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Clone lineages of grape phylloxera differ in their performance on *Vitis vinifera*

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Abstract

Grape phylloxera, *Daktulosphaira vitifoliae* Fitch, is an important pest of grapevines (*Vitis vinifera* L.) (Vitaceae). The distribution and frequency of phylloxera clone lineages vary within infested regions of Australia, suggesting the introduction of separate lineages of *D. vitifoliae* with host associations. Virulence levels of particular phylloxera clones may vary on *V. vinifera*, but much of this evidence is indirect. In this study, we directly tested the performance of phylloxera clones on *V. vinifera* using an established excised root assay and a new glasshouse vine assessment. In the root assay, grape phylloxera clones differed in egg production and egg to adult survivorship. In the vine assay, clones differed in the number of immature and adult life stages on roots. In addition vine characteristics, including mean stem weight, root weight, leaf chlorophyll and leaf area, were affected by different phylloxera clones. The two most widespread clones displayed high levels of virulence. These results point to only some phylloxera clones being highly virulent on *V. vinifera*, helping to explain patterns of field damage, phylloxera distributions and continued survival and production of *V. vinifera* vines in some infested areas.

Keywords: *Daktulosphaira vitifoliae*, clone lineages, microsatellite, *Vitis*, plant-host relationships, parthenogenesis

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Introduction

Grape phylloxera, *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae), is an important pest of grapevines worldwide, with feeding damage known to result in rapid loss of production and, in extreme cases, the eventual demise of a vineyard. *Daktulosphaira vitifoliae* are phylloxerids with a host range limited to the genus *Vitis*, forming galls on the leaves and roots of this plant (Granett *et al*., 2001). On grapevines, *D. vitifoliae* are obligate/functionally parthenogenetic, with the suppression of meiosis during oogenesis resulting in offspring expected to be genetically identical to their parent (Hales *et al*., 1997). As a result, populations generally consist of ecologically distinct clone lineages (Corrie, 2003; Vorwerk & Forneck, 2007). This mode of reproduction is different to the mode used by many members of the closely related Aphididae family, which often undergo holocycly (cyclic parthenogenesis), although obligate parthenogenesis also occurs in this group (Hales *et al*., 1997).

In Australia, *D. vitifoliae* has been contained mostly within seven geographic regions following the introduction

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of strict quarantine restrictions in 1917 (De Castella & Brittlebank, 1917). Two regions are in New South Wales and five are in Victoria. One region in northeast Victoria (which includes Rutherglen, Glenrowan, Milawa) contains a total of 83 genotypic classes defined by microsatellite markers (Corrie *et al*., 2002; Corrie & Hoffmann, 2004; Umina *et al*., 2007). Phylloxera resistant rootstocks are used in these regions. These were bred from grapevines of American *Vitis* parentage and have been available and utilised as the primary phylloxera management option for over a hundred years in Australia. In the other north east and central Victoria regions, there are two dominant and widespread genotypic classes, G1 and G4 (Corrie *et al*., 2002) referred to as 'superclones'; and, in these regions, there remain vineyards planted predominantly with phylloxera-susceptible *V. vinifera* vines not grafted onto resistant rootstocks. This pattern of genetic diversity where there are a few common genotypes is also characteristic of some members of the Aphididae following invasion into Australia (e.g. Vorburger *et al*., 2003).

Although phylloxera can feed on the roots of most grapevines, this is generally restricted in resistant American *Vitis* varieties to the primary root system. Attack on the secondary roots and subsequent gall formation is believed only to occur on susceptible grapevine varieties (Granett *et al*., 2001). While the mechanisms of resistance are poorly understood (Granett *et al*., 2001; Kellow, 2001), this fundamental difference between grape parentage has enabled the economic production of *V. vinifera* through grafting onto resistant rootstocks in phylloxera-infested regions. Nevertheless, the majority of plantings in Australia are on susceptible ungrafted *V. vinifera* rather than resistant rootstocks.

Variation in feeding and reproductive performance among phylloxera populations has previously been documented (Song & Granett, 1990; De Benedictis & Granett, 1993; Hawthorne & Via, 1994; Martinez-Peniche & Boubals, 1994; Omer *et al*., 1999; Corrie *et al*., 2003). In addition, host-adapted biotypes have been characterised; particular phylloxera biotypes are better able to utilise vine hosts from where they are found than other vine hosts (Song & Granett, 1990; Hawthorne & Via, 1994; De Benedictis *et al*., 1996). However, little is known about the association between such genetic variability and performance on *V. vinifera*. Using excised root bioassays, Corrie *et al*. (2003) showed that *D. vitifoliae* collected from three regions where different genotypic classes predominated varied in their performance on ungrafted vines, but clone genotypes were not defined. Moreover, the performance of clones on intact vines has never been assessed.

In this study, we test whether the two widespread clones (G1 and G4) differ from other phylloxera clones in an excised root assay. We also describe an assay for assessing the performance of phylloxera clones on intact vines in the glasshouse and use this assay to compare the performance of the two widespread clones (G1 and G4) against a combination of two other clone lineages (G29 and G46). An advantage of the intact vine assay is that we could assess phylloxera effects on plant performance, by measuring vine parameters as well as the abundance of the different phylloxera life stages. Implications of these findings for assessing the impact of phylloxera on vines, as well as control recommendations, are discussed.

Materials and methods

Clones

Phylloxera clone types were collected from *Vitis vinifera* vines by digging up root material from vineyards located in Upton, King Valley and Rutherglen in Victoria, Australia. The Upton and King Valley phylloxera each consist of a single lineage (G1 and G4, respectively); these lineages are, by far, the most common in southeastern Australia, while Rutherglen vineyards contain a mix of lineages. In the laboratory, insects were maintained on excised roots from the vine hosts from which they were collected, and eggs were obtained directly from the roots within a few days. Clone types were identified using nuclear DNA microsatellite loci scored on samples stored in 100% ethanol at −20°C. Genomic DNA was extracted from single adults using Chelex-100 (BioRad) following Corrie *et al*. (2002), and amplification of six microsatellite loci (DVIT1-6) was performed as described in Corrie *et al*. (2002) and Umina *et al*. (2007).

Daktulosphaira vitifoliae *survival on excised* Vitis vinifera

This assay considered the impact of *Vitis vinifera* on survival and reproduction of phylloxera clones. A 30-day excised root assay was performed on the two dominant clones, G1 and G4, as well as clone lineages G3, G19, G20 and G30 (see Umina *et al*., 2007). These six clones represent genetically diverse lineages, based on phylogenetic analyses, and each clone was the most common genotype in the vineyard blocks from which they were sampled (Corrie & Hoffmann, 2004). These clones are root galling, with the exception of G3 which is also leaf galling. The root assays were performed on *V. vinifera* (cv. Sultana) as described elsewhere (Corrie *et al*., 2003). Sultana was used because this was the only variety available for isolating root pieces, although phylloxera perform equally well on other varieties of *V. vinifera* (e.g. Corrie *et al*., 2003). Briefly, a single excised root piece was washed and placed in a Petri dish lined with filter paper. Eggs (from a single phylloxera clone) were placed on each replicate root piece with a paintbrush. An assay was undertaken to allow all the clones to be characterized. Ten eggs of each of the clones were placed onto each root piece. Egg hatch occurred within seven days. Developmental stage (instar/adult) was recorded every five days for only 30 days, by which time instars had either (i) reached the adult stage or (ii) died and the next generation of immatures was absent. Once phylloxera reached adulthood, most laid eggs, and these were counted (total number per replicate) within the same 30-day interval in which development was monitored. Bioassays were performed at 23°C ± 1°C in sealed containers to restrict light and prevent cross contamination of clones. All clones were verified before commencement and at completion of the study using the DNA microsatellite markers as described above. Data from five to 20 replicates of each vine × clone combination were obtained, depending on availability of suitable root material and phylloxera numbers.

Feeding damage of D. vitifoliae *on* V. vinifera

This experiment considered the impact of phylloxera clones on intact vines. Twenty-four one-year-old grapevine

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(*V. vinifera* cv. Sauvignon Blanc clone FV5V10) rootlings were potted into autoclaved 20-cm plastic pots using a sterilised soil-perlite composite (80% potting mix, 20% perlite). Vines were kept in a shadehouse for nine months and transferred in early January to a glasshouse prior to phylloxera inoculation. The trial was conducted over an eight-month (245-day) period in a temperature-controlled glasshouse, cycling between 24°C ± 2°C (0600–1800 h) and 20°C ± 2°C (1800–0600 h). Vines were watered daily via an automatic dripper system, and relative humidity was maintained between 60–70%. Growth lights were used for 12 h each day (0600–1800 h) with an additional spike from 0000–0100 h to offset dormancy due to changes in day length.

The trial comprised of four phylloxera treatments: infestation with G4 phylloxera, G1 phylloxera, a mixed population of G29 and G46, and uninfested controls. Unfortunately, only G1 and G4 can be reliably sourced from vineyards because they are invasive; other clones are present as mixtures in vineyards from a restricted region (Corrie *et al*., 2002), and the clones available for experiments cannot be ascertained until a molecular analysis is completed. Six vines planted individually in pots were set up for each treatment. The treatments were placed in a randomised block arrangement. Each pot was sealed in a 45 mm × 35 mm draw-string bag with mesh aperture of 53 μm, secured at the base of the vine trunk. To prevent phylloxera cross-contamination, Tanglefoot™ (a sticky compound that prevents the movement of crawling insects) was applied around the neck of the bag where contacting the vine trunk.

G1 and G4 phylloxera clones were sourced from commercial vineyards (King Valley and Upton regions, respectively) and reared *in vitro* on excised root pieces based on the method of Granett *et al*. (1985). The Rutherglen populations, G29 and G46, had been previously collected from *V. vinifera* roots in a commercial vineyard and maintained on *V. vinifera* vines because they cannot be reared easily on root pieces, although all infestations involved eggs to minimize acclimation. All clones were verified before commencement and at completion of the study using the microsatellite markers described above. Two infestation rates were used. Three vines were infested with a total of 60 eggs per vine (subsequently referred to as a low (L) infestation rate) while the three remaining vines were infested with 600 eggs per vine (subsequently referred to as a high (H) infestation rate) in an attempt to reflect the variable numbers of 1st instar phylloxera typically encountered on an infested root (e.g. Herbert *et al*., 2006). Grapevine roots were exposed and eggs were placed on moist filter paper in close contact with the vine root system.

Life-stage quantification

Vines were removed from pots and the root system scored for level of phylloxera damage (Boubals, 1966). Uninfested vines were also inspected to ensure that no phylloxera contamination had occurred. Three samples of roots, approximately 1 g in wet weight, were randomly taken from the root system of each vine and washed with tap water through a 60 μm mesh, and the collected filtrate (containing phylloxera) was stored in 70% ethanol. Phylloxera life-stages were determined, using a dissector microscope at 10× magnification, and classified into the following categories: (i) egg, (ii) 1st instar (or crawler) and (iii) intermediate and adult life stages (this was a combined category comprising of 2nd–4th instars and apterous and alate adults). Root pieces were oven-dried and weighed to adjust for the amount of root tissue sampled.

Leaf and vine assessments

Average measurements of leaf colour, leaf chlorophyll and leaf area were obtained for six leaves taken from each cane. The youngest mature leaf was defined as the 5th leaf from the growing tip. This leaf and the next five leaves inwards towards the vine trunk were sampled.

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

Leaf chlorophyll measurements were obtained with a hand-held Minolta™ SPAD-502 chlorophyll meter. This determines the relative amount of chlorophyll present by measuring optical density differences in the chlorophyll wavelength regions (400–500 nm and 600–700 nm regions), with no transmittance in the infra-red region. Using these two transmittances, the meter calculates a numerical SPAD value (within ±1.0 SPAD unit at room temperature), which is proportional to the amount of chlorophyll present in the given leaf. Measurements were taken by inserting the leaf and closing the measuring head. Two readings were taken for each leaf, with the total readings for six leaves averaged to give a single chlorophyll value per vine. Total leaf area was determined for each vine using a Paton™ electric planimeter. All vine leaves, including those that had sprouted from pruned secondary canes, were included in this assessment.

Vine trunk diameter measurements were taken at 5, 7 and 9 cm from the base of the vine. Measurements were taken using a CE electronic™ digital calliper. Node length and number were recorded from the primary cane that had been standardised to ten nodes prior to commencement of the study. Total oven-dried (50°C for 12 h) vine stem and total root mass were also determined.

Statistical analysis

In the excised root assay, performance of the clones as assessed by the proportion of eggs reaching adulthood, total number of eggs produced (both measured per single root piece) and the number of eggs per surviving female was compared using one-way ANOVAs. Prior to analysis, egg counts were log + 1 transformed and proportions were angular transformed to ensure normality and homogeneity of variances. To maintain biological meaning, means and standard deviations are presented untransformed. Tukey's

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b post hoc tests were used to compare the performance of specific clones.

For the potted vine trial, one-way ANOVAs and Tukey's-*b post hoc* tests were undertaken to assess the differences between treatments (the controls and each of the three clones at two infestation levels) for the different traits measured. We then specifically assessed the effects of clone and infestation level (and their interaction) by running two-way ANOVAs excluding the controls. Because of the number of leaf/vine characteristics compared, probability values were adjusted for multiple comparisons using the Bonferroni procedure. Leaf and vine traits were not transformed for analysis.

Results

Survival of D. vitifoliae *clones on excised* V. vinifera

The laboratory excised root assay was used to screen six genetically diverse lineages (G1, G3, G4, G19, G20 and G30). All six phylloxera clones were able to feed, reach adulthood and reproduce on *V. vinifera* cv Sultana (fig. 1). However, clone lineages differed in their performance. There were significant differences between clones for the proportion of eggs to reach adulthood ($F = 5.74$; df = 5, 35; $P < 0.001$), total egg number ($F = 5.69$; df = 5, 35; $P < 0.001$) and number of eggs laid per surviving female ($F = 11.38$; df = 5, 35; $P < 0.001$). *Post hoc* tests for egg-to-adult survival indicated that there was no statistical difference between G1 and G4, while G1 was also not different from the other clones apart from G19; but G4 differed from all other clones. For total egg number and eggs per surviving female, G1 and G4 outperformed all other clones (fig. 1) and did not differ significantly from each other.

Effects of D. vitifoliae *clone lineages feeding on in planta* V. vinifera

Life stage numbers

The numbers of phylloxera recorded at each life-stage on *V. vinifera* depended on clone (fig. 2). G4 numbers were relatively higher than those of the other clones for all life-stages, particularly the G29 and G46 combination. There were significant differences among the clones for the number of eggs ($F = 9.78$; df = 2, 12; $P = 0.003$), 1st instars ($F = 14.36$; df = 2, 12; $P = 0.001$) and intermediate/adult life-stages ($F = 18.25$; df = 2, 12; $P < 0.001$). *Post hoc* tests indicated that, for eggs and intermediate/adult life-stages, the G4 high treatment was significantly different from the other treatments; while, for crawler numbers, the G4 and G1 high treatments differed from both G29 + G46 treatments. As expected, there was a significant difference between the high and low infestation levels for all three life-stages (eggs, $F = 9.18$; df = 1, 12; $P = 0.01$; 1st instar, $F = 10.80$; df = 1, 12; $P = 0.006$; and intermediate/adults, $F = 15.43$; df = 1, 12; $P = 0.002$). There was also an interaction between infestation level and clone for the intermediate/adult life-stages ($F = 7.96$; df = 2, 12; $P = 0.006$); the relative difference between the low and high infestations tended to be greater for G4 than for the other clones (fig. 3).

Leaf assessments

Leaf characteristics tended to differ between treatments but differences between phylloxera clones were not as evident as in the assessment of life stages (fig. 3). For hue angle, there were significant differences between treatments ($F = 16.82$; df = 6, 17; $P < 0.001$) when all treatments, including the controls, were compared. When only infested vines were considered, there was a significant effect of clone ($F = 9.03$; df = 2, 12; $P = 0.004$) and infestation level ($F = 30.36$; df = 1, 12; $P < 0.001$). In *post hoc* tests, angles for the G1 (high) and G4 (high) treatments differed to those in the G29 + G46 treatments (which had similar values to the controls). For chlorophyll content, treatments differed significantly overall ($F = 6.93$; df = 6, 17; $P < 0.001$). However, an apparent difference between clones ($F = 4.18$; df = 2, 12; $P = 0.043$) was not significant after correction for the number of leaf comparisons. In the *post hoc* tests, controls only differed from the G4 (high) treatment. For leaf area, there was a difference between treatments overall ($F = 19.66$; df = 6, 17; $P < 0.001$) and a significant effect of clone when only infested vines were considered ($F = 9.43$; df = 2, 12; $P = 0.003$); *post hoc* tests indicated a significant difference between G4 (high) and the

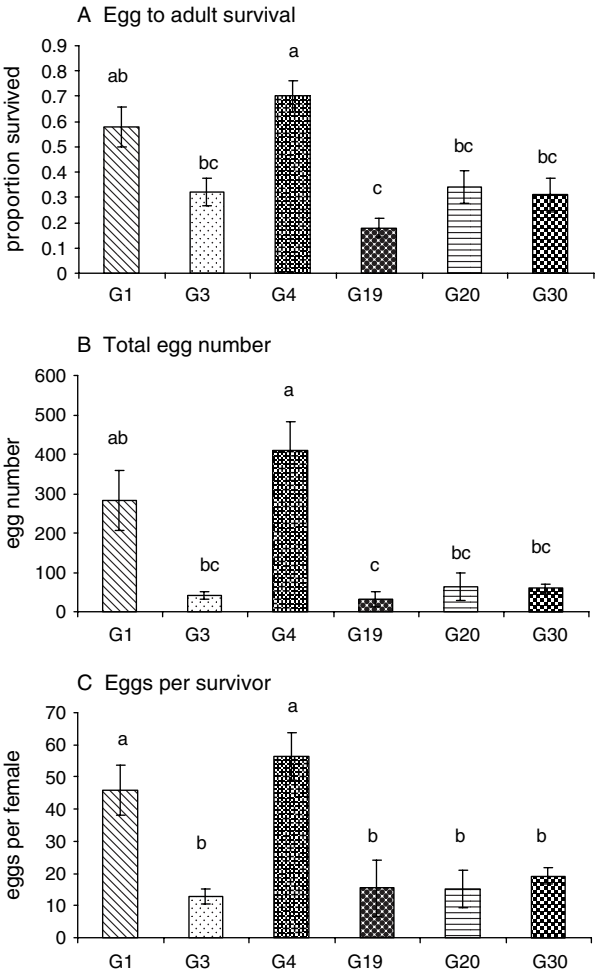

Fig. 1. Means of (a) proportion of eggs that survived and developed to adulthood for phylloxera lineages (G1, G3, G4, G19, G20 and G30), (b) the number of eggs produced on ungrafted Sultana in the first root assay and (c) number of eggs produced per survivor. Standard error bars are shown and letters indicate significant differences by *post hoc* tests.

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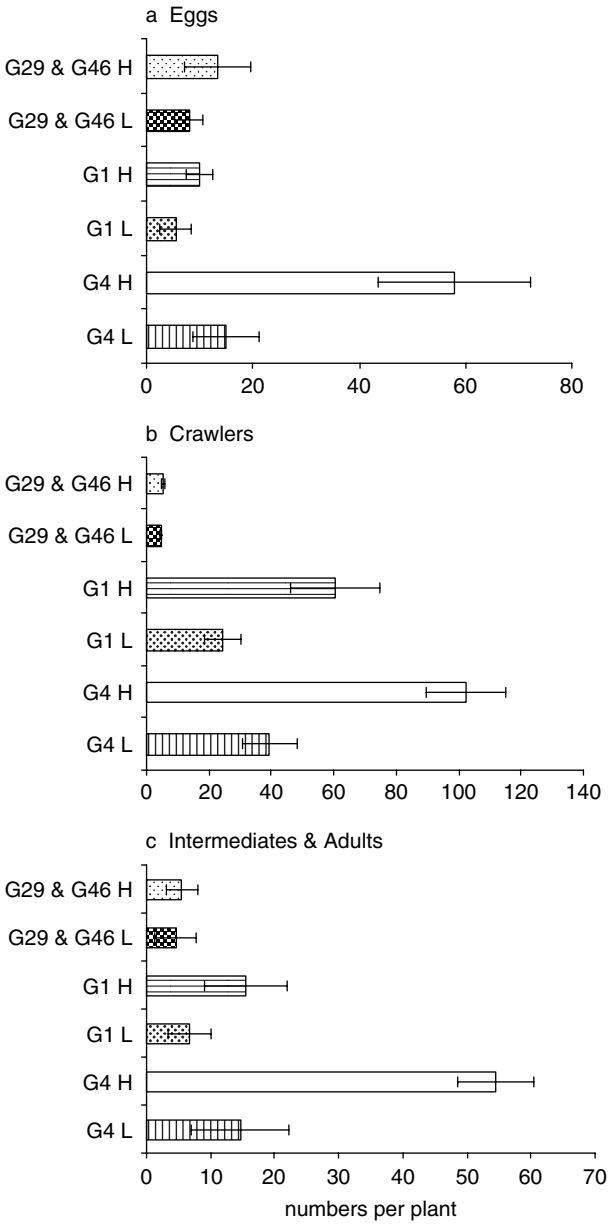

Fig. 2. Mean life stages (per gram oven-dried (OD) root weight) for different phylloxera genotypes and two infestation rates (L, low; H, high) on *V. vinifera*.

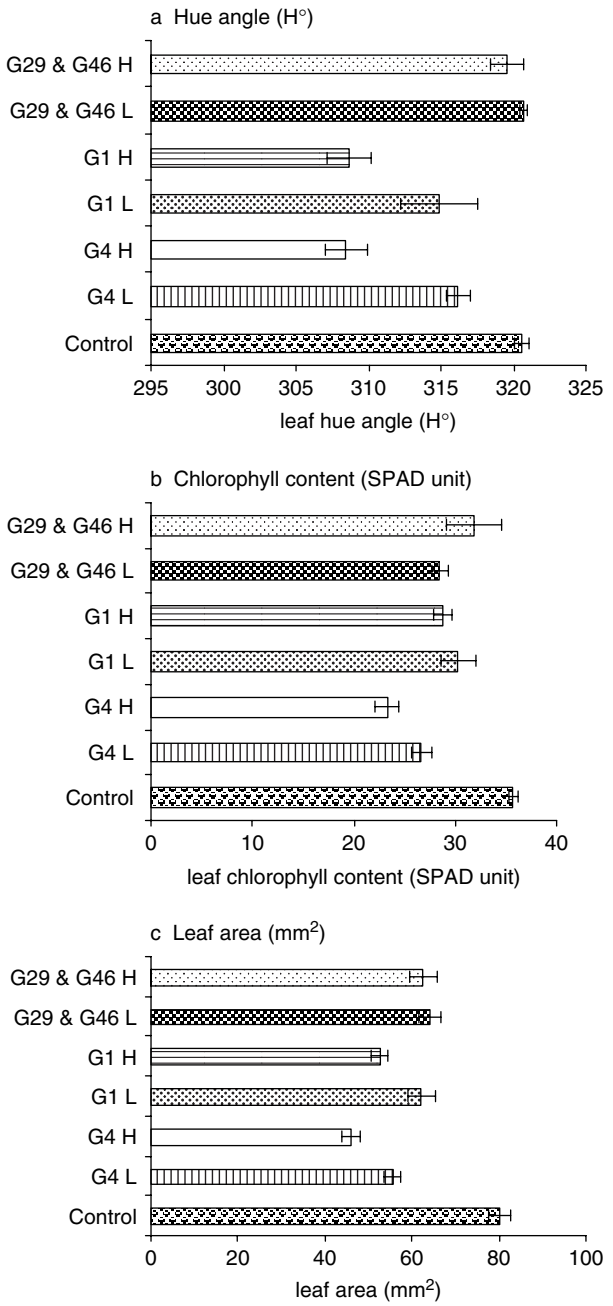

Fig. 3. Leaf assessments of (a) hue angle, (b) chlorophyll content and (c) total leaf area, following infestation of different phylloxera genotypes at two infestation levels (L, low; H, high). The 'control' treatment represents vines that were not infested with phylloxera.

G29 + G46 treatments, as well as between the controls and all phylloxera treatments (fig. 3). Phylloxera infestations, therefore, influenced leaf area, leaf colour and, to a lesser extent, chlorophyll content; and effects were more evident when plants were infested with G1 and, particularly, G4 than with the other combined clones.

Vine assessments

There were highly significant differences among treatments for the vine measures of root mass ($F = 5.169$; df = 6, 17; $P = 0.003$) and stem weight ($F = 6.475$; df = 6, 17; $P = 0.001$), but differences for trunk diameter, node length or node number were not significant after correction for multiple comparisons ($P > 0.03$ in ANOVAs). For root mass, there was a significant effect of clone ($F = 12.27$; df = 2, 12; $P = 0.001$) and also infestation ($F = 12.61$; df = 1, 12; $P = 0.004$) when only infested vines were considered. Root mass was highest for the G29 + G46 (low infestation) treatments, followed by the control, G4 and G1 treatments, respectively (fig. 3), although

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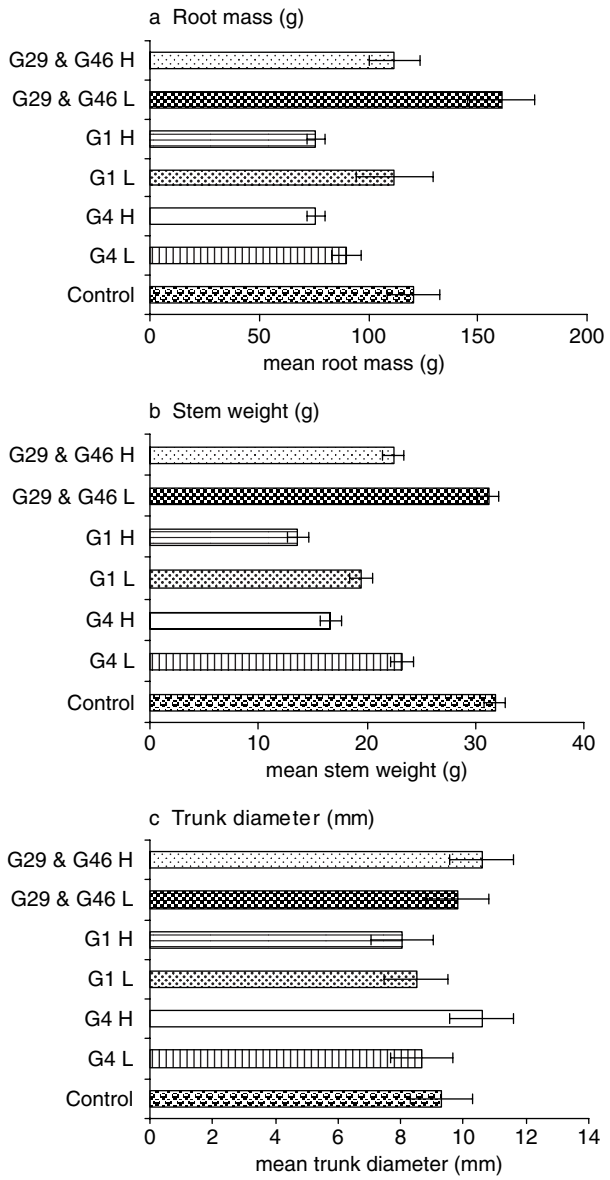

Fig. 4. Vine assessments of (a) mean root mass, (b) mean stem mass and (c) trunk diameter, following infestation of different phylloxera genotypes at two infestation levels (L, low; H, high). The 'control' treatment represents vines that were not infested with phylloxera.

only the G4 (high and low) and G1 (high) treatments differed from G29 + G46 in *post hoc* tests. Marked levels of root decline were particularly evident for the G1 (high infestation) and G4 treatments. For mean stem mass, effects of clone ($F = 4.93$; df = 2, 12; $P = 0.027$) and infestation level ($F = 6.69$; df = 1, 12; $P = 0.024$) were not significant after correction for multiple comparisons. In *post hoc* tests, controls did differ from high infestations of G1 and G4. For trunk diameter, there was a significant effect of clone ($F = 9.43$; df = 2, 12; $P = 0.002$) when infested vines were considered. Vines infested with G1 (high infestation) tended to have a reduced trunk diameter compared with other clones (fig. 4). Clone and infestation effects did not significantly influence node characteristics.

Discussion

The assays showed differences between the phylloxera clones with respect to their ability to survive on *V. vinifera* cv. Sultana. There were marked differences between clone lineages, with G1 and G4 outperforming all other phylloxera lineages in the excised root assay. These patterns were further supported by data collected from the glasshouse assay and highlight that G4, and to a lesser extent G1, perform well on ungrafted *V. vinifera* vines and cause physiological deterioration of the vines. Consequently, damage levels within vineyards of *V. vinifera* are likely to be more apparent when infested by these two clones.

These results help explain the distribution of both G1 and G4 phylloxera clones in south-eastern Australia. G1 or G4 are the most common genotypes and are found across several regions where ungrafted rootstocks are planted (Corrie *et al.*, 2003). These lineages appear to be the most invasive because they are found in the most recently infested vineyards in the King Valley (G4), Upton (G1), Murchison (G1), Lancefield (G1) and several outbreaks in the Yarra Valley (G1), all in the state of Victoria (Umina *et al.*, 2007; Herbert *et al.*, 2008; Powell, 2008). In contrast, other genotypic lineages are mostly confined to three regions, Rutherglen, Glenrowan and Milawa, with the Rutherglen region containing the greatest amount of genetic diversity (Corrie, 2003).

In the Rutherglen region, ungrafted vineyards have been infested for greater than 40 years with no apparent visible symptoms or yield loss. In contrast, newly infested vineyards may become commercially uneconomic after only 12 years (e.g. Herbert *et al.*, 2006). G1 and G4 are found at some vineyards in Rutherglen (Corrie *et al.*, 2003; Umina *et al.*, 2007). It is unclear why G1 and G4 infestations have failed to kill the ungrafted vines in this region. Factors like soil conditions might have an impact on phylloxera effects (Powell *et al.*, 2003; Reisenzein *et al.*, 2007; Bruce *et al.*, 2009), or perhaps there are interactions among the phylloxera genotypes.

Results from excised root assays will often differ to findings from assays involving whole vines. The excised root assay measures the physiological interaction between an insect and an isolated root piece. Vine resistance, tolerance and susceptibility may involve responses from other vine components (Kellow, 2001). Nevertheless, the results from the whole vine assays here support the findings of the excised root assays. The whole vine assay also has the advantage of being able to assess how grape phylloxera influence vine health. Classical symptoms of grape phylloxera-associated decline are first seen as decreased cane growth, followed by premature leaf yellowing, potassium deficiency, root system galling and decay, and increasing yield decline as the overall vine health deteriorates (Granett *et al.*, 2001). Differences in leaf and stem parameters, such as hue angle, leaf area and chlorophyll content were observed in the current glasshouse study over a time period of eight months, suggesting rapid declines when vines are infested with either G1 or G4. These findings suggest that leaf assessments, in particular, have the sensitivity required to detect subtle changes in vine physiology that precede the classic phylloxera visual symptoms that typically take at least two to three

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years to manifest (Herbert *et al*., 2003). Leaf assessments have previously been used to detect the presence of phylloxera and might be developed into an effective monitoring tool (Tucker *et al*., 2007). In contrast, node and trunk assessments appear less sensitive to phylloxera damage and for detecting differences among clones.

Early detection studies focussing on changes in leaf physiology and chemistry may have the potential to distinguish phylloxera infested vines from healthy vines even when obvious classic vine symptoms are absent. The data presented here indicate that physiological changes in vines reflect levels of infestation by phylloxera. Research by Blanchfield *et al*. (2006) on the potential of high spectral resolution spectroscopy analysis to detect very subtle 'pre-visual' differences in leaf chlorophyll and photoprotective pigment content have shown that chlorophyll levels are an important indicator of phylloxera presence on roots.

Currently, the most widely adopted method of testing phylloxera responses to vines involves excised root bio-assays, but these over-estimate grape phylloxera virulence and under-estimate rootstock resistance (Granett *et al*., 2001). The whole-vine assay used in this study may provide an alternative means of testing resistance levels, providing quantitative information on both insect fecundity as well as damage levels of grape vines. This approach could provide a useful system for testing the resistance of *Vitis* spp. to representatives of the full range of phylloxera types known to be present in vineyards. The present data indicate that phylloxera lineages differ in their performance on *V. vinifera* in whole vine assays. These differences match results from excised root assays and are consistent with patterns of damage by clone lineages in the field.

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