiments 9 and 10 was increased to pH 4.5 in diet experiment 12. The grape phylloxera mean and maximum survival times were increased in the artificial diet formulations *5% sucrose pH 4.5 plus amino acids* and *5% glucose pH 4.5 plus amino acids*.

The meridic artificial diet formulation containing the unfiltered *grapevine root extract and 5% sucrose pH 4.5 plus amino acids* (diet experiment 15) did not increase grape phylloxera survival time in comparison with the water control diet. The mean survival time for the meridic artificial diet formulation was reduced in comparison with the holidic artificial diet formulation *5% sucrose pH 4.5 plus amino acids*.

## *4.3 STYLET INTERACTIONS*

In all grape phylloxera artificial diet experiments, the location and feeding status of the insects was monitored while recording insect survival time. Although ingestion of the artificial diet solution by the insects was not determined, insect probing activity on the diet membrane and stylet interactions observed within the artificial diet solution suggested a level of attraction by grape phylloxera to the diet membrane and the artificial diet formulation.

### *4.3.1 INSECT PROBING ACTIVITY*

Grape phylloxera observed rates of probing activity on artificial diet membranes did not correlate with mean survival times. In diet experiments based on the filter paper chamber, a significant difference in the proportion of probing insects between control diets and artificial diet formulations was identified in diet experiment 1 and 5 (Table 7). In diet experiment 1, the artificial diet formulations *5% sucrose pH 7.0* and *20% sucrose pH 7.0* both recorded a higher proportion of probing insects in comparison with the no-diet control, however only *20% sucrose pH 7.0* was significantly different to the water control. Although both artificial diet formulations recorded a mean survival time that was higher than both control diets, neither the *5% sucrose pH 7.0* or the *20% sucrose pH 7.0* diet formulation were significantly different (Table 2). In diet experiment 5, a significantly higher proportion of insects were observed in probing activity (in comparison with the water control) for the artificial diet formulations *5% sucrose pH 7.0* and *5% sucrose pH 7.0 plus five amino acids* (Table 7). However both artificial diet formulations recorded (non-significant) lower mean survival times in comparison with the water control. The only filter paper chamber artificial diet to record a significant difference in the mean survival rate was diet experiment 4, however no probing activity was observed on either the no-diet control or the *5% sucrose pH 7.0* artificial diet formulation.

**Table 7.** Grape phylloxera probing activity in artificial diet experiments conducted using the filter paper chamber; life stage, diet formulation and probing rate indicated. Number of insects per diet formulation indicated by the life stage (n) value.

| diet number | life stage (n) | | artificial diet formulation and probing rate | | | | | overall log*it* p-value |
|---|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | e | |
| 1 | hatchery first instar | formulation | **no-diet** | **water pH 7.0** | ***5% sucrose pH 7.0*** | 10% sucrose pH 7.0 | ***20% sucrose pH 7.0*** | |
| | (25) | mean (n) | 0.31 (4) | 0.71 (10) | 0.76 (13) | 0.60 (9) | 1.5 (19) | ***0.003*** |
| | | control log*it* | | | ***0.033 :*** 0.47 | 0.35 : 0.54 | ***< 0.001 : 0.017*** | |
| 2 | first and intermediate instar | formulation | *first instar* **water pH 7.0** | *first instar* 10% sucrose pH 7.0 | *intermediate* **water pH 7.0** | *intermediate* 10% sucrose pH 7.0 | | |
| | (15) | mean (n) | 0.22 (2) | 0 (0) | 0.11 (1) | 0 (0) | | 0.26 |
| | | control log*it* | | 0.12 | | 0.28 | | |
| 3 | first instar | formulation | *no pressure* **10% sucrose pH 7.0** | *water pressure* 10% sucrose pH 7.0 | | | | |
| | (15) | mean (n) | 0.43 (3) | 0.86 (6) | | | | 0.13 |
| | | control log*it* | | 0.13 | | | | |
| 4 | first instar | formulation | **no-diet** | 5% sucrose pH 7.0 | | | | |
| | (15) | mean (n) | 0 (0) | 0 (0) | | | | NA |
| | | control log*it* | | NA | | | | |

**Table 7 cont.**

| diet number | life stage (n) | | artificial diet formulation and probing rate | | | | | overall log*it* p-value |
|---|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | e | |
| 5 | hatchery first instar | formulation | **water pH 7.0** | ***5% sucrose pH 7.0*** | ***5% sucrose pH 7.0 + 5AA[1]*** | 5% sucrose pH 6.0 | 5% sucrose pH 4.5 | |
| | (16) | mean (n) | 0.22 (2) | 0.80 (8) | 0.67 (10) | 0.64 (7) | 0.18 (2) | ***0.009*** |
| | | control log*it* | | ***0.013*** | ***0.015*** | 0.066 | 0.91 | |
| 6 | hatchery first instar | formulation | **water pH 7.5** | root extract[2] pH 5.0 | 5% sucrose pH 7.5 | 5% glucose pH 7.5 | 5% fructose pH 7.5 | |
| | (20) | mean (n) | 1.8 (16) | 1.8 (14) | 2.9 (20) | 1.8 (11) | 2.1 (19) | 0.30 |
| | | control log*it* | | 0.30 | 0.34 | 0.53 | 0.80 | |

Control diets for each experiment are highlighted in **bold**, diet formulation characteristics in *italic* indicate experiment variances not based on the artificial diet solution. Mean number probing per monitoring period and total number probing insects for experimental period (n) are presented. Logistic regression (log*it*) p-values presented for overall analysis and individual diet formulation pair-wise comparison with control diet; if >1 control diet, both control pair-wise values are presented. Significant log*it* p-values are highlighted in ***bold italic***, diet formulations significantly different from the control diet are also highlighted.

[1] diet formulation containing the five amino acid profile denoted as '+ 5AA'

[2] oligidic grapevine root extract diet formulation

**Table 8.** Grape phylloxera probing activity in artificial diet experiments conducted using the humidity chamber; life stage, diet formulation and probing rate indicated. Number of insects per diet formulation indicated by the life stage (n) value.

| diet number | life stage (n) | | artificial diet formulation and probing rate | | | | overall log*it* p-value |
|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | |
| 7 | intermediate instar | formulation | **water pH 7** | ***5% sucrose pH 7.0 + 5AA[1]*** | | | |
| | (15) | mean (n) | 0.15 (4) | 0.68 (25) | | | ***< 0.001*** |
| | | control log*it* | | ***< 0.001*** | | | |
| 8 | intermediate instar | formulation | **water pH 7.5** | 5% sucrose pH 7.5 | 5% fructose pH 7.5 | 5% glucose pH 7.5 | |
| | (10) | mean (n) | 0.06 (1) | 0.13 (1) | 0.07 (1) | 0.08 (1) | 0.99 |
| | | control log*it* | | 0.79 | 0.99 | 0.93 | |
| 9 | intermediate instar | formulation | **water pH 7** | 5% fructose pH 6.5 + AA[2] | 5% sucrose pH 6.5 + AA[2] | | |
| | (20) | mean (n) | 0.04 (1) | 0.04 (1) | 0.17 (5) | | 0.20 |
| | | control log*it* | | 0.95 | 0.13 | | |
| 10 | intermediate instar | formulation | **water pH 7** | 5% glucose pH 6.5 + AA[2] | 10 AA pH 6.5 | | |
| | (30) | mean (n) | 0 (0) | 0 (0) | 0 (0) | | NA |
| | | control log*it* | | NA | NA | | |

**Table 8 cont.**

| diet number | life stage (n) | | artificial diet formulation and probing rate | | | | overall log*it* p-value |
|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | |
| 11 | intermediate instar | formulation | **water pH 4.5** | ***5% sucrose pH 4.5 + AA²*** | | | |
| | (30) | mean (n) | 0.54 (13) | 1.2 (33) | | | ***0.002*** |
| | | control log*it* | | ***0.002*** | | | |
| 12 | intermediate instar | formulation | **no-diet** | **water pH 4.5** | ***5% sucrose pH 4.5 + AA²*** | ***5% glucose pH 4.5 + AA²*** | |
| | (40) | mean (n) | 0.91 (38) | 1.2 (52) | 1.8 (120) | 1.6 (70) | ***< 0.001*** |
| | | control log*it* | | | ***<0.001*** : ***<0.001*** | ***0.003*** : 0.15 | |
| 13 | intermediate instar | formulation | **water pH 4.5** | ***5% sucrose pH 4.5 + AA²*** | | | |
| | (40) | mean (n) | 0.52 (22) | 0.27 (11) | | | ***0.037*** |
| | | control log*it* | | ***0.037*** | | | |
| 14 | hatchery first instar | formulation | **water pH 4.5** | 5% sucrose pH 4.5 + $AA^2$ | | | |
| | (30) | mean (n) | 1.0 (14) | 0.75 (12) | | | 0.42 |
| | | control log*it* | | 0.42 | | | |

**Table 8 cont.**

| diet number | life stage (n) | | artificial diet formulation and probing rate | | | | overall log*it* p-value |
|---|---|---|---|---|---|---|---|
| | | | a | b | c | d | |
| 15 | first instar | formulation | **water pH 4.5** | 5% sucrose pH 4.5 + AA[2] | ***5% sucrose pH 4.5 + AA[2] + root extract[3]*** | | |
| | (40) | mean (n) | 0 (0) | 0.11 (3) | 0.33 (9) | | ***0.002*** |
| | | control log*it* | | 0.053 | ***< 0.001*** | | |

Control diets for each experiment are highlighted in **bold**, diet formulation characteristics in *italic* indicate experiment variances not based on the artificial diet solution. Mean number probing per monitoring period and total number probing insects for experimental period (n) are presented. Logistic regression (log*it*) p-values presented for overall analysis and individual diet formulation pair-wise comparison with control diet; if >1 control diet, both control pair-wise values are presented. Significant log*it* p-values are highlighted in ***bold italic***, diet formulations significantly different from the control diet are also highlighted.

[1] diet formulation containing the five amino acid profile denoted as '+ 5AA'

[2] diet formulation containing the ten amino acid profile denoted as '+ AA'

[3] meridic grapevine root extract diet formulation

Pressurising the artificial diet solution, trialled in diet experiment 3, resulted in no significant increase in mean survival time (Table 2) or proportion of probing insects (Table 7). However there was a general observation of more insect activity on the diet membrane in the water pressurised filter paper chamber as indicated by the higher mean probing value. The water weight pressurisation system was maintained in the artificial diet set up as it was essential in the humidity chamber design to enclose the artificial diet solution and to keep the two Parafilm® M membranes aligned.

Based on the humidity chamber design, the artificial diet formulations in five diet experiments (7, 11, 12, 13 and 15) were observed to have a significantly higher proportion of insects undertaking probing activity in comparison with the control (Table 8). All five diet experiments recorded a significantly higher proportion of probing activity on artificial diet formulations in comparison with control diets; except for diet experiment 13 where a higher level of probing activity was recorded on the water control (Table 8). In diet experiment 15, the meridic artificial diet containing the *grapevine root extract and 5% sucrose pH 4.5 plus amino acids* recorded a significantly ($p < 0.001$) higher proportion of insects probing the diet formulation in comparison with the water control; the artificial diet formulation *5% sucrose pH 4.5 plus amino acids* was marginally different ($p = 0.053$). Generally, the mean survival time of all artificial diet formulations from diet experiments 7, 11, 12, 13 and 15 were higher than controls; however only diet experiment 12 recorded significant differences (Table 3).

Diet experiment 12 was the only humidity chamber artificial diet to record a significant difference in the mean survival rate; the artificial diet formulation *5% sucrose pH 4.5 plus amino acids* was significantly different to the no-diet and water controls. There was also a significantly ($p < 0.001$) higher proportion of probing activity on this diet formulation in comparison with both control diets (Table 8); the *5% glucose pH 4.5 plus amino acids* was only significantly different from the no-diet control ($p = 0.003$). The

level of probing activity was consistently higher in the *5% sucrose pH 4.5 plus amino acids* artificial diet formulation throughout the diet experiment (Figure 6).

The significance in the proportion of probing insects in comparison with control diets was highest in the artificial diet formulations containing 5% sucrose plus amino acids. Across all diet experiments, the *5% sucrose pH 7.0 plus five amino acids* was used in two diet experiments (5 and 7) and the proportion of probing insects was significantly higher compared with the water control in both experiments (Tables 7 and 8). The artificial diet formulation *5% sucrose pH 4.5 plus amino acids* was used in five diet experiments (11-15); the proportion of probing insects was significantly higher compared with control diets in diet experiments 11 and 12. In diet experiment 13 there was a higher proportion of probing insects recorded for the water control (Table 8). The *5% sucrose pH 6.5 plus amino acids* was only trialled in diet experiment 9; and although there was a higher mean number of probing insects, the proportion of probing insects was not significantly different from the water control.

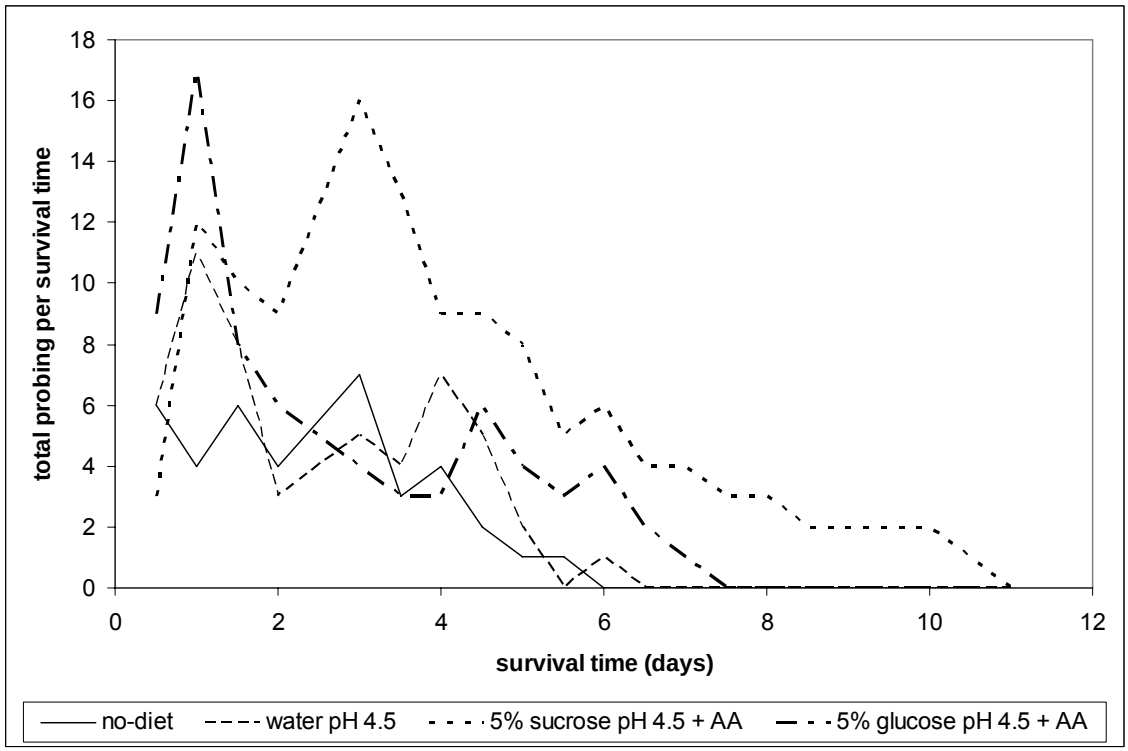


**Figure 6.** Grape phylloxera probing activity in artificial diet experiment 12; total number of insects probing per survival time (days). Intermediate life stage in the humidity chamber feeding on 'no-diet', 'water pH 4.5', '5% sucrose pH 4.5 plus amino acids (+ AA)' or '5% glucose pH 4.5 plus amino acids (+ AA)'.

### 4.3.2 *DIET SOLUTION CRYSTALLISATION*

Some grape phylloxera insects identified as being involved in probing activity in the artificial diet experiments were observed on the diet membrane with the stylet visibly extended from the labium and penetrating the diet membrane. A crystallisation of the artificial diet solution was observed at the tip of the stylet of intermediate instar insects (n = 7) in diet experiments 11 and 12 (Figure 7). The crystallisation was only observed when the insects were exposed to the artificial diet formulation *5% sucrose pH 4.5 plus amino acids*. Insects exposed to the *5% sucrose pH 4.5 plus amino acids* diet formulation were also observed to move their stylets in a 'backwards and forwards' movement in the artificial diet solution.

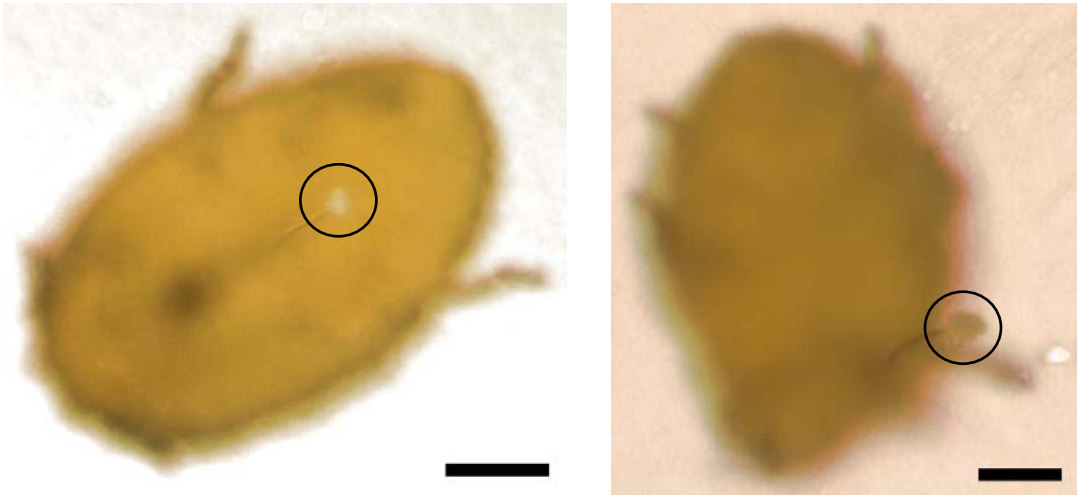

**Figure 7.** Two intermediate grape phylloxera observed in artificial diet experiment 11, feeding on diet *5% sucrose pH 4.5 plus amino acids*. The insects were positioned on the underside of the diet membrane with their stylets extended from the labium into the diet solution, a crystallisation of the diet solution formed at the tip of the stylet (circled). Scale bar 100 µm.

# 5 DISCUSSION

The development of an artificial feeding system for radicicolae grape phylloxera was impacted by a number of factors, including chamber design and insect base level survival on control diets. Grape phylloxera survival rates and probing activity levels were affected by sugar and amino acid composition of the artificial diet formulation, and the pH value of the solution. Life stage may also affect the apparent success of an artificial diet.

Grape phylloxera survival on the artificial feeding systems was obtained for a maximum period of 11 days. The variation in survival time for the artificial diet formulation *5% sucrose pH 4.5 plus amino acids* in diet experiments 11-15 highlighted the impact of the natural population variation within the diet experiments, and emphasised why statistical analysis was only comparable within individual diet experiments. Although grape phylloxera probing activity increased with the addition of amino acids and the reduction of the artificial diet formulation pH, in general, variation in the artificial diet formulation had a limited impact on grape phylloxera survival in comparison with control diets. No significant increases in survival time were reproducible.

Current knowledge of the amino acid and sugar composition of grape phylloxera infested grapevine material assisted in the development of the artificial diet formulations, however more defined research on the chemical composition of the parenchyma cell food source is required to further the development of an optimal diet solution. The artificial diet experiments presented provided an indication of the essential nutritional and pH requirements for grape phylloxera survival; however the limited success of the artificial diet formulations prevented the trialling of possible control agents for the future management of the pest insect.

### *5.1 ARTIFICIAL DIET CHAMBER DESIGN*

The environmental conditions of the insect chamber are critical for the success of an artificial diet system, and to be able to confidently attribute diet failure to the artificial diet formulation (Cohen, 2003). Grape phylloxera survival rates are affected by temperature and relative humidity levels. Buchanan (1990) found that at 25°C, 85% relative humidity was required for a radicicolae grape phylloxera survival rate of greater than 50%. The condensed droplets of water observed in both the filter paper and humidity chambers indicated that the relative humidity level within the insect chambers was high, and therefore was not a factor in grape phylloxera survival.

The condensed droplets of water on the diet membrane of the insect chambers did however impede insect movement, especially in the filter paper chamber. Water droplets were larger in the filter paper chamber, where first instar insects were used. First instar grape phylloxera measure 326 µm in length, and the intermediate life stages have a mean length of 492 µm (from light microscopy measurements, Chapter 2). The relatively small size of first instar grape phylloxera increased the difference in size ratio between the insect and the water droplet, and may be a factor for the increased number of immobilised insects on the diet membrane of the filter paper chamber. Some first instar and intermediate instar insects in the humidity chamber were observed to be surrounded by water droplets, although movement across the diet membrane surface was still possible.

The filter paper chamber design, with the enclosure of the diet solution within a double layer Parafilm® M sachet, has been successfully used for the artificial feeding systems of a range of plant-sucking insects, including leafhoppers, planthoppers (Powell *et al.*, 1993), and aphids (Mittler, 1988). However more automated designs of artificial feeding systems are required for sedentary feeding insects, where a low level of disturbance is required once the insects are settled on the artificial diet food source

(Mittler, 1988). Due to this complication, the humidity chamber design was considered more appropriate for grape phylloxera due to the ability to renew the diet solution without disrupting insect probing activity.

The humidity chamber design offered a simplified chamber design from the model previously used by Forneck and Wöhrle (2003), which was based on a design by Akey and Beck (1971). For the gallicolae grape phylloxera artificial feeding system, Forneck and Wöhrle (2003) used a 35 mm glass chamber for both the insect and diet chambers, and a number of specialised design features: (1) silicone rings were glued to the glass chambers to prevent the stretched Parafilm® membrane from tearing; (2) the Parafilm® membrane only covered the diet chamber; (3) the insect chamber was placed on top of the diet chamber to allow the gallicolae grape phylloxera access to the diet solution; (4) a ring of filter paper was required as a gasket between the Parafilm® membrane and the silicone ring to prevent sticking; (5) two silicone tubes were attached to the diet chamber to create a flow through chamber; (6) the diet solution (5 ml) was renewed by flushing the diet chamber with fresh diet solution via the silicone access tubes.

The humidity chamber design used for radicicolae grape phylloxera allowed for the same level of non-disturbance for probing insects during artificial diet renewal, but did not involve the use of any specialised equipment. The assembly of the humidity chamber design only required a 35 mm plastic petri dish, two layers of Parafilm® M membrane, a 0.5 ml tube lid, a circle of fine mesh and 50 µl of diet solution. A water weight was required in the humidity chamber design to keep the two layers of Parafilm® M aligned, and to pressurise the diet solution. Although the level of diet pressurisation applied by the water weight did not significantly affect the rate of insect survival, this was only examined on one artificial diet formulation (*10% sucrose pH 7.0*) in the filter paper chamber design, and the result may have been influenced by the diet formulation

more than the level of diet pressurisation. However regardless of impact of diet pressurisation on dietary uptake and survival, the water weight was a critical component of the humidity chamber design for the alignment of the diet and lid membranes.

Compared with the systems of Akey and Beck (1971) and Forneck and Wöhrle (2003), the simplified design of the humidity chamber decreased the complexity, and the requirement of specialised resources, for future artificial diet studies involving sedentary feeding insects.

### *5.2 SURVIVAL RATES ON CONTROL DIETS*

The extended survival time of grape phylloxera on control diets (no-diet maximum survival time 7 days and water 9 days) increased the difficulty of surveying artificial diet formulations as several days of the diet experiment would pass before any indication of possible increases in insect survival (due to the diet formulation) was evident. In some cases (diet experiments 2, 5, 8, 10 and 11) the control diet survival time was longer than the experimental artificial diet formulation. The survival rate of grape phylloxera on control diets was generally reduced on no-diet controls (compared with water) and with experiments involving first instars (compared with intermediate instars), although there was no significance in these trends.

Both the filter paper and humidity chamber contained condensed droplets of water within the insect chamber. This high humidity environment removed dehydration as a factor for insect death, and provided access to water for ingestion by insects exposed to a no-diet control. The potential for non-direct artificial diet interaction was highlighted by the significant survival analysis result in diet experiment 4, where an absence of probing in either the no-diet control or the *5% sucrose pH 7.0* artificial diet formulation indicated that the insects may have ingested fluid from the condensed droplets of water within the diet chamber for survival. As there was no extension in the

maximum survival time between the control and diet formulation, the significant improvement in survival time was likely to be due to natural population variation rather than a reflection of the 'success' of the diet formulation.

Higher levels of probing activity on water control diets in comparison with no-diet controls (although not significant) suggested that the water control was a more robust method for comparison of survival times with experimental diet formulations. If a water control was included in the diet experiment 4, it is speculated that no significant difference in mean survival time would have been detected. The weakness of the no-diet control in comparison with the water control was demonstrated in diet experiments 1 and 12. Diet formulation *5% sucrose pH 4.5 plus amino acids* in diet experiment 12 was significantly different from both the no-diet and water controls for both survival time and the proportion of insects probing, however there was a stronger level of significance for the no-diet compared with the water control. The diet formulation *5% glucose pH 4.5 plus amino acids* was not significantly different from either control for mean survival time, although there was a significantly higher proportion of insects probing the diet formulation in comparison with the no-diet control. The same relationship between significance in probing activity and the no-diet and water controls was also present in diet experiment 1. The higher levels of probing activity on the water controls compared with the no-diet controls (which resulted in reduced levels of significance) indicated that water was a more stringent control diet as it more closely reflected insect activity on diet formulations.

Although diet experiment 2 indicated no significant difference in grape phylloxera survival time between first and intermediate instars, variation in insect survival time based on the same diet formulation during different diet experiments (with different life stages) indicated that intermediate instars survived longer than first instars. The advantage of having food in the digestive system of the intermediate instars, due to the

previous exposure to grapevine excised root material, enhanced survival rates and fitness levels in comparison with first instars. The digestive system of grape phylloxera contains modifications that allow for the storage of food (from light microscopy section, Chapter 2). This storage system could explain the trend of intermediate instars surviving longer than first instars within the artificial feeding system, and also the presence of moulted cuticles within the intermediate instar diet chambers. The presence of moulted cuticles in intermediate instar, but not in first instar, diet chambers suggested that the insect development was due to pre-feeding on grapevine root material, and the slow digestion of this food source, as opposed to feeding on the diet formulation. The storage of food in the digestive system of grape phylloxera also provided an explanation for the extended survival time of grape phylloxera on control diets, and on experimental artificial diet formulations where limited probing activity was observed.

The survival time of radicicolae grape phylloxera on control diets experienced in the current study were longer than the survival time (6 days) of fourth instar gallicolae grape phylloxera on a water control diet (Forneck and Wöhrle, 2003). However in both systems, the grape phylloxera survival time was atypical to Aphididae artificial feeding systems where very limited survival times are recorded on water control diets. During examination of the influence of sucrose concentration on the dietary uptake of *A. pisum,* Srivastava and Auclair (1971) identified that the insect survived only one day on a 0% sucrose diet, and a maximum of three days if pre-fed on a 30% sucrose diet.

### *5.3 ARTIFICIAL DIET FORMULATION*

Grape phylloxera feed solely on species of *Vitis*, and the development of a successful artificial diet formulation for a specialised feeder like grape phylloxera requires detailed consideration of the natural diet of the insect (Davis, 1972). The natural food source of grape phylloxera are parenchyma cells within the root cortex (Kellow *et al.*, 2004);

however initial artificial diet formulations involved variations in sugar concentration and form based on the diet solutions of Aphididae that feed on phloem sap. There were variable results (based on insect survival time and probing activity) from the inclusion of sucrose, fructose or glucose in the artificial diet formulations; however sucrose was the main sugar used in the diet formulations due to elevated sucrose levels recorded in grape phylloxera infested root material (Ryan *et al.*, 2000). Glucose was re-examined in later diet experiments due to evidence that a glucose complex (starch) accumulated at radicicolae grape phylloxera feeding sites during early gall development (Forneck *et al.*, 2002). In diet experiment 12, intermediate instars exposed to the artificial diet formulation *5% sucrose pH 4.5 plus amino acids* survived significantly longer, with a higher level of probing activity, than insects exposed to the *5% glucose pH 4.5 plus amino acids* diet formulation. Carbohydrates (sugars), as an energy source, are typically a major part of an insect diet (Chapman, 1969), and sucrose appeared to be the preferred form of sugar for grape phylloxera. The preference of radicicolae grape phylloxera for diet formulations containing sucrose was further supported by the significant levels of probing activity observed on the *5% sucrose pH 7.0* and *20% sucrose pH 7.0* diet formulations in diet experiments 1 and 5.

Protein as amino acids are another major component of insect artificial diets, and the absence of any one of the 10 essential amino acids, or their inclusion at too low a concentration, usually results in poor growth rates (Chapman, 1969). The inclusion of amino acids in the artificial diet formulations for grape phylloxera generally improved the survival rates of the insect, and significantly increased the proportion of insects probing the diet membrane in a range of diet experiments. The parenchyma cell natural food source of grape phylloxera contains relatively high concentrations of protein and low concentrations of carbohydrate in comparison with phloem sap (Ponsen, 1997). Amino acids are therefore considered an important factor for feeding stimulation, and a chemo-stimulation effect may be the cause for the high rate of

significant probing activity identified in a number of artificial diet formulations involving the addition of amino acids to a sucrose base. The non-correlation of probing activity to insect survival rates indicated that the diet formulations did not fully satisfy the nutritional requirements of grape phylloxera, and modifications are required for future studies.

The addition of grapevine root extracts as an oligidic or a meridic diet formulation was also expected to impact upon insect survival rates. Natural food source complexes added to the artificial diet may act as stimuli to improve diet uptake due to the inclusion of nutrients essential for attracting the insect to the specialised food source (Cohen, 2003). Filtered and unfiltered root extracts were trialled as a precaution against the accidental removal of a feeding stimulant by the filtration process; however neither artificial diet formulation including a root extract solution had a significant impact on grape phylloxera survival rates. There was however a significantly higher proportion of insects probing the diet membrane in the meridic diet formulation containing the unfiltered *grapevine root extract and 5% sucrose pH 4.5 plus amino acids*. The parenchyma cell food source of radicicolae grape phylloxera may have been diluted in the homogenised mix of whole ground root material, and therefore may be a contributing factor for the reduced survival rates.

The acidic pH of the grapevine root (pH 5.0) was similar to the *5% sucrose pH 4.5 plus amino acids* diet formulation (diet experiment 12) that significantly enhanced grape phylloxera survival time. A pH similar to the natural food source would be expected to be the preferred diet formulation; however the non-reproducibility of this result makes this problematic to conclude. The pH of grapevine root xylem sap varies with water treatment and time of day (Stoll *et al.*, 2000), and presumably would also be influenced by other vineyard management strategies. Also, the specific pH of parenchyma cells was unknown, and may vary from xylem sap or a whole root extract. Therefore,

although this study implied that grape phylloxera prefer an acidic pH, they may be able to feed at a range of pH values.

Grape phylloxera were observed to probe the diet membrane, although ingestion of the artificial diet solution was not proven. A possible correlation method to prove ingestion is the observation of waste excretion as honeydew, however if honeydew production occurs in grape phylloxera, it is at very low levels and is not easily observable (Chapter 2). The crystallisation near the tip of the stylet for insects feeding on the *5% sucrose pH 4.5 plus amino acids* diet formulation however provided evidence that the insects were piercing the diet membrane and contacting the diet solution. The composition of the crystallisation was unknown, but may represent the precipitation of a chemical reagent from the diet solution. A component of the amino acid mix (suspected to be L-isoleucine), dissolved less readily during the preparation of this artificial diet formulation and may have come out of solution in response to grape phylloxera salivation. The movement of the grape phylloxera stylets within the diet solution also confirmed that the stylet was capable of piercing the diet membrane, and that the insects were sampling the diet solution.

### *5.4 COMPARISON WITH PREVIOUS RESEARCH*

Forneck and Wöhrle (2003) obtained significant improvements in gallicolae grape phylloxera survival (in comparison with a water control) for two artificial diet formulations: 5% sucrose and 5% sucrose plus 10 amino acids. The amino acid mix contained essential amino acids (methionine, threonine, valine, isoleucine, leucine, phenylalanine, and histidine) and non-essential amino acids (arginine, asparagine and glutamine). The total concentration of amino acids in the gallicolae grape phylloxera artificial diet was less than 0.2% (Wöhrle, 1999), and individual amino acid concentrations were 25-300 times less than the amino acid mix trialled with radicicolae grape phylloxera, which was based on *A. pisum* (Akey and Beck, 1971). Additionally,

the 5% sucrose concentration used for both gallicolae and radicicolae grape phylloxera diet formulations was low compared with optimal concentrations for *A. pisum* of 20-25% sucrose (Srivastava and Auclair, 1971).

The pH of the gallicolae grape phylloxera artificial diet formulations were not reported (Wöhrle, 1999; Forneck and Wöhrle, 2003), however the pH of the diet solutions were not adjusted after the addition of the individual chemical reagents (per. com. Astrid Forneck). An estimated solution pH may be determined from the initial pH value of comparable solutions used for radicicolae grape phylloxera artificial diet formulations. The starting pH of 5% sucrose solutions was generally neutral (mean pH 6.8), and the starting pH for 5% sucrose plus (five or ten) amino acids was acidic (mean pH 4.5). Although the amino acid mix varied in composition and concentration between the gallicolae and radicicolae formulations, these values provide a pH for comparison with the artificial diet experiments presented in the current study for radicicolae grape phylloxera.

The survival time for gallicolae grape phylloxera on the 5% sucrose and 5% sucrose plus 10 amino acids was a maximum of 14 days (Wöhrle, 1999). Radicicolae grape phylloxera on comparable artificial diet formulations in diet experiments 4 and 12 (*5% sucrose pH 7.0* and *5% sucrose pH 4.5 plus amino acids*) survived significantly longer than the control diets, however survival times were less than gallicolae grape phylloxera. These two separate studies indicate that grape phylloxera may be able to feed on a range of pH values, and therefore artificial diet formulation pH may not be a critical factor.

The amino acid composition and concentration levels used for the gallicolae grape phylloxera artificial diet formulation were inferred from a study by Rilling *et al.* (1975) on the amino acid composition of grape phylloxera leaf galls (Forneck and Wöhrle, 2003). A similar study that identified the changes in amino acid concentration with radicicolae

grape phylloxera infestation of grapevine roots was conducted by Kellow *et al.* (2004). Glutamine was the dominant amino acid in both profiles, however the amino acid concentrations presented by the Rilling *et al.* (1975) and Kellow *et al.* (2004) studies differed, indicating the amino acid composition of the food source for gallicolae and radicicolae grape phylloxera may not be the same. The current study also identified a difference in pH between micropropagated grapevine leaf and root material. The high level of glutamine observed in the infested grapevine material indicated that this amino acid should be considered 'essential' for future grape phylloxera artificial diet formulations, along with the ten essential amino acids highlighted by Cohen (2003).

The amino acid concentrations used in the radicicolae grape phylloxera artificial diet formulations were in excess of the grapevine root profile in order to prevent amino acid availability from being a limiting factor in the diet solution. The total concentration of amino acids in the five amino acid mix was 1.4%, and 1.9% for the ten amino acid mix. The fluid uptake of artificial diet formulations for the phloem feeding Aphididae *Myzus persicae* (Sulzer) were optimal with total amino acid concentrations of 3%, with poor ingestion rates observed on diet solutions with less than 1% total amino acids (Mittler, 1967). Grape phylloxera may feed on parenchyma cell contents which contain a higher level of amino acids compared with phloem tissue, suggesting that the total amino acid concentration in the grape phylloxera artificial diet formulations should be increased. However a 10-fold diluted Aphididae artificial diet formulation was more successful than the original concentrated solution when adapted to a member of the Cicadellidae family (Wais and Kuo-Sell, 1990). Therefore an amino acid composition that reflected the relative amino acid concentrations within an infested grapevine root may be beneficial for future radicicolae grape phylloxera artificial diet studies. Further work is required to determine the optimum amino acid concentration for the composition of the diet formulation for radicicolae grape phylloxera artificial feeding systems.

Grape phylloxera are related to the Aphididae, but due to differences in diet nutrition, parenchyma cells compared with phloem sap, the direct application of an Aphididae diet to grape phylloxera was not successful. An additional complication in relying upon Aphididae artificial diet formulations for application to grape phylloxera was due to the Aphididae association with the endosymbiont *Buchnera*. Aphididae with an endosymbiont relationship are capable of surviving on an artificial diet formulation lacking essential amino acids due to the production of the amino acids by *Buchnera*; however aposymbiotic insects require all of the essential amino acids to be present in the diet formulation in order to survive (Douglas, 2006). Radicicolae grape phylloxera may contain a transient bacterial symbiotic relationship (Chapter 3), however it was not expected that the symbiont would be essential for survival, or supply the insect with essential nutrients not obtained in the natural diet. Therefore, due to the absence of a nutritionally supportive endosymbiont in radicicolae grape phylloxera, all essential nutritional requirements must be obtained solely from the artificial diet formulation.

The development of the first artificial feeding system for radicicolae grape phylloxera resulted in a simplified insect chamber that provided a suitable environment for insect survival, and a procedure for renewing diet solutions with minimal disturbance to insect feeding behaviour. The survival time of radicicolae grape phylloxera in response to artificial diet formulations was variable; however variable survival results are anticipated with the development of an artificial feeding system for a novel insect species. The survival rates of grape phylloxera in an artificial feeding system are expected to improve as knowledge of the essential dietary requirements of the insect increase.

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# CHAPTER 5

# APPLICATION OF ELECTRICAL PENETRATION GRAPH TO GRAPE PHYLLOXERA FEEDING BEHAVIOUR STUDIES

Sections of this chapter are currently *in press* for publication as part of the *Proceedings of the Third International Phylloxera Symposium*:

Kingston, K. B., Powell, K. S. and Cooper, P. D. (2007). Characterising the root-feeding habits of grape phylloxera using electrical penetration graph. *Acta Horticulturae*.

# 1 SUMMARY

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) feed solely on species of *Vitis*, and although the population dynamics of the insect have been investigated on a variety of *Vitis*, the feeding behaviour of the insect is unknown. Electrical penetration graph (EPG) has been applied to the grape phylloxera – grapevine model to investigate the feeding behaviour of three apterous life stages on susceptible *Vitis vinifera* L. (Vitaceae). Due to the size and root-feeding habit of grape phylloxera, modifications to the EPG system were required. Successful EPG recordings were obtain from grape phylloxera feeding on grapevine excised root pieces and the roots of micropropagated tissue culture grapevines. Waveform groupings (14) were identified based on continual feeding and probing categories. Differential feeding behaviour was determined between the gall-initiating first instars, the sedentary feeding intermediate instars and the reproductive adult. Grape phylloxera feed on parenchyma cell contents with successive puncturing of new cells once a food source is depleted. EPG recordings capturing the complete probe event, including plant penetration and continual feeding waveforms, supported this feeding pattern with evidence of repeated probing attempts prior to continual feeding waveforms. Once established in a feeding cell, grape phylloxera may exhibit continual feeding waveforms for up to 5 hours. Limited 'proof of concept' analysis was also conducted on grape phylloxera resistant and immune grapevine rootstock material.

## 2 INTRODUCTION

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) feed only on *Vitis* species, causing economic loss to vineyards planted with the susceptible host-plant, *Vitis vinifera* L. (Vitaceae). Feeding induces the development of galls on the leaves and roots of grapevines. Gall development is typically associated with a localised increase in sugar, protein and lipids (Gullan and Cranston, 2005), and in grape phylloxera root galls, or nodosities, high levels of starch, free amino acids, amides, and enzyme activity have been reported (Forneck *et al.*, 2002; Kellow *et al.*, 2004). Sectioning of grape phylloxera induced nodosities tracked the stylet of the insect on an intracellular pathway through the cortex, with the tip of the stylet settling within a single parenchyma cell around five cells below the epidermis (Kellow *et al.*, 2004). In contrast with Aphididae, the phloem was never penetrated, and cells surrounding the 'feed' cell did not appear damaged.

The grafting of American *Vitis* species to *V. vinifera* as resistant rootstocks remains the main management option for radicicolae grape phylloxera in infested vineyards; however there are reports in America and Europe of adaptation by grape phylloxera leading to the breakdown of this resistance (Granett *et al.*, 1987; Boubals, 1994; Porten *et al.*, 2000). Radicicolae grape phylloxera attempt to feed on most resistant rootstock varieties, therefore resistance ratings are based on insect survival rates, the non-formation of nodosities and the appearance of root necrosis (Kellow *et al.*, 2002). Interpretation of the variable feeding behaviour of grape phylloxera on susceptible and resistant host-plants is important for ensuring the long-term success of resistant rootstocks as a management option against the pest insect.

Insect feeding behaviour covers a wide range of feeding-motivated activities, including ingestion, stylet penetration, surface exploration and dispersal. Electrical penetration graph (EPG) may be applied to an insect-plant model to investigate probing and non-

probing behaviours (Tjallingii, 2000). EPG amplifies an electrical signal that is created when the stylet mouthpart of an insect penetrates into a food substrate. The EPG amplifier detects changes in electrical conductivity within the stylet canal of the insect and the plant tissue of the food source (Tjallingii, 2000). Stylet penetration and probing are synonyms for the act of stylet insertion into a food source, and incorporate all subsequent feeding behaviours including stylet movement, salivation, and ingestion (Backus, 2000). The separate use of the term 'ingestion' is restricted to the process of food uptake into the alimentary tract past the true mouth.

Since the development of the EPG concept (McLean and Kinsey, 1964), and further development by Tjallingii (1978), the technique has become established as a methodology suitable for studying the feeding behaviour of a range of piercing insects. EPG analysis can differentiate between the feeding activities of stylet penetration, stylet pathway to the food source, salivation and ingestion, and may assist with determining host-plant resistance mechanisms and virus transmission (van Helden and Tjallingii, 2000). The majority of published research in the field involves Hemipteran taxa, including members of the families: Aphididae (Sandanayaka and Hale, 2003), Pemphigidae (Cole *et al.*, 1993), Miridae (Cline and Backus, 2002), Cicadellidae (Backus *et al.*, 2005), and Aleyrodidae (Jiang and Walker, 2003). Studies have also been undertaken on insects from other orders, including Thysanoptera: Terebrantia (Kindt *et al.*, 2003), and Acarina: Tetranychidae (Guo and Zhao, 2000). The EPG technique has previously been applied to only one member of the Phylloxeridae family, *Phylloxera coccinea* Heyden (Harrewijn *et al.*, 1998).

Before commencement of this research, anticipated difficulties with the application of the EPG technique to grape phylloxera were based on the relatively small size of the insect, its root-feeding habit and the anticipated food source of parenchyma cell contents. A major concern was how these factors would impact on (and potentially

reduce) the strength of the EPG signal. EPG has however previously been applied to a range insect-plant models where each of these factors were individually overcome, including: (1) similar-sized whitefly (Hemiptera: Aleyrodidae) nymphs and adults (Lei *et al.*, 1996; Lei *et al.*, 1997); (2) aphids (Homoptera: Pemphigidae) feeding on lettuce roots (Cole *et al.*, 1993); and (3) western flower thrips (Thysanoptera: Terebrantia) feeding on non-vascular plant tissue (Harrewijn *et al.*, 1996).

The aim of the current study was to determine the feeding activity required by grape phylloxera for the establishment of the grapevine root feeding site, root gall initiation and insect population development. EPG was applied to assist with interpreting grape phylloxera host-plant interactions and feeding behaviour. Determination of the grape phylloxera feeding location may also be assisted by EPG studies. No publications exist describing the application of EPG to the grape phylloxera – grapevine model; therefore an initial understanding of the waveforms generated during grape phylloxera feeding was essential for the future application of this technique.

EPG was primarily used to investigate the feeding behaviour of radicicolae grape phylloxera on susceptible *V. vinifera*. Life stages were statistically compared to determine if a differential feeding behaviour was exhibited with life stage development. Recordings of the probe penetration feeding behaviour of grape phylloxera on *V. vinifera* were also analysed. There were limited recordings of feeding behaviour on resistant rootstocks, however the intention was to expand the study in the future to include resistant rootstock varieties in order to interpret host-plant resistance mechanisms. Two plant recording systems were trialled in the radicicolae grape phylloxera EPG recordings.

# 3 MATERIAL AND METHODS

## *3.1 INSECT AND PLANT MATERIAL*

Radicicolae grape phylloxera were originally collected from infested vineyards in the King Valley, Victoria, Australia. Grape phylloxera from this region have been identified as a single genotypic class, G4 (Corrie *et al.*, 2002). Populations were maintained at the Department of Primary Industries – Rutherglen Centre on excised *V. vinifera* root pieces (approximately 1 cm width x 10 cm length), prepared using a protocol slightly modified from Granett *et al.* (1985). The modifications were: (1) roots washed clean of attached soil with a soft brush under running water; (2) roots soaked for 5 minutes in Ridomil® Gold Plus systemic fungicide solution (2.3 g/L); (3) roots triple rinsed with sterile distilled water prior to air drying; (4) both ends of roots wrapped in moist cotton wool; and (5) the size of the petri dish was increased to 15 cm, with the increase in area reducing the level of water condensation within the chamber. Grape phylloxera populations were incubated in the dark at a constant temperature of 25 ± 3°C prior to EPG recording.

Only apterous radicicolae grape phylloxera were used for this study. Grape phylloxera were selected for EPG recording based on life stage and location on the excised root piece. For selection from excised root pieces, developmental life stages were determined by comparative increases in size. The presence of eggs located externally near the posterior end of the insect confirmed the adult reproductive stage. If present, neighbouring moulted cuticles were counted to confirm the insect life stage. The apterous radicicolae grape phylloxera life stages used for EPG analysis were first instars, third and fourth instars (grouped as 'intermediate instars'), and adults. Insects feeding in an isolated location were preferentially connected to the EPG insect electrode in order to record individual grape phylloxera – grapevine root interactions.

However isolated insects were not exclusively used due to the group distribution of grape phylloxera on gall sites limiting their availability.

Lignified grapevine root material was sourced from phylloxera-free vineyards to prevent the potential cross contamination of G4 with other grape phylloxera genotypic classes, and excised root pieces were prepared as outlined above (modified from Granett *et al.*, 1985). Susceptible (*V. vinifera* cv. Sultana and Shiraz), resistant (*V. riparia* x *V. rupestris* cv. Schwarzmann and *V. champini* cv. Ramsey) and immune (*V. cinerea* x *V. riparia* cv. Börner) lignified root material were utilised for grape phylloxera feeding behaviour trials. Sterile micropropagated grapevines (Kellow *et al.*, 2002) of *V. vinifera* cv. Shiraz and *V. cinerea* x *V. riparia* cv. Börner were also established. Slight modifications from the Kellow *et al.* (2002) protocol included: (1) agar medium was not supplemented with benzyl aminopurine or napthaleneacetic acid; (2) plantlets were not transferred to a second agar medium prior to the perlite-based medium; and (3) after transfer to the perlite-based medium, tissue culture vines were not inoculated with grape phylloxera eggs.

### *3.2 PLANT ELECTRODE AND EPG RECORDING SYSTEMS*

The plant electrode was modified from the stiff, uninsulated copper wire connected to the output wire and inserted into a potted plant outlined in the standard EPG system (Figure 1). The modified plant electrode consisted of a 1 cm brass pin which could be used with both the 'excised root' and 'tissue culture' recording systems. Excised roots were used to maintain the grape phylloxera populations in the laboratory, and provided the simplest plant set up for data acquisition. The tissue culture method allowed recording of grape phylloxera feeding on the roots of a whole plant, in the absence of a soil environment.

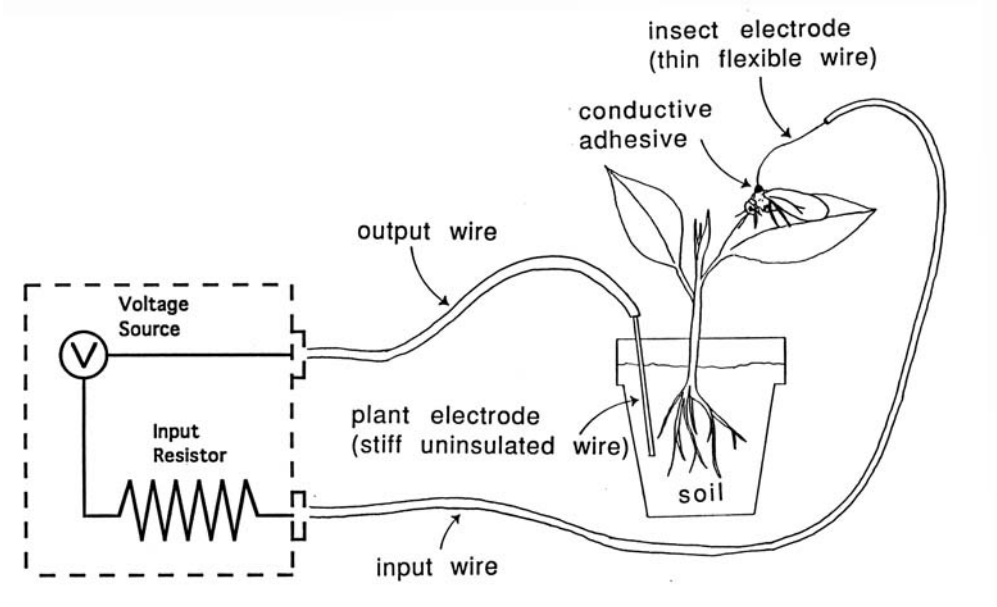


**Figure 1.** Diagram of the main EPG components, where the amplifier (represented by the dashed box) is connected to the insect-plant system (from Walker, 2000). The featured components of the system are labelled with arrows.

Outline of the standard EPG system (from Walker, 2000): The EPG amplifier consists of two electrical components, the voltage source and the input resistor. An electrical current travels from the voltage source, through the output wire, to the plant electrode, which is inserted into the moist soil of a potted plant. The insect is connected to an insect electrode, consisting of a short length of 8-20 µm gold wire, with conductive adhesive (silver paint). The insect electrode is then connected via the input wire to the input resistor. When the insect inserts the stylet into the plant, an electrical circuit is completed and the electrical current flows from the voltage source, through the plant-insect biological connection, through the input resistor and back to the voltage source. The biological connection introduces both variable resistance and variable voltage into the circuit. These signals are recorded by the EPG amplifier and are used to interpret insect feeding behaviour.

### *3.2.1 EXCISED ROOT RECORDING SYSTEM*

Excised root pieces were typically 10 cm in length x 6-10 mm in diameter. Standardisation of the root piece diameter was dependent upon variability of the lignified root material available. Excised root pieces were prepared from lignified grapevine root material as per Granett *et al.* (1985), with modifications as outlined earlier. Grape phylloxera populations were established on the excised root pieces prior to use for EPG recording. Both ends of the excised root pieces were wrapped in water dampened cotton wool, with the modified plant electrode placed between the root exterior surface and the cotton wool at one end (Figure 2a). Wetting the cotton wool provided a suitable level of conductivity for the movement of an electrical current during EPG recording.

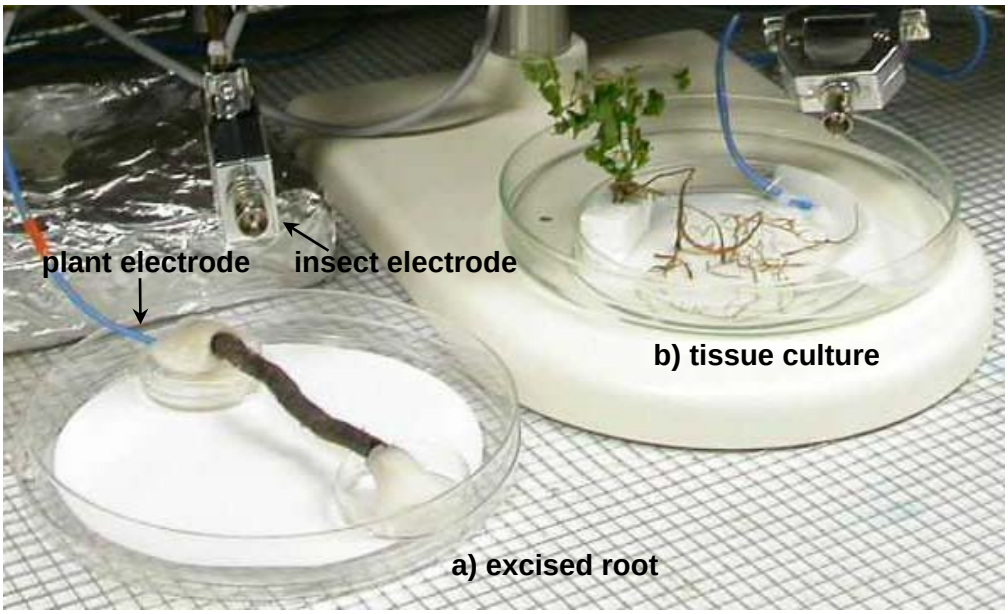


**Figure 2.** The EPG recording set up for the **a)** excised root and **b)** tissue culture root systems. The plant electrode (EPG output wire) and insect electrode (EPG input wire) are labelled on the excised root system.

### *3.2.2 TISSUE CULTURE RECORDING SYSTEM*

Micropropagated grapevines were prepared as per Kellow *et al.* (2002), with modifications as outlined earlier. Once the tissue culture vines had established, they were removed from the perlite-based medium and positioned on a 15 cm petri dish lined with filter paper. The modified plant electrode was placed in contact with the filter paper, which was kept wet with water throughout the recording (Figure 2b). Wetting the filter paper assisted in maintaining the structure of the tissue culture vine and provided a suitable level of conductivity for the movement of an electric current.

## *3.3 INSECT ELECTRODE AND EPG RECORDING POSITIONS*

The EPG insect electrode consisted of 3-5 cm of 12.5 µm diameter gold wire (Sigmund Cohn, Mount Vernon, NY) connected with conductive silver paint (water-based) to the input wire. Using a stereo microscope, the end of the gold wire was repeatedly dipped into the silver paint until a droplet formed. The gold wire was held with self-closing forceps to prevent the wire from slipping. The silver paint droplet was then carefully placed onto the abdomen of the insect and held for a few seconds until dry and the gold wire was fixed in place to the dorsal surface (Figure 3). Due to the survival of grape phylloxera for up to 9 days without nutritional intake (from artificial diet experiments, Chapter 3), individuals used for EPG recording were not starved prior to set up.

Two insect recording positions were developed for grape phylloxera EPG recordings. The ‘active feeding’ insect position was recorded to gain an initial understanding of grape phylloxera feeding waveforms as no published EPG data was available for comparison. The ‘probe initiation’ insect position was recorded to understand the active feeding waveforms in relation to the complete feeding behaviour profile (stylet penetration, stylet pathway and ingestion) required by grape phylloxera for feeding establishment and survival on *Vitis* species.

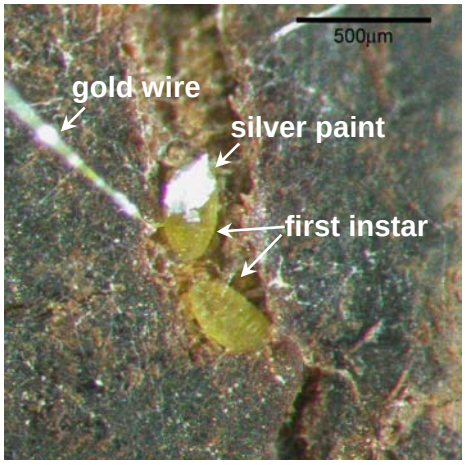


**Figure 3.** First instar grape phylloxera on an excised root piece in the active feeding position and connected to the insect electrode. The main features of the insect electrode are labelled with arrows, including the short section of 12.5 µm gold wire and the conductive silver paint. Scale bar 500 µm.

### *3.3.1 ACTIVE FEEDING RECORDING POSITION*

Grape phylloxera were individually wired to the insect electrode without disturbance from the established excised root food source. The insect was then connected to the EPG amplifier for recording. Due to the predominately sedentary feeding nature of grape phylloxera, it was assumed that these insects were actively feeding at the time of data acquisition. If the insect was accidentally disturbed during set up, and the stylet was removed from the excised root food source, then the insect was reclassified as being in a probe initiation EPG recording position.

### *3.3.2 PROBE INITIATION RECORDING POSITION*

Grape phylloxera individuals were wired to the insect electrode and then gently disturbed from the established excised root food source with a sable-haired paintbrush. The insect was left hanging from the insect electrode for a short period of time until attached to the EPG amplifier and positioned onto a new food source. A stereo microscope was used to assist in this positioning. Probe initiation EPG recordings were performed using both the excised root and tissue culture recording systems.

## *3.4 EPG EQUIPMENT*

A DC (direct current) voltage EPG amplifier was used for all recordings. The Giga-8 series EPG amplifier (Wageningen University, Laboratory of Entomology), with the potential to simultaneously record the feeding activity of eight insects, operated under an input resistance of 1 giga Ohm ($10^9\Omega$), amplification range x 50-100 and an input bias current < 1 pA. The number of channels utilised per recording varied from 1-6. All recordings were performed at room temperature (23 ± 2°C) within a Faraday cage covered with black cardboard to provide a dark environment for the radicicolae grape phylloxera (Figure 4). There was no standardisation for the timing of the recordings over the 24 hour/day period, and recording length varied from 0.5-8 hours (the majority of recordings were five hours in length).

## *3.5 WAVEFORM CHARACTERISATION*

EPG data was captured with a DATAQ® Di700 A/D data acquisition card (DATAQ® Instruments) and analysed using PROBE 3.0 software (Wageningen University, Laboratory of Entomology). Prior to waveform analysis, EPG recordings were reviewed to identify common waveforms. The majority of waveforms were defined from insects in the active feeding position, and are categorised as 'continual feeding waveforms'. Waveforms only associated with the probe initiation were categorised as 'probe waveforms'; probe waveforms occurred between non-penetration and continual

feeding waveform events. Continual feeding waveforms that were observed relatively infrequently during waveform analysis were initially categorised as undefined, and reviewed post analysis to determine additional waveform groupings. Waveforms that occurred only once during all recordings remained classified as 'undefined' (labelled waveform 7). Only recordings from insects on the excised root recording system were analysed for waveform characterisation.

**Figure 4.** The faraday cage used for all EPG recordings; the access door was opened during the set up of the plant and insect electrodes, and closed during recordings to reduce the level of background electrical interference. The pictured set up contains the tissue culture plant system with the stereo microscope (right), and two excised root systems (left). The wire exterior of the faraday cage was covered with black cardboard to provide a dark environment for the radicicolae grape phylloxera.

Continual feeding waveforms were characterised by amplitude (height of wave presented in mV), wave duration (width of wave in seconds), repetition rate (number of waves per second), and a general description of the waveform pattern (Figure 5). For waveform measurements, 5 second views of the EPG recordings were examined. Waveform duration was measured for all complete waves within the 5 second view, and converted (1/width of wave in seconds) into Hertz (Hz). The number of complete waves for the same 5 second view was divided by five for a per second repetition rate (Hz), and the amplitude of the wave was recorded. Five 5-second views were measured per recording. When the waveform occurred for a sufficient period of time, there was a 5 second gap between consecutive measurements of the same recording. The 5 second gap between measurements was decreased or increased as required (depending upon waveform occurrence) to allow for the measurement of five 5-second views. Where possible, the same waveform was reviewed at a range of voltage levels to provide information about the signal origin, resistance (R) or electromagnetic force (emf), of the wave.

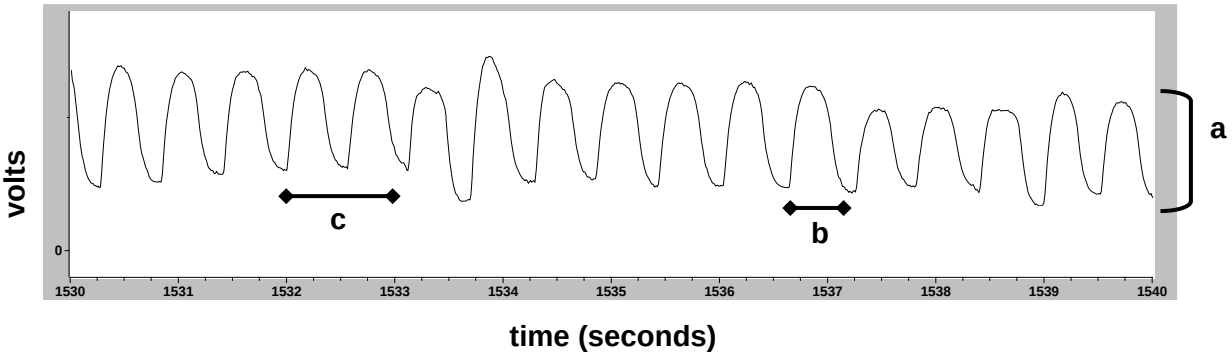


**Figure 5.** Key points for waveform characterisation during EPG analysis included: **a)** amplitude or height of wave presented in mV, **b)** wave duration or width of wave in seconds, **c)** repetition rate or number of waves per second, and a general description of the pattern.

EPG recordings were reviewed to determine regular waveform sequence transitions and the frequency of waveforms. No correlation studies were performed to identify biological function of the waveforms; therefore a waveform numbering system was applied to prevent the implication of biological meaning.

Probe waveforms were only characterised by wave amplitude and signal origin due to the absence of duration or repetition in the high amplitude patterns. The amplitude of continual feeding waveforms that occurred following probe waveforms were calculated as a percentage of the amplitude for the pre-continual feeding waveform pattern (waveform 9). Individual waveform 9 events were defined as 100%, and the amplitude of continual feeding waveforms occurring within each probe event were calculated as a percentage of this amplitude, and presented as %V. By definition, non-penetration (waveform 1) was 0 %V of waveform 9.

### *3.6 WAVEFORM ANALYSIS*

PROBE 3.0 was used to monitor the duration and occurrence of each of the characterised waveforms within each EPG recording. Minor waveform interruptions of 1-2 waves were ignored and the dominant waveform was recorded. 'Silent periods' between waveforms were considered part of the previous waveform duration, unless there was a change in baseline voltage and evidence for waveform transition. A silent period was defined as a temporary return to the baseline voltage level between waves, but for EPG analysis did not infer a change in waveform or the removal of the insect stylet from the food source.

Waveform transition was generally defined as the continued occurrence of the new pattern for more than 5 seconds. Exceptions were made for short period, high amplitude waves. This rule reduced the complexity of the analysis, although some information may have been excluded. As this was the initial application of EPG to the feeding behaviour of grape phylloxera, and no correlation data identifying biological

function and feeding activity was available, there was a deliberate decision not to over analyse the data.

Most recordings were five hours in length; recordings less than five hours in length were analysed for the entire recording time. Eight hour recordings were only analysed for five hours to standardise the recording length; no new waveforms were observed in the three hours not analysed.

Excised root pieces were pre-colonised by a population of grape phylloxera, while the roots of the tissue culture plants had not previously been exposed to grape phylloxera feeding activity. Duplicate recordings of the same insect and root material combination were discarded so each recording analysed represented a new insect-plant interaction, although the same root material may have been used for more than one insect recording. Progressive life stage recordings (first instar – intermediate instar – adult) were made on the same excised root piece, resulting in the low possibility that a duplicate recording was made with the same individual insect on the same excised root piece at a different life stage. Individual insects on the root pieces were not identifiable.

The plant and insect EPG recording structure was used to determine the relative success of the trialled procedures, but did not define groupings for waveform analysis. Recordings were therefore analysed dependent upon waveform occurrence. 'Continual feeding recordings' contained continuous feeding waveforms only, while 'probe recordings' contained probe and continuous feeding waveforms. If probe waveforms only occurred for a short period of time at either the beginning or the end of the recording, they were excluded to allow for analysis as a continuous feeding recording. All probe recordings contained more than one 'probe – continuous feeding' waveform transition event.

### *3.7 WAVEFORM NON-SEQUENTIAL PARAMETERS*

PROBE 3.0 collected data was transferred to Microsoft® Excel to calculate waveform duration values. GenStat-Eighth Edition© (Lawes Agricultural Trust) was used to calculate summary statistics for the data, including the non-sequential parameters: total time spent in each waveform (total time), number of occurrences of each waveform (frequency), mean time spent in each waveform (mean time) and the percentage of total recording time spent in each waveform (% recording time). The mean time and percentage of total recording time calculations removed any time bias introduced by variable EPG recording length. Where possible, the non-sequential parameters were grouped by life stage to calculate the *'grouped mean'* of these values for the total number of recordings that displayed each waveform.

All waveform events were included in the analysis, including the final waveform occurrence which was prematurely ended due to the completion of the EPG recording time. Because of the regular occurrence of the common waveforms, the artificial ending of the final waveform was not expected to impact on analysis.

For individual insect comparisons, Microsoft® Excel stacked columns were used to present waveform distribution as a percentage of total recording time. The *'grouped mean'* percentage of total recording time for life stage groupings were corrected to represent the percentage of recording time across all recordings (the grouped mean was calculated from the insect sample number that displayed each waveform, which may not have equalled the whole life stage group sample number). The *'corrected'* percentage of total recording time values were presented as Microsoft® Excel pie charts. Pie charts were also used to display the percentage of total recording time of a single EPG recording when comparisons with other recordings were not made.

### *3.7.1 STATISTICAL ANALYSIS*

Statistical analysis was performed to compare the waveform distribution of grape phylloxera life stages displaying continual feeding waveforms on susceptible excised root pieces. Due to low sample numbers, EPG recordings from susceptible *V. vinifera* cv. Sultana and Shiraz excised root pieces were combined for continual feeding waveform analysis. Both *V. vinifera* cv. Sultana and Shiraz were used for EPG recordings due to the limited supply of standardised grapevine root material. EPG recordings with continual feeding waveforms were grouped into life stage categories for statistical analysis. Data for intermediate (third and fourth) instars were pooled to represent a 'base-level' feeding behaviour in comparison with first instar and adults. GenStat-Eighth Edition© summary of statistics generated the mean time (and variance) spent in each waveform for the analysed continual feeding waveform recordings. These values were sorted into first instar, intermediate instar and adult categories, and from lowest to highest values. The data was visually compared to confirm that there was no grouping of either the Sultana or Shiraz root material, or the intermediate life stages, prior to further statistical analysis.

A total of 21 recordings were analysed for insects feeding on susceptible excised root pieces with continual feeding waveforms; seven recordings were analysed for each life stage. Sample numbers within life stage groupings were too low for statistical analysis, and descriptive data only was presented. In comparing all life stages, GenStat-Eighth Edition© general analysis of variance (ANOVA) was used to determine if there was a difference in waveform mean time and percentage of total recording time with life stage development. ANOVA was only possible on waveforms that occurred in the majority of recordings. Statistical significance for ANOVA was based at $p < 0.05$.

Sample numbers for EPG recordings involving probe waveforms on susceptible excised root pieces (n = 3), probe waveforms on susceptible tissue culture roots (n = 1)

and resistant rootstock material (n = 3) were too low for statistical analysis. Due to limited duplication of these EPG recordings, there was no grouping of separate recordings into life stage categories for waveform analysis. Characterisation of waveforms and waveform distribution data are presented, although no conclusive statements were able to be made regarding the feeding behaviour of grape phylloxera from these recordings.

### *3.8 WAVEFORM SEQUENTIAL PARAMETERS*

EPG recordings containing probe waveforms were analysed to determine sequential parameters for non-penetration, and probe (probe and continual feeding waveforms) events. Probe events were further grouped as short or long probes. Short probe events were classified as the occurrence of the probe waveform followed by a single continual feeding waveform before the EPG pattern returned to the non-penetration pattern. Long probe events were classified as the occurrence of the probe waveform followed by more than one continual feeding waveform. The length of time for the continual waveform occurrence was not a factor in probe event classification.

Sequential parameters were defined as the number of short probes before the first long probe and the total time required to observe the first long probe. The longest probe event was defined as the 'principal probe'. The principal probe was characterised by the number of general probes occurring prior, the total time required to observe the principle probe, and the total length of time for the principal probe. Using the non-penetration and probe event classifications, additional non-sequential parameters (number of events, total time, mean time and event occurrence as a percentage of the total recording time) were also calculated for the waveform groupings.

# 4 RESULTS

EPG recordings were successfully acquired from both the excised root and tissue culture recording systems. Grape phylloxera in the active feeding position remained stationary and appeared to continue feeding once connected to the EPG amplifier, indicating that tethering the insect to the gold wire did not deter feeding. Grape phylloxera in the probe initiation position were observed to move along the root surface once connected to the gold wire, although free movement was restricted by the rigidity and length of the wire. Once insects had initiated feeding, there was no indication of disturbance to normal feeding behaviour caused by connection to the EPG amplifier. EPG recordings were successfully performed on all apterous life stages of grape phylloxera attempted.

## *4.1 EPG RECORDING SUCCESS RATE*

EPG success was determined by the presence of recognisable waveforms. Failed recordings were identified as receiving no EPG signal (a flat 0V line), a non-reactive test calibration pulse or a high voltage noise rhythm (unable to be reduced by using the PROBE 3.0 'smooth' function) that masked feeding waveforms. The majority of recordings were performed with insects on the excised root recording system in the active feeding position (Table 1). On susceptible root material, 28 out of 40 (70%) attempted recordings were successful, and two out of eight (25%) attempted recordings were successful on resistant root material. No EPG recordings were attempted with insects in the active feeding position on the tissue culture recording system as the tissue culture plants were not previously colonised by a grape phylloxera population.

EPG recordings with insects in the probe initiation position were performed using both the excised root and the tissue culture recording systems (Table 1). On the excised root system, probe initiation recordings were successful for five out of 20 (25%)

attempted recordings on susceptible root material. No successful probe initiation recordings were obtained from the two attempts on resistant excised root material (Table 1). Combining both susceptible and resistant tissue culture root material, probe initiation recordings were successful for three out of 12 (25%) recordings.

**Table 1.** Number of successful grape phylloxera EPG recordings comparing plant recording system (excised root and tissue culture) with insect position (active feeding and probe initiation).

| | **excised root** | | | **tissue culture** | | |
|---|---|---|---|---|---|---|
| | susceptible | | resistant | susceptible | | resistant |
| | Shiraz | Sultana | | Shiraz | Sultana | |
| active feeding | 8 (10) | 20 (30) | 2 (8) | NA | NA | NA |
| probe initiation | 0 (1) | 5 (19) | 0 (2) | 2 (11) | NA | 1 (1) |

Susceptible (Shiraz and Sultana) and resistant (combined) root material indicated. Total number of attempted recordings presented in parentheses; not all plant system-insect position combinations were attempted (NA).

A comparison of the success rate for grape phylloxera life stages was made by examining the plant and insect recording system with the highest sample number: the excised root system with insects in the active feeding position. First instar insect recordings had a success rate of 63% from 16 attempted recordings, intermediate instars 77% from 13 attempts, and the adult life stage was successful for 73% of the 11 attempted recordings on susceptible excised root pieces (Table 2). As EPG recording success rates were similar for all grape phylloxera life stages, life stage was not a factor for EPG recording success.

The EPG plant system and insect position success rate did not equal the EPG recording analysis number due to the complexity of the pattern in some recordings. For recording analysis, groupings were based on the EPG plant system (susceptible excised root or tissue culture; or resistant rootstock) and waveform occurrence (continual feeding waveforms only or probe and continual feeding waveforms).

**Table 2.** Number of successful grape phylloxera EPG recordings on susceptible excised root pieces with insects in the active feeding position; recordings differentiated by life stage.

| life stage | **susceptible** | | combined |
|---|---|---|---|
| | Shiraz | Sultana | total |
| first instar | 0 (1) | 10 (15) | 10 (16) |
| intermediate | 5 (5) | 5 (8) | 10 (13) |
| adult | 3 (4) | 5 (7) | 8 (11) |
| totals | 8 (10) | 20 (30) | 28 (40) |

Total number of attempted recordings presented in parentheses.

## *4.2 WAVEFORM CHARACTERISATION*

Analysis of grape phylloxera recordings involving continual feeding waveforms identified 11 re-occurring waveform groups (Table 3). Three additional probe waveforms were observed in recordings containing probe – continuous feeding waveform transition events (Table 4). EPG recordings only containing continual feeding waveforms were termed 'continual feeding recordings', and recordings also containing probe waveforms were termed 'probe recordings'. Waveforms were labelled numerically, with sub-numbering indicating a variation within the main waveform. A visual representation of the continual feeding waveforms (Figures 6 and 7) and the probe waveforms (Figure 8) highlighted the variation in appearance of grape phylloxera EPG patterns. No correlation studies were performed on these waveforms to infer biological function, and ingestion was not confirmed.

### *4.2.1 CONTINUAL FEEDING WAVEFORMS*

The most common continual feeding waveforms were waveforms 2, 3 and 4 (Figures 6 and 7). Waveform 2 displayed a regular monophasic pattern, with an irregular biphasic interruption. Waveform 2 regularly interchanged with waveform 3, which was a biphasic pattern with an irregular silent period. The overall amplitude of waveform 3 was twice the value of waveform 2 (Table 3), although there was variation in the relative size of the positive and negative peaks of waveform 3. Waveform 4 displayed a similar biphasic pattern to waveform 3, although the absence of a silent period between waves resulted in a higher repetition rate and wave duration value (Table 3). The increase in wave repetition rate was evident by comparing waveform 3 and 4 at a 30 second timeframe (Figure 7). Waveform 4 regularly interchanged with waveform 3, and occasionally with waveform 2. Both waveforms 3 and 4 were of emf signal origin, and did not vary in pattern shape with EPG voltage.

Waveforms 2.2 and 2.4 were short interruption variations to waveform 2 (Figure 6). The amplitude value for waveform 2.2 was twice the value of waveform 2 (Table 3). Waveform measurements for waveform 2.4 were similar to waveform 2, although the waveforms were differentiated by a short term change in the EPG baseline voltage. Waveform 3.4 displayed a similar change in the baseline voltage which occurred during waveform 3.

Waveform 5 was a more complex monophasic pattern in comparison with waveform 2 (Figure 6), and interchanged with waveform 2 during recordings. Waveform 6 was a composite of the characteristics of waveforms 2 and 3. Waveform 6 interchanged between waveforms 2 and 3, but occurred at increased amplitude in comparison with these waveforms (Table 3). A complete waveform 6 was defined as including both the monophasic and biphasic pattern, resulting in a decreased repetition rate and wave duration value in comparison with waveforms 2 and 3.

Waveform 7 represented a grouping of 'undefined' waveforms, where patterns occurred only once during all recordings. The resulting complex mix of undefined patterns therefore required further observation in additional recordings in order to be classified. Due to the variation in the pattern, no visual image of the waveform was presented.

Not represented as unique changes in wave shape, waveforms 8 and 10 indicated a change in EPG recording amplitude (Figure 7). Waveform 8 displayed a 3 V change in the baseline of the recording without any interruption to other waveforms, and interchanged between waveforms 2, 3, 7 and 6. Waveform 10 was a high voltage interruption to waveforms 2 and 3. With a resistance (R) signal origin, waveform 10 was a positive high amplitude interruption at a positive EPG voltage, and a negative interruption at a negative voltage.

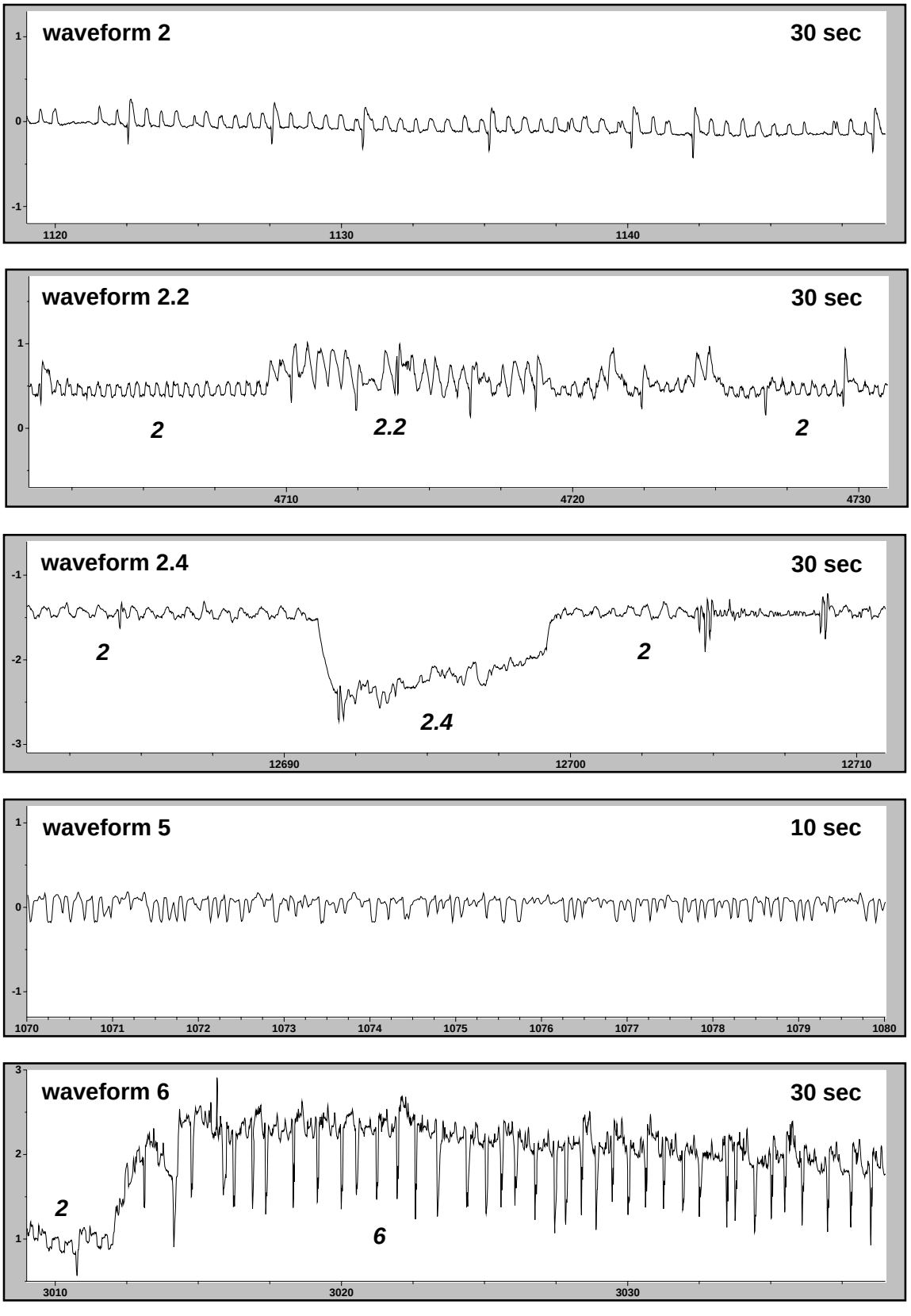


**Figure 6.** Visual representation of grape phylloxera continual feeding monophasic EPG waveforms (including the mono-biphasic combination waveform 6); timeframe and waveform transitions indicated.

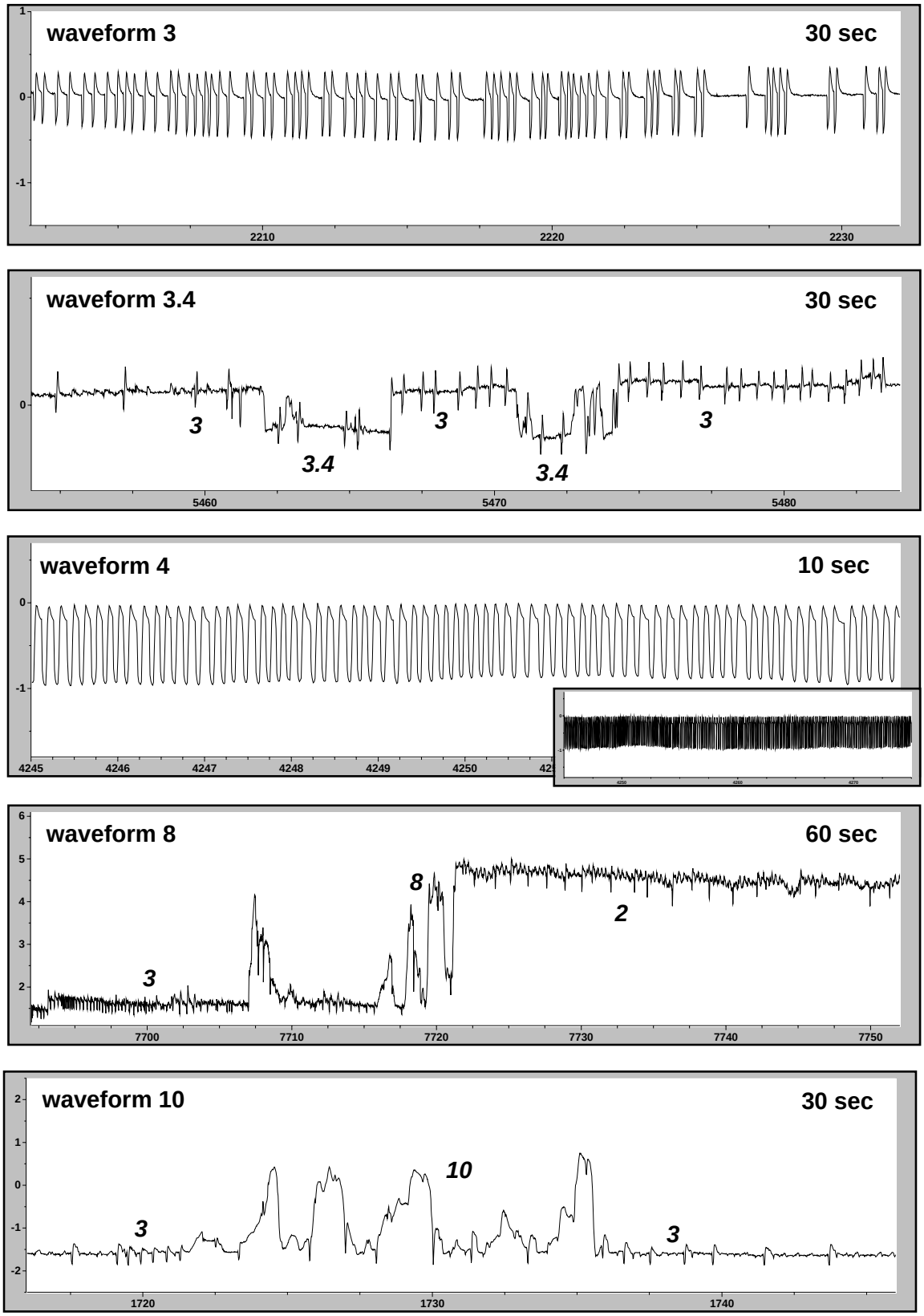


**Figure 7.** Visual representation of grape phylloxera continual feeding biphasic and high voltage EPG waveforms; timeframe and waveform transitions indicated. Waveform 4 insert is the pattern at a 30 second timeframe.

**Table 3.** Grape phylloxera continual feeding EPG waveform characteristics and measurements. Waveforms defined by type, amplitude (mV and %V), repetition rate (Hz), duration (Hz), signal origin and a description of the waveform; definitions in text. Amplitude (mV and %V) n value indicated the number of recordings viewed for measurements; repetition rate n value indicated the number of 5 second views measured, and wave duration n value indicated the number of waves measured.

| waveform | | waveform measurements | | | | | waveform characteristics |
|---|---|---|---|---|---|---|---|
| label | type | amplitude (mV) | amplitude (%V) | repetition rate (Hz) | duration (Hz) | signal origin (R/emf) | description |
| 2 | Monophasic | 295 ± 239 (n = 6) | 12 ±10 (n = 3) | 3.4 ± 1.3 (n = 90) | 6.2 ± 2.9 (n = 1526) | R/emf | sharp positive peak with waves in baseline, irregular biphasic interruption |
| 2.2 | Monophasic | 600 ± 361 (n = 3) | 18 ± 5.9 (n = 1) | 2.1 ± 0.85 (n = 8) | 4.0 ± 1.8 (n = 83) | unknown | increased amplitude interruption to waveform 2, momentary change in baseline voltage |
| 2.4 | Monophasic | 138 ± 18 (n = 2) | 11 ± 10 (n = 2) | 2.2 ± 0.35 (n = 3) | 6.8 ± 4.3 (n = 33) | unknown | continuation of waveform 2, momentary change in baseline voltage |
| 3 | Biphasic | 657 ± 334 (n = 6) | 20 + 10 (n = 3) | 1.9 ± 0.9 (n = 83) | 5.7 ± 1.1 (n = 768) | emf | sharp positive and negative peak, irregular silent period between waves |
| 3.4 | Biphasic | 413 ± 301 (n = 2) | NA | 1.6 ± 0.95 (n = 5) | 7.5 ± 3.8 (n = 41) | unknown | continuation of waveform 3, momentary change in baseline voltage |
| 4 | Biphasic | 703 ± 237 (n = 4) | 20 ± 10 (n = 2) | 7.1 ± 0.88 (n = 40) | 7.6 ± 1.0 (n = 1413) | emf | sharp positive and negative peak, no silent period between waves |

**Table 3 cont.**

| waveform | | waveform measurements | | | | | waveform characteristics |
|---|---|---|---|---|---|---|---|
| label | type | amplitude (mV) | amplitude (% V) | repetition rate (Hz) | duration (Hz) | signal origin (R/emf) | description |
| 5 | Monophasic | 370 ± 98 (n = 5) | NA | 4.3 ± 0.67 (n = 14) | 5.9 ± 3.0 (n = 301) | unknown | sharp positive peak with waves along peak |
| 6[1] | Mono-Biphasic combination | 1317 ± 633 (n = 3) | 103 ± 0 (n = 1) | 0.8 ± 0.43 (n = 15) | 1.5 ± 0.65 (n = 60) | unknown | composite of waveforms 2 and 3 with deep negative peak, increased amplitude |
| 7 | Undefined | variable | variable | variable | variable | unknown | mixed group of single occurrence patterns |
| 8 | Altered baseline voltage | mV change 3396 ± 246 (n = 2) | NA | NA | NA | unknown | approximate 3 V amplitude change in baseline voltage, continuation of waveforms |
| 10 | High amplitude interruption | % increase 337 ± 149 (n = 5) | 16 ± 0 (n = 1) | variable | variable | R | high amplitude interruption to waveforms, no change in baseline voltage |

%V values only presented for waveforms occurring within probe recordings, other waveforms %V presented as NA; all other waveform measurements were from continual feeding recordings. Waveform measurements not presented due to the complexity of the wave, or if the measurement was not applicable, are presented as NA

[1] Waveform 6 measurements based on a complete mono-biphasic wave

**Table 4.** Grape phylloxera probe EPG waveform measurements and characteristics. Waveforms defined by type, amplitude (mV and %V), repetition rate (Hz), duration (Hz), signal origin and a description of the waveform; definitions in text. Amplitude (mV and %V) n value indicated the number of recordings viewed for measurements; repetition rate and wave duration measurements were not attempted.

| waveform | | waveform measurements | | | | | waveform characteristics |
|---|---|---|---|---|---|---|---|
| label | type | amplitude (mV) | amplitude (%V) | repetition rate (Hz) | duration (Hz) | signal origin (R/emf) | description |
| 1 | Non-penetration | NA | 0 | NA | NA | NA | 0V, flat line pattern |
| 9 | Altered baseline voltage | 1162 ± 1245 (n = 3) | 100 ± 0 (n = 3) | NA | NA | R | high amplitude pattern, transition pattern from waveform 1 to continual feeding waveforms |
| 9.1 | High amplitude interruption | 1295 ± 1209 (n = 3) | 101 ± 41 (n = 3) | NA | NA | R | high amplitude pattern similar to waveform 9 but not followed by a change in waveform |

All waveform measurements from probe recordings. Waveform measurements not presented due to the complexity of the wave, or if the measurement was not applicable, are presented as NA

### *4.2.2 PROBE WAVEFORMS*

Grape phylloxera EPG recordings containing probe – continuous feeding waveform transition events were observed to have three re-occurring probe waveforms that were not observed in the recordings that displayed continual feeding waveforms only (Table 4). Waveform 1 represented non-penetration, and the EPG pattern displayed a non-responsive, 0 V, flat line that indicated there was no electrical contact in the insect-plant biological system. Waveform 9 was a high voltage pattern observed as a transition between waveform 1 and the continual feeding waveforms (Figure 8a). Waveform 9 represented electrical contact with the plant and penetration of the stylet into a food source. At the end of the continual feeding patterns the EPG pattern returned to waveform 1, and indicated that there was no longer electrical contact between the insect and the plant. Waveform 9.1 was similar in structure to waveform 9, and occurred prior to the continual feeding waveforms (Figure 8b). Waveform 9.1 also occurred during continual feeding patterns without the EPG pattern returning to waveform 1. Both waveform 9 and 9.1 were of R signal origin.

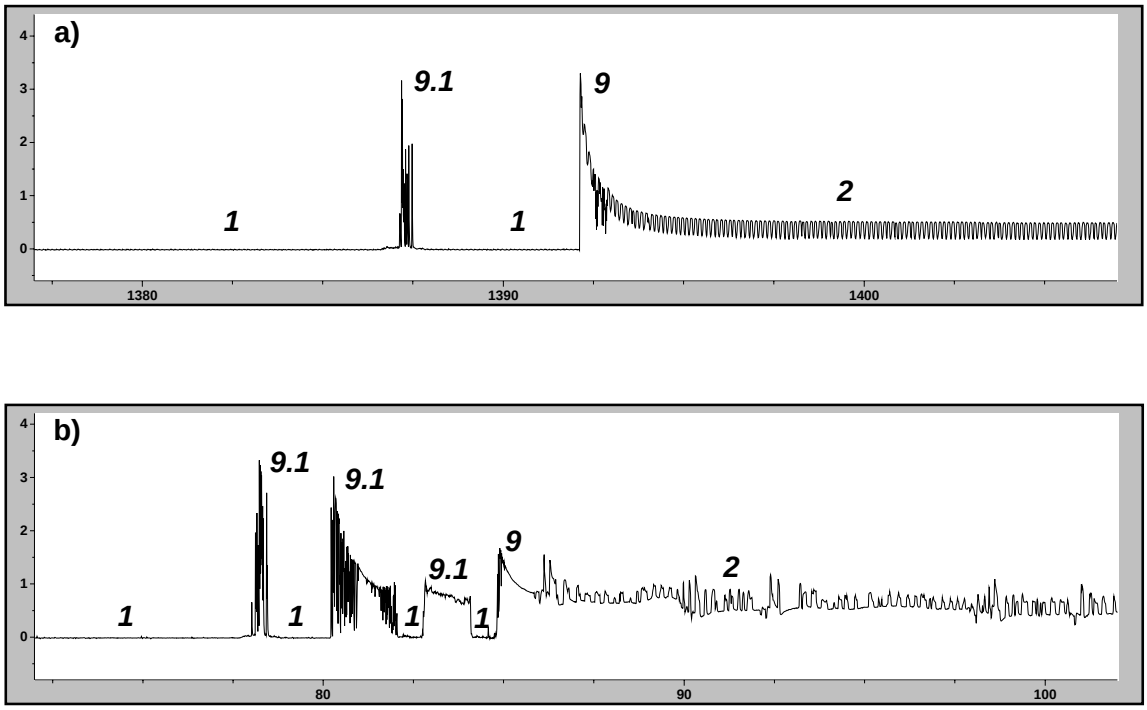


**Figure 8.** Visual representation of grape phylloxera probe waveforms; **a)** 30 second overview showing waveform 1 interrupted by waveform 9.1, followed by waveform 9 and the continual feeding waveform 2; **b)** 30 second overview showing multiple interruptions of waveform 1 by waveform 9.1, prior to waveform 9 and the continual feeding waveform 2. Waveform transitions indicated.

### *4.2.3 WAVEFORM INCIDENCE*

Combining all grape phylloxera EPG recordings analysed, there was over a 90% incidence of waveforms 2 and 3 (Table 5). Waveform 4 occurred in 64% of recordings, and all three waveforms were present in all life stages of grape phylloxera analysed (first instar, intermediate instar, adult). Waveforms 7 and 10 were the next most common waveforms, occurring across all life stages and in around 30% of recordings. The only other continual feeding waveform to occur in all life stages was waveform 2.4; however the rate of incidence was only 14%.

Waveforms 5, 6 and 8 were observed only in recordings from intermediate instar and adult grape phylloxera, and occurred at a relatively low incidence rate of between 14-18% (Table 5). Waveform 2.2 was observed at a similar incidence rate, but only in first instar and intermediate instar recordings. Waveform 3.4 was the only continual feeding waveform observed in a single grape phylloxera life stage, first instar.

The probe waveforms 1, 9 and 9.1 were observed in probe recordings on susceptible excised root material, as well as first instar EPG recordings on Börner excised roots and Shiraz tissue culture (Table 5). The intermediate instar on Ramsey excised root material and the adult insect on Börner tissue culture were already established in continual feeding waveform patterns at the beginning of EPG recording and therefore did not display probe waveforms. By definition, probe waveforms were not observed in the susceptible excised root continual feeding recordings.

**Table 5.** Waveform incidence from grape phylloxera EPG recordings on the excised root and tissue culture recording systems; totals indicate the number of individual waveforms occurring for each grape phylloxera category.

| waveform | **excised root – susceptible** | | | | | **excised root – resistant** | | **tissue culture** | |
|---|---|---|---|---|---|---|---|---|---|
| | continual feeding recordings | | | probe recordings | | Börner | Ramsey | Shiraz | Börner |
| | first instar (7) | intermediate (7) | adult (7) | intermediate (1) | adult (2) | first instar (1) | intermediate (1) | first instar (1) | adult (1) |
| **continual feeding waveforms (11)** | | | | | | | | | |
| 2 | 7 | 7 | 7 | 1 | 2 | 0 | 1 | 0 | 1 |
| 2.2 | 1 | 3 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 2.4 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| 3 | 7 | 7 | 7 | 1 | 2 | 0 | 1 | 1 | 1 |
| 3.4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 3 | 7 | 5 | 0 | 2 | 0 | 1 | 0 | 0 |
| 5 | 0 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 7 | 2 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| 8 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| 10 | 1 | 3 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| totals | 8 | 10 | 6 | 6 | 5 | 1 | 5 | 3 | 5 |

**Table 5 cont.**

| waveform | **excised root – susceptible** | | | | | **excised root – resistant** | | **tissue culture** | |
|---|---|---|---|---|---|---|---|---|---|
| | continual feeding recordings | | | probe recordings | | Börner | Ramsey | Shiraz | Börner |
| | first instar (7) | intermediate (7) | adult (7) | intermediate (1) | adult (2) | first instar (1) | intermediate (1) | first instar (1) | adult (1) |
| **probe waveforms (3)** | | | | | | | | | |
| 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 |
| 9 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| 9.1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 |
| totals | 0 | 0 | 0 | 3 | 3 | 2 | 0 | 3 | 0 |

Recordings differentiated by waveform analysis (continual feeding or probe) or *Vitis* variety (Börner, Ramsey or Shiraz), and life stage (first instar, intermediate instar or adult); sample number for each category presented in parenthesis.

### *4.3 CONTINUAL FEEDING RECORDING ANALYSIS*

Waveform analysis was performed on continual feeding recordings from insects on susceptible *V. vinifera* cv. Sultana and Shiraz excised root material. To ensure equal units for statistical analysis, life stage sample number was limited by the life stage with the lowest number of recordings analysed, adults. Seven adult recordings were suitable for continual feeding waveform analysis; therefore 21 recordings were analysed across all life stage groupings.

In all life stages of grape phylloxera, continual feeding waveforms were observed for up to 5 hours, however the total EPG recording time of the analysed recordings varied. First instar EPG recordings were 203 ± 97 (mean ± standard deviation) minutes in length, intermediate instars 248 ± 99 minutes, and adults 140 ± 112 minutes. To correct for the recording time variation, waveform distribution between life stages was compared by the non-sequential parameters of grouped mean time and the grouped mean percentage of total recording time spent within each waveform (Table 6).

Waveforms 2, 3, 4, 7 and 10 occurred in all life stage groupings of grape phylloxera; however ANOVA statistical analysis was only possible on waveforms that occurred in the majority of recordings. Waveforms 2 and 3 were present in 100% of recordings, and waveform 4 in 71% of recordings (Table 5); waveforms 7 and 10 were observed in less than 25% of all recordings and were excluded from the statistical analysis.

ANOVA indicated a marginally significant ($p = 0.063$) decrease in the percentage of total recording time spent in the waveform 2 from first instars to the adult life stage (Table 6). There was no significant difference in life stage mean time for waveform 2, or between either values for waveform 3. Waveform 4 occurred for significantly ($p = 0.036$) less mean time in the first instar life stage in comparison with adults, and all life stages were significantly different ($p = 0.001$) when comparing the percentage of total recording time.

**Table 6.** Grape phylloxera EPG continual feeding recordings on susceptible (*V. vinifera* cv. Sultana and Shiraz) excised root material; life stage comparison of waveform non-sequential parameters presented as grouped mean values for observed insect recording number. Not all waveforms were observed in all recordings (NA).

| waveform | life stage | total time (sec) | frequency | mean t (sec) | % recording time |
|---|---|---|---|---|---|
| 2 | first instar | 9012 ± 4549 | 77 ± 46 | 133 ± 68 | 75 ± 13[a] |
| | intermediate | 9186 ± 4041 | 68 ± 31 | 155 ± 53 | 59 ± 10[ab] |
| | adult | 4055 ± 3923 | 38 ± 27 | 109 ± 71 | 48 ± 26[b] |
| | **ANOVA p** | | | **0.30** | **0.063** |
| 2.2 | first instar | 27 ± 0 | 2 ± 0 | 14 ± 0 | 0.36 ± 0 |
| | intermediate | 59 ± 34 | 4 ± 3.5 | 19 ± 13 | 0.33 ± 0.2 |
| | adult | NA | NA | NA | NA |
| 2.4 | first instar | 13 ± 0 | 2 ± 0 | 6.5 ± 0 | 0.084 ± 0 |
| | intermediate | 6 ± 0 | 3 ± 0 | 2.1 ± 0 | 0.034 ± 0 |
| | adult | NA | NA | NA | NA |
| 3 | first instar | 2925 ± 2338 | 82 ± 56 | 34 ± 11 | 23 ± 11 |
| | intermediate | 3971 ± 1886 | 82 ±36 | 53 ± 19 | 28 ± 6.7 |
| | adult | 2060 ± 2076 | 44 ± 38 | 45 ± 48 | 21 ± 17 |
| | **ANOVA p** | | | **0.45** | **0.60** |
| 3.4 | first instar | 13 ± 13 | 2 ± 1.4 | 5.3 ± 3.0 | 0.16 ± 0.20 |
| | intermediate | NA | NA | NA | NA |
| | adult | NA | NA | NA | NA |
| 4 | first instar | 154 ± 230 | 13 ± 20 | 11 ± 2.6[a] | 0.93 ± 1.3[a] |
| | intermediate | 1177 ± 972 | 22 ± 18 | 55 ± 29[ab] | 10 ± 8.7[b] |
| | adult | 2005 ± 1889 | 25 ± 25 | 109 ± 84[b] | 21 ± 12[c] |
| | **ANOVA p** | | | **0.036** | **0.001** |
| 5 | first instar | NA | NA | NA | NA |
| | intermediate | 2012 ± 0 | 22 ± 0 | 91 ± 0 | 11 ± 0 |
| | adult | 1399 ± 1598 | 13 ± 6.9 | 84 ± 82 | 29 ± 33 |
| 6 | first instar | NA | NA | NA | NA |
| | intermediate | 207 ± 199 | 1 ± 0 | 207 ± 199 | 1.2 ± 1.1 |
| | adult | NA | NA | NA | NA |

**Table 6 cont.**

| waveform | life stage | total time (sec) | frequency | mean t (sec) | % recording time |
|---|---|---|---|---|---|
| 7 | first instar | 629 ± 578 | 2 ± 1.4 | 556 ± 682 | 4.5 ±2.1 |
| | intermediate | 737 ± 0 | 2 ± 0 | 369 ± 0 | 4.1 ± 0 |
| | adult | 26 ± 0 | 1 ± 0 | 26 ± 0 | 0.36 ± 0 |
| 8 | first instar | NA | NA | NA | NA |
| | intermediate | 59 ± 70 | 3 ± 1.4 | 16 ± 16 | 0.33 ± 0.39 |
| | adult | NA | NA | NA | NA |
| 10 | first instar | 14 ± 0 | 2 ± 0 | 7.2 ± 0 | 0.19 ± 0 |
| | intermediate | 37 ± 35 | 3.3 ± 2.1 | 13 ± 8.9 | 0.26 ± 0.27 |
| | adult | 169 ± 0 | 14 ± 0 | 12 ± 0 | 2.4 ± 0 |

Parameters presented (total time, frequency, mean time and percentage of total recording time), are the grouped mean ± standard deviation. ANOVA p value presented in **bold** for 'waveform x life stage' comparisons of mean time and mean percentage of recording time.

Waveforms 2.2 and 2.4 were only observed in first instar and intermediate instar recordings, and waveform 5 was only observed in intermediate instar and adult recordings. Waveform 3.4 was observed in first instar recordings, and waveforms 6 and 8 in intermediate instar recordings. The intermediate instars experienced the highest diversity in waveform occurrence, with 10 of the 11 continual feeding waveforms observed across the seven recordings (Table 5).

Within grape phylloxera life stage groupings, there was variation in waveform distribution as a percentage of total recording time (Figure 9). All seven replicates of the first instar life stage were dominated by waveforms 2 and 3 (Figure 9a). Waveforms 2, 3 and 4 occurred in all seven replicates of the intermediate instar life

stage (Figure 9b); although in insect 2 the observed percentage of total recording time for waveform 4 was less than 1%. Waveform 5 was only observed in insect 5 of the intermediate instar recordings. The highest level of variation in waveform distribution was present in the adult recordings (Figure 9c). Waveforms 2 and 3 were present in all recordings and waveform 4 in all except insects 5 and 6. Waveform 5 was present in insects 1-4, although in insect 3 the observed percentage of total recording time value was less than 1%. Waveform 5 was the predominant waveform in insects 1 and 2.

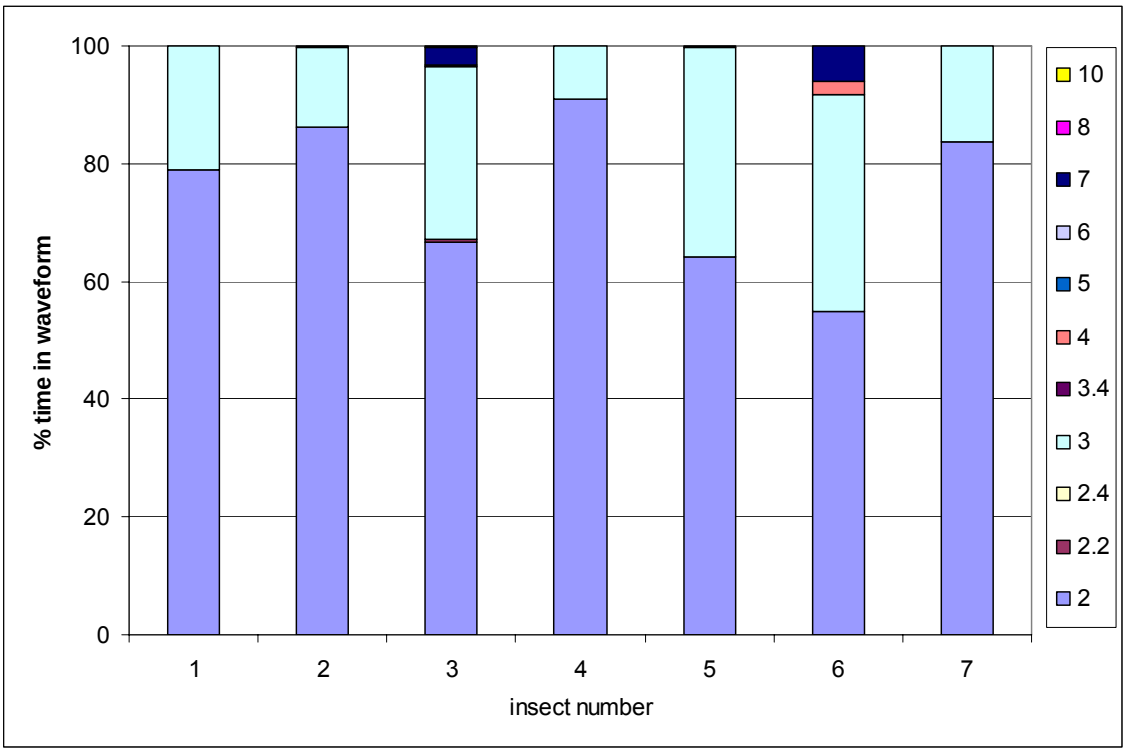


**Figure 9a.** First instar grape phylloxera continual feeding waveform distribution as a percentage of total recording time for seven individual insects; on susceptible (*V. vinifera* cv. Sultana and Shiraz) excised root material. Individual waveforms are indicated by the colour coded key.

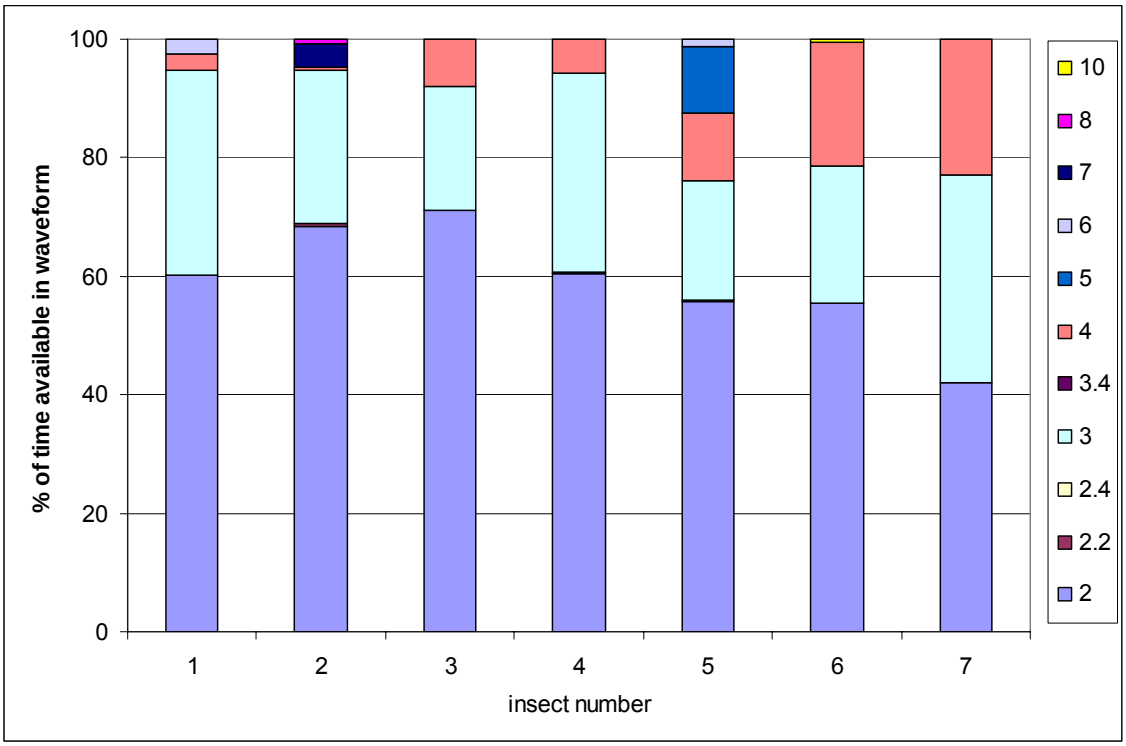


**Figure 9b.** Intermediate instar grape phylloxera continual feeding waveform distribution as a percentage of total recording time for seven individual insects; on susceptible (*V. vinifera* cv. Sultana and Shiraz) excised root material. Individual waveforms are indicated by the colour coded key.

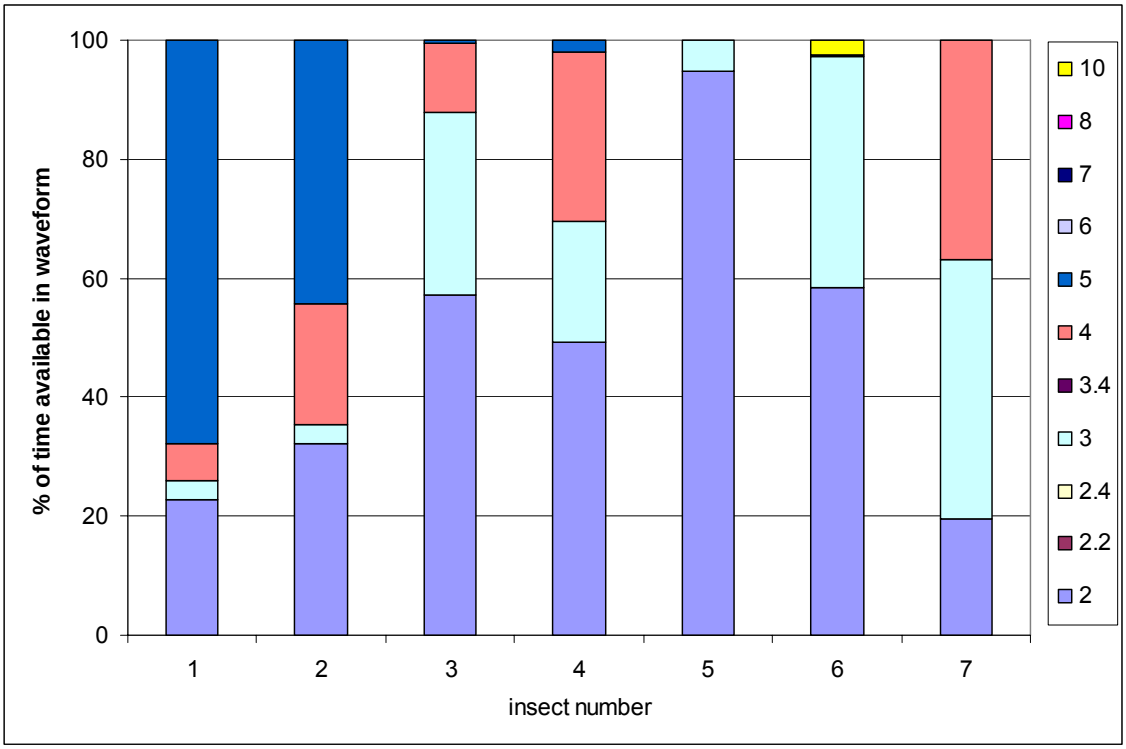


**Figure 9c.** Adult grape phylloxera continual feeding waveform distribution as a percentage of total recording time for seven individual insects; on susceptible (*V. vinifera* cv. Sultana and Shiraz) excised root material. Individual waveforms are indicated by the colour coded key.

Due to the individual variation within the grape phylloxera life stage categories, it was necessary to calculate the corrected percentage of total recording time values to display the overall variation in waveform distribution (Figure 10). Waveform distribution in first instar grape phylloxera was dominated by waveforms 2 and 3, which occurred for a combined corrected total of 98% of the total recording time (Figure 10a). Waveforms 2 and 3 were also the most dominant waveforms in intermediate instars; however the higher level of waveform diversity reduced the combined corrected percentage of total recording time value to 87% (Figure 10b). The only other waveform to occur for more than 2% of the corrected percentage of total recording time was waveform 4 (10%). A more even distribution of waveforms was observed in the adult life stage, where four out of six observed waveforms occurred for at least 15% of the corrected percentage of total recording time (Figure 10c). Waveforms 2 and 3 combined were observed for 69% of the corrected percentage of total recording time, and remained the most dominant within the waveform distribution chart. Waveforms 4 and 5 were observed for 15% and 16% (respectively) of the corrected percentage of total recording time.

During all of the grape phylloxera EPG continual feeding recordings analysed, probe waveforms 1 and 9 were not observed to indicate stylet removal by the insect. Continual feeding waveforms were recorded for up to 8 hours, although a maximum of 5 hours was examined for analysis.

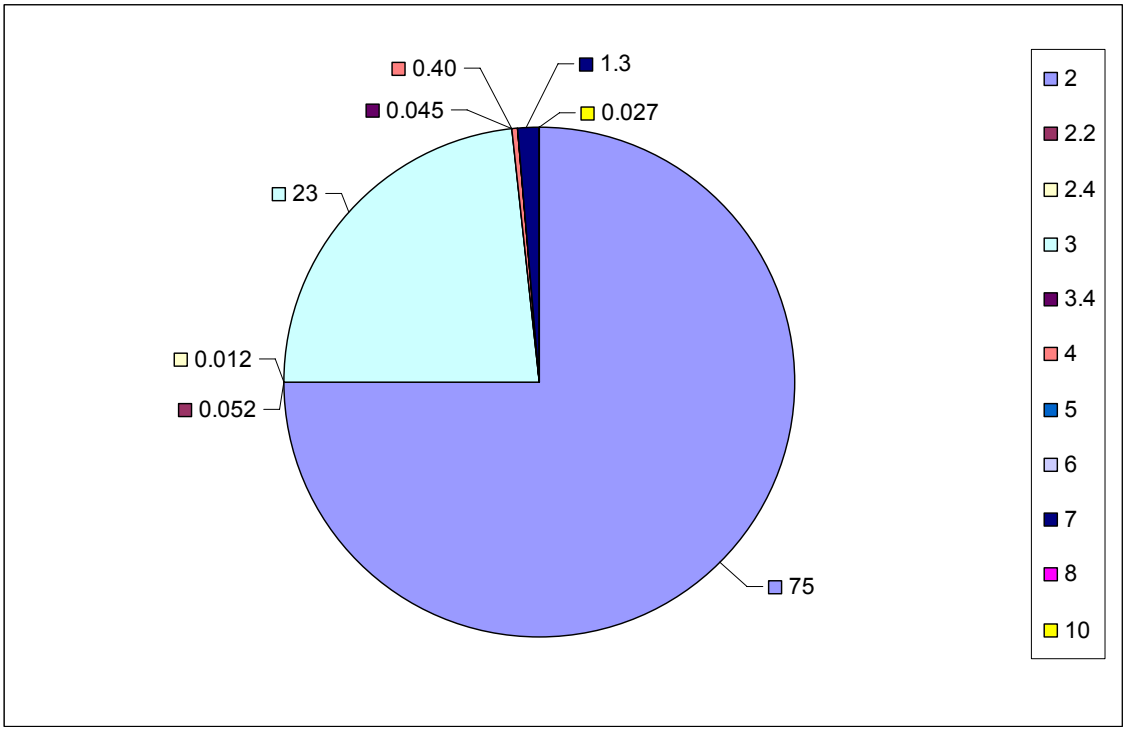


**Figure 10a.** First instar grape phylloxera continual feeding waveform distribution on susceptible (*V. vinifera* cv. Sultana and Shiraz) excised root material; values presented as the corrected percentage of total recording time, n=7. Individual waveforms are indicated by the colour coded key, and values are linked with the pie chart.

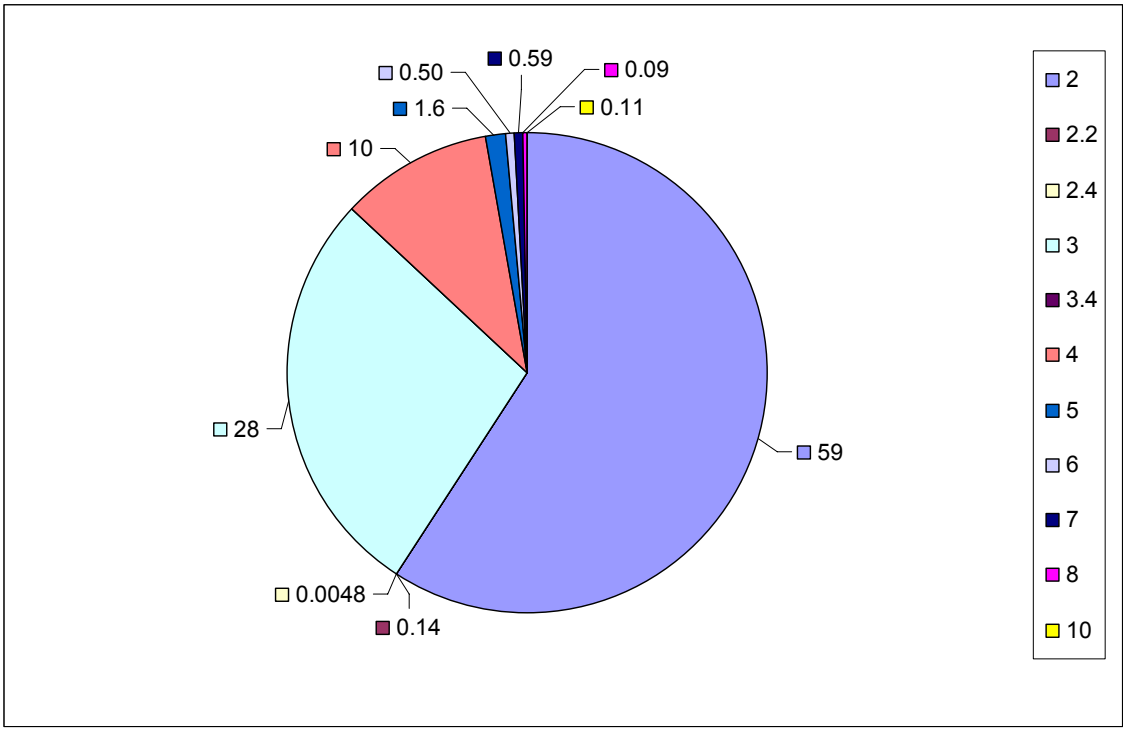


**Figure 10b.** Intermediate instar grape phylloxera continual feeding waveform distribution on susceptible (*V. vinifera* cv. Sultana and Shiraz) excised root material; values presented as the corrected percentage of total recording time, n=7. Individual waveforms are indicated by the colour coded key, and values are linked with the pie chart.

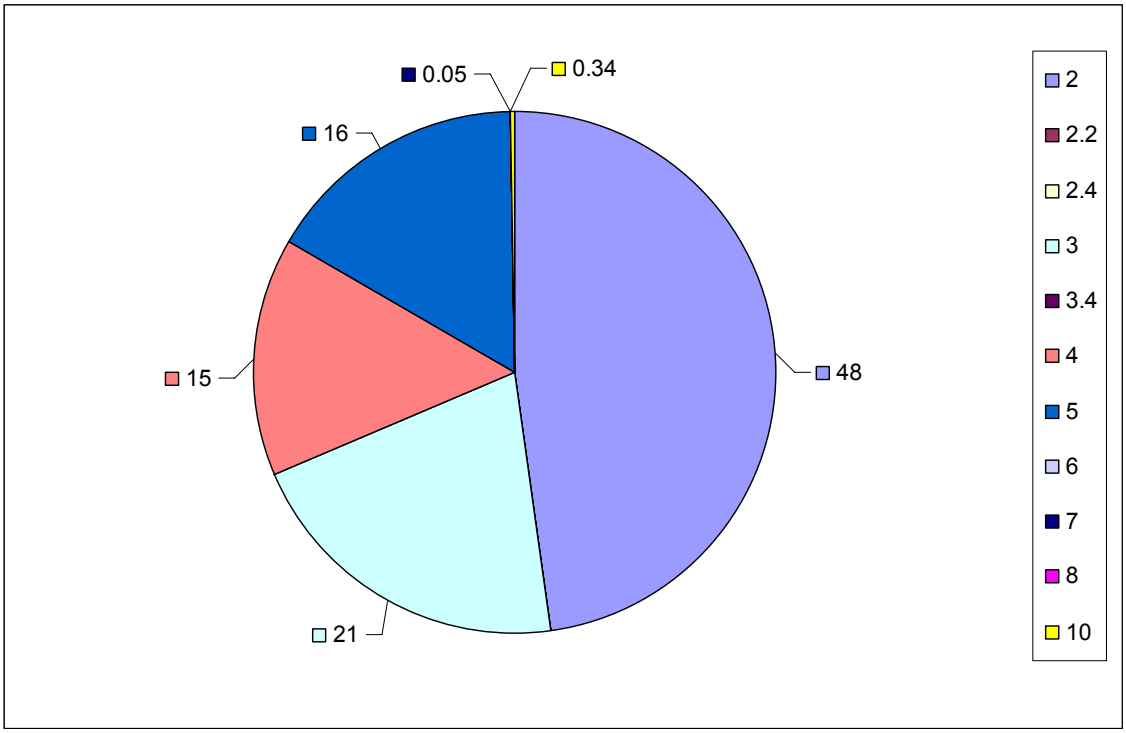


**Figure 10c.** Adult grape phylloxera continual feeding waveform distribution on susceptible (*V. vinifera* cv. Sultana and Shiraz) excised root material; values presented as the corrected percentage of total recording time, n=7. Individual waveforms are indicated by the colour coded key, and values are linked with the pie chart.

### *4.4 PROBE RECORDING ANALYSIS*

Waveform analysis was performed on three probe recordings from insects on susceptible *V. vinifera* cv. Sultana excised root pieces. Additional recordings displayed potential probing patterns, however further analysis was not possible due to uncertainty over the presence of continual feeding waveforms, the complexity of the patterns and interference by electronically induced background noise in the EPG system. The three analysed recordings were of different life stages; two insects were adult (labelled -1 and -2) and one an intermediate instar.

In each of the recordings, there were several probe – continual feeding waveform transition events, therefore all of the probe waveforms (1, 9 and 9.1) were observed in the three recordings (Table 7). Waveforms 2 and 3 were the only continual feeding patterns observed in all three recordings. An additional two waveforms occurred in the adult recordings; waveform 4 occurred in both, waveform 6 in adult-1 and 2.4 in adult-2. The intermediate instar recorded four additional waveforms; 2.2, 2.4, 7 and 10. Continual feeding waveforms 3.4, 5 and 8 were not observed in any of the probe recordings, therefore the percent amplitude (%V) values for these waveforms were not be calculated (Table 3).

Excluding the probe waveforms, the distribution of the continual feeding waveforms for the probe recordings (Figure 11) are similar to the waveform distributions observed in the continual feeding recordings (Figure 9). All recordings were on susceptible excised root pieces.

**Table 7.** Grape phylloxera EPG probe recordings on susceptible (*V. vinifera* cv. Sultana) excised root material; comparison of continual feeding and probe waveform non-sequential parameters. Not all waveforms were observed in all recordings (NA).

| waveform | life stage | total time (sec) | frequency | mean t (sec) | % recording time |
|---|---|---|---|---|---|
| 2 | adult-1 | 1696 | 11 | 154 ± 177 | 50 |
| | adult-2 | 1165 | 19 | 61 ± 80 | 11 |
| | intermediate | 3505 | 92 | 38 ± 68 | 49 |
| 2.2 | adult-1 | NA | NA | NA | NA |
| | adult-2 | NA | NA | NA | NA |
| | intermediate | 286 | 35 | 8.2 ± 7.2 | 4.0 |
| 2.4 | adult-1 | NA | NA | NA | NA |
| | adult-2 | 12 | 5 | 2.4 ± 0.96 | 0.11 |
| | intermediate | 18 | 5 | 3.6 ± 2.4 | 0.25 |
| 3 | adult-1 | 390 | 14 | 28 ± 38 | 11 |
| | adult-2 | 47 | 8 | 5.8 ± 2.9 | 0.44 |
| | intermediate | 32 | 3 | 11 ± 2.3 | 0.45 |
| 4 | adult-1 | 704 | 9 | 78 ± 130 | 21 |
| | adult-2 | 1114 | 14 | 80 ± 155 | 10 |
| | intermediate | NA | NA | NA | NA |
| 6 | adult-1 | 47 | 1 | 47 ± 0 | 1.4 |
| | adult-2 | NA | NA | NA | NA |
| | intermediate | NA | NA | NA | NA |
| 7 | adult-1 | NA | NA | NA | NA |
| | adult-2 | NA | NA | NA | NA |
| | intermediate | 54 | 1 | 54 ± 0 | 0.76 |
| 10 | adult-1 | NA | NA | NA | NA |
| | adult-2 | NA | NA | NA | NA |
| | intermediate | 73 | 16 | 4.5 ± 3.4 | 1.0 |

**Table 7 cont.**

| waveform | life stage | total time (sec) | frequency | mean t (sec) | % recording time |
|---|---|---|---|---|---|
| 1 | adult-1 | 456 | 9 | 51 ± 60 | 13 |
| | adult-2 | 8427 | 15 | 562 ± 1286 | 78 |
| | intermediate | 3092 | 35 | 88 ± 387 | 43 |
| 9 | adult-1 | 8.9 | 3 | 3.0 ± 2.8 | 0.26 |
| | adult-2 | 15 | 5 | 3.0 ± 2.9 | 0.14 |
| | intermediate | 46 | 26 | 1.8 ± 1.5 | 0.64 |
| 9.1 | adult-1 | 113 | 7 | 16 ± 34 | 3.3 |
| | adult-2 | 20 | 13 | 1.5 ± 1.1 | 0.19 |
| | intermediate | 93 | 22 | 4.2 ± 4.0 | 1.29 |

Sample number n = 1, absolute parameters (total time, frequency, and percentage of total recording time) are presented as totals; mean parameters (mean time) are the mean ± standard deviation.

Time allocation comparisons between recordings of waveform sequential parameters were made by grouping waveforms into non-penetration and probe events (Table 8). Probe events (including continual feeding waveforms) as a percentage of the total recording time varied from 22-79%. Probe events were categorised as either short probes (waveform 9 followed by a single continual feeding waveform), or long probes (waveform 9 followed by more than one continual feeding waveform). Generally, several (2-5) short probes were observed as a precursor event to a long probe (Figure 12a). Additionally, waveform 9.1 was observed to occur repeatedly prior to a probe event (Figure 12b). For simplicity, waveform 9.1 was grouped with waveform 1 as a non-penetration event for the purpose of waveform sequential parameter analysis. Waveform 9.1 was interpreted to represent contact between the insect and the plant

but not complete penetration of the plant surface as continual feeding waveforms did not follow the wave event. Therefore in the absence of penetration, waveform 9.1 was categorised as a non-penetration event.

Pre-probe attempts were also observed before the principle probe, which varied in length from 27-32 minutes (Table 8). Waveforms 2 and 4 were both observed immediately following waveform 9, although extended periods of feeding activity were only observed when waveform 2 was the first continual feeding pattern.

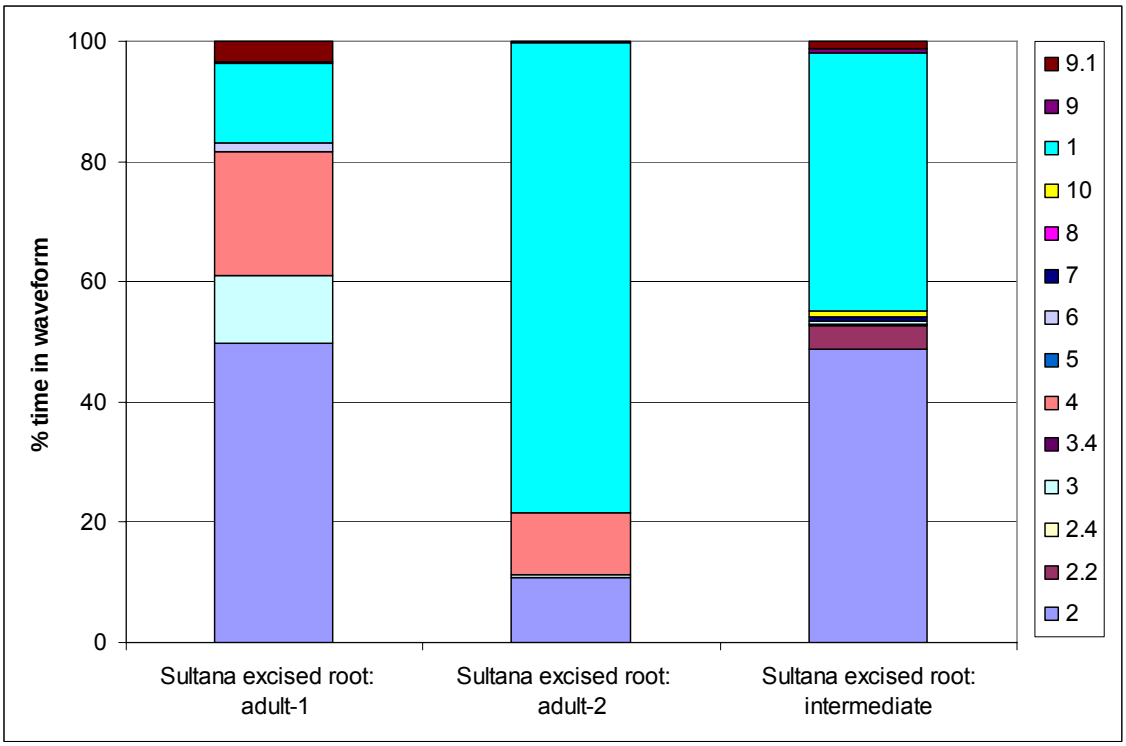


**Figure 11.** Grape phylloxera EPG probe recordings on susceptible (*V. vinifera* cv. Sultana) excised roots, waveform distribution as a percentage of total recording time. Individual waveforms are indicated by the colour coded key.

**Table 8.** Grape phylloxera EPG probe recordings on susceptible (*V. vinifera* cv. Sultana) excised root material, comparison of waveform sequential parameters. Data presented for the non-penetration (np) waveform, and probe events (total, short probe, long probe and principle probes).

| recording parameter | adult-1 | adult-2 | intermediate |
|---|---|---|---|
| **np characteristics** | | | |
| number np events | 3 | 6 | 26 |
| total duration np | 568 | 2359 | 3162 |
| mean duration np | 189 ± 124 | 1407 ± 1805 | 122 ± 450 |
| np % recording time | 16 | 78 | 44 |
| **probe characteristics – total** | | | |
| number of probe events | 3 | 5 | 26 |
| total duration of probe | 2847 | 2359 | 4037 |
| mean duration of probe | 712 ± 814 | 472 ± 791 | 155 ± 386 |
| probe % recording time | 79 | 22 | 56 |
| **short probe characteristics** | | | |
| number short probes | 2 | 1 | 17 |
| total duration short probe | 51 | 14 | 354 |
| mean duration short probe | 25 ± 12 | 14 ± 0 | 21 ± 27 |
| number short probes before first long probe | 2[a] | 0 | 5 |
| **long probe characteristics** | | | |
| number of long probes | 2 | 4 | 9 |
| total duration long probe | 2797 | 2345 | 3698 |
| mean duration long probe | 1398 ± 319 | 586 ± 864 | 409 ± 595 |
| total recording time prior to first long probe | 619[b] | 85 | 561 |
| **principal probe characteristics** | | | |
| number probes before the principal probe event | 2 (short) | 3 (long) | 5 (short) |
| total recording time to the principal probe event | 1976 | 1694 | 561 |
| duration of principal probe event | 1624[c] | 1871 | 1921 |
| total recording time | 3600 | 10800 | 7200 |

**Table 8 footer.** Sample number n = 1, absolute parameters (number of events, total duration, and event occurrence as a percentage (%) of total recording time) are presented as totals; mean parameters (mean duration time) are the mean ± standard deviation. Time values are in seconds.

[a] continual feeding waveforms at beginning of recording, therefore this value is the number of short probes before 2nd long probe (1st observed long probe in recording)

[b] continual feeding waveforms at beginning of recording, therefore this value is the time between the end of the 1st long probe and the beginning of the 2nd long probe event (1st observed probe initiation in recording)

[c] waveform was artificially terminated by the end of the EPG recording

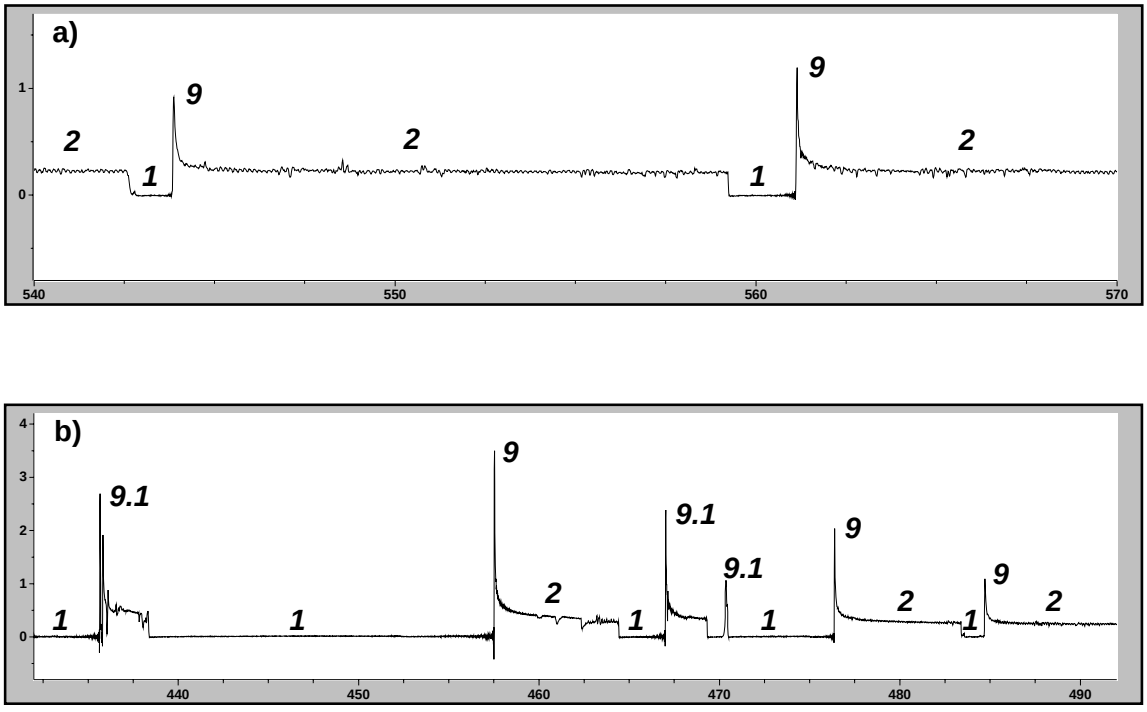


**Figure 12.** Grape phylloxera EPG probe patterns on susceptible (*V. vinifera* cv. Sultana) excised roots; **a)** 30 second overview showing two short probe events (waveform 9 followed waveform 2), interrupted by waveform 1, prior to a long probe event at 561 seconds; **b)** 60 second overview showing multiple occurrences of waveform 9.1 and short probe events, a pattern that was regularly observed prior to a long probe event. Waveform transitions are indicated.

## 4.5 TISSUE CULTURE RECORDING ANALYSIS

Limited EPG recordings on the tissue culture plant system were successful (Table 1), and this analysis was preformed as a 'proof of concept' of the set up only. Waveform analysis was performed on a first instar recording on susceptible *V. vinifera* cv. Shiraz tissue culture roots. The first instar EPG recording displayed probe (1, 9 and 9.1) and continual feeding (3, 7 and 10) waveforms (Figure 13). During the five hour recording, there was only one probe event which lasted for approximately 5 minutes (Table 9), where waveform 3 regularly interchanged with waveform 10. The non-penetration event was observed for 98% of the total recording time, during which waveform 1 regularly interchanged with waveform 9.1.

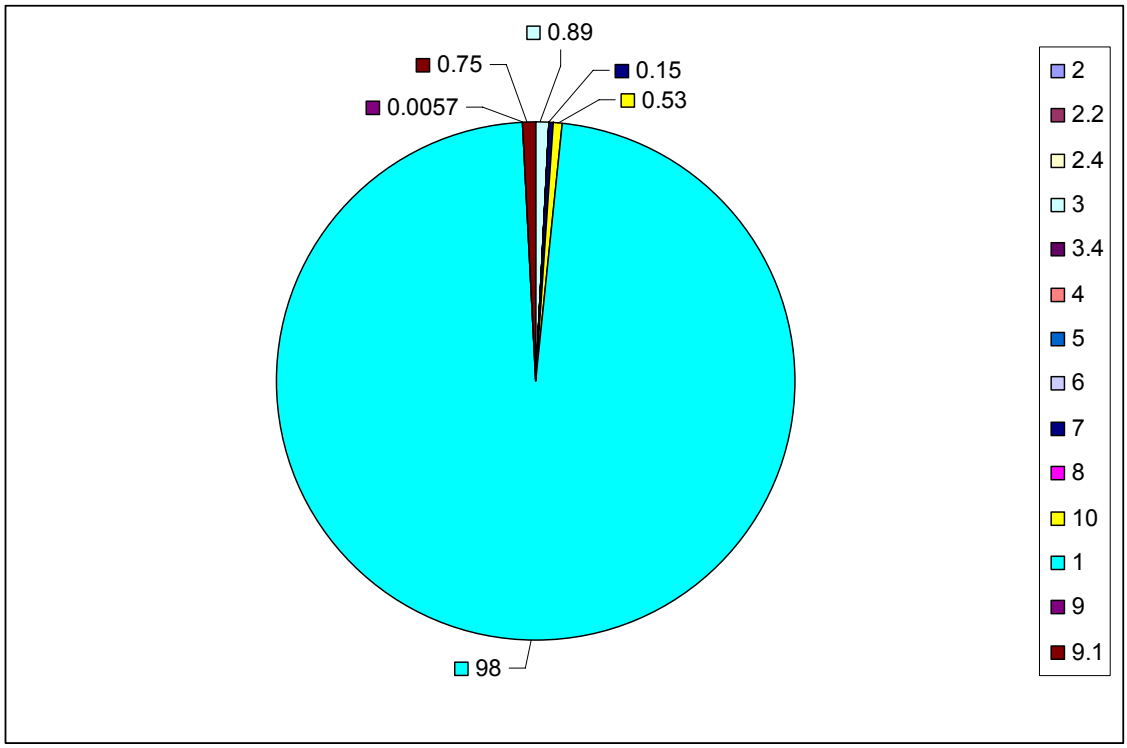


**Figure 13.** First instar grape phylloxera EPG recording on susceptible (*V. vinifera* cv. Shiraz) tissue culture roots, waveform distribution as a percentage of total recording time. Individual waveforms are indicated by the colour coded key.

**Table 9.** First instar grape phylloxera EPG recording on susceptible (*V. vinifera* cv. Shiraz) tissue culture root material; probe and continual feeding waveform non-sequential and sequential parameters.

| waveform | total time (sec) | frequency | mean t (sec) | % recording time |
|---|---|---|---|---|
| *non-sequential parameters* | | | | |
| **probe waveforms** | | | | |
| 1 | 17109 | 35 | 489 ± 1190 | 98 |
| 9 | 1 | 1 | 0.84 ± 0 | 0.0057 |
| 9.1 | 132 | 33 | 4.0 ± 4.5 | 0.75 |
| **continual feeding waveforms** | | | | |
| 3 | 156 | 19 | 8.2 ± 6.0 | 0.89 |
| 7 | 27 | 1 | 27 ± 0 | 0.15 |
| 10 | 93 | 19 | 4.9 ± 3.5 | 0.53 |
| *sequential parameters* | | | | |
| **np characteristics** | | | | |
| number np events | | | 2 | |
| total duration np | | | 17241 | |
| mean duration np | | | 8621 | |
| np % recording time | | | 98 | |
| **probe characteristics** | | | | |
| number of probe events | | | 1 | |
| total duration of probe | | | 277 | |
| probe % recording time | | | 1.6 | |
| total recording time to probe event | | | 6155 | |

Sample number n = 1, absolute parameters (total time, frequency, and percentage of total recording time) are presented as totals; mean parameters (mean time) are the mean ± standard deviation. Time values are in seconds.

### *4.6 RESISTANT ROOTSTOCK RECORDING ANALYSIS*

Limited (11) EPG recordings were attempted on the root material of resistant *Vitis* species (Table 1). Waveform analysis was performed on three of these recordings: two from *V. cinerea* x *V. riparia* cv. Börner and one from *V. champini* cv. Ramsey. Due to differing life stages, root types and EPG plant recording systems, there was no replication within the resistant rootstock recordings. The analysis was performed for an initial interpretation of grape phylloxera feeding behaviour, and as a 'proof of concept' for the application of EPG to grape phylloxera resistant rootstock studies. No conclusions were made regarding grape phylloxera feeding behaviour on resistant roots in comparison with susceptible root material.

The analysed resistant rootstock recordings were from a first instar on Börner excised root (5 hour recording), an intermediate instar on Ramsey excised root (5 hour recording), and an adult insect on Börner tissue culture (1 hour recording). No single common waveform was observed in all three recordings. The most common continual feeding waveforms, waveforms 2 and 3, were observed in the intermediate instar-Ramsey excised root and adult-Börner tissue culture insect-plant combinations (Table 10). Waveforms 8 and 10 also occurred in both recordings. Waveform 4 was only observed in the intermediate instar-Ramsey recording, while an undefined waveform 7 was identified in the adult-Börner recording. Although the Ramsey recording was on excised root material, and the Börner recording on tissue culture roots, the waveform distribution of both resistant rootstock recordings (Figure 14) were similar to susceptible excised root material (Figure 9). Waveforms 2 and 3 were the most dominant, occurring for 93% of the total recording time in the intermediate instar-Ramsey excised root recording, and for 87% of the time in the adult-Börner tissue culture recording.

**Table 10.** Grape phylloxera EPG recordings on resistant rootstock (Börner and Ramsey) excised root and tissue culture root material, comparison of continual feeding and probe waveform non-sequential parameters. Only one life stage presented per plant system: first instar-Börner excised root; intermediate instar-Ramsey excised root; adult-Börner tissue culture.

| waveform | resistant rootstock | total time (sec) | frequency | mean t (sec) | % recording time |
|---|---|---|---|---|---|
| 2 | Börner excised | NA | NA | NA | NA |
| | Ramsey excised | 11939 | 68 | 176 ± 188 | 67 |
| | Börner tissue | 2649 | 28 | 95 ± 243 | 75 |
| 3 | Börner excised | NA | NA | NA | NA |
| | Ramsey excised | 4535 | 84 | 54 ± 149 | 26 |
| | Börner tissue | 428 | 31 | 14 ± 32 | 12 |
| 4 | Börner excised | NA | NA | NA | NA |
| | Ramsey excised | 1208 | 20 | 60 ± 82 | 6.8 |
| | Börner tissue | NA | NA | NA | NA |
| 7 | Börner excised | 74 | 6 | 12 ± 7.4 | 0.41 |
| | Ramsey excised | NA | NA | NA | NA |
| | Börner tissue | 91 | 6 | 15 ± 8.4 | 2.6 |
| 8 | Börner excised | NA | NA | NA | NA |
| | Ramsey excised | 12 | 1 | 12 ± 0 | 0.068 |
| | Börner tissue | 11 | 2 | 5.4 ± 2.3 | 0.31 |
| 10 | Börner excised | NA | NA | NA | NA |
| | Ramsey excised | 36 | 2 | 18 ± 12 | 0.20 |
| | Börner tissue | 345 | 21 | 16 ± 21 | 9.8 |
| 1 | Börner excised | 8400 | 115 | 73 ± 139 | 47 |
| | Ramsey excised | NA | NA | NA | NA |
| | Börner tissue | NA | NA | NA | NA |
| 9.1 | Börner excised | 9522 | 121 | 79 ± 115 | 53 |
| | Ramsey excised | NA | NA | NA | NA |
| | Börner tissue | NA | NA | NA | NA |

Root type and plant system combinations (n = 1), absolute parameters (total time, frequency, and percentage of total recording time) are presented as totals; mean parameters (mean time) are the mean ± standard deviation. Not all waveforms were observed in all recordings (NA).

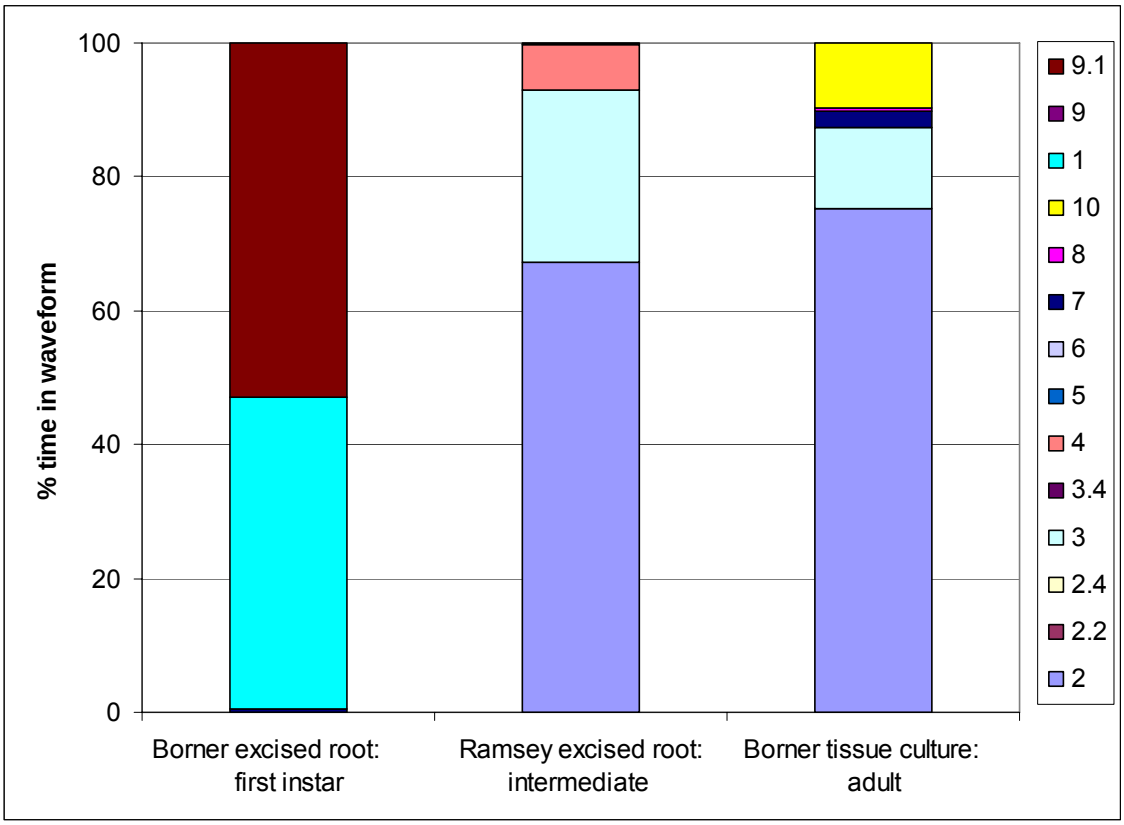


**Figure 14.** Grape phylloxera resistant rootstock (Börner and Ramsey) EPG recordings waveform distribution as a percentage of total recording time. Individual waveforms indicated by the colour coded key.

Probe waveforms 1 and 9.1 were observed in the first instar-Börner excised root recording (Figure 14), with limited 'possible feeding' attempts observed by the occurrence of an undefined waveform 7 for less than 1% of the total recording time (Table 10). The high voltage probe waveforms observed in the first instar-Börner excised root recording were in contrast to the continual feeding waveforms observed in the adult-Börner tissue culture recording (Figure 15).

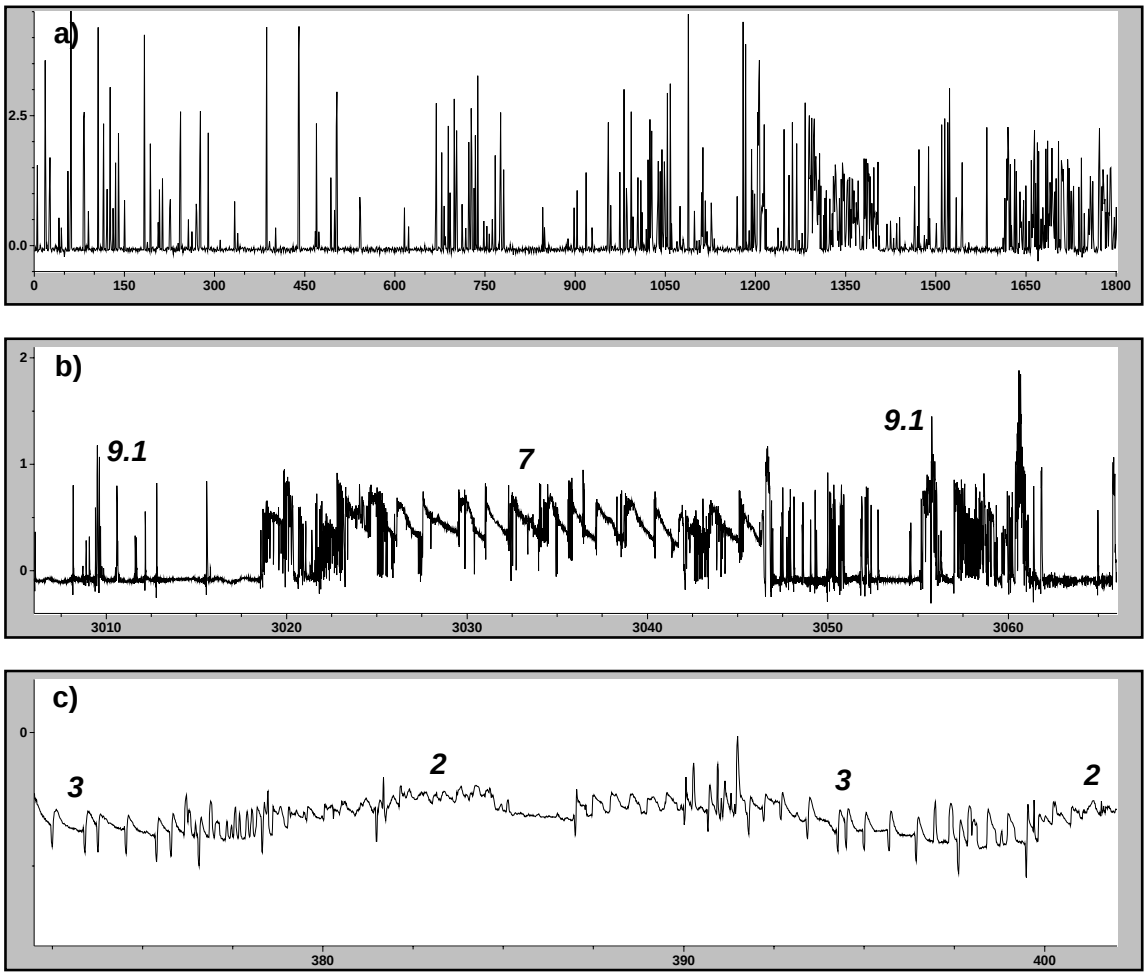


**Figure 15.** Grape phylloxera EPG patterns on Börner rootstock plant material; **a)** 30 minute overview of a first instar recording on Börner excised root, the high amplitude probe waveform 9.1 was the most dominant pattern; **b)** 60 second overview of a first instar possible feeding attempt (waveform 7) on Börner excised root; and **c)** 30 second overview of an adult feeding pattern on Börner tissue culture. Waveform transitions indicated.

# 5 DISCUSSION

EPG recordings were successfully performed on all feeding life stages of radicicolae grape phylloxera, represented as first instar, intermediate instar and adult. Data acquisition was achieved using both the excised root and tissue culture recording systems, and with insects in both the active feeding and probe initiation positions. EPG recordings of grape phylloxera had not previously been documented, and these results validated the EPG technique as an application suitable for grape phylloxera feeding behaviour studies, overcoming several anticipated difficulties highlighted earlier.

## *5.1 GRAPE PHYLLOXERA CONSTRAINTS*

Radicicolae grape phylloxera are relatively small insects; adults measure 690 µm in length, and first instars 326 µm (from light microscopy measurements, Chapter 2). However insect life stage (and size) did not impact on EPG recording success rate. It was important to obtain EPG recordings from life stages of grape phylloxera that display biologically different associations with the grapevine food source. First instars are important for insect distribution and establishment, being active along the grapevine root surface before becoming sedentary feeders once a feeding site is established. Feeding by first instars initiates gall development, which acts as a nutrient sink and sustains the development of the other life stages (De Klerk, 1974). The adult life stage does not move during the course of parthenogenetic egg development, limiting the available nutrition to the current feeding site.

The root feeding habit of grape phylloxera presented challenges in the set up of the plant electrode system. Potted plants would be the optimal set up as they provide a whole plant response in a natural soil environment, however EPG studies on the *Pemphigus bursarius* L., lettuce root aphid (Homoptera: Pemphigidae) identified that side-roots and bare soil cause the EPG signal to short-circuit in contact with the gold wire of the insect electrode (Cole *et al.*, 1993). Although limitations have been reported

for using an excised plant system in place of a whole, intact plant (van Helden and Tjallingii, 2000), the excised root and tissue culture plant recording systems were the preferred options for grape phylloxera. The use of excised root pieces and tissue culture vines for grape phylloxera laboratory studies had previously been validated in a number of population dynamics and rootstock screening experiments (Granett *et al.*, 1985; Forneck *et al.*, 1996; Kellow *et al.*, 2002; Powell *et al.*, 2006). Both plant recording systems obtained successful data acquisition, and sufficient EPG signal strength for waveform analysis.

Grape phylloxera are sedentary feeders, with sufficient nutrition on susceptible grapevines being obtained from a single feeding site (Kellow *et al.*, 2004). Uncertainty therefore existed over the re-establishment of feeding from an insect that was forcefully disturbed from their original food source for probe initiation EPG recordings. The success rate (and sample number) for insects in the probe initiation position was reduced in comparison with insects maintained in an active feeding position, however probe initiation data was acquired. EPG recordings of the complete insect feeding behaviour, obtained from the probe initiation position, are important for the interpretation of grape phylloxera – grapevine interactions. Probe EPG recordings are required to understand the processes of site selection and feeding establishment, therefore the validation of this insect EPG recording position was an important advancement for the future application of EPG technology to investigating grape phylloxera feeding behaviour.

Intermediate instar and adult life stages analysed for probing patterns indicated repeated probing attempts prior to settling on a feeding site. These recordings confirmed that intermediate instar and adult life stages can re-establish feeding if disturbed from an established location. In Australia, first instars are considered the highest contamination risk for human assisted transfer to non-infested vineyards due to

above ground dispersal activity during summer (Powell *et al.*, 2000). First instar grape phylloxera initiate feeding sites and grapevine galling for subsequent life cycle development and population maintenance. By confirming that later life stages are also able to establish new feeding locations, the risk is increased that any radicicolae grape phylloxera life stage, if relocated by human assistance, may establish a new colony in a previously non-infested vineyard. Human assisted transfer of radicicolae grape phylloxera may occur due to the movement of infested grapevine material, or contaminated soil, footwear, clothing and machinery (King and Buchanan, 1986; Dunstone *et al.*, 2003). Wind may also be a factor for grape phylloxera dispersal.

### *5.2 INSECT AND PLANT ELECTRODE DESIGN*

Multiple recording systems were trialled for the application of the EPG technique to the grape phylloxera – grapevine model. The excised root plant recording system was more successful in obtaining usable EPG data acquisition than the tissue culture plant system. However this direct comparison was misleading as fewer recordings were made on tissue culture, and all insects used for the tissue culture recordings were in the probe initiation position. By comparing probe initiation recordings, there are similar success rates between the excised root and tissue culture recording systems, therefore validating both procedures for application to the grape phylloxera – grapevine model.

On the excised root recording system, the active feeding position obtained a higher percentage of successful data acquisitions than the probe initiation position. A higher success rate of insects in the active feeding position may be expected due to the non-disruption of insect feeding behaviour, however insect electrode tethering may also be a factor. Insect tethering to the gold wire of the insect electrode has been shown to influence insect feeding behaviour, especially impacting upon an insects ability to walk over the plant surface (van Helden and Tjallingii, 2000). Insects in the active feeding position typically did not move from the feeding site during EPG recording, and were

therefore less impeded by attachment to the insect electrode. Although grape phylloxera in the probe initiation position were observed to walk along the root surface, the impact of tethering to the insect electrode may have had an impact on the success rate of these EPG recordings. A reduction to the possible impacts of insect tethering on EPG success rates may be achieved with the use of a finer insect electrode connection with ultra-thin platinum wire. During EPG research involving whitefly (Homoptera: Aleyrodidae), Walker and Janssen (2000) found that the 12.5-25 µm diameter gold wire used for Aphididae studies impeded whitefly walking behaviour, and continued future studies with a 2.5 µm diameter Wollaston process platinum wire.

The success rate for the tissue culture recording system may also be improved with future modifications to the current procedure. The tissue culture system was difficult to keep dry as the thin roots of the grapevine were in close contact with the wet filter paper. EPG research into the feeding behaviour of *P. bursarius* identified the importance of keeping the root of the host-plant dry during EPG recording as a non-conductive surface on the food source was critical for the detection of the 'true' probing signal from the plant-insect biological connection (Cole *et al.*, 1993). High levels of noise and false signals restricted the number of tissue culture recordings able to be analysed, therefore a more refined set up may reduce the level of conductive interference and improve the success rate of the tissue culture recording system.

### *5.3 GRAPE PHYLLOXERA EPG WAVEFORMS*

The repeated occurrence of the same EPG waveforms independent of the plant recording system (excised root or tissue culture) and the insect recording position (active feeding or probe initiation) validated the application of EPG for grape phylloxera feeding behaviour studies. The distribution of the continual feeding waveforms was similar in both continual feeding recordings and probe recordings, and indicated that the EPG insect position did not impact on grape phylloxera feeding behaviour once a

feeding site was established. There was limited success of the tissue culture recording system with susceptible grapevine material, however the occurrence of the same continual feeding waveforms on the Börner tissue culture recording indicated that the excised root and the tissue culture recording systems produced comparable EPG patterns and grape phylloxera feeding behaviour. The repeated occurrence of the same EPG waveforms also supported the classification of the waveforms for the current study.

There are limited examples for the application of EPG technology to determine differential feeding behaviour between the life stages of an insect species. The feeding behaviour of whitefly instars have been compared (Lei *et al.*, 1996; Jiang and Walker, 2003), however no Aphididae studies have compared adult EPG feeding patterns with instars (van Helden and Tjallingii, 2000). Due to the importance of first instar grape phylloxera to population dispersal and feeding site establishment (Powell *et al.*, 2000), and questions relating to the continual intake of food, and maintenance of the reproductive load in adults (from light microscopy sectioning, Chapter 2), a comparative approach was taken for grape phylloxera EPG recordings. The intermediate instar grouping provided a base level interpretation of grape phylloxera feeding in a sedentary position on an established feeding site and in the absence of reproductive pressures.

The differential occurrence of the 11 continual feeding waveforms observed in grape phylloxera EPG recordings implied the existence of variable feeding requirements for different life stages investigated. Waveforms 2 and 3 were consistently the most dominant across all life stages; waveform 4 also dominated the waveform distribution in intermediate instar and adult recordings. The significant differences between life stages in the mean percentage of total recording time for waveforms 2 and 4 implied the potential for EPG waveform analysis to differentiate between the feeding behaviour of grape phylloxera life stages. There was variation within life stage categories;

however the variation in waveform distribution between life stages highlighted the value of the EPG technique for investigating grape phylloxera – grapevine host-plant interactions.

First instars recorded the lowest level of within life stage variation. The standardisation of biology and plant interaction experienced by first instars due to the requirement of establishing a feeding location was reflected by the dominance of only two waveforms (2 and 3) in the waveform distribution chart. Within life stage variation increased for intermediate instar EPG recordings, potentially reflecting variable plant interactions due to changing biology and energy requirements during life stage development.

The highest within life stage variation in the continual feeding waveforms was observed in adult grape phylloxera. Adults parthenogenetically oviposit several eggs per day that are approximately 40% the length of the adult and occupy 6% of the body cavity (Chapter 2). The internal development of eggs result in the compression of the digestive system, and potentially restrict food intake. Although the continual feeding waveforms recorded for adult grape phylloxera did not indicate the withdrawal of the stylet to restrict foot intake, periodic feeding may still occur. The length of the silent period within and between waveforms was not analysed, but may be worth investigating as an indicator for a 'pause' in adult feeding behaviour as evidence for periodic feeding. The adults recorded in the current study laid eggs during the EPG recording time; evidence for oviposition was observed as an increase in the number of eggs present near the adult insect at the end of recording time. Variations in individual insect biology depending upon the stage of egg production may account for the diversity of EPG waveforms in adults.

### *5.4 Interpretation of Continual Feeding Waveforms*

The waveforms presented in Table 3 appear visually similar to waveforms that have been correlated to biological activity for the phloem-leaf feeding Aphididae (Reese *et*

*al.*, 2000). However EPG waveforms generated from grape phylloxera represent data based on a different insect family (Phylloxeridae), with a putative different primary food source (parenchyma cell contents) and at a different feeding location (roots). In a review written by van Helden and Tjallingii (2000), they stress that correlation studies must be completed on each new taxon group studied in order to be able to assign biological meaning to the EPG data. Previous Phylloxeridae research involving *P. coccinea* does present similar EPG waveforms from a taxon closely related to grape phylloxera (Harrewijn *et al.*, 1998), however no correlation studies were performed in the study to apply definition of biological meaning to the current EPG data.

Correlation studies relate insect activity (penetration, stylet pathway, ingestion, and oviposition) to the EPG waveforms using a number of established techniques (Walker, 2000). Many of the correlation techniques used for Aphididae, including stylectomy and 'honeydew clocks', may not be applicable to grape phylloxera due to the parenchyma cell food source. The non-pressurised parenchyma cells would not exude the plant sap that is collected from phloem tissue following an Aphididae stylectomy. Grape phylloxera are not reported to produce honeydew (Ponsen, 1997), therefore the collection of a 'honeydew clock' would not be possible for correlation of food ingestion. Correlation techniques most likely applicable to the grape phylloxera – grapevine model include histological studies into the location of the stylet during specific waveform events, and split-screen video observation of feeding activity during EPG recording on plant tissue and artificial feeding systems (Walker, 2000). Comparisons of grape phylloxera EPG waveform patterns from plant and artificial diet recording systems would also determine if the origin of the signal was insect based or due to the plant and/or the plant-insect biological connection (Tjallingii, 1985).

Due to a lack of correlation studies, all grape phylloxera waveform analysis was based on visual characterisation and not biological function. An initial interpretation of

possible biological meaning to the grape phylloxera waveforms has been inferred from studies based on insects with similar feeding habits, and patterns that are commons across a range of insect taxonomic groups. However in the absence of correlation studies these interpretations must be viewed with caution.

Waveform 2 was visually similar to waveform G2 observed in *P. coccinea* EPG recordings (Harrewijn *et al.*, 1998). Harrewijn *et al.* (1998) interpreted waveform G2 as the biological function of parenchyma cell content ingestion. *P. coccinea* waveform G1 was identified as salivation, and was similar in structure to grape phylloxera waveform 6. Parenchyma feeders, like grape phylloxera, continuously secrete saliva during feeding (Kloft, as cited in Pollard, 1973), however waveform 6 was only observed in three intermediate instar recordings out of the 28 EPG recordings analysed (across all life stages and set ups). Waveform 6 occurred for less than 1% (corrected percentage of total recording time) of the intermediate instar continual feeding recordings on susceptible excised roots; an insufficient occurrence to exclusively represent the essential biological function of salivation. Due to the dominance of grape phylloxera waveforms 2 and 3 across all EPG recordings, and the regular interchange (and interruption) between the two waveforms, waveform 3 potentially represented salivation in grape phylloxera EPG recordings, and waveform 2 ingestion. Salivation and ingestion are expected to be the main biological functions occurring during insect feeding.

Waveforms 2.2, 2.4 and 3.4 represented unexplainable variations within the regular patterns of waveforms 2 and 3 (respectively). Waveform 5 was a more complex monophasic pattern than waveform 2, with the inversion of waves between the positive peaks from the baseline to the top of the peak. From continual feeding recordings on susceptible excised roots, waveform 5 occurred in four adult EPG recordings, and one intermediate instar recording. The origin of the waveform was unknown, but the

uniqueness of waveform 5 to later developmental life stages indicated that it may be associated with an altered ingestion pattern, possibly due to the compression of the digestive system and restriction of food intake, caused by the internal development of parthenogenetic eggs (from light microscopy sections, Chapter 2). As waveform 5 occurred predominately in the adult life stage, another possible cause for the EPG pattern was the oviposition of moist eggs during the recording period.

Waveform 4 appeared visually similar to waveform 3, but occurred at an increased repetition rate. Waveforms 3 and 4 regularly interchanged and occurred in sequence with waveform 2. It could be proposed therefore that waveforms 3 and 4 were involved in the same biological function, with waveform 4 occurring at a more rapid rate. Both waveforms were recorded when the EPG amplifier was adjusted to near 0 V, where waveforms are detected that result from insect muscle contraction and fluid streaming potentials (Harrewijn *et al.*, 1998). The strong negative peaks of waveform G1 observed in *P. coccinea* reflected the large salivary pump present in Phylloxeridae species (Harrewijn *et al.*, 1998). If, like *P. coccinea* waveform G1, grape phylloxera waveform 3 was representative of salivation, then an increase in activity of the salivary pump would potentially explain the increased repetition rate observed in waveform 4.

Waveform 8 represented a 3 V change in the amplitude of the EPG pattern, and most likely indicated a transition of the grape phylloxera stylet from an intracellular to an extracellular position within the grapevine food source. Similar abrupt changes were recorded in *Bemisia argentifolii* Bellows and Perring (Hemiptera: Aleyrodidae) nymphs. The *B. argentifolii* EPG pattern changed from a (relatively) high voltage position to a low voltage position with the movement of the insects stylet tip from the extracellular space between cells, to within the plant cell in an intracellular position (Jiang and Walker, 2003). Waveform 8 occurred in two intermediate instar grape phylloxera EPG recordings; waveform 8 occurred twice in the first recording, and four times in the

second. In all occurrences, waveform 8 indicated an increase in amplitude and was followed by the limited occurrence of a second waveform (2, 6 or 7) for a period of between 200-500 seconds. The second waveform (2, 6 or 7) was followed by another waveform 8 and resulted in a decrease in amplitude and a return to the original recording voltage level. Waveforms 2 and 3 occurred on both sides of the waveform 8 patterns at the original recording voltage level. Based on *B. argentifolii* observations (Jiang and Walker, 2003), waveform 8 may represent a short disruption of intracellular feeding in the main continual feeding waveforms 2 and 3. The stylet of grape phylloxera may have momentarily moved to an extracellular position before returning to intracellular feeding. The cause of this disruption was unknown.

Waveform 10 was a variable pattern that involved a high amplitude interruption to a continual feeding waveform. The R signal origin of waveform 10 indicated that the positive or negative direction of the high amplitude interruption was dependent upon the EPG voltage setting. A range of phytophagous Aphididae species displayed a similar sudden change in signal potential, which lasted 5-15 seconds prior to a sudden return to the original voltage level. This short potential drop (pd) was associated with the puncturing of parenchyma cells (Tjallingii, 1985). If grape phylloxera waveform 10 represented the penetration of the parenchyma feeding cell, then the observation of the waveform in five of the 21 continual feeding recordings on susceptible excised root material indicated that once grape phylloxera established a feeding location, there was limited movement. When waveform 10 did occur, continual feeding waveforms 2 and 3 were associated on either side. This sequence indicated that grape phylloxera feed on a single parenchyma cell, and then puncture a connecting cell to continue feeding. This observation supported histological studies of radicicolae grape phylloxera nodosities where a successive number of adjacent parenchyma cells were damaged along the stylet track to the feeding site (Kellow *et al.*, 2004). Few cells surrounding the feeding cells appeared to be damaged. An additional long pd associated with the

penetration of the sieve elements for phloem feeding by the Aphididae (Tjallingii, 1985) were not observed in grape phylloxera EPG recordings.

The probe waveforms 9 and 9.1 observed in grape phylloxera were visually similar to the penetration pattern identified in *P. coccinea* (Harrewijn *et al.*, 1998) and in *Frankliniella occidentalis* Pergande, western flower thrips (Thysanoptera: Thripidae) (Harrewijn *et al.*, 1996). In both insect-plant models, repeated probing events occurred prior to the establishment of a feeding pattern. As with waveform 10, the R signal origin of waveforms 9 and 9.1 indicated that the positive or negative direction of the high amplitude interruption was dependent upon the EPG voltage setting. Although labelled independently based on their occurrence in either continual feeding or probe recordings, waveform 10 and 9.1 may represent the same biological event of puncturing parenchyma cell walls and causing momentary interruptions to the EPG pattern. Waveform 9.1 was more common during initial plant exploration, as reflected by interruptions to the non-penetration waveform 1, although waveform 9.1 was also observed during continual feeding waveforms. Waveform 9 differed from waveform 9.1 as the high amplitude interruption initiated a continual feeding waveform event. Waveforms 9 and 9.1 represented the potential drop generated by the puncturing of the parenchyma cell wall during the stylet pathway to the feed cell.

### *5.5 EVIDENCE FOR FEEDING SITE LOCATION*

Radicicolae grape phylloxera have been reported to penetrate the epidermis and cortex of the root on an intracellular pathway to the feeding location of parenchyma cells contents (Petri, 1907; Forneck *et al.*, 2002; Kellow *et al.*, 2004). Within the parenchyma cell, branching of the stylet track has been observed (Petri, 1907; Forneck *et al.*, 2002), although Kellow *et al.* (2004) only observed branching of the stylet track with multiple probing events on resistant rootstocks. A single stylet track was observed when radicicolae grape phylloxera were feeding on susceptible *V. vinifera*, although

there was evidence of successive puncturing of adjacent parenchyma cells as the stylet progressed deeper into the cortex. Pollard (1973) displayed the pathway of the grape phylloxera stylet as the continual puncturing of cells (and their walls) until the insect reached the parenchyma feeding site.

The high voltage probe waveforms 9 and 9.1 represented the initial electrical contact in the insect-plant biological system. The repeated occurrence of waveform 9.1 suggested that the insect was involved in pre-probing and stylet re-positioning activity prior to the establishment of sustained continual feeding waveforms that were prefixed by waveform 9. Waveform 9 and 9.1 (and waveform 10) are proposed to represent the intracellular stylet pathway to the parenchyma feeding cell within the grapevine root. The occurrence of waveforms 9.1 and 10 within continual feeding waveforms may indicate the puncturing of a new parenchyma cell wall during the same probing event. The waveform sequence of continual feeding waveforms interrupted by waveforms 9.1 or 10 supported the observations by Kellow *et al.* (2004) and Pollard (1973) of successive cell wall puncturing during the intracellular pathway of grape phylloxera to a new parenchyma feeding cell.

Grape phylloxera are sedentary feeders, with a single feeding site supporting growth, development and the production of 100-200 eggs (Kellow *et al.*, 2004). Feeding on an individual parenchyma cell would not be expected to be able to support the insect throughout these processes, however the continued probing of new parenchyma cells at the same feeding location may provide sufficient nutrition. Grape phylloxera continual feeding waveforms were observed in all life stages for a period of up to 5 hours without the occurrence of the high amplitude waveforms 9.1 or 10. This observation indicated that an extensive period of time was required to exude the nutritional value out of a single parenchyma cell. The high protein content of parenchyma cell contents may assist the maintenance of grape phylloxera survival.

However probe recordings indicated that grape phylloxera initiating a new feeding location take a period of time to establish a suitable feeding site. The principle probe recorded in grape phylloxera EPG probe recordings were preceded by a number (2-5) of shorter probes, and the principle probe events were terminated within 27-32 minutes.

### *5.6 RESISTANT ROOTSTOCK SCREENING*

Grape phylloxera EPG recordings on resistant roots indicated continual feeding patterns on Ramsey and Börner root material. Börner was reported as be immune to grape phylloxera feeding (Schmid and Rühl, 2003), although Kellow *et al.* (2002) labelled Börner as 'highly resistant' as grape phylloxera attempted to feed on the micropropagated tissue culture grapevine roots but could not initiate nodosities. The first instar insect EPG recording displayed only high voltage, probe-like activity with very limited evidence of feeding waveforms on the Börner excised root material. However the adult insect on the Börner tissue culture root displayed continual feeding waveforms. This establishment may have been assisted by the fleshier, softer roots of the tissue culture grapevine compared with the lignified excised root pieces, and by life stage.

Resistant rootstock trials (Powell *et al.*, 2006) involve placing grape phylloxera eggs onto the root material of grapevines, which then hatch into first instars. If the first instars do not successfully establish (as with Börner), then the survival of adult insects was not tested. The EPG results presented here highlight the possibility that post-first instar grape phylloxera insects may be capable of establishing feeding sites on the 'immune' rootstock Börner. In future resistant rootstock trials, EPG technology could be used to determine the feeding behaviour of all life stages on the resistant root material, removing the reliance upon the initial egg hatching and first instar establishment survival rates. Individual life stage mechanical features, including the

size of the insect and the length of the stylet (from microscopy measurements, Chapter 2), may have an impact on grape phylloxera feeding establishment; once a feeding site, and the associated 'nutrient sink', has been established on resistant rootstocks by a later life stage, earlier life stages may experience higher feeding success, leading to a grape phylloxera population becoming established on the resistant rootstock food source.

Alternatively, the continual feeding waveforms recorded by the adult insect on Börner tissue culture roots may have occurred prior to the necrotic response by the grapevine to grape phylloxera feeding. El-Nady and Schroder (2003) identified two forms of necrosis in Börner in response to grape phylloxera feeding on excised root material. Type I necrosis developed within 12-24 hours of grape phylloxera feeding, and Type II necrosis within 2-5 days. As a result of both forms of necrosis, the tissue surrounding the feeding site died, preventing the successful establishment of the insect. Although the adult insect recorded continual feeding waveforms on the Börner root material, the insect would have established the feeding site on the tissue culture roots during the set up of the EPG equipment for recording. As a consequence, the continual feeding waveforms recorded possibly represented feeding behaviour prior to the Börner necrotic response. The long term survival of the adult life stage on the Börner tissue culture roots was not recorded.

As implied by EPG analysis, resistant rootstock trials indicate that grape phylloxera do establish feeding sites on Ramsey root material; insect development has been reported to reach the adult life stage with the production of fertile eggs (Powell *et al.*, 2006). Kellow *et al.* (2002) labelled Ramsey as ‘resistant’ due to the low survival and reproduction rate of grape phylloxera insects, and the relatively small development of nodosities on tissue culture grapevines. Differential insect population numbers were observed for different grape phylloxera genotypic classes on Ramsey excised roots

and potted grapevines (Powell *et al.*, 2006). Only one genotypic class of grape phylloxera (G4) was used for these EPG experiments to limit the biological variation displayed in the EPG patterns. Future EPG studies comparing feeding behaviour of different grape phylloxera genotypic classes may provide insight into the observed differential survival rates of grape phylloxera genotypic classes in resistant rootstock trials.

The current study has shown that the EPG technique can be applied to radicicolae grape phylloxera to investigate feeding behaviour characteristics on both susceptible and resistant grapevine varieties. Fourteen waveforms were characterised for grape phylloxera feeding on susceptible *Vitis vinifera*, including continual feeding and probe EPG waveforms. Grape phylloxera life stages vary in feeding behaviour, and are able to be differentiated by waveform occurrence within an EPG recording. The potential of EPG to improve knowledge of grape phylloxera – resistant rootstock interactions was highlighted by the differential feeding behaviour of the first instar and the adult insect on Börner root material. Further studies are now required to correlate these waveforms with biological activity of the grape phylloxera to identify interactions with the *Vitis* host-plant. Once these correlation studies on susceptible *V. vinifera* have been completed, the EPG technology may be adapted to resistant rootstock studies, and potentially develop resistance ratings for rootstocks based on active feeding behaviour and establishment.

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# CHAPTER 6

# GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The aim of this thesis was to address a number of questions relating to the biology, nutritional requirements and feeding behaviour of radicicolae grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae). The major conclusions from the research undertaken during the study are summarised below, and discussed in relation to the interrelated research areas. Possible future directions to further extend our knowledge in these areas are highlighted.

# 1 SUMMARY OF DATA

## *1.1 CHAPTER 2: STRUCTURE OF THE DIGESTIVE SYSTEM OF GRAPE PHYLLOXERA*

The structure of the digestive system of radicicolae grape phylloxera was atypical to the general Aphidoidea model due to the split function of the midgut and the absence of a filter chamber. The midgut was blind-ending in the posterior region, with a midgut-hindgut junction in a dorsal position in the medial region. The passage of the hindgut was intertwined with the ovaries, and extended to the posterior region of the insect in a dorsal position to the gonopore. If food is stored in the posterior region of the midgut prior to absorption in the anterior region of the split function midgut, the structure of the digestive system may allow grape phylloxera to survive during dispersal periods to new *Vitis* host-plants.

Due to the absence of observed honeydew excretion by live insects, the anal opening observed with scanning electron microscopy could not be confirmed as the site for waste excretion. However the presence of a droplet of fluid at the posterior end of two adult insects implied that the hindgut may contain waste material. In the absence of anal dorsal muscles to contract the anal opening and excrete the liquid waste, the contraction of the longitudinal muscles was proposed as an alternative action for the excretion of waste material. The compression of the fluid in the hindgut chamber with

the contraction of the longitudinal muscles, together with the pressure caused by oviposition in the adult life stage, could theoretically result in the expulsion of anal waste material. The low level of waste excretion observed in grape phylloxera may be a consequence of the high utilisation of nutrients from the parenchyma cell food source. Measurements of the stylet length of all apterous life stages of radicicolae grape phylloxera confirmed that vascular tissue feeding was unlikely to occur in this species.

### *1.2 CHAPTER 3: INVESTIGATING BACTERIAL SYMBIOTIC RELATIONSHIPS IN GRAPE PHYLLOXERA*

A bacterial association with radicicolae grape phylloxera confirmed by microbiological and molecular studies. The morphology and molecular analysis of the bacteria indicated that the bacterial species differed from the bacteria associated with gallicolae grape phylloxera. The 91% identity of the radicicolae grape phylloxera bacterial DNA sequence with uncultured bacterium confirmed the successful amplification of the 16S rRNA gene, however the level of identify was too low to classify the species. Amplification of the bacterial DNA with 16S rDNA primers was transient, suggesting that the association was not essential for grape phylloxera survival; however there appeared to be a relationship between the insect root collection source and the health or fitness of the root and the insect population. The transient bacterial association may therefore impact on grape phylloxera development under field conditions, and could potentially influence resistant rootstock screening under laboratory conditions.

### *1.3 CHAPTER 4: DEVELOPMENT OF AN ARTIFICIAL FEEDING SYSTEM FOR GRAPE PHYLLOXERA*

A number of experimental factors were found to impact on grape phylloxera mobility, survival and probing activity within the two diet chambers trialled. The humidity chamber design was the most suitable for observation of grape phylloxera and reduced

the impact of condensed droplets of water on the small insect. The survival rate of radicicolae grape phylloxera in an artificial feeding system was variable, with natural population fitness most likely having an effect on survival time. Significant improvements in insect survival times were recorded for two diet formulations, although neither result was reproducible and the artificial diet formulation is not currently optimised. However significant increases in survival rate and probing activity on the artificial diet formulations did validate the application of an artificial feeding system to grape phylloxera studies. There was a general response by grape phylloxera to diet formulations that included amino acids in the diet solution and were adjusted to an acidic pH. Sucrose appeared to be the most suitable form of sugar for the pest insect.

The maximum survival time recorded by a radicicolae grape phylloxera insect feeding on an experimental diet formulation was 11 days; however the significance of survival rates was reduced by the extended survival time of insects on control diets (maximum survival 9 days). Survival time may be affected by grape phylloxera life stage, with intermediate instars generally surviving longer than first instars. The extended survival time of intermediate instars may be a consequence of the larger size of the insects, and an ability to store food and fat during grapevine feeding.

### *1.4 CHAPTER 5: APPLICATION OF ELECTRICAL PENETRATION GRAPH TO GRAPE PHYLLOXERA FEEDING BEHAVIOUR STUDIES*

Electrical penetration graph (EPG) recordings were successfully performed on three apterous life stages (first instar, intermediate instar, and adult) of radicicolae grape phylloxera. Plant systems (excised root and tissue culture) that reflected established methods for rearing the insect under laboratory conditions, and screening for rootstock resistance, were successfully trialled for grape phylloxera EPG recordings. Two different insect recording positions were also trialled for application to grape phylloxera EPG studies, and both the active feeding and the probe initiation positions were

successful. The strength of the EPG signal was sufficient for grape phylloxera feeding behaviour analysis despite anticipated difficulties based on the small size of the insect and the root parenchyma cell food source.

Re-occurring EPG patterns were identified in the grape phylloxera recordings and defined as representing probe and continual feeding waveforms. Grape phylloxera life stages were determined to have differential feeding behaviour on susceptible *V. vinifera* based on the percentage of feeding time spent in the continual feeding waveforms. The differential feeding behaviour was expected to reflect the varying host-plant relationships and biological requirements of first instar, intermediate instar and adult life stages. Correlation studies are required to assign biological meaning to the grape phylloxera EPG waveforms, however the two most common waveforms (2 and 3) are likely to represent ingestion and salivation.

Grape phylloxera are sedentary feeders, and this was supported by the recording of EPG continual feeding waveforms for up to 5 hours without any evidence of stylet removal, relocation or cell penetration. However probe recordings confirmed that grape phylloxera can successfully re-establish a feeding site if they are forcefully removed from an existing feeding location. Multiple high voltage probe events (representing cell puncturing) during the establishment of a new feeding site were expected to correlate with the parenchyma cell feeding location of grape phylloxera.

## 2 IMPLICATIONS OF RESEARCH

Phylloxeridae taxonomically belong in the Aphidoidea superfamily, with the sister groups Aphididae and Adelgidae. Evolutionary divergence of the Aphididae resulted in differential feeding behaviour between the three families, with the Aphididae predominately feeding on the phloem sap of vascular tissue, and the Adelgidae and Phylloxeridae on non-vascular parenchyma cells (Ponsen, 2006). The Phylloxeridae and Adelgidae represent a more ancient lineage than the Aphididae (Heie, 1987), and the essential association with the *Buchnera* endosymbiont is isolated to the Aphididae (Martinez-Torres *et al.*, 2001). Ponsen (2006) made a histological comparison of the three families, and identified a number of similarities between the digestive system of the Adelgidae and Phylloxeridae; the current study provided additional knowledge of specific grape phylloxera characteristics.

The compartmentalisation of the radicicolae grape phylloxera midgut has not previously been reported. The simplified structure of the grape phylloxera digestive system, in comparison with the Aphidoidea model, is presumably a consequence of the high utilisation of the parenchyma cell food source. The Aphididae have evolved a filter chamber to assist with the excretion of the high fluid, low nutritional waste products from the phloem sap diet. Adelgidae do not contain a filter system, but unlike Phylloxeridae, are reported to excrete honeydew (Ponsen, 2006). The production of low volumes of honeydew was not confirmed in grape phylloxera, however it is speculated that the high utilisation of the high protein and low sugar diet reduced the volume of waste excretion to non-detectable levels.

The *Buchnera* endosymbiotic relationship is specific to Aphididae as a consequence of their diet of phloem sap. The parenchyma feeding Adelgidae have a different symbiont relationship, although Phylloxeridae feeding on the same food source are reported not to have a symbiotic relationship (Ponsen, 2006). The identification of a transient

bacterial symbiont in radicicolae grape phylloxera may represent an evolutionary link between the absence of bacterial associations and the symbiotic relationship observed in the parenchyma feeding Adelgidae. The endosymbiont association present in the Aphididae represents an essential and mutalistic association not present in the other sister groups.

The absence of a nutritional symbiotic relationship in radicicolae grape phylloxera potentially impacted on the survival rate of insects in the artificial feeding system. *Buchnera* supplement the diet of Aphididae with amino acids that may be absent from a diet formulation (Douglas, 2006). The grape phylloxera natural food source of parenchyma cells, together with the absence of amino acid producing symbionts, increased the necessity of the artificial diet formulation to contain the correct amino acids at ideal concentrations in order to stimulate insect feeding and influence survival rates. As a consequence, the formulation of artificial diet solutions may be more critical for a Phylloxeridae artificial feeding system compared with an Aphididae diet formulation.

The EPG analysis of radicicolae grape phylloxera feeding behaviour supported the proposed intracellular feeding location of parenchyma cells. In the development of a feeding site, it is generalised that all Aphidoidea produce a stylet sheath (Miles, 1987), where the sheath seals the pathway of the stylet to the feeding location. However due to the sedentary feeding behaviour of grape phylloxera, and EPG evidence for the maintenance of the single cell feeding location without the penetration of an adjacent cell for up to 5 hours, an alternative mechanism of nutritional transport may be present in this insect. Kellow *et al.* (2004) proposed that the accumulation of starch and amides in root galls induced by grape phylloxera feeding activity may represent phloem unloading in order to maintain the 'nutrient sink' at the feed site. A similar mechanism has been proposed in the parenchyma feeding Coreidae (Heteroptera), where an

'osmotic pump' is formed due to the secretion of salivary enzymes and the 'leakage' of phloem fluids into the parenchyma feed site (Miles and Taylor, 1994). Further investigation of the feeding behaviour of grape phylloxera with EPG may provide evidence for the mechanism of nutritional transport, and therefore rationalisation for the sustained survival of the sedentary feeding insect.

The data presented in this thesis also has a number of implications for current grape phylloxera management strategies and research methods. First instars are the main dispersal life stage of grape phylloxera, and are currently considered the main 'risk' for the movement of the pest insect in Australian viticulture (Dunstone *et al.*, 2003). Previously it was unknown how long grape phylloxera could survive without feeding on *Vitis* plant material; however the artificial diet experiments highlighted that under ideal temperature and humidity conditions, survival was possible for several days. First instar grape phylloxera survived for up to 7 days on the water controls in the artificial diet experiments, and intermediate instars for up to 9 days. These survival times confirmed that grape phylloxera are capable of surviving human-assisted transport, either directly on people or on machinery, to a vineyard for the establishment of a new infestation site.

Currently first instars are considered the highest risk of human-assisted transfer due to the active movement of the insect, the relative abundance of the life stage in the canopy of the grapevine, and the role the life stage plays in establishing a new feeding site and gall development on the grapevine root (De Klerk, 1974; Powell *et al.*, 2000). Focusing the risk of spread by grape phylloxera predominately on the first instar life stage may however be an over simplification as the later life stages may also be 'dispersed' to new infestation sites by human assistance. The probing activity observed by intermediate instars using the artificial diet system, and the successful re-establishment of a feeding site observed by intermediate and adult life stages during

EPG recordings confirmed that all apterous life stages of grape phylloxera pose the risk of establishing a new infestation site. The later life stages are also able to survive longer than first instars in the absence of grapevine material, possibly due to the storage of food in the digestive system. Although the later life stages are less active in the grapevine canopy in comparison with first instars (Powell *et al.*, 2000), they may be present in soil and root samples collected from a grape phylloxera infested vineyard. The survival of grape phylloxera for several days in the absence of food intake and the ability of the insect to re-establish a feeding site following disturbance, reinforces the need for the maintenance of quarantine protocols in Australia to restrict the spread of the pest insect.

Current grape phylloxera resistant rootstock screening trials only infest root material with eggs (Kellow *et al.*, 2002; Powell *et al.*, 2006), and therefore require the survival of first instar insects in order to establish a feeding site and grape phylloxera population. The differing feeding behaviour of first instar and adult grape phylloxera observed using EPG on Börner root material highlighted the differential response of the life stages to the same resistant rootstock. This result demonstrated the value of EPG studies for understanding grape phylloxera host-plant interactions. Future grape phylloxera resistant rootstock trials may be enhanced by the inclusion of EPG analysis to determine the feeding behaviour of a range of life stages on a range resistant root varieties. Differential feeding behaviours from grape phylloxera developmental life stages may also assist with interpreting the mechanism, and sustainability, of rootstock resistance.

The biological basis for the variable survival and fecundity rates identified in radicicolae grape phylloxera genotypic classes (Viduka *et al.*, 2003; Powell *et al.*, 2006) is currently unknown. The bacterial association identified in the G4 genotypic class may differ for other grape phylloxera genotypic classes. Therefore, grape phylloxera host-plant

interactions, and the differential impact of grape phylloxera genotypic classes, may be influenced by the transient bacterial symbiotic relationship identified in the current study.

A number of new methods have been validated during the course of the current study which will be of assistance to future grape phylloxera research: (1) Cold stage SEM was determined to be the optimum preparation for viewing the external and internal structure of the soft-bodied grape phylloxera; (2) The artificial diet humidity chamber design developed for radicicolae grape phylloxera studies was an important improvement on previous designs which require more complicated equipment. The simplified chamber design will also be of value to other sedentary insect artificial feeding systems; (3) The EPG recording systems validated different approaches for the design of plant and insect electrodes for root feeding insects. Both the excised root and the tissue culture recording systems allowed for the recording of comparable EPG patterns in the absence of a soil environment.

## 3 FUTURE DIRECTIONS

Further definition of the feeding site of grape phylloxera, and primarily the chemical composition of the food intake, is an essential requirement for the continuation of research in the areas of radicicolae grape phylloxera biology, nutrition and feeding behaviour. The feeding location of radicicolae grape phylloxera is currently defined as *Vitis* parenchyma cells; however the composition of the food ingested is not definitively known. The chemistry of the nutrient sink induced by grape phylloxera feeding is broadly defined as increases in the concentration of sugars and amino acids in the locality of the gall tissue; however this does not define how the chemistry of the ingested material from the feeding site alters during gall development and subsequent changes in root morphology.

A higher level definition of the feeding location for radicicolae grape phylloxera is important for understanding a number of factors relating to the current study, including: (1) food intake as a basis for the processes of food digestion and absorption; (2) the food intake basis for waste excretion requirements; (3) the nutritional requirements of the insect, (4) the chemistry of the food intake as a reference point for the development of an optimal artificial diet formulation; (5) the correlation of EPG waveforms with plant cell location; (6) and the direction of future developments in alternative management options, where water and nutrient management may result in changes to the chemistry of the grape phylloxera feeding site. Chemical analysis of the composition of grapevine root parenchyma cells may be sufficient for a range of nutrition based issued raised above, however EPG correlation studies require further analysis of the grape phylloxera feeding site. In order to determine the exact feeding location of grape phylloxera and the position of the stylet during ingestion, transmission electron microscopy studies are required. Previous studies (Kellow *et al.*, 2004) based on light microscopy were unable to determine if the stylet was positioned inside the cell wall or the plasmalemma.

Additional future directions relating to the individual areas of study covered by this thesis are highlighted below.

### *3.1 GRAPE PHYLLOXERA BIOLOGY*

Scanning electron microscopy studies of the external morphology of radicicolae grape phylloxera included observations of sensory pits at the end of the antennae. The function of these sensory pits is currently unknown, but their role is assumed to involve a chemosensory function allowing the location of a *Vitis* plant for the establishment of a feeding site based on chemical cues. However, the chemosensory cues exhibited by *Vitis* are currently unknown. Further investigation of the function of the antennal sensory pits, together with use of electroantennograms for the examination of the

behavioural responses to host-plant volatiles and exudates released by *Vitis*, would assist in understanding how grape phylloxera are able to locate the *Vitis* food source during population dispersal events. Examination of host-plant associations between susceptible and resistant *Vitis* species based on chemosensory cues may assist with interpreting the mechanisms of grape phylloxera resistance.

The structure of the radicicolae grape phylloxera digestive system has been described, however the issue of anal waste excretion still requires resolution. Artificial diet studies incorporating tracking dyes would allow for the pathway of food ingested by grape phylloxera to be followed through the digestive system, and determination of the possible excretion of honeydew waste through an anus. If waste is excreted via the salivary glands back into the plant cell, as proposed by Schaller (1960), then the combination of salvia and waste products would have an impact on host-plant interactions and may be involved with the process of gall formation. Therefore the resolution of the pathway of waste excretion in radicicolae grape phylloxera remains an important factor for investigating the host-plant response to grape phylloxera feeding.

An investigation of the function and locality of the main digestive enzymes involved in grape phylloxera feeding is also important for understanding host-plant interactions. Identification of the main digestive enzymes may lead to the development of a novel management option for the pest insect, where specific enzyme inhibitors could be manipulated to interfere with normal function of the digestive system. The artificial feeding system would provide a model assay for examining the effect, and optimising the efficacy, of enzyme inhibitors on grape phylloxera feeding, growth and development.

The transient identification of a bacterial association with radicicolae grape phylloxera suggested the bacteria species was acquired from the environment and the association may be impacted by grapevine health and insect fitness. However there is evidence of

maternal transmission for the bacterial association in gallicolae grape phylloxera (Vorwerk *et al.*, 2007). To confirm the method of transmission in grape phylloxera, molecular studies are required to include radicicolae grape phylloxera parthenogenetically produced eggs from populations feeding on a range of grapevine root material, including field grown grapevines, potted grapevines, excised root pieces and tissue culture grapevines. Confirmation of the method of transmission would assist in interpreting the necessity of the bacterial association for radicicolae grape phylloxera survival, as maternal inheritance would imply that the symbiotic relationship was beneficial for the insect. Artificial feeding studies with symbiotic and aposymbiotic insects would also assist in interpreting the necessity of the relationship, and enable examination of the nutritional associations.

The current bacterial symbiont study has focused on one grape phylloxera genotypic class, G4. Bacterial DNA has also been detected with 16S rDNA primers in the genotypic classes G1 and G20 (data not shown); however further investigation is required to determine if there is a relationship between the bacterial association and the virulence or developmental rate of the genotypic classes. Sequencing of the bacterial DNA from a range of radicicolae grape phylloxera genotypic classes would determine the specificity of the bacterial association and if more than one bacteria species was involved.

### *3.2 GRAPE PHYLLOXERA NUTRITIONAL REQUIREMENTS*

The future direction of the radicicolae grape phylloxera artificial feeding system is related to the long-term purpose of the system, and whether the artificial diet is required as a model system to test novel control agents, or for more extensive nutritional studies. If the main function of the artificial diet is as a routine bioassay for the testing of novel control agents (including enzyme inhibitors, lectins, protease inhibitors and amylase inhibitors) on the biology of the insect, then an extensive

chemical analysis may not be required. Although the root extract diet formulations did not extend survival time in the current study, further improvements in methodology and isolation of the grapevine root material could provide a suitable food source. A more refined root extract may provide the simplest artificial diet system to allow examination of insect survival rate in comparison with novel control agents, and therefore ensure the success of the bioassay method. Root extracts could be obtained from resistant rootstocks to compare grape phylloxera feeding behaviour with susceptible root material. Analysis of the chemical composition of susceptible and resistant root extracts, based on the parenchyma cell food source rather than a complete homogenised mix, may assist with interpreting the mechanisms of grape phylloxera resistance.

If however the aim of the artificial diet is to examine the essential nutritional requirements of grape phylloxera, then a fully defined holidic diet formulation is required. The artificial diet formulation of *5% sucrose pH 4.5 plus amino acids* provides a starting point for increased definition of the grape phylloxera diet solution; however information on the natural dietary intake of the insect is required to further the progress in this area. Therefore, the optimisation of the artificial feeding system for either the application as a bioassay or for the purpose of identifying the nutritional requirements of the insect requires additional studies. A fully optimised artificial diet would assist with examining the impacts of changes to grapevine root chemistry on grape phylloxera survival. Changes in grapevine physiology induced by vineyard management could include the impacts of nutrient and water stress on grapevine physiology. The comparative nutritional requirements of different radicicolae grape phylloxera genotypic classes could also be examined with a fully optimised artificial diet formulation.

For future applications of the artificial feeding system, a tracking dye is required to prove ingestion of the diet solution. Current observations of probing activity are

indicative of ingestion; however the uptake of a tracking dye incorporated in the diet solution is required to prove that ingestion is occurring. The artificial feeding system could also be incorporated into EPG studies to investigate the feeding behaviour of grape phylloxera on an artificial diet solution. The impacts of novel control agents on the feeding behaviour of the insect could also be monitored with EPG.

### *3.3 GRAPE PHYLLOXERA FEEDING BEHAVIOUR*

Further studies are required to correlate grape phylloxera EPG waveforms with biological activity in order to further interpret the feeding behaviour of the insect. Possible correlation techniques for the grape phylloxera – grapevine model include: histological studies examining the location of the stylet during specific waveform events; split-screen video observation of feeding activity during EPG recording; and combined artificial diet – EPG studies to view stylet and salivary pump activities for definition of ingestion and salivation waveforms. Once completed, EPG has the potential to investigate a number of host-plant interactions involving grape phylloxera genetic diversity and *Vitis* resistance mechanisms.

Resistant rootstocks display variable levels of resistance to radicicolae grape phylloxera depending upon the genetics of the insect (Viduka *et al.*, 2003; Powell *et al.*, 2006). Current resistant rootstock trials based on excised roots and glasshouse experiments only determine the survival rate of grape phylloxera populations on the resistant rootstocks, and are unable to determine differences in feeding behaviour. EPG has the potential to define the differential feeding behaviour of grape phylloxera on resistant rootstocks based on insect genetics and developmental life stage. This application of the EPG technology would assist in determining the resistance mechanisms of the rootstocks, and would potentially increase the processing time of resistant rootstock screening in comparison with current glasshouse experiments and field-based validation trials.

EPG could examine the observed variable impact of radicicolae grape phylloxera on *Vitis* species based on genotypic classification (Powell *et al.*, 2006). Umina *et al.* (2007) identified 83 genotypic classes in Australian vineyards, with G1 and G4 being the most common. Viduka *et al.* (2003) identified higher levels of survival and fecundity in these two genotypic classes in comparison with four other classes examined. EPG has the potential to determine the biological basis to the differential survival and fecundity rates presented by G1 and G4, assisting in future management of these 'super-clones'.

Resistant rootstocks are currently the only recommended long-term management option for the viticulture industry, therefore interpreting resistance mechanisms, and highlighting potential areas of resistance breakdown is a high priority to the industry. Current resistant rootstock screening methods are based on excised root, tissue culture or potted grapevine systems (Kellow *et al.*, 2004; Powell *et al.*, 2006). However higher survival rates of grape phylloxera have been noted on the excised root system in comparison with whole plants, implying that excised roots overestimate the impact of grape phylloxera feeding and therefore underestimate rootstock resistance levels (Granett *et al.*, 2001). This observation questions the effectiveness of the current methods for screening rootstock resistant for providing results representative of field based outcomes, where the survival of grape phylloxera is also influenced by soil type, climate and vineyard management practices. The modification of the grape phylloxera EPG system to allow for EPG recording on a potted grapevine, within a soil based environment, would allow for comparison in the feeding behaviour of grape phylloxera dependent upon the excised or attached nature of the root food source.

# 4 GENERAL CONCLUSION

Radicicolae grape phylloxera continues to pose a threat to the viticulture industry due to the economic damaged caused by feeding on the susceptible European grapevine, *Vitis vinifera* L. (Vitaceae). However due to the worldwide success of resistant rootstocks in the management of grape phylloxera, a number of basic features of the insects biology, nutritional requirements and feeding behaviour have not been extensively studied. This thesis reports on a number of advances in our knowledge of grape phylloxera in these areas, and provides the background required to continue studies into host-plant interactions and the resistance mechanisms of resistant rootstocks. The continued investigation of the biology, nutrition and feeding behaviour of radicicolae grape phylloxera is essential for the maintenance of current management strategies against the pest insect, and for the development of alternative management methods.

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