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Research Article

Performance of a Leaf-Galling Phylloxera (*Daktulosphaira vitifoliae*) on Roots of Diverse *Vitis* spp. Rootstocks in North East Victoria, Australia

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Background and Aims. Grape phylloxera in Australia comprises diverse genetic strains that feed on roots and leaves of *Vitis* spp. The G38 phylloxera strain was detected on roots of *Vitis* spp., for the first time in North East Victoria in 2015. Prior to 2015, G38 phylloxera was only known to feed on leaves. The aim of this study was to evaluate the survival and development of G38 phylloxera on roots of diverse *Vitis* spp. under field, controlled laboratory, and greenhouse conditions. *Methods and Results*. In the field, emergence traps quantified first instars and alates emerging from roots of diverse rootstocks and *Vitis vinifera* L. High numbers of phylloxera were collected in traps placed at vines of rootstocks 101-14, 3309 Courderc and Schwarzmann. Nodosity were also observed on roots of 101-14, 3309 Courderc and Schwarzmann in the field and in-pot vines experiments. The better performance of G38 phylloxera on these three rootstocks compared to *V. vinifera* in the field and in potted vines parallelled the excised roots experiments. *Conclusions*. The relatively high performance of G38 phylloxera on the 101-14, 3309 Courderc and Schwarzmann rootstocks suggest a susceptible response and could be associated with rootstock parentage. Further investigation is warranted to determine implications for rootstocks development. *Significance of the Study*. These findings are fundamental for decision-making in phylloxera risk assessment and rootstock selection. The study reaffirms the need for triphasic (in vitro, in planta, and in-field) rootstock screening protocols for phylloxera.

1. Introduction

Grapevine phylloxera, *Daktulosphaira vitifoliae* (Fitch), is a pest of major economic significance to viticulture worldwide. Phylloxera feed on roots and leaves of *Vitis* spp. [1, 2]. The phylloxera root-feeding form, called the radicicole is typical on ungrafted cultivated varieties of *Vitis vinifera* (L.) ssp. *Sativa* throughout the world. On young nonlignified root tips, phylloxera root feeders induce hooked galls (called nodosities) and malformed swellings (called tuberosities) on older lignified roots. Nodosities and tuberosities are a source of nutrition and sites for oviposition and larval development [3–5]. Individual insects attach at feedings sites and develop through four intermediate stages to adults that lay up to 400 eggs [6]. Nodosities impede root growth and tuberosities cause splits on the roots. The splits lead to decay because of secondary fungal infections eventually causing vine death [7, 8]. The leaf-feeding form, called the gallicole, not only feeds on leaves of North American *Vitis* species [2, 9] but can also attack leaves of *V. vinifera* [1, 10–12].

The phylloxera life cycle is complex and varies in different viticultural environments. High genotypic diversity and reproduction that is predominantly asexual have been revealed in *D. vitifoliae* populations in Europe [3] and Australia [13–15]. In Europe, separate introductions from the North East coast of North America can be traced back to

two genetic groups of the dominant American species *V. riparia* and *V. labrusc*a [16]. A significant deviation from Hardy–Weinberg equilibrium suggests that parthenogenesis is the primary reproductive mode of reproduction for Californian *D. vitifoliae* populations [17]. The asexual females feed on either roots (radicicole) or leaves (gallicole). During the growth season, gallicoles and radicicoles undergo several asexual generations where the parthenogenetic females lay several hundred eggs [3, 18, 19]. The first instars are mobile and move to the leaves and/or roots where they establish new feeding sites on growing shoots or young root tips. Radicicoles overwinter as first instar nymphs under the bark of roots [18, 20] and can give rise to winged adults (called alates) that emerge from the ground to disperse and find new feeding sites.

In Australia, management of phylloxera is achieved in part through quarantine [21]. For long term management, North American *Vitis* spp. that have coevolved with phylloxera are broadly used due to host responses that resist or tolerate infestations [2, 21]. Lack of complete resistance and, in some cases, potential breakdown in resistance limits the uptake and durability of rootstocks. Studies in Europe and the USA indicate that biotype-C phylloxera is adapted to feeding on rootstocks with the *riparia* parentage, including the Teleki rootstocks, 101-14 and Schwarzmann [22]. As a result, vineyard productivity in some regions may be lower where biotype-C populations predominate. To date, several phylloxera resistant loci have been identified in the Borner rootstock [23], *V. cinerea* C2-50 [24], *Muscadine rotundifolia,* and a complex hybrid [25]. DNA-markers linked to these phylloxera resistance loci can now be used to breed durable resistant rootstocks harbouring two or more resistant loci [26]. Aside from phylloxera resistance, rootstocks are also used to counteract other soilborne pests, such as nematodes, as well as maintain vine productivity in response to abiotic stress, soil pH, and porosity [27].

Phylloxera resistance or tolerance attributes of rootstocks is determined by insect genotype-plant interactions [28, 29]. On tolerant rootstocks, phylloxera can feed and then reproduce and maintain a population for several years without causing yield loss or vine death [30]. Previous rootstock screening studies in Australia have focused on six root-galling phylloxera strains using a triphasic screening approach [28, 31]. This encompasses a suite of protocols that integrate laboratory (excised roots) and greenhouse (potted vines) trials to screen new and existing rootstocks for phylloxera resistance [1, 21] and to analyse the ability of phylloxera to survive and develop on rootstocks [1, 32]. Small-scale field trials in commercial vineyards are conducted where possible [22, 33, 34]. Together, this information is incorporated into management decision-making tools, such as the Grapevine Rootstock Selector, to provide information that enables growers to select rootstocks that are resistant or tolerant to both phylloxera and root-knot nematodes [35].

In Australia, phylloxera has been established in the North East Victoria Phylloxera Infested Zone (PIZ) since the early 1900s [13, 14, 36]. Most vineyards are infested by parthenogenetic lineages that feed on roots, and there is insufficient evidence to support the classical life cycle [37]. Gallicoles predominate on *Vitis* spp. in North America, Europe, and South America [3, 19, 38, 39]. In Australia, gallicoles have only been observed infrequently in the North East Victoria Phylloxera Infested Zone (PIZ) [37, 40]. The winter egg has been recorded only once in Australia [41], and it is not clear how the leaf-galling populations are sustained from year to year. Corrie and Hoffmann [37] found some genotypes of phylloxera on leaves and roots with evidence that those on roots arose from sexual reproduction in leaves. Eighty-three diverse phylloxera genetic strains were characterised in the early 2000s, of which 49 were identified as root feeders, 23 leaf feeders, and 11 that feed on both leaves and roots [14, 42]. Recent extensive surveys on vine roots conducted in the King Valley region of the North East Victoria PIZ, where only G4 phylloxera strain had been reported [13], identified 32 newly characterised genetic strains [15].

This publication reports on a case study conducted over three consecutive years (2015–2018) in a commercial vineyard named BGM located 274 km north-east of Melbourne, Australia in the North East Victoria Phylloxera Infested Zone (PIZ) [36]. This site was of interest because of the detection of G38 phylloxera strain previously reported only as leaf-galling under field conditions. The G38 phylloxera was first characterised from a leaf-galling sample that was collected near the Warby Ranges and Mount Glenrowan south east Australia on the AxR#1 rootstock [13, 37, 43]. The results of that study implied that G38 could be the outcome of sexual reproduction between individuals of G2 and G3 phylloxera that were sampled from both leaf and root [43].

The vines in this study site were planted in 1988 on uncultivated soil without a previous history of grapevine cultivation. The vines used in this study were in four rows, planted in a randomised complete block trial in which the plots were *V. vinifera* on own roots and grafted to different rootstocks. In the summer of 2015, the vines were observed to have yellowing leaf symptoms comparable to phylloxera infestations for the first time. Root inspection revealed nodosities and yellow clusters similar to phylloxera colonies on several rootstock cultivars and *V. vinifera*. The insects from the roots were collected for morphological and molecular identification. They were confirmed as phylloxera and characterised as the G38 genetic strain using six nuclear DNA microsatellite markers [14, 44]. No leaf galls were observed on the canopy of vines. The detection of the G38 phylloxera strain on roots at a site with existing replicates of *V. vinifera* vines on own roots or on different rootstocks offered a serendipitous opportunity to study the survival and development of the G38 phylloxera strain under field conditions and subsequently under controlled environments using insects collected from the BGM vineyard.

2. Methods

2.1. Insect Stock Cultures. In February 2016, adults, eggs, and first instars of the G38 phylloxera strain were randomly collected from roots of grafted rootstocks and *V. vinifera* vines in the BGM vineyard described in the introduction.

The insects were collected from four rows where the G38 phylloxera strain was first detected. Several single adult lineages of the G38 phylloxera were cultured and established in the laboratory under controlled conditions (25 ± 2°C; 12 h L : D). The genetic lineage of the insects was confirmed as the G38 phylloxera strain using six nuclear DNA microsatellite markers as per methods by Umina et al. [14] and Agarwal et al. [44] before the cultures were multiplied.

To produce a stock culture for laboratory and glasshouse experiments, insects were maintained under quarantine and mass reared on excised roots of *V. vinifera* cv. Chardonnay as per methods by Kingston [45].

2.2. Description of Vines in Four Rows at BGM Vineyard Used for In-Field Assessments. The four rows of vines used for in-field assessments (Table S1) were established in 1991 from callused rootstock cuttings of the varieties Schwarzmann, 101-14, 3309 Courderc, 125AA Kober, 1103 Paulsen, 5A Teleki, SO4, R99 and Sori and callused cuttings of own root Cabernet Sauvignon (clone LC10), were established through 600 mm wide polythene film in 1991. The rootstock cuttings were sourced from the Murray Valley Vine Improvement Association; and the Cabernet Sauvignon (clone LC10) were sourced from a local vineyard in North East Victoria. In the following season the rootstocks were subsequently field grafted to Cabernet Sauvignon (clone LC10) and trained up to form a bilateral cordon. The own rooted Cabernet Sauvignon vines were also trained up in the same season to the equivalent system. Both the Cabernet grafted rootstock vines and the own rooted Cabernet Sauvignon vines were reworked to Sangiovese (clone MAT 7) in the spring of 2005.

2.3. Plant Material Used for Laboratory and Glasshouse Experiments. Vines used for controlled glasshouse and laboratory experiments were sourced as dormant cuttings from the Yalumba Nursery, Nuriootpa, South Australia which is in a Phylloxera Exclusion Zone and free from phylloxera. The cuttings were planted in 4.5L plastic black pots using heat sterilised 80% general purpose potting mix (Spotswood Potting Mixes and Fertilisers, Yarra Glen, Victoria) and 20% perlite (Peards Nursery, Albury, NSW) and fertilised with 3.5 g Osmocote™ per pot. The vines comprised the following varieties; *V. vinifera* (own rooted Pinot Noir), Ramsey (Pinot Gris), Schwarzmann (Saperavi), 101-14 (Malbec 1056 FSAC), 3309 Courderc (Cabernet Sauvignon), 5BB Kober (Pinot noir), 110 Richter (Shiraz), 1103 Paulsen (Cabernet Sauvignon), 140 Ruggeri (Shiraz), Börner (Shiraz), and 420A (Shiraz) (Table 1). Once planted, vines were kept in a shade house and drip irrigated for two min daily over 12 months to allow optimal root development before inoculating with G38 phylloxera eggs from stock cultures.

2.4. In-Field Assessment of G38 Phylloxera

2.4.1. Emergence Trapping. The development of the G38 phylloxera on diverse *Vitis* spp. was assessed under field conditions on four rows of vines at the BGM vineyard (Table 1). The assessment was conducted using cylindrical 4-litre durable clear plastic containers (Décor™) emergence traps that were installed at vines as per methods by [48]. To install the traps, a container was placed on levelled ground at approximately 10 cm from the base of trunk on each of the experimental vine and secured with metal tent pegs.

Trapping commenced over the vegetation period from mid-Spring until mid-Autumn (November–April) of 2015/2016 following the first detection of phylloxera on roots of vines in the vineyard and continued for three subsequent summer seasons (2015/2016, 2016/2017, and 2017/2018) during phylloxera peak activity [49]. Vines on which traps were placed were randomly selected from four rows of a block of mixed rootstock and own rooted vines (*V. vinifera*) (Figure 1). One trap was placed per vine. Eight to ten replicate vines were selected for each rootstock cultivar and *V. vinifera*. Ten *Vitis* varieties were selected for the study (Table S1).

Emergence trap samples were collected once every month over the three seasons. Sample collection involved rinsing the traps condensate with 80% ethanol and pouring contents into sampling containers. The traps were thereafter rinsed with tap water and replaced. Trap samples were transported to a quarantine laboratory at Agriculture Victoria, Rutherglen. The samples were observed under a low power dissecting microscope and first instars and alate adults found in each trap were quantified (Table S2). Total number of first instars and alates in traps over the three years were summed up for each *Vitis* spp. cultivar.

2.4.2. Root Inspection–Confirming Presence/Absence of Phylloxera on Vines Roots. Insects may have been caught in traps placed on resistant vines through the movement of first instars along the roots from nearby susceptible vines. Roots were, therefore, visually inspected to record the presence or absence of phylloxera life stages from a sample of vines where traps were placed for each *Vitis* spp. Visual detection of adults with eggs and 2^{nd}–4^{th} instars intermediate stages confirmed the successful development and completion of an asexual generation (Table S3). To conduct the visual assessment, a quantitative destructive technique was used. A dozen lignified and nonlignified root sections (5–30 cm in length) per vine were dug up to a depth of 5–20 cm and roots and soil samples were collected. Roots of eight replicate vines per rootstock type and *V. vinifera* on own roots were visually inspected. The samples were transported to a quarantine laboratory at Agriculture Victoria, Rutherglen where they were inspected for presence of phylloxera life stages with the aid of a low power dissecting microscope. Visual leaf symptoms noted as chlorosis on leaves were observed at veraison on experimental vines during the third year of study (Figure S1).

2.5. Performance of G38 Phylloxera in Potted Vines. The development of G38 phylloxera on diverse *Vitis* spp. was studied using potted vines. The experiment used a completely randomised block design with eight replicate vines

Table 1: Description of *Vitis* spp. used to study the survival and development of the G38 phylloxera.

Vine variety	Names of breeder (year*)	Country of origin	*Vitis* parentage	Method and resistance rating		
				In-field	Potted vine	Excised roots
Vitis vinifera	Domesticated	Mediterranean	Own root	Susceptible	Tolerant	Tolerant
101-14	Millardet and de Grasset (1882)	France	*V. riparia* × *V. rupestris*	Susceptible	Susceptible	Susceptible
Schwarzmann	Schwarzmann (1891)	Czech republic	ND × *V. riparia* "*Gloire de Montpellier*"	Tolerant	Tolerant	Tolerant
3309 Couderc	Couderc (1881)	France	ND	Tolerant	Tolerant	Tolerant
1103 Paulsen	Paulsen (1895)	Italy	*V. berlandieri* '*Rességuier 2*' × *V. rupestris* "*du Lot*"	Resistant	Tolerant	Resistant
140 Ruggeri	Ruggeri (1896)	Italy	*V. berlandieri* "*Boutin*" × *V. rupestris* "*du Lot*"	NT	Resistant	Resistant
110 Richter	Richter (1902)	France	*V. berlandieri* "*Boutin*" × *V. rupestris* "*du Lot*"	NT	Resistant	Tolerant
Richter 99	Richter (1902)	France	*V. berlandieri* "*Rességuier 2*" × *V. rupestris* "*du Lot*"	Resistant	NT	NT
5A Teleki	Teleki (1900)	Hungary	*V. berlandieri* "*Rességuier 2*" × *V. riparia* "*Gloire de Montpellier*"	Tolerant	NT	NT
SO4	Teleki and Fuhr (1896)	Germany	*V. berlandieri* "*Rességuier 2*" × *V. riparia* "*Gloire de Montpellier*"	Tolerant	NT	NT
125AA Kober	Teleki and Kober (1896)	Austria	*V. berlandieri* "*Rességuier 2*" × *V. riparia* "*Gloire de Montpellier*"	Tolerant	NT	NT
420A	Millardet and de Grasset (1886)	France	*V. berlandieri* × *V. riparia*	NT	Resistant	NT
5BB Kober	Teleki and Franz Kober (1896)	Austria	*V. berlandieri* "*Rességuier 2*" × *V. riparia* "*Gloire de Montpellier*"	NT	Resistant	Tolerant
Ramsey	Thomas Munson (1900)	USA	*V. candicans* × *V. rupestris*	NT	Resistant	Tolerant
Börner	Börner (1936)	Germany	*V. riparia* "*Gm183*" × *V. cinerea* "*Arnold*"	NT	Tolerant	Tolerant
Sori	Seeliger (1925)	Germany	*V. solonis* × *V. riparia*	Tolerant	NT	NT

*Maul and Töpfer [46]; McGovern et al. [47]; ND = not determined. NT =not tested. Vines used for in-field assessments are presented in bold text. Vines were classified from evidence presented in the literature cited as resistant if nodosities, tuberosities and phylloxera adults were absent; tolerant if nodosities were present with phylloxera life stages except for adults with eggs; susceptible if nodosities and/or tuberosities were present and, phylloxera survived to reproductive adult.

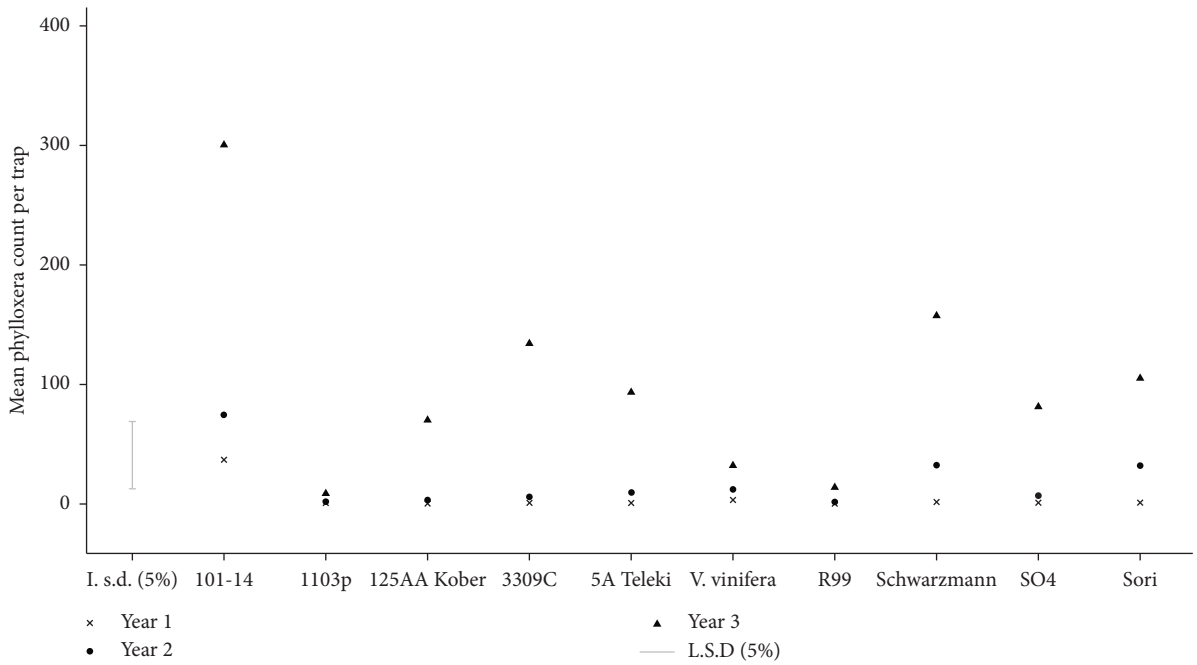

Figure 1: Mean numbers of G38 phylloxera (sum of first instars and alates) that were trapped from vines of nine rootstock cultivars and *V. vinifera* from 2015 to 2018. Least significant differences (L.S.D) between means ($p = 0.05$).

comprising of *V. vinifera* (susceptible control) and ten *Vitis* rootstock varieties. The rootstocks had various scion varieties and comprised of Ramsey (Pinor Gris), Schwarzmann (Saperavi), 101-14 (Malbec 1056 FSAC), 3309 Courderc (Cabernet Sauvignon), 5BB Kober (Pinor Gris), 110 Richter (Shiraz), 1103 Paulsen (Cabernet Sauvignon), 140 Ruggeri (Shiraz), Börner (Shiraz), and 420A (unknown) (Table 1). Sori and Richter 99 were not available for the potted trials. The vines were obtained from Yalumba nursery, Nuriootpa in South Australia.

Vines were infested with the G38 phylloxera as per methods by Forneck et al. [1] and Korosi et al. [31]. Briefly, the vines were removed from pots and a lignified root (5 mm in diameter) and surrounding fibrous roots enclosed in a muslin cloth. The roots were inoculated with 20 eggs of G38 phylloxera, and a small amount of potting mix immediately placed gently around the roots. The ends of the cloth were carefully tied with a cable tie to enclose the eggs, roots, and soil into a "root pocket." A sticky gum-based insect barrier, Tanglefoot™, was applied along the edges of the cloth to stop phylloxera from escaping and the vine repotted by topping up with a potting mix and perlite mixture. The root pockets were repotted with the rest of the roots and the vines thereafter fertilised with 3.5 g Osmocote™ and 500 ml Thrive™ per potted vine. The vines were maintained in the glasshouses at $22^{\circ} \pm 2^{\circ}$C and a relative humidity of 60–70% and drip irrigated for 2 min daily.

The experiment ran for eight weeks. Vines were removed from pots and the root pocket snipped to assess phylloxera development and damage on roots. Assessments were done under a low power dissecting microscope. The following parameters were calculated: (i) total insects summed up as the phylloxera developmental stages (eggs, first instars, intermediate instars, adults and alate); (ii) count of nodosities; and (iii) counts of tuberosities (Table S4).

2.6. Performance of G38 Phylloxera on Excised Roots. Roots of 10 *Vitis* varieties (*V. vinifera*, Ramsey, Schwarzmann, 101-14, 3309 Courderc, 5BB Kober, 110 Richter, 1103 Paulsen, and 140 Ruggeri and Börner) were cut into 4-5 cm lengths and placed in plastic Petri dishes (90 × 25 mm). Ten 1–4-day old eggs of G38 phylloxera were placed on a root piece using an artists' paint brush. A Petri dish with one excised root and 10 eggs was considered a replicate. Each treatment (rootstocks and *V. vinifera*) consisted of five replicates. Petri dishes were sealed with cling film and then aluminium foil to restrict light filtering through and maintained in the growth room at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Over the entire experiment, cotton wool on the end of the roots was saturated with 2 mL ultrapure water once every week to keep the roots hydrated and viable.

The number of eggs that hatched was recorded at day 8. First, instars that established feeding sites and subsequently survived to adults were recorded at days 18, 25 and 32. Adults were distinguishable from intermediates if they had eggs in proximity. Eggs that were laid on days 18 and 25 were counted and removed from the roots to discount for overlapping generations. Parameters were calculated as follows: (i) survival was calculated as the number of adults that developed from the initial eggs inoculated on roots; (ii) average fecundity was calculated as the number of eggs produced per adult per day after the first adult detection; (iii) gross fecundity was calculated at day 32 as the total eggs laid over the lifetime of all adults per root. This was the sum of

the number of adults in each root for each of days 18, 25 and 32; (iv) proportion of inoculated eggs reaching the adult stage was calculated as adults at day 32 out of total eggs inoculated on roots ($n = 10$) (Table S5).

2.7. Statistical Analysis. Generalised Linear Models (GLMs) with Poisson distributions were used to compare the total number of insects caught in traps over the three years across the *Vitis* spp. A GLM with Poisson distribution was used to compare trap catches over three years with the trapping year and rootstock fitted I the model. A GLM was also used to compare the total number of nodosities, and total number of insects (eggs, intermediates, and adults) recorded among *Vitis* spp varieties in pots, net and gross fecundity among excised root varieties. The proportion of eggs that reached the adult stage among excised root varieties was analysed using a GLM and model was fitted with a binomial distribution. All models were built in *R* using the "MASS" package and evaluated using loglikelihood tests in the "epiDisplay" package.

Tukey's *post hoc* tests with corrections were used to compare different treatments using the "lsmeans" package where our GLM model was significantly different to a null model as determined by the loglikelihood tests. All residual values were examined graphically to ensure normality and homogeneity of variances. Observations with standardised residuals greater than 3.0 were excluded from analyses.

For observations from roots, the number of phylloxera caught in traps over the three years was modelled using logistic regression, and the odds ratio was calculated. From the odds ratio, the probability of phylloxera reaching adulthood for each rootstock was calculated using the formula, $p = (e^z/1 + e^z)$ where p = probability and z is the relevant odds ratio [50]. The analysis was performed in GenStat 18th Edition [51].

For each trial type, phylloxera survival from eggs to adults on the vines was used to define rootstock resistance and susceptibility ratings. Rootstocks were rated resistant if neither insect nor nodosities and tuberosities were present on roots, tolerant if nodosities, first instars at low numbers (<5) and intermediate stages without adults were present on roots and susceptible if nodosities and tuberosities and all phylloxera life stages were present on roots.

3. Results

3.1. In-Field Assessments of G38 Phylloxera

3.1.1. Emergence Traps. Phylloxera was captured in emergence traps across all rootstock treatments during the study (Table 2). The GLM revealed that the total number of insects caught in traps was significantly different across the vine cultivars ($\chi^2 = 44503$; d.$f = 9$; $p < 0.0001$). Traps placed at the 101-14 vines consistently caught the highest numbers of phylloxera over the three years (Table 2). In comparison, less than 10 first instars were caught in traps collected from the 1103 Paulsen and Richter 99 rootstock vines (Table 2). There were no statistical differences between trap catches for Sori, Schwarzmann, 5A Teleki, 3309 Couderc, SO4, 125AA Kober, and *V. vinifera* (Table 2). Alates were caught in trap samples from *V. vinifera* and all rootstock cultivars except for 1103 Paulsen and Richter 99 (data not shown).

Table 2: Mean numbers (±standard error) of total G38 phylloxera (first instars and alates) caught in traps placed at vines of nine rootstock cultivars and *V. vinifera* over three consecutive years (2015, 2016 and 2017).

Rootstock	n	Mean total insects
101-14	9	137 ± 22c
Sori	9	46 ± 9ab
Schwarzmann	9	64 ± 17b
5A Teleki	9	35 ± 9ab
3309 Couderc	9	47 ± 16ab
SO4	9	30 ± 12ab
125AA Kober	9	25 ± 7ab
V. vinifera	10	16 ± 3ab
1103 Paulsen	9	4 ± 1a
Richter 99	9	5 ± 2a
d.f		44503
Chi square probability		<0.001

Means with different letters are significantly different (post hoc Tukey's test $p < 0.05$).

The number of insects captured in traps increased significantly over the three seasons following the initial detection of the G38 phylloxera and differences were attributable to vine cultivar ($\chi^2 = 3973$; d.$f = 29$; $p < 0.001$; Figure 1). Traps placed on vines with the 101-14 rootstock captured highest number of insects during the third year of the study, followed by Schwarzmann, 3309 Courderc, Sori, Teleki 5A, SO4, and 125AA Kober (Figure 1). Collectively across all the rootstocks and *V. vinifera*, phylloxera catches in traps were highest in December and April (data not shown), indicative of multiple generations per season.

3.1.2. Root Inspection–Confirming Presence/Absence of G38 Phylloxera Stages. We confirmed successful development from the egg to the adult stage of G38 on each rootstock cultivar. The G38 phylloxera successfully developed and reproduced on all rootstock cultivars except for Richter 99 (Table 3). Scions of *V. vinifera*, Schwarzmann, 101-14, Sori, 3309 Courderc, 125AA Kober, and SO4 showed yellowing symptoms consistent of those of phylloxera infestations by the third year of study (Table 3; Figure S1).

3.2. Performance of G38 Phylloxera in Potted Vines. There were no significant differences among rootstocks for the number of nodosities ($P > 0.05$; Table 4). Rootstocks typically had less than ten nodosities expect for 420A which developed no nodosities 8 weeks after inoculation with G38 phylloxera eggs (Table 4). Tuberosities on lignified roots were observed on Schwarzmann, Börner, 1103 Paulsen, 101-14, Ramsey, and 110 Richter (Table 4), and no statistical differences were observed between the rootstocks ($P > 0.05$; Table 4).

There were significant differences among rootstocks for the number of insects recorded in root pockets (d.$f = 10$; $p < 0.003$; Table 4). Successful development as determined

Table 3: Probability of G38 phylloxera survival (egg to adult with eggs) on roots of neighbouring rootstock cultivars and *V. vinifera.*

Rootstock type	Adults	Eggs	Intermediates
V. vinifera *	1.000	1.000	0.874
Schwarzmann*	1.000	1.000	0.799
101-14*	0.857	0.857	0.857
Sori*	0.799	0.799	0.599
3309 Courderc*	0.666	0.666	0.666
125AA Kober*	0.599	0.599	0.599
SO4	0.666	0.666	0.500
5A Teleki	0.334	0.334	0.334
1103 Paulsen	0.199	0.199	0.199
Richter 99	0.001	0.001	0.001

*Indicates scion-rootstock *Vitis* spp that showed yellowing due to phylloxera infestations by the third year of study. The odds ratio, estimated from regression analysis, was used to calculate each probability.

Table 4: Mean (±standard errors) of nodosities, tuberosities and total count of phylloxera (eggs, intermediates and adults) on roots of eleven *Vitis* spp ($n = 8$). The roots were contained in muslin cloth pockets and cocultivated with the G38 phylloxera for 8 weeks.

Rootstock	Nodosities	Tuberosities	Total count of insects
3309C	11 ± 4	0	3 ± 2ab
Schwartzmann	9 ± 3	8 ± 6	16 ± 8a
Vitis vinifera	8 ± 2	0	1 ± 1b
Borner	7 ± 6	4 ± 0	0b
1103 Paulsen	5 ± 2	6 ± 4	1 ± 1b
101-14	5 ± 2	3 ± 0	2 ± 2b
140 Ruggeri	3 ± 2	0	0b
Ramsey	2 ± 1	2 ± 0	0b
5BB Kober	2 ± 1	0	0b
110 Richter	1 ± 1	10 ± 0	0b
420A	0 ± 0	0	0b
d*f*	10	5	10
Chi square probability	<0.057	0.06	<0.001

Means with different letters are significantly different (post hoc Tukey's test $p < 0.05$).

by an asexual generation (egg to adult) of the G38 phylloxera was recorded in root pockets of 3309 Couderc, Schwarzmann, and 101-14 (Table 4). Eggs were recorded on Schwarzmann ($n = 21$), 3309C ($n = 5$) and 101-14 ($n = 3$) while no eggs were found on all the other rootstocks. The high count of eggs on Schwarzmann indicated a potential peak in generation during the eight weeks incubation period.

3.3. Performance of G38 Phylloxera on Excised Roots. On excised roots, the G38 phylloxera showed significant differences in survival on diverse rootstock cultivars. The proportion of eggs that reached the adult stage was 0–0.4 and differed among the rootstock varieties (GLM: $\chi^2 = 22$; d.$f = 9$; $p = 0.008$; Table 5). Survival of inoculated eggs to the adult stage also differed among vine cultivars (GLM: $\chi^2 = 221$; d.$f = 9$; $p < 0.001$; Table 5). Eggs inoculated on rootstock 101-14 had the highest proportion of eggs surviving to the adult stage (40%). Although at least 60% of eggs inoculated on 110 Richter, 1103 Paulsen, and 140 Ruggeri hatched, first instars neither established feeding sites nor developed to adulthood (data not shown). A single egg developed to the adult stage for the Börner, 5BB Kober, Ramsey and *V. vinifera,* and 3309 Courderc rootstocks (Table 5). The G38 phylloxera did not survive on rootstocks 110 Richter, 1103 Paulsen, and 140 Ruggeri (Table 5).

Average fecundity was affected by the *Vitis* varieties ($\chi^2 = 1278$; d.$f = 9$; $p < 0.001$; Table 5) and was highest on the 101-14 vines (Table 5). The average fecundity was below 10 egg/adult/day on Schwarzmann, *V. vinifera*, Ramsey, 5BB Kober, 3309 Courderc, and Börner vines (Table 5) and was associated with ow numbers (<2) of insects developing to reproductive adults on these *Vitis* spp. Only one first instar insect developed to an adult that reproduced a single egg on 1103 Paulsen, Börner, 3309 Courderc, 110 Richter, and 1103 Paulsen. Gross fecundity was also affected by the rootstock varieties (GLM: $\chi^2 = 9$; d.$f = 5701$; $p < 0.0001$; Table 5), with the G38 phylloxera multiplying nine-fold on 101-14 by day 32 (Table 5).

3.4. Susceptibility Rating. G38 phylloxera reproduced freely on 101-14 under field conditions, consistent with these vines being classified as susceptible to this strain (Table 1). The rootstocks Sori, Schwarzmann, 5A Teleki, 3309 Couderc, SO4, and 125AA Kober appeared to tolerant the G38 phylloxera strain. Traps placed on vines with rootstocks 1103 Paulsen and Richter 99 caught the least number of insects and thus appear to be resistant to the G38 phylloxera strain (Table 1). Furthermore, the probability of G38 phylloxera completing an asexual generation on 1103 Paulsen and Richter 99 rootstocks was negligible.

Neither nodosities nor reproductive adults were observed on potted vines of rootstocks 110 Richter and 1103 which further supports classification of these rootstocks as being resistant to G38 phylloxera (Table 1). Survival and completion of an asexual generation by the G38 phylloxera accompanied by nodosities and/or tuberosities upon root inspection were recorded for 3309 Courderc, Schwarzmann, Sori, and 101-14, with means being higher than those recorded for *V. vinifera* on own roots. Potted vines of these rootstocks are, therefore, likely to tolerate G38 phylloxera. Survival and reproduction of the G38 phylloxera on excised roots was greatest for rootstock 101-14 compared to the other nine rootstocks tested, consistent with this rootstock being classified as susceptible to strain G38.

4. Discussion

This study reports that G38 phylloxera, which is known to be a leaf-galling strain also occurs as root galling on diverse rootstocks and *V. vinifera*. This finding presented an opportunity to examine the performance of a phylloxera strain that feeds on both roots and leaves on diverse rootstocks, employing the triphasic screening approach, which utilises field trials together with more controlled laboratory and greenhouse trials. No leaf galls were observed on vines in the block under study during the three years. An important outcome from the field trial was that the rootstock cultivars, 101-14, 3309 Courderc, Sori, 5A Teleki, and Schwarzmann,

Table 5: Proportion of inoculated eggs that reached the adult stage, survival, average, and gross fecundity of G38 phylloxera on excised roots of ten *Vitis* cultivars.

Vitis species	Proportion of eggs reaching adulthood	Survival to adult	Average fecundity	Gross fecundity
101-14	0.43 ± 0.05a	4.2 ± 0.5a	23.4 ± 4.0a	98.3 ± 39.2a
Schwarzmann	0.15 ± 0.03b	1.5 ± 0.3b	9.3 ± 3.9b	19.6 ± 10.1b
V. vinifera	0.08 ± 0.04bc	0.8 ± 0.4cb	2.6 ± 1.2cb	4.5 ± 3.1b
Ramsey	0.06 ± 0.02bc	0.6 ± 0.2cb	2.8 ± 1.5cb	2.5 ± 1.9b
5BB Kober	0.06 ± 0.02bc	0.6 ± 0.2cb	1.5 ± 0.7cb	3.0 ± 2.0b
3309 Courderc	0.01 ± 0.01c	0.1 ± 0.1c	0.7 ± 0.5cb	0.9 ± 0.9b
Börner	0.01 ± 0.01c	0.1 ± 0.1c	0.4 ± 0.4c	0.6 ± 0.5b
110 Richter	0c	0c	0c	0b
1103 Paulsen	0c	0c	0c	0b
140 Ruggeri	0c	0c	0c	0b
d*f*	9	9	9	9
Chi square probability	0.008	<0.001	<0.001	<0.001

Data are means ± standard error of insects living on roots at days 18, 25, and 32. Means with different letters are significantly different (post hoc Tukey's test $p < 0.05$).

hosted relatively higher numbers of G38 phylloxera compared to *V. vinifera* on own roots, which is generally considered susceptible to phylloxera. This is the first record of G38 phylloxera on roots of these rootstocks.

Formation of galls and tuberosities was observed on some rootstocks under this study. Phylloxera survival is dependent on gall formation for nutrition and development of immature stages [49–56]. Rootstocks that showed a propensity to form galls, such as 101-14, Schwarzmann, Sori, and 3309 Courderc, could be a preferred food source favouring phylloxera reproduction. Subsequently, several generations may occur continuously with significant overlap in developmental stages, as evidenced by the trapping of alate stages. The production of alates suggests dispersive movement across rootstock vines that could be triggered by overcrowding or deteriorating root quality [2, 49, 57]. The dispersive movement of G38 phylloxera could explain why resistant rootstocks such R99 and 1103 Paulsen had positive trap samples yet neither nodosities nor reproductive adults were found when the roots were inspected. This observation suggests that some tolerant rootstocks, if planted near to those that are susceptible to strains such as the G38 phylloxera, could provide alternative food source, thus favouring the spread of phylloxera to neighbouring vines in blocks and vineyards.

Results from the three types of assays used in this study demonstrated that the rootstock 101-14 is susceptible to G38 phylloxera, and this phylloxera strain may be selected under diverse rootstock plantings [58–60]. Using excised root bioassays, the gross fecundity of G38 phylloxera was highest on 101-14 rootstock compared to the other nine *Vitis* varieties tested. Though excised root bioassays do not indicate a complete plant response, they are considered to be a suitable measure for phylloxera biotyping [55]. Results suggest that the G38 phylloxera possibly belongs to the biotype-C group, which is a classification that includes strains that show superior performance on nodosities on roots of rootstocks derived from American *Vitis* spp. such as 5C Teleki and 101-14 and 3309 Courderc and reduced ability to establish on *V. vinifera* roots [22, 61, 62]. Rootstocks that had very low populations, such as was observed in 1103 Paulsen, 140 Ruggeri, Ramsey, 5BB Kober, and 110 Richter using potted vines assays, may use hypersensitivity as a resistance mechanism [63]. The root response to phylloxera expressed as tissue browning, indicative that the oxidation of phenolic compounds and nodosities are unsuitable as feeding sites for developing nymphs [64].

Rootstocks with parentages that are susceptible to certain phylloxera types have caused enormous economic losses. For instance, the rootstock AXR#1 (*V. vinifera* × *V. rupestris*) succumbed to infestations of virulent phylloxera biotypes in California, causing loss in production of between US$1 billion and $6 billion [65, 66]. The failure of AXR#1 led to caution concerning the use of rootstocks with insufficient tolerance to phylloxera, especially those with parentages that are highly susceptible. In this study, several rootstock cultivars had the riparia parentage, but it appears that only those with *V. riparia* × *V. rupestris* were susceptible or tolerant to the G38 phylloxera. The 1103 Paulsen and Richter 99 both with *V. berlandieri* × *V. rupestris* parentage had low insect populations consistently across the three experimental methods and are potentially tolerant to the G38 phylloxera. Our findings corroborate those in California that have shown phylloxera biotypes adapted to feeding on rootstocks with *V. riparia* parentage, such as 101-14 and Schwarzmann and some Teleki hybrids [22, 67, 68]. There is evidence to suggest that two genes are involved in nodosity formation based on findings from a study of traits related to phylloxera susceptibility from the *V. vinifera* and *V. rupestris* hybrid AXR1 [69]. There is also a report of a hybrid of *V. riparia* and *V. cinerea* Arnold that shows high resistance to phylloxera [23].

The emergence of biotype-C genetic strains that are adapted to feeding on *V. riparia* rootstocks has been highlighted as a concern when selecting rootstocks due to the potential for resistance breakdown [24]. The ability of phylloxera strains to utilise rootstocks as a food source and their interactions with hybrids such as *V. riparia* in conferring resistance needs to be studied further to enable access

to suitable traits for rootstock breeding programs. Our understanding of phylloxera genetic diversity in Australia is limited by lack of extensive surveys, especially in regions where phylloxera has existed since the early 1900s, which could explain why G38 phylloxera may not have been found on roots before. Recent surveys in the King Valley region, however, found a higher phylloxera genetic diversity than was previously thought [15].

In the current study, Börner rootstock, a hybrid from *V. riparia* and *V. cinerea* "Gm183," was found to tolerate infestations by G38 phylloxera in potted vines and excised roots assays. Generally, Börner is considered to resist phylloxera infestations [23, 70, 71] and high level resistance has been demonstrated against some phylloxera genetic strains [21, 28, 72, 73]. This result is consistent with the findings of Powell [21] and Powell and Krstic [28] who found Börner to be tolerant to the root-galling G19 phylloxera strain under field assessments and tolerant to the G30 phylloxera strain *in vitro*. In the study of Powell and Krstic [28], Börner had higher numbers of G19 phylloxera in traps compared to other rootstocks and *V. vinifera* under field conditions. Since Börner is considered an attractive genetic resource for rootstock breeding due to its good grafting characteristics and adaptation to varied soil types [24, 72, 74], further studies are needed to confirm the contribution of *V. riparia* to incomplete resistance of plant accessions to some phylloxera strains such as the G38.

Screening for resistance of rootstocks to different phylloxera strains forms an essential component of rootstock selection and provides planting recommendations to the grape and wine industry. The three screening methods employed each have their disadvantages and advantages, such as ease of replication and/or applicability to the field, and differences in overall findings have been observed in the past when comparing the three screening methods [15, 28, 61]. In this study, rootstocks such as 1103 Paulsen and 110 Richter led to different findings when the results of the three trials were compared, implying that environmental conditions in the field could affect the response of these rootstocks to phylloxera. Potted vines always had optimal water content and relative humidity was high compared to likely conditions in the field. We did, however, observe necrotic nodosities on roots within the pockets, and this could contribute to restrict feeding due to deteriorating nutrition leading to insect mortality. We, therefore, recommend inoculating eggs on the roots of potted vines without the pocket enclosure. Furthermore, the G38 phylloxera was cultured on Chardonnay. The original feeding host could have influenced the ability of the G38 phylloxera to feed on different *Vitis* spp. varieties [75].

The reliance of the Australian grape and wine industry on a few popular rootstock varieties is of concern, as it is currently the only recommended management approach for phylloxera. For instance, growers favouring 101-14 risk having their rootstocks succumb to infestations by phylloxera genetic strains such as G38 phylloxera, which show relatively high virulence and preference for particular rootstocks. With the uptake of new rootstocks and growers preferring certain rootstocks over others, phylloxera tolerant vines—if widely planted—may inadvertently promote the spread of phylloxera feeding forms such as G38, which could spread quickly across certain rootstocks. Phylloxera strains such as G38, which exist as both root and leaf galling forms and show high levels of galling and fecundity on rootstocks, ought to be included as tests strains in screening trials that evaluate rootstock resistance. Future studies could explore whether situations exist in vineyards that might promote adaptive changes in feeding and phylloxera fitness. An improved understanding on these parameters would shape the grape rootstock breeding programs with the development of new rootstocks that are better adapted to situations in Australian vineyards. Selection of rootstocks with broad resistance to multiple phylloxera strains will also reduce the risk of phylloxera dispersal and hence strengthen quarantine.

5. Conclusion

The relatively high numbers of G38 phylloxera on rootstocks indicate that host preference is a survival mechanism with potential to increase the risk of phylloxera spreading to uninfested vineyards in Australia under poor containment practices. The introduction of unknown phylloxera lineages that feed on both leaves and roots thus presents a potential additional risk factor in the management of phylloxera. Management efforts in Australia should not only focus solely on the radicicole phylloxera forms but also the gallicole forms. The results from this study should initiate further investigations into the persistence of phylloxera strains that exist as both root and leaf because their impact on rootstocks is likely to be underestimated due to limited research. Human assisted and potentially natural dispersal of leaf-galling phylloxera forms between regions is a high risk for the industry and their management needs to be considered in existing quarantine protocols.

Data Availability

The data used to support the findings of this study are included within the supplementary information files.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

Supplementary 1. Table S1: Four rows of grafted rootstock and *V. vinifera* vines at the BGM vineyard located in the North East Victoria Phylloxera Infested Zone where field assessments were conducted. Emergence traps were placed at base of trunk of selected vines marked in bold font. Supplementary 2. Table S2: Counts of first instars and winged adults (alates) collected in emergence traps over three summer seasons (in 2015, 2016 and 2017). Supplementary 3. Table S3: Visual inspection of roots for phylloxera life stages on selected rootstocks and V. vinifera. Visual inspections were performed by digging and assessing roots for presence of eggs, first instars, and intermediate stages and adults (winged and wingless) in the 3rd year of study to confirm that the G38 phylloxera completed an asexual generation cycle on the vine at which traps were placed. Supplementary 4. Table S4: Counts of phylloxera eggs, first instars, intermediates and adults, nodosities and tuberosities eight weeks after infestation with the G38 phylloxera genetic strain using potted vine experiments. Supplementary 5. Table S5: G38 phylloxera living on excised roots of various rootstock varieties and *V. vinifera* on days 18, 25, and 32. Supplementary 6. Figure S1: Visual symptoms of rootstock vines that were monitored for G38 phylloxera infestations using bucket traps. Images were taken at veraison during the third year of study. (*Supplementary Materials*)

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