

Grape Phylloxera External Morphology Observations under Scanning Electron Microscopy

K.B. Kingston and K.S. Powell
Primary Industries Research Victoria
Department of Primary Industries
RMB 1145, Chiltern Valley Road
Rutherglen, Victoria 3685
Australia

P.D. Cooper
School of Botany and Zoology
Australian National University
Canberra, ACT 2600
Australia

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Abstract

Grape phylloxera is a major viticulture pest present in most grape-growing regions of the world. Radicolae grape phylloxera feed on the root system of grapevines, and may result in the death of susceptible vines. During an investigation into the biology of grape phylloxera, scanning electron microscopy was used to examine the insects' external morphology. During incomplete metamorphosis, grape phylloxera moult four times with a sequential increase in size with each instar. Parthenogenetic egg production in adult grape phylloxera result in the swelling of the abdomen and the posterior region near the gonopore. A posterior opening appears to be present for excretion, however honeydew production has not been observed during feeding. The stylet length of grape phylloxera indicated that the insect feeds on non-vascular parenchyma tissue. This high protein and low sugar food source is the basis for the differing morphological and physiological traits between grape phylloxera and the closely related Aphididae.

INTRODUCTION

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae), has been a significant pest of the viticulture industry since the late 1800s. Despite being the focus of research for over 100 years, there are still a number of uncertainties pertaining to the biology of the insect. Grape phylloxera is closely related to the Aphididae and is often described as an "aphid-like" insect. However, the two families differ in a number of feeding and digestion characteristics, and the terminology "aphid-like" has resulted in a number of false assumptions regarding the biology of grape phylloxera.

The digestive system of grape phylloxera has previously been investigated, with most researchers concluding that the insect does not have a complete digestive system, and therefore lacks the capacity for anal waste excretion. Evidence supporting the lack of anal excretion includes the intestinal tract, or midgut, ending blindly (Federov, 1959), and the absence of an anal opening (Dreyfus, as cited in Ponsen, 1987). Alternative proposed methods of waste excretion have included the use of modified salivary glands (Schaller, 1960; Sobetskiy and Derzhavina, 1973), waste excretion during oviposition (Rilling, as cited in Forneck et al., 2001) and the use of extra intestinal digestion removing the need for waste excretion (Federov, 1959). The absence of sugary honeydew production (Riley, 1870) is associated with the absence of dorsal anal muscles as evidence for an incomplete digestive system (cited in Ponsen, 1997). However, a more recent study by Ponsen (1997) indicated the presence of an anal opening and a complete digestive system. As a consequence of a lack of consistency in previous reports, the structure of the grape phylloxera digestive system, and the method of waste excretion, remains unclear.

Grape phylloxera feeding induces the development of galls on the leaves and roots of grapevines. Sectioning of grape phylloxera induced galls, or nodosities, have tracked the stylet of the insect on a path through the cortex, with the tip of the stylet settling within a single non-vascular parenchyma cell around five cells below the epidermis (Kellow et al., 2004). The Aphididae food source of vascular phloem tissue was never

penetrated, and cells surrounding the parenchyma feed cell appeared not to be damaged. However, the level of resolution obtained from this study was unable to determine if the stylet tip was positioned within the cytoplasm or the vacuole of the feeding cell. This lack of detail leaves definition of grape phylloxera dietary intake unresolved.

Adult grape phylloxera lay 3-6 eggs per day during a reproductive period of 1-2 months (Granett et al., 1983). The sessile feeders remain attached to the same feeding location during life stage development, and during oviposition accommodate for the continual production of eggs by repositioning the posterior end (De Klerk, 1974). Uncertainty over the digestive system and feeding location of grape phylloxera raises questions as to how the insect is able to maintain this reproductive output. As part of a study into the biology of grape phylloxera, we investigated the external morphology of radicolae grape phylloxera using scanning electron microscopy (SEM). The use of SEM was validated by comparing external body measurements with previous light microscopy research, to confirm the absence of artefact development and minimal sample distortion during the SEM preparation. The SEM fine detail on external features of grape phylloxera was used to build upon and clarify existing knowledge regarding the feeding location and methods of waste excretion for the insect.

MATERIALS AND METHODS

Radicolae grape phylloxera were originally collected from infested vineyards in Victoria, Australia, and maintained at the Department of Primary Industries – Rutherglen Centre on excised grapevine (*Vitis vinifera* L.) root pieces. Excised root piece (approximately 1 cm width x 10 cm length) preparation was slightly modified from Granett et al. (1985). Modifications were: roots washed clean of attached soil with a soft brush prior to a 5 minute soak in Ridomil® Gold Plus systemic fungicide solution (2.3 g/L); roots triple rinsed with distilled water prior to drying; and Petri dish lids did not have a mesh covered hole. Grape phylloxera populations were incubated in the dark at a constant temperature of $25 \pm 3^\circ\text{C}$. Insects were handled using a moist sable-haired paintbrush to avoid damage.

All developmental stages of the apterous radicolae life cycle were collected, with nymphal life stage determined by comparative increases in size, and the presence of nearby eggs indicated adult reproduction. If present, neighbouring moulted cuticles were counted to confirm the insects' life stage.

Insects were collected into 2 ml cryo-tubes, snap frozen in liquid nitrogen and stored at -80°C until required. Cold stage SEM was performed in a Cambridge Instruments Stereoscan 360 SEM. 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. External morphology of the insects was examined using a 15 kV electron beam, with images collected digitally. Measurements of external body features ($\pm 1 \mu\text{m}$) were made using ImageJ public domain software (Rasband, 2005). Stylet length was 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.

Sample numbers used for each life stage measurement and observation were variable due to the loss of samples 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 morphology observation.

GenStat-Eighth Edition® (Lawes Agricultural Trust) was used to calculate summary statistics, and standard deviation (Microsoft® Excel) as an indicator of variation in determining differences between life stages. Statistical analysis was not possible due to the low sample number.

RESULTS

Grape phylloxera experience incomplete metamorphosis during life stage development from egg to adult. The nymphs go through four successive moults during development, resulting in four instar life stages between the egg and adult. There was limited change in the external morphology of radicolae grape phylloxera during life stage development (Fig. 1), except for the continual increase in size of the external body measurements. Instars increased in length by approximately 15% and width by 25% with each successive life stage, while adults increased in size (length and width) by approximately 80% from the fourth instar life stage (Table 1). In length, eggs were 32% the size of a reproductive adult. Oviposition occurred over a period of 0.30-1.45 hours, with subsequent egg laying activity observed within 2.30 hours (unpublished data). The change in external morphology induced by the internal presence of eggs in a reproductive adult was evident (Fig. 1e). There was a visual increase in size of the adult insect, and an expansion, or swelling, of the posterior end due to the oviposition of eggs.

A posterior opening was identified as an indentation with a lateral-lateral orientation in the posterior region of the insect (Fig. 2a). The length of the posterior opening did not vary for the first – third instars, however the fourth instar recorded the largest posterior opening (Table 1). 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 adult grape phylloxera insects (Fig. 2b) also inhibited observation of a posterior opening.

The ratio of labium and stylet length to external body length decreased as life stage increased, due to no increase in the labium and stylet lengths between the four instars. The adult labium and stylet length was 36% and 90% (respectively) larger than the longest instar measurement (Table 1). There was no stylet length measurement for first instar insects as they were collected prior to feeding, and due to the lack of probing activity the stylet was still enclosed within the labium structure.

There was a general increase in the length of the antennae with life stage development (Table 1); however the ratio of antennae length to insect length decreased as life stage increased. Sensory pits were observed at the end of the antennae (Fig. 3).

DISCUSSION

External body measurements of radicolae grape phylloxera displayed a continual increase in size with each life stage development; however the largest increase was observed during transition from fourth instar into adult. This increase in size (80%) may be in part representative of the observation by Davidson and Nougaret (1921) of a growth phase during the fourth instar life stage. Davidson and Nougaret reported that between the third and fourth moult (indicating the fourth instar life stage), the fourth instar was observed to increase in length (25%) and width (17%). Adult insects used for measurement presented here were typically the largest insects within the population; smaller insects were observed that could be classified as adults, but they were not collected. Selecting the larger adult insects, along with the possibility of collecting early moult fourth instars, would have exacerbated the variation in size between the fourth instar and adult life stage beyond what was expected within a natural population.

In comparison with previous external body measurements made by light microscopy (Davidson and Nougaret, 1921; Buchanan, 1990), the SEM measurements for first instar and adult insects were of comparable size, however second, third and fourth instars were recorded at smaller sizes in this study. This variation may be due to an unavoidable distortion of the insect with angle placement on the SEM stub, and also the quality of the food source. As noted by Davidson and Nougaret (1921), adult insects feeding on fleshy and succulent roots measured 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 this study were collected from excised root populations maintained in the laboratory. These populations are smaller in size to insects collected from potted vine populations (unpublished data), and are not expected to grow to a size comparable with

field collected insects as environmental and nutritional factors are limiting. Egg measurements (using light microscopy) have previously been reported with an average length of 299 μm and width of 149 μm (De Klerk, 1974). The measurements presented in this study are within range of these averages, although the width dimension may have been affected by a portion of the egg being sunken into the SEM stub mounting paste, therefore affecting the accuracy of the values (Fig. 1f).

Comparisons between grape phylloxera external body measurements via previous light microscopy research and the SEM measurements presented did not completely align, however there was similarity in the external body measurements of eggs, first instars and adults. Current light microscopy measurements of all life stages of grape phylloxera grown on laboratory excised roots also aligned with the SEM measurements (unpublished data). The combination of comparisons with previous and current light microscopy measurements was sufficient to validate the use of SEM to investigate the fine detail of the external structures of grape phylloxera.

The location of a posterior opening was investigated as evidence for anal waste excretion. Adult grape phylloxera had an enlarged gonopore posterior opening for egg oviposition. In a limited number of SEM images, the presence of a second posterior opening, dorsal to the gonopore, was also visible. The location of this second posterior opening aligned with the posterior opening observed in all instar life stages of grape phylloxera. The size of the posterior openings was variable in size, but the observation did provide evidence for the external termination point of an anal opening, and for anal waste excretion. The location of the posterior openings also aligned with the expected termination point of the hindgut of grape phylloxera from longitudinal sectioning (Kingston et al., 2004).

An absence of honeydew production (Riley, 1870) has been cited as evidence that grape phylloxera does not have a system of anal waste excretion (Ponsen, 1997). Honeydew was not visible when observing insects actively feeding on grapevine roots, however the possible presence of honeydew was observed as a droplet of fluid at the posterior end of adult grape phylloxera. The droplet of fluid was potentially the result of pressure applied to the abdomen during preparation. However, the exact exit point of the fluid was unable to be determined, and it is possible that the fluid in the SEM image was from the gonopore passage.

Reported observations in the closely related *Phylloxera coccinea* Heyden (Hemiptera: Phylloxeridae) however provide support for the theory that the droplet of fluid observed in grape phylloxera was associated with waste excretion. *Phylloxera coccinea* do not display visible levels of honeydew production, however a similar droplet of 'honeydew' has been observed with the application of pressure to the abdomen (Ponsen, 1997). Ponsen inferred that Phylloxeridae feeding on parenchyma cells have a reduced fluid intake in comparison to the phloem feeding, and honeydew generating, Aphididae. The waste excretion levels generated by grape phylloxera may be too low for visible detection under standard conditions.

The feeding location of grape phylloxera has been reported to be a single parenchyma cell within the cortex tissue of the grapevine root (Kellow et al., 2004). Stylet length was measured as an indicator of the possible feeding location of 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 this study are similar to earlier light microscopy data by Davidson and Nougaret (1921), and can imply possible feeding locations within the grapevine root. By comparing stylet length with the distance to parenchyma and phloem cells, it may be possible to determine where grape phylloxera can feed in the host plant.

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 penetration required for phloem feeding. The thickness of the cortex in *V. vinifera* ranges from 200-300 μm , or 7-12 cell layers deep (Kellow, 2000). Stylet penetration from the

root surface to the food source 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 in a straight line, the stylet measurements recorded indicate that only adult individuals would be capable of feeding on phloem cells. These measurements therefore support the expectation that grape phylloxera primarily feed on grapevine root cortex tissue.

The ratio of grape phylloxera antennae length to external body length was greatest for the first instar life stage. The function of the sensory pore located at the end of each antenna was unknown, but may be important during dispersal and host plant displacement. 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., 2001); therefore a high level sensory system would be important for the location of a new host plant. The antennae measurements presented in this study are similar to those presented by Davidson and Nougaret (1921) from light microscopy.

Grape phylloxera internal morphology studies, together with cross sectioning of grapevine root feeding sites, are required to better describe the grape phylloxera digestive system and feeding location. However investigating the external morphology characteristics has contributed to our understanding of some feeding processes. Although the location of an anal opening is not definitive, the evidence of a posterior opening in all life stages is indicative of the potential for anal waste excretion. Alternative methods of waste excretion have not been discussed, however with grape phylloxera reported to feed on a single parenchyma cell (which is supported by stylet length measurements and no visible honeydew detection), excretion of waste by the salivary glands back into the food source seems an unlikely mechanism.

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Tables

Table 1. Apterous radicolae grape phylloxera morphology measurements from SEM images, all life stages. Mean values $\pm$ standard deviation, n value in brackets.

Life stage	External body (μm)		Posterior opening (μm)	
	length ¹	width ²		
adult	894 $\pm$ 79 (2)	550 $\pm$ 7.0 (3)	25 $\pm$ * (1)	
fourth instar	474 $\pm$ 23 (3)	306 $\pm$ 47 (3)	37 $\pm$ 7.8 (2)	
third instar	414 $\pm$ 37 (4)	242 $\pm$ 2.1 (4)	14 $\pm$ 3.8 (3)	
second instar	361 $\pm$ 33 (5)	183 $\pm$ 8.6 (5)	18 $\pm$ 2.5 (3)	
first instar	315 $\pm$ 40 (5)	152 $\pm$ 20 (5)	23 $\pm$ 1.6 (5)	
egg	284 $\pm$ 21 (2)	126 $\pm$ 22 (2)	NA	

Life stage	Mouthparts (μm)		Antennae (μm)	
	labium	stylet	right	left
adult	226 $\pm$ 51 (2)	349 $\pm$ * (1)	147 $\pm$ * (1)	118 $\pm$ * (1)
fourth instar	123 $\pm$ * (1)	182 $\pm$ 46 (2)	86 $\pm$ * (1)	105 $\pm$ * (1)
third instar	133 $\pm$ 26 (3)	162 $\pm$ 28 (4)	100 $\pm$ 2.9 (3)	87 $\pm$ 14 (3)
second instar	164 $\pm$ 57 (2)	149 $\pm$ 19 (3)	90 $\pm$ 2.5 (3)	91 $\pm$ 6.4 (2)
first instar	166 $\pm$ 19 (5)	NA	82 $\pm$ 18 (5)	91 $\pm$ 4.2 (5)
egg	NA	NA	NA	NA

¹ anterior – posterior measurement

² lateral measurement

* n = 1 therefore no standard deviation value

Figures

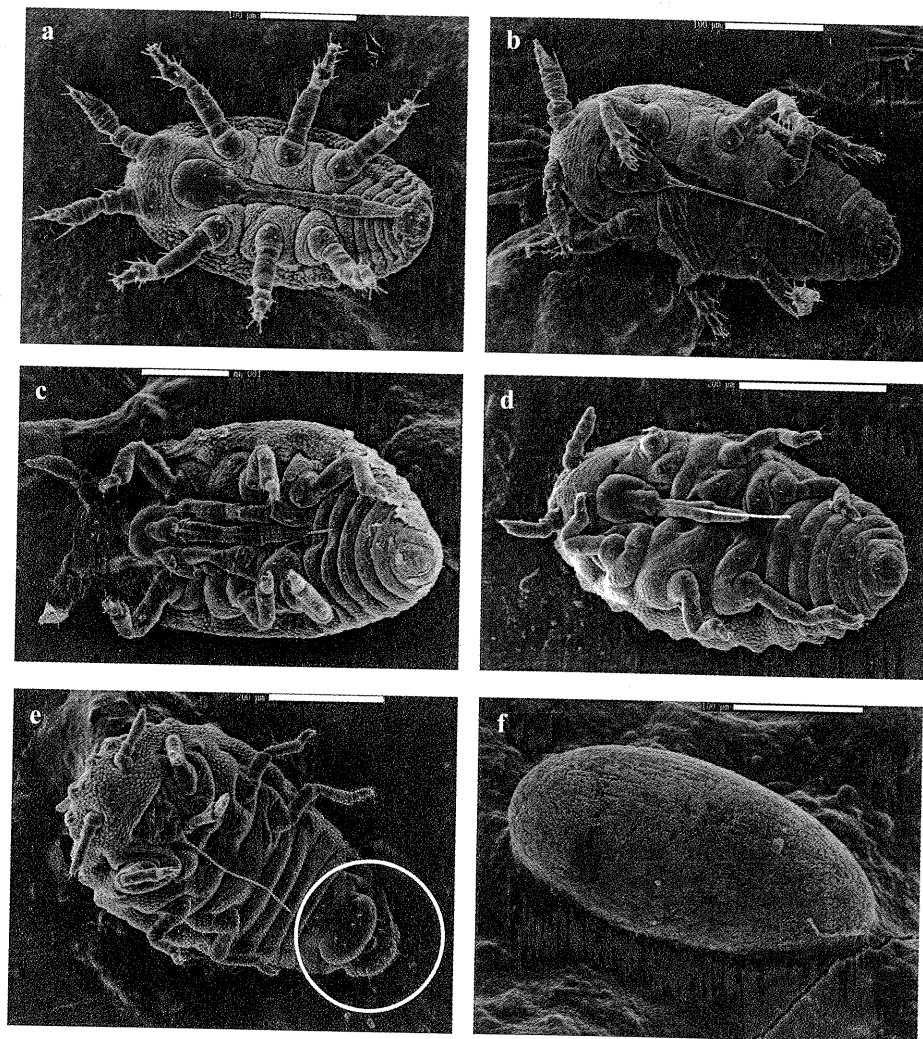


Fig. 1. SEM images of all life stages of apterous radicicolae grape phylloxera. (a) first instar, scale bar 100 μ m. (b) second instar, scale bar 100 μ m. (c) third instar, scale bar 100 μ m. (d) fourth instar, scale bar 200 μ m. (e) adult, the circle highlights the swelling of the posterior region, scale bar 200 μ m. (f) egg, note that the base is sunken into the stub mounting paste, scale bar 100 μ m.

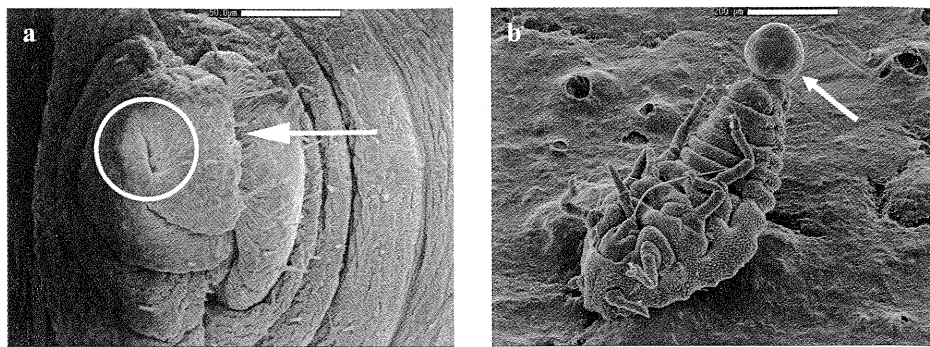


Fig. 2. SEM image of apterous radicolae adult grape phylloxera. (a) the posterior opening measured as evidence for an anal waste excretion; the posterior opening is circled, the gonopore is indicated by the arrow, scale bar 50 μm . (b) droplet of fluid (indicated by the arrow) at the posterior end of the insect, scale bar 200 μm .

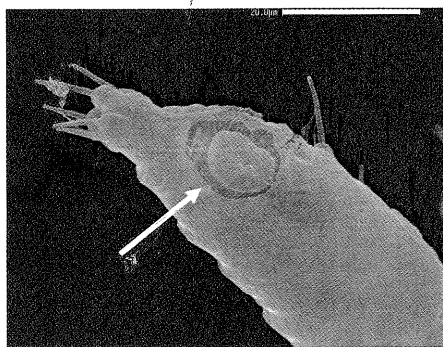


Fig. 3. SEM image of the sensory pit (indicated by the arrow) at the end of the antennae of all life stages (first instar – adult) of apterous radicolae grape phylloxera, scale bar 20 μm .