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Mealybugs Demonstrate Feeding Preference Differences Between Different Grapevine Varieties

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Summary

Rootstocks are almost universally used for grapevine because they provide protection against the root pest phylloxera. We are interested in other roles that rootstocks might provide, in particular the possibility of deterring mealybug feeding. Mealybugs feed by drawing sap from the plant's phloem tissue. While phloem feeding can weaken the plant, it is of particular concern because it efficiently enables the transmission of viruses. Mealybug feeding/survival was initially tested on eight grape varieties. Five varieties were then selected with varying responses and these were used to test mealybug feeding under a range of environmental and experimental conditions. Although the absolute numbers of insects surviving varied between treatments the ranking of the varieties remained constant. This information is now informing the development of a bioassay for mealybug feeding on grape. It is also being used to study the possible chemical basis of feeding deterrence in this material.

INTRODUCTION

European grapevine (*Vitis vinifera* L.) propagates readily by dormant hardwood cuttings. Given the large number of known varieties, their widespread distribution, and the mention of varieties in early writings, the striking of cuttings clearly dates back at least to the time of the Roman Empire and probably much further still. However, in the mid-1800s a series of pest and disease incursions struck European vineyards with devasting impact. The causal organisms were of American origin, introduced unintentionally by plant collectors who were gathering species from around the globe and bringing them to Europe for display and use. The full impact of these 'American Plagues' is unknown, but it is recorded that 40% of French vineyards died within a 10-year period, between 1863 and 1873 (Campbell, 2006).

Two foliar diseases: downy mildew (caused by *Plasmopara viticola*) and powdery mildew (caused by *Erysiphe necator*) and an insect pest called phylloxera (*Daktulosphaira vitifoliae* Fitch) proved to be the most difficult to control. Foliar sprays of sulfur and copper were subsequently found to control the mildew diseases, but the root pest phylloxera was largely immune to any applied substance. The solution to phylloxera control came with the discovery that some American grape species could be used as rootstocks, providing enough resistance to the pest that no other measure of control was required. The American species found to be resistant to phylloxera were *V. riparia*, *V. rupestris* and *V. berlandieri* (Munson, 1909). Although each species can be used as a rootstock alone, each also has limitations. *Vitis riparia*, for instance was easily grafted but not suitable for use on alkaline soils, which are common in Europe. Conversely, *V. berlandieri* grows well on alkaline soils but is hard to graft (Jackson, 2008).

In response, hundreds of crosses were conducted throughout the late 1800s, testing a wide range of species in different combinations to develop interspecific rootstocks that provided protection against phylloxera, as well as ease of propagation and good survival in a range of soil types. The rootstocks produced at that time are the same ones used today in most of the world. Phylloxera was first identified in New Zealand vineyards in 1902 by the notable viticulturist Romeo Bragato (Bragato, 1906). He immediately introduced the new European rootstocks. Subsequently, grafting became a standard practice for the establishment of commercial vineyards. Even today the main rootstocks used in New Zealand are Couderc 3309 (*V. riparia* x *V. rupestris*) bred in 1881, Schwarzmann (*V. riparia* x V. *rupestris*) bred in 1891, Millardet et De Grasset 101-14 (*V. riparia* x *V. rupestris*) bred in 1882, and *V. riparia* variety Gloire (https://www.vivc.de/index.php).

Our interest in grape rootstocks began with the observations that mealybugs appear to prefer some grape varieties more than others, and also that plants on different rootstocks vary in their rate of virus infection. Two mealybug species are common in New Zealand vineyards: the long-tailed mealybug (*Pseudococcus longispinus*) and the citrophyllus mealybug *(P. calceolariae*). Both feed by drawing sap from plant phloem tissue. While feeding can result in damage when infestation levels are high, a far greater concern is the transmission of viruses, mediated by this feeding behaviour (Petersen and Charles, 1997; Tsai et al.,

2010; Sandanayaka et al., 2013). In New Zealand, the virus of greatest concern is grapevine leafroll-associated virus 3 (GLRaV3). Infection by GLRaV3 leads to lost vigour, reduced yield and changes juice chemistry (Bell et al., 2021). GLRaV3 infection is incurable, so control options focus on the early detection of the disease, the replacement of diseased vines and insect vector control. The aims of the current study were to quantify differences in insect feeding on different varieties of grape, then to explore the potential metabolic basis for this phenomenon, with the long-term aim of developing mealybug-resistant rootstocks.

MATERIALS AND METHODS

Eight grape cultivars were used in the study: Cabernet Franc (the susceptible control), Malbec, *V. riparia* Riparia Gloire, Couderc 3309, Millardet et De Grasset 101-14, Schwarzmann, *V. labrusca x V. vinifera* Isabella, and Siebel 5437. Potted plants from each variety were propagated from dormant canes and maintained under glasshouse conditions until the vines contained a minimum of six leaves. One mealybug species was used, *P. calceolariae*, with insects sourced from a laboratory colony maintained on sprouting potatoes. *Pseudococcus calceolariae* was chosen as it is known to colonise both the root and shoot tissues of grape, while *P. longispinus* typically colonises grape shoots (Charles et al., 2006).

Mealybug feeding and survival

A longitudinal study was conducted to determine survival from neonate settlement through to sexual maturity. It also provided an estimate of the length of time required for mealybugs to complete their life cycle under the assay conditions, and to explore possible differences between a diverse set of grape varieties. The cultivars used for this experiment were: Cabernet Franc, Malbec, *V. labrusca x V. vinifera* Isabella, Siebel 4986 and Siebel 5437. An excised-leaf assay was used to facilitate the frequent counting of live insects. Individual leaves, removed from potted plants, were held in Perspex chambers with the leaf base immersed in a vial of water. Mealybug neonates were released onto greenhouse-grown leaves and the numbers of surviving insects recorded every 3–5 days. The detached leaves needed to be replaced every 3–4 weeks throughout the study period with the insects moved onto the fresh leaves on each occasion.

An attached-leaf, no-choice greenhouse study was then conducted to confirm the preference results observed in the detached leaf assay. The grape cultivars tested were: the rootstocks Millardet et De Grasset 101-14, Schwarzmann and Riparia Gloire; and the scion cultivars Cabernet Franc and Malbec. Ten healthy plants from each variety were chosen. Six leaves from each plant were then selected and labelled prior to the inoculation of mealybugs. The greenhouse was maintained at a minimum of 10°C and a maximum of 28°C, under natural lighting. An Eppendorf tube containing approximately 30, newly emerged first instar mealybugs was attached to the abaxial side of each individual leaf close to the base of the midrib with a small amount of Blu-Tack. Once attached to the leaf, the lid of the tube was opened to allow the mealybugs to find their food source and the leaf was then covered with a zip-lock fine mesh net bag (13 x 16 cm) (**Fig. 1**). Two weeks after the inoculation, the first infested leaf from the bottom of each grape plant was removed

from the stem and brought into the laboratory to examine the settlement and development of mealybugs. Accordingly, a mealybug infested leaf from the bottom of each grape plant was removed once a week to examine for mealybug development; 2nd leaf after 3 weeks from inoculation and 3rd, 4th, 5th and 6th leaves were removed after 4, 5, 6, and 7 weeks, respectively.

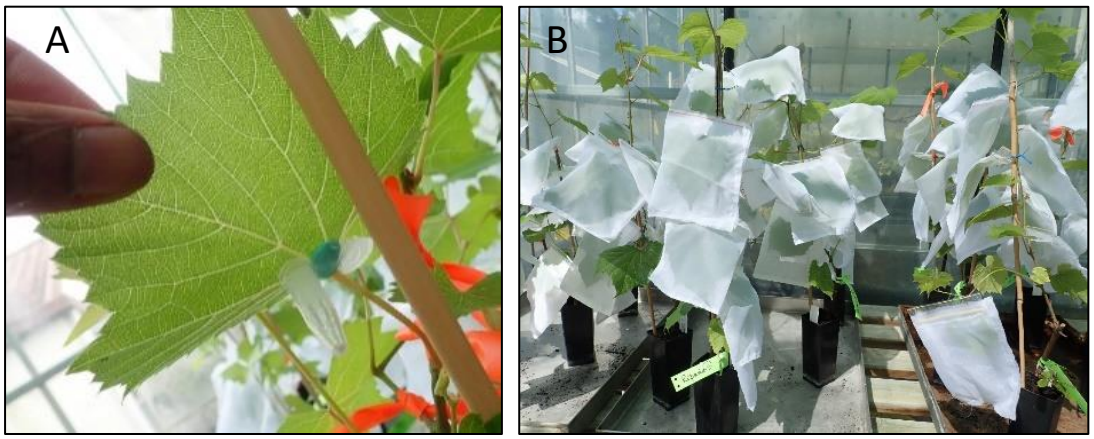

Figure 1. Handling of mealybugs in the greenhouse. A) Release of mealybugs onto a leaf. B) mesh bags used to contain the insects during the study period.

A further (no-choice) study of survival was conducted using field-grown plants to test whether the greenhouse study was also representative of field insect survival. Field grown plants of: Cabernet Franc (the susceptible control), Malbec, Millardet et De Grasset 101-14, and Schwarzmann were used. Plants of Riparia Gloire were unavailable for this study. Neonates were transferred to leaves within mesh bags on the trial plants and insect survival was assessed from the second week, for 7 weeks after inoculation.

To determine whether the insects would demonstrate a preference for different varieties when given a choice, plants of Millardet et De Grasset 101-14, Schwarzmann, Riparia Gloire, Cabernet Franc and Malbec were grown together within a mesh cage and neonate insects were introduced onto a paper platform that touched each plant. Insect colonisation of each variety was then assessed 4 and 8 weeks after inoculation.

A final study was performed using root tissues. Neonate insects were bound within a mesh bag, tied around a fleshy root on each replicate plant. The varieties used were Millardet et De Grasset 101-14, Schwarzmann, Riparia Gloire, Cabernet Franc and Malbec. Mealybug survival on the roots was assessed at a single timepoint, 12 weeks after inoculation.

Phytochemistry

To analyse whole leaf chemistry, leaves were collected from two sites (Lincoln field and Auckland glasshouse) and freeze dried. In addition, leaf surface waxes were extracted from the Lincoln vines. Duplicate extractions were analysed throughout the study. For the whole leaf samples approximately 10 mg Dry Weight of the sample was mixed per 1mL solvent and for the wax analysis 1 mg wax was mixed with 1mL

solvent. The phytochemistry was then analysed following ethanol extraction, followed by liquid chromatography–mass spectrometry (LCMS). A 2 µL aliquot of each prepared extract was separated with a mobile phase consisting of 0.1 % formic acid in type 1 water (A) and 0.1 % formic acid in acetonitrile (B) by reverse phase chromatography, maintained at 40°C with a flow rate of 400 µL/min. A gradient was applied: as 0-1 min/5%B, 7-10 min/95%B, 11-14min/5%B. The eluent was scanned from 1-11 minutes by API-MS (Orbitrap) with heated electrospray ionisation (HESI) in the negative and positive mode. Data were acquired for precursor masses from *m/z* 110–1200 amu at 70K resolution with data dependent ms/ms for product ions generated by normalised collision energy (NCE:30) at 17.5K resolution. Data were processed with the aid of Xcalibur®4.1 and Compound Discoverer 2.1. Each dataset was compared using Principal Components Analysis (PCA).

RESULTS

Insect Feeding Assays

The results of the longitudinal study of mealybug survival on different varieties are summarised in **Figure 2**, panel A. Mealybug neonates suffered significant losses immediately after transfer to the leaves of all the varieties tested but after the first week numbers stabilised. The highest survival rate was for the *V. labrusca* hybrid Isabel, indicating that this species is an unlikely source of mealybug resistance. The lowest survival rate was for the *V. vinifera* scion variety Malbec. On susceptible plants, at the time of settlement, the insects typically grouped together over a leaf vein (**Fig. 2, panel B**), while this was not seen on the more resistant varieties (**Figure 2, panel C**). At the end of the study (8 weeks after neonate transfer), male cocoons and fourth instar females were seen, indicating that 7–8 weeks of observations are required to follow development through the complete life cycle of these insects under the experimental conditions.

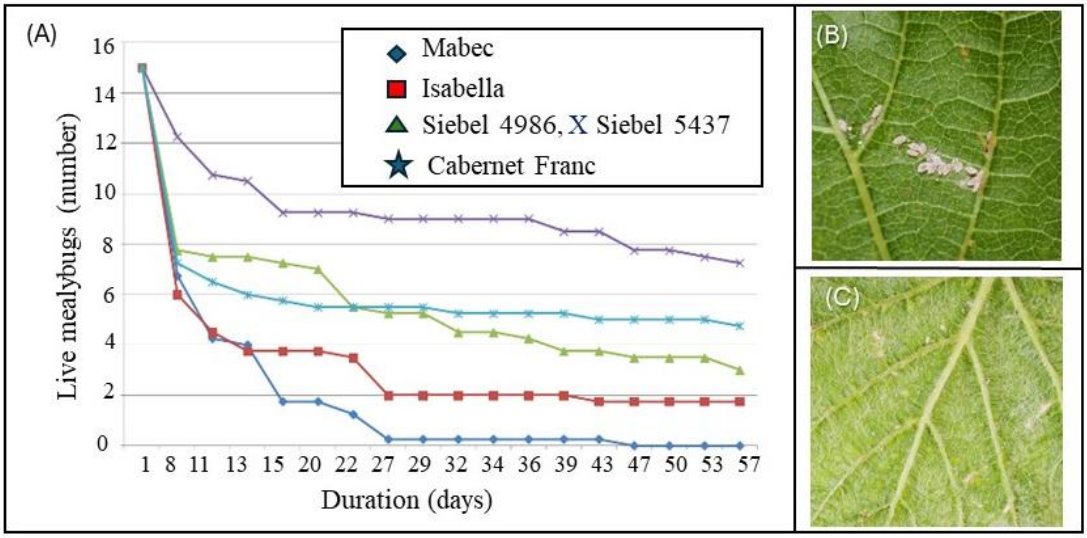

Figure 2. Mealybug survival on detached leaves. A) Longitudinal study of survival over 8 weeks. B) Mealybugs on susceptible variety Cabernet Franc after 4 weeks. C: Mealybugs on resistant variety Malbec after 4 weeks.

The results of the no-choice attached-leaf assay are summarised in **Figure 3**. At the 2-week time-point, no significant differences in mealybug settlement were apparent for the five varieties studied. However, after 7 weeks of observation, significantly more insects were surviving on the Cabernet Franc and Riparia Gloire plants than on Millardet et De Grasset 101-14, Schwarzmann, or Malbec.

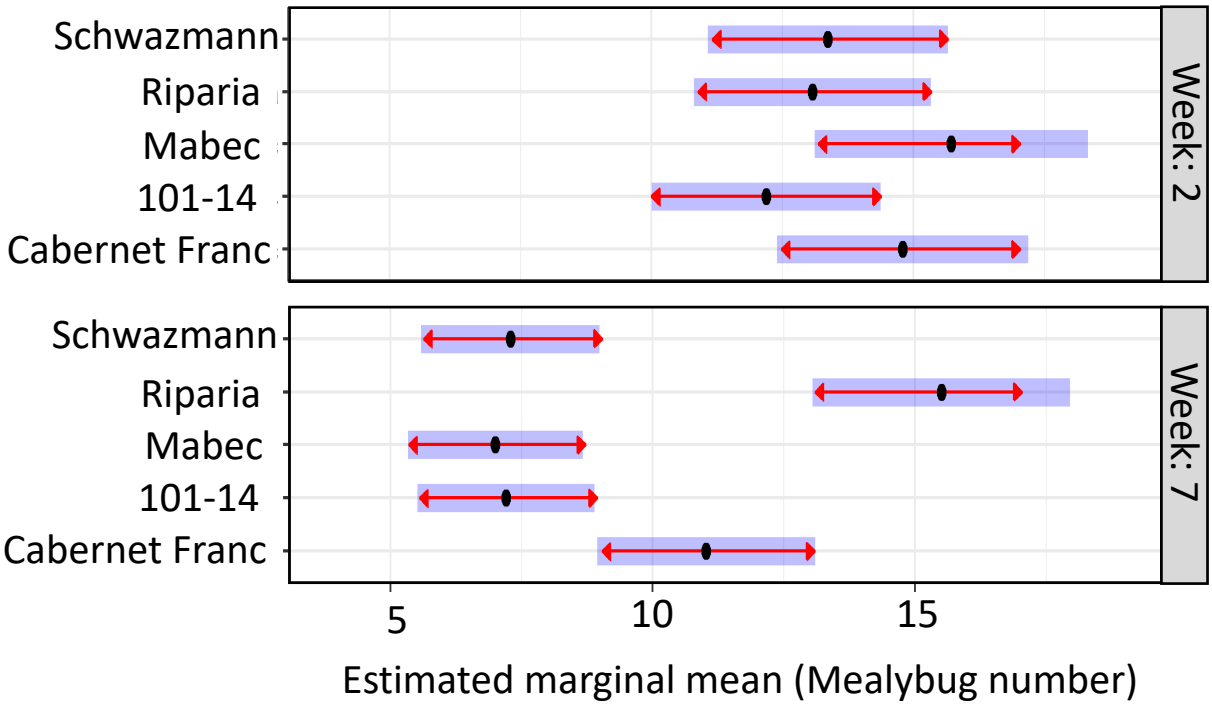

Figure 3. Attached leaf no-choice assay. Estimated means of mealybug numbers on leaves of five different grapevine rootstock varieties after 2 and 7 weeks from inoculation under no-choice conditions. The purple bars show confidence intervals for the estimated marginal means, and the red arrows are used for comparisons among them. If an arrow from one mean overlaps an arrow from another group, the difference is not significant.

Similar results were observed for the field-based no-choice leaf test **(Fig.4)**. Overall, mealybug survival on all varieties was lower in the field than in the glasshouse and the development rate of mealybugs in the field was slower. However, the rankings of the varieties were similar with insects having the highest rate of survival on Cabernet Franc and the lowest survival on Schwarzmann and Millardet et De Grasset 101-14.

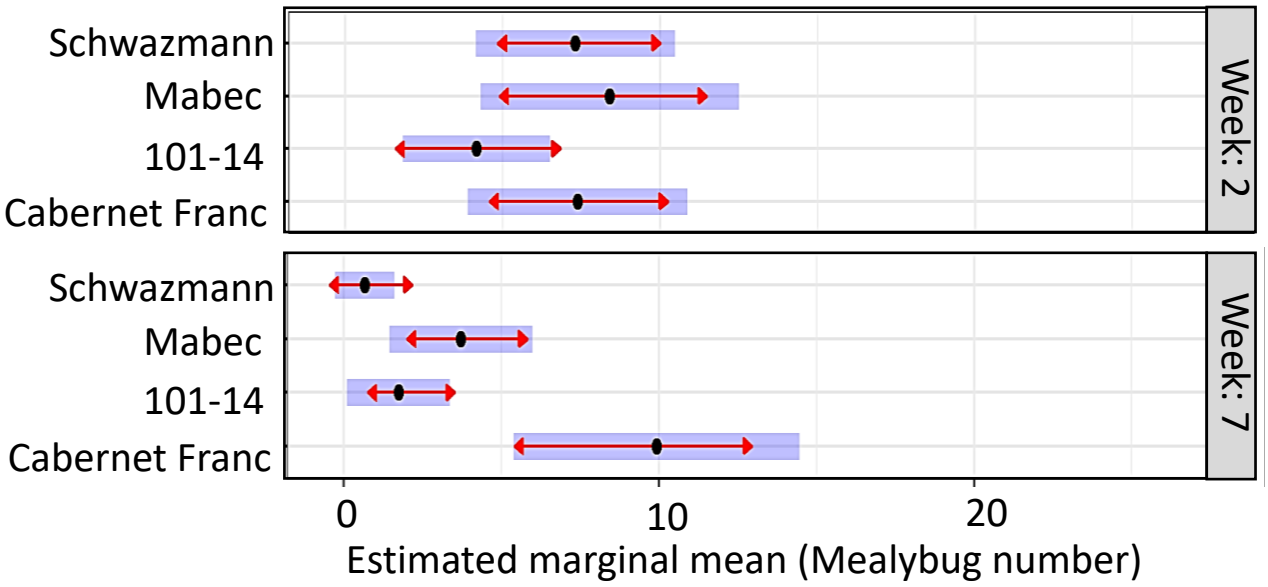

Figure 4. Field-based leaf no-choice assay. Estimated means of mealybug numbers on leaves of five different grapevine rootstock varieties after 2 and 7 weeks from inoculation under no-choice conditions. The purple bars show confidence intervals for the estimated marginal means, and the red arrows are used for comparisons among them. If an arrow from one mean overlaps an arrow from another group, the difference is not significant.

The results for the glasshouse choice test are summarised **in Figure 5**. At the 2-week observation point, insect preference for Riparia Gloire and Cabernet Franc is apparent as seen in the no-choice test, but this effect was no longer seen at the 8-week observation point.

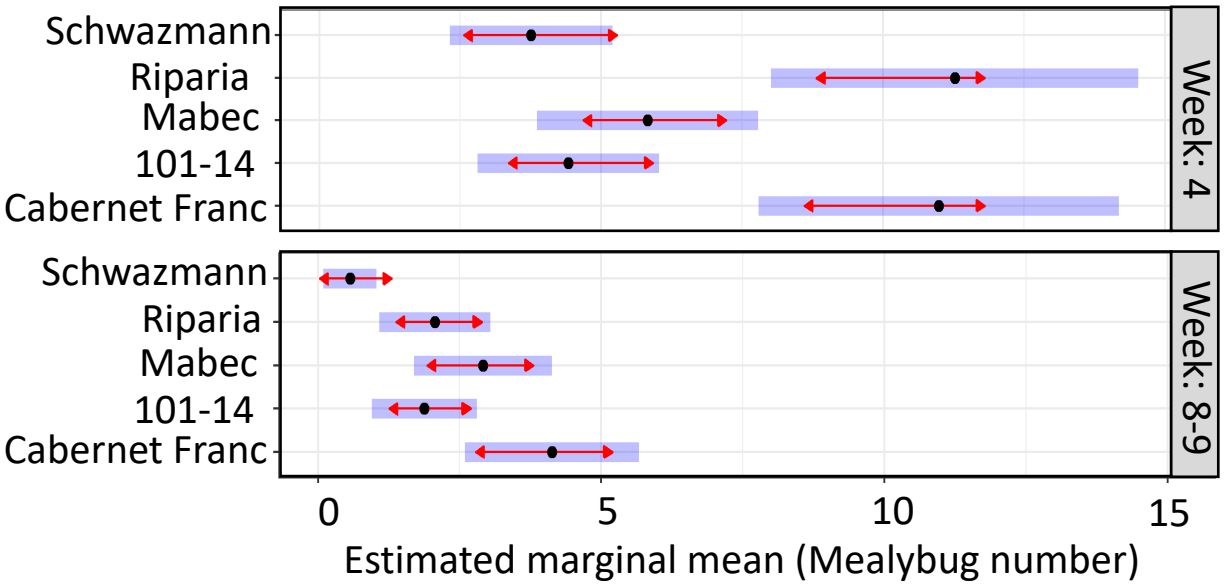

Figure 5. Glasshouse leaf no-choice assay. Estimated means of mealybug numbers on leaves of five different grapevine rootstock varieties after 4 weeks and then 8–9 weeks from inoculation under no-choice conditions. The purple bars show confidence intervals for the estimated marginal means, and the red arrows are used for comparisons among them. If an arrow from one mean overlaps an arrow from another group, the difference is not significant.

Finally, the results of the root, no-choice survival test are presented in **Figure 6**. A preference for the roots of Cabernet Franc is clearly demonstrated.

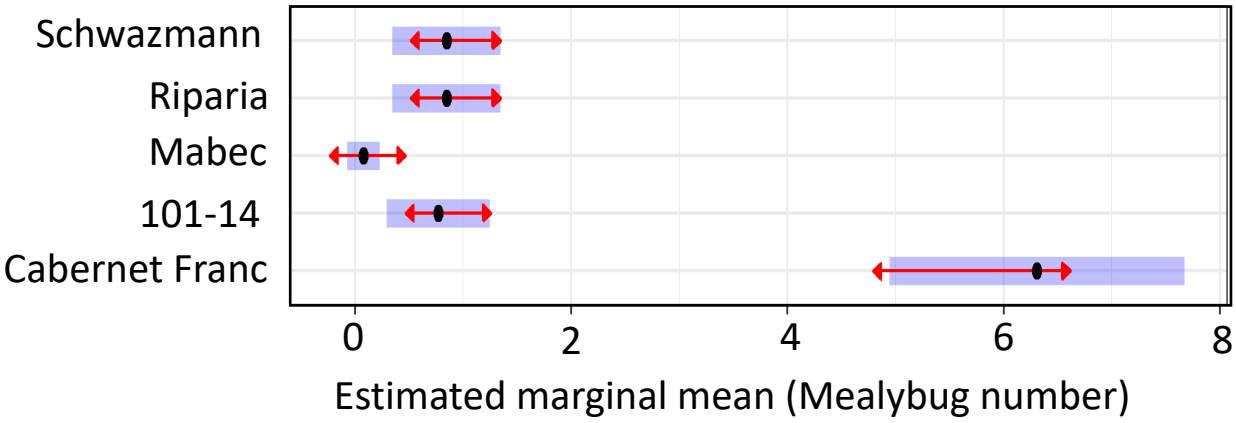

Figure 6. Root no-choice assay. Estimated means of mealybug numbers on roots of five different grapevine rootstock varieties after 3 months from inoculation under no-choice conditions. The purple bars are confidence intervals for the estimated marginal means. If a red arrow from one group overlaps an arrow from another group, the difference is not significant.

Phytochemistry

The results of the mealybug feeding assays were used to rank varieties by insect preference (**Table 1**) to determine whether any classes of metabolic chemical markers correlated with the observed feeding preference scores.

Table 1. Vine and grape relationship to mealybug feeding preference scores

Vine ID	Grape variety	Mealybug feeding preference
VID1159	Cabernet Franc	High
VID305	Malbec	Low
VID848	Millardet et De Grasset 101-14	Low
VID888	*Vitis riparia* gloire	High
VID890	Schwartzman	Low
VID858	Couderc 3306	Unknown

Chromatographic data from negative (Cn) ion mass features were used in a principal component analysis (Figure 7), that showed the Cn data representing polar small molecules, better explained (74%) the data variation compared to Cp (46%), across the first two principal components. The selected mass features from these reverse phase LC-MS analyses were then isolated and manually interpreted to identify their structure. This process identified hydrolysable tannins and some tentatively identified phenolics (see below) as compounds whose levels negatively correlated with mealybug feeding preference. The hydrolysable tannins of greatest significance were confirmed to be ellagitannins which are derived from β-

1,2,3,4,6- pentagalloyl-D-glucose. The key components of the extracted wax associated with low feed preference were tentatively identified as hydroxy tyrosol (HT) derived straight chain lipid esters of palmitic (C16), stearic (C18) and arachidic (C20) acids.

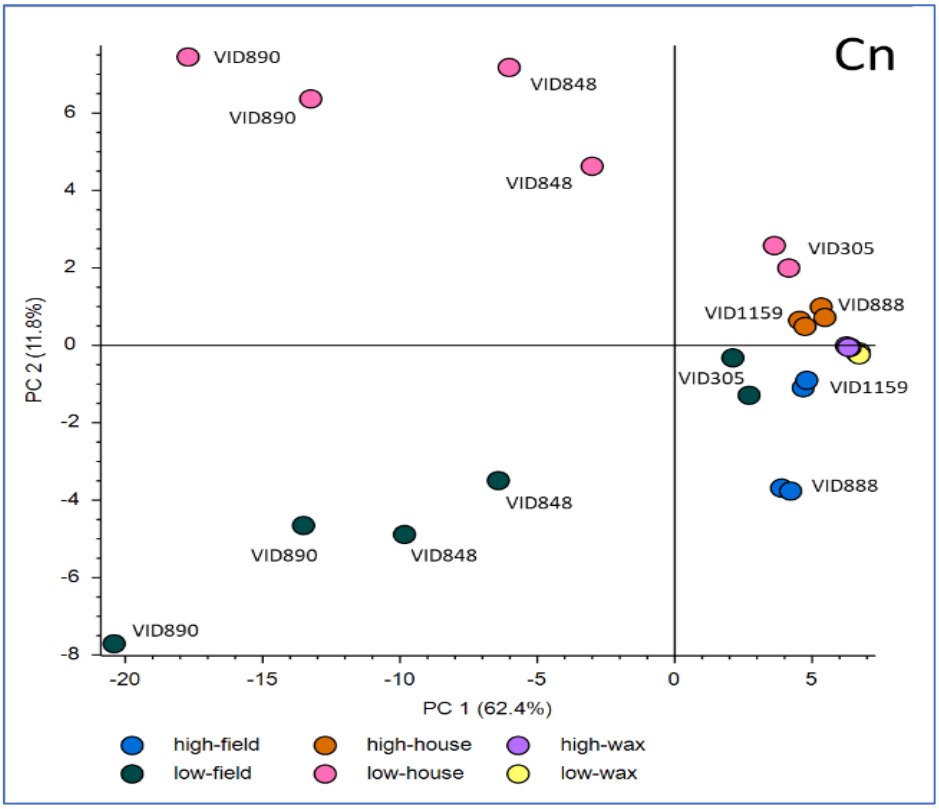

Figure 7. Principal Components analysis of negative (Cn) ion mode liquid chromatography-mass spectrometry.X-axis=Principal Component 1 and Y-axis = Principal Component 2, of leaf extracts from field (Lincoln vineyard) or house (glasshouse at PFR Auckland) and wax (Lincoln field leaf surface extracts) with high and low mealybug feeding preference; VID =vine identification (Cn, only).

DISCUSSION

Five feeding assays were designed to test mealybug preference, survival and developmental progress under a range of environmental conditions, including laboratory (detached leaf assay), greenhouse and field-grown plants. Also, both root and shoot tissues were inoculated, and both choice and no-choice tests were conducted to determine whether these factors influenced insect survival. An initial longitudinal study indicated that mealybugs complete a single generation in 8 weeks, under laboratory conditions. Subsequent tests were, therefore, typically analysed for mealybug survival at week 7. The exception was the root test, which for practical reasons was analysed between weeks 8 and 9.

Absolute mealybug survival rates varied significantly between the different feeding assays. The highest survival rates

were observed in the greenhouse leaf assay, whilst the lowest were in the root and field assays. However, although the absolute values differed, the relative rankings of insect survival on the different cultivars tested remained similar throughout. In order of preference (from highest to lowest), the varieties ordered: Cabernet Franc = Riparia Gloire > Malbec > (Schwarzmann = Millardet et De Grasset 101-14). Cultivars Isabella, Couderc 3309, and Siebel 5437 were only used in a limited number of the assays so are not listed. The consistency of preference/survival rankings suggests a standardised test could be used as a 'bioassay' for mealybug feeding in grape. We recommend the leaf, no-choice, greenhouse test for this purpose as it had the highest differences in survival rates between varieties after 7 weeks (Figure 3) and it was among the easiest test to perform.

The consistency of the rankings between environments also suggests that genetic and/or metabolic factors are important in determining mealybug feeding preference and survival on grape hosts. A preliminary metabolomic study was, therefore, conducted to identify constituent chemistries that correlated with the mealybug feeding preference. From this study soluble tannins were implicated as potential feeding deterrents of importance. The roles of tannins in plant herbivore defence can be grouped into three functional mechanisms: 1/ protein precipitation capacity (PPC); 2/ reducing nitrogen (N) digestibility and 3/ oxidative activity at high pH (Marsh et al 2020). Mapping our data onto this classification system, it appears that polar ellagitannins and hydrolysable tannins (Karl et al 1983) play a role in grape herbivore defence. The mode(s) of functional defence would most likely be oxidative activity (OA) from the polar HHDPs with some protein precipitation PPC from the less polar HHDPs and their derivatives.

Leaf surface chemistry may also be important in grape plant defence. Higher levels of hydroxytyrosol fatty acids were seen to correlate with mealybug feeding deterrence in the current study. Structurally similar phenolic lipids were implicated as antifeedants against caterpillars using a leaf disc choice assay post, sprayed with isolated natural compounds (Sharma et al 2007). The esters of hydroxy tyrosol have also been shown to reduce nitrous oxide production in biological assays (Plastina et al 2019) which could further support the biological defence hypothesis. It is important to note that our chemical analyses were preliminary and exploratory in nature. Only limited replication was used and only a limited number of analyses were undertaken. Consequently, it is inappropriate to claim any discovery at this stage and future research is planned to validate the findings.

In conclusion, herbivore defence was observed in different grape varieties and the results suggest that this may be due to the presence of hydrolysable tannins and phenolic lipid wax components. Further research work is planned to explore the potential causative links between the marker metabolites and mealybug feeding preference. If causative links are demonstrated, the chemical markers will be used to guide a grape rootstock breeding effort, aimed at blocking the transfer of GLRaV3 and other insect-vectored viruses into the grape plant by discouraging insect feeding.

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