GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 PERSISTENCE IN *VITIS VINIFERA* REMNANT ROOTS

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SUMMARY

Grapevine leafroll-associated Virus 3 (GLRaV-3) adversely alters quantitative and qualitative parameters of wine production. New Zealand grape growers respond to this economic threat by replacing virus-infected grapevines with certified virus-tested vines. When vines are removed, most roots remain *in situ*, potentially acting as long-term reservoirs of GLRaV-3. In New Zealand this disease is vectored by three species of pseudococcid mealybugs (Hemiptera: Pseudococcidae), i.e. *Pseudococcus longispinus*, *P. calceolariae* and *P. viburni*, with the two latter species frequently found on roots of host plants. Viruliferous mealybugs moving from GLRaV-3 infected remnant roots to newly planted vines form a probable pathway explaining the relatively rapid re-appearance of the disease in replanted vineyards. We conducted four field studies to determine the status of GLRaV-3 in remnant roots after applying herbicide and/or leaving ground fallow for variable intervals following vine removal. In vineyard A, one of three herbicides (glyphosate, triclopyr, or metasulfuron) was applied to freshly cut vine stumps. One year after treatment, roots and mealybugs found tested positive for GLRaV-3. In vineyard B, no herbicide was applied to cut vines, which were removed and the ground left fallow for 12 months. Twenty-six weeks after vine removal, mealybugs found on remnant roots tested positive for GLRaV-3 and after 12 months, virus was detected in 97% of the roots. The absence of any real decline in the proportion of roots with GLRaV-3 prompted testing at vineyard C, where 4 years earlier vines were cut and the stumps swabbed with glyphosate. Despite this fallow period, two thirds of root samples tested positive for GLRaV-3. Before vine removal in vineyard D, glyphosate was applied to leaves. Six months later 87% of root samples tested positive for GLRaV-3. The implications of these results for the management of leafroll virus are discussed.

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) imposes severe economic costs in all major grape-growing regions of the world (Walker *et al*., 2004; Charles *et al*., 2006; Nimmo-Bell 2006; Freeborough and Burger, 2008; Golino *et al*., 2008). Included in the genus *Ampelovirus* (family *Closteroviridae*) (Martelli *et al*., 2005), GLRaV-3 is phloem-limited and is one of nine recognised serologically distinct viruses associated with grapevine leafroll disease (Martelli and Boudon-Padieu, 2006; Golino and Almeida, 2008). Physiological symptoms in advanced infections include declines in yield, delayed crop maturity and lower soluble solids, reduced berry anthocyanin, and elevated berry titratable acidity (Charles *et al*., 2006), which result in reduced wine quality (Mannini *et al*., 1998). GLRaV-3 occurs only in *Vitis* and affects both white and red grape varieties. It is visually most apparent in the latter where it is characterised by dark-red, downward rolling leaves with green veins (Golino *et al*., 2008).

In New Zealand, a 'leafroll disease' was recognised in the 1960s, with visual symptoms observed in many vineyards (McKissock, 1964). Subsequent studies quantified the effects of the disease on vine performance and wine quality (Chamberlain, 1967; Chamberlain *et al*., 1970; Over de Linden and Chamberlain, 1970a, 1970b; Thomas, 1976), and although not formally classified, the symptoms described were probably those of GLRaV-3. The momentum generated by these studies was not maintained, resulting in a lack of cohesive strategies to manage the disease and its rate of spread. Indeed, a nationwide survey of growers in 2005 revealed that few were conducting optimal controls to minimise the threat of leafroll virus spread (Bonfiglioli and Stewart, 2005). GLRaV-3 is now regarded as the most common and destructive virus disease affecting New Zealand grapevines (Bonfiglioli *et al*., 2002; Bonfiglioli and Hoskins, 2006).

Two factors exacerbate the spread of GLRaV-3: the

use of infected propagating material (Golino *et al*., 2008) and insect vectors (Charles *et al*., 2006). To manage the quality of new plantings for New Zealand's rapidly growing wine industry, the sector implemented the New Zealand Grafted Grapevine Standard (Nimmo-Bell, 2005). One objective was to deliver high-health rootstock and scion wood that had been rigorously tested for the absence of GLRaV-3. This initiative, coupled with recent publicity, raised awareness of GLRaV-3 to a point where few New Zealand growers would now risk using non-certified planting stock.

Pivotal to the success of the grafted grapevine standard is reducing the level of pressure exerted by insect species capable of transmitting GLRaV-3. Especially problematic are mealybugs (Hemiptera: Pseudococcidae), which are phloem feeders. Worldwide, a number of mealybug species have been identified as vectors of GLRaV-3. In New Zealand, three species have been recorded in vineyards: *Pseudococcus longispinus*, *P. calceolariae*, and *P. viburni* (Charles, 1993). All are vectors of GLRaV-3 (Petersen and Charles, 1997; Golino *et al*., 2002).

Today, mealybugs are regarded as the most important pest group in New Zealand vineyards. While some aspects of the biology of these species are known (Charles, 1981; Wakgari and Giliomee, 2003) other aspects remain poorly understood. For example, *P. calceolariae* is frequently found on grapevine roots but the proportion of the vineyard population on roots at any point in time and its relative mobility in this environment remain unknown. This subterranean behaviour confounds efforts to monitor populations on grapevines and may also mitigate biological control and the effects of contact insecticides (e.g. buprofezin) (Godfrey and Pickel, 1998; Walton and Pringle, 2004).

Following the removal of vines infected with leafroll virus, New Zealand growers often report visual symptoms of the disease appearing within 12–18 months of a block being re-planted. New sources of infection typically appear as random occurrences but because of the grafted grapevine standard and adherence to sourcing vines from accredited nurseries only, we believe it is increasingly unlikely the disease was introduced via the nursery. In South Africa, a similar distribution of young virus-infected vines was found to be spatially correlated with an earlier vineyard that contained a high incidence of GLRaV-3 (Pietersen, 2004). The same author (Pietersen, 2004) argued that this mode of disease spread might be attributed to the survival of viruliferous mealybugs on residual vine roots during the interval between the removal of the old vineyard and the re-establishment of the new one. In other words, remnant roots may act as long-term reservoirs of GLRaV-3.

Until very recently, roots were not considered to be an important part of vine removal. Vines were either cut (leaving all roots behind) or pulled from the ground (removing perhaps only 20% of the roots). For cut vines, it was widely believed that an immediate application of herbicide to the surface of a freshly cut stump would kill the roots. Historically, the herbicide of choice was glyphosate, and, although its use was specifically aimed at preventing shoot re-growth from rootstocks, in recent years it was thought it might also offer prospects for eliminating leafroll virus. However, there is no efficacy information supporting the use of glyphosate to kill grapevine stumps and roots (Young, 2009) and we could find nothing in the literature linking its use to the elimination of GLRaV-3. Given the New Zealand wine sector's reliance on glyphosate, it was important to establish the efficacy of this product when developing protocols for managing this disease.

In this paper we report on four field studies undertaken during 2007 and 2008, following the removal of vineyard blocks of vines that were infected with GLRaV-3. In each vineyard, differing protocols were used to facilitate vine death and/or vine removal. The objective of each study was to determine the GLRaV-3 status in remnant vine roots at variable intervals following the respective treatments adopted at each site.

MATERIALS AND METHODS

Study sites and trial designs. The vineyards were located around Hastings, Hawke's Bay, on the east coast of New Zealand's North Island (39°65′S 176°83′E). One vineyard was a small (0.27 ha) non-commercial research property with cv. Chardonnay vines on rootstock 3309, which were planted in 1993. The other three vineyards were commercial properties where treatment strategies or vine removal protocols were decided by vineyard managers. The studies reported here were therefore designed around the unique circumstances of each vineyard operation.

Comparison of stump-applied herbicides. At the non-commercial vineyard, budwood from each of the 48 vines used in the study was virus tested (as described below) in early May 2007. All samples tested positive for GLRaV-3. Two weeks later, the trunk of each vine was cut *ca*. 15 cm above ground and a vertical 1-cm diameter hole was drilled 2 cm into the cut surface to create a reservoir. One of three herbicides, Roundup® Renew (200 ml/200 ml water; active ingredient glyphosate), Answer® (15 g/l; a.i. metasulfuron) or Grazon® (50 ml/l; a.i. triclopyr) was applied with a small paint brush at 5 ml per stump for all treatments, to stumps within 30 seconds of a trunk being cut. An organo-silicone spreader-penetrant (Boost® Penetrant, 1 ml/l) was added to each herbicide. The study included an untreated control with each treatment replicated four times. Pending root extraction, the stumps re-

mained undisturbed for the duration of the study.

To measure virus persistence over time, root sampling was undertaken six days after treatment and again at weeks 9, 20 and 51. In week 51, two samples returned a negative ELISA result, so these were re-tested using real-time RT-PCR.

Fallow study 1. In August 2007, a 1.0-ha block of 25-year-old own-rooted cv. Gewürztraminer vines heavily infected with GLRaV-3 was removed from a commercial vineyard. Herbicide was not applied before vine removal and the block was to remain unplanted (fallow) for 12 months. Before vine removal, budwood was collected from 10 vines within each of three randomly selected plots (9 x 12 m), separated from each other by 50 m. Budwood was tested for GLRaV-3 and all 30 samples were virus-positive.

The vines were removed using a large 'L'-shaped steel blade mounted onto the bucket of a front-end loader. The roots were cut to a depth of *ca.* 300 mm and *ca.* 150 mm out from the vine trunk on both sides of the row. This technique successfully removed all aerial parts but only a relatively small proportion of the root system, particularly of older vines.

On 11 February 2008 (6 months after vine removal), remnant roots were extracted from the three plots using a 3-tonne digger (n=10 samples per plot). Each sample consisted of two root fragments with a minimum length of 150–200 mm and a minimum diameter of *ca.* 10 mm. In all the studies reported here, root samples collected were first checked for the presence of mealybugs. Any found were transferred into individually numbered vials for species identification and real-time RT-PCR testing for GLRaV-3.

The block remained fallow until a second root extraction was undertaken on 23 July 2008 (49 weeks after vine removal). However, by this time, the infrastructure in the block (posts, wires, irrigation) had been re-established, which meant the digger had difficulty manoeuvring within the three plots described above. Instead, remnant roots were recovered at *ca.* 10 m intervals along one of the four vine rows. All root samples collected were tested for the presence of GLRaV-3, as described below.

Fallow study 2. This large commercial vineyard contained many blocks with high rates of GLRaV-3 infection. In 2004, the proprietors progressively removed blocks as part of a programme to eliminate leafroll virus. Remnant roots were sampled from a 1.0-ha own-rooted cv. Merlot block planted in 1981. The vines were removed in August 2004 and the block had remained fallow since. No formal assessment of GLRaV-3 infection rates was made before the vines were removed, but at the time it was estimated that >95% of the vines were infected with this disease. Hence it was assumed that remnant roots would have originated from diseased vines.

In 2004, the vine trunks were cut *ca.* 150 mm above ground and *ca.* 5 ml of glyphosate (200 ml/200 ml water) was swabbed directly onto the cut surface of each stump. During the 4-year interval between vine treatment and root extraction, the block was periodically cultivated, but a formal programme to remove vine roots was not undertaken. In late July 2008, a 3-tonne digger was used to extract remnant roots from 10 randomly selected plots. Each sample consisted of at least two root fragments with a minimum length of 150-200

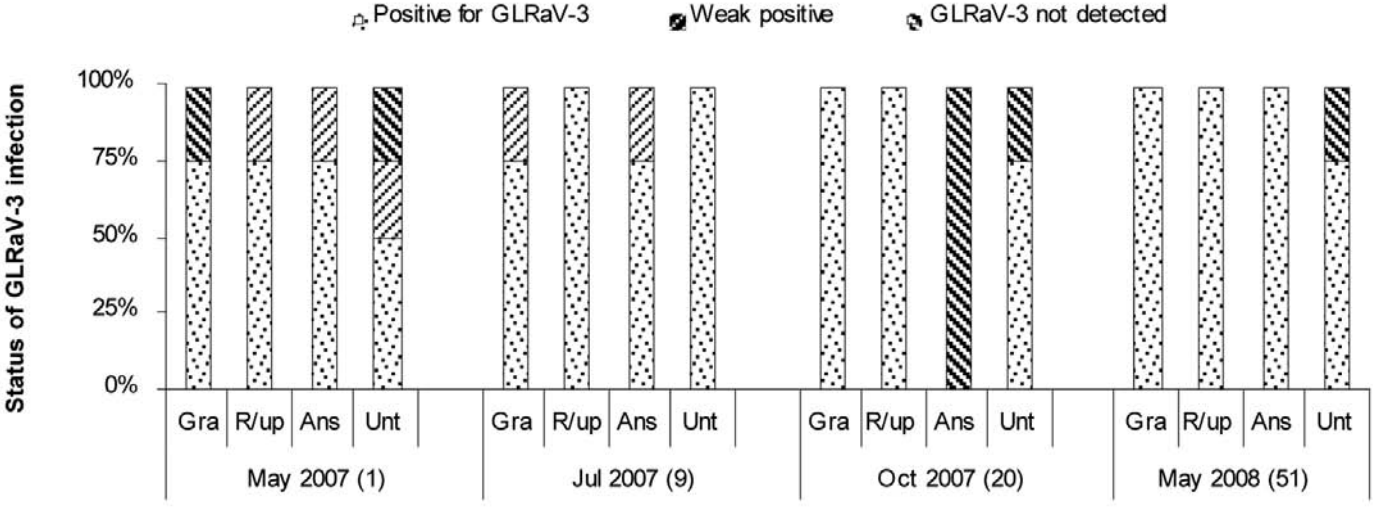

Fig. 1. GLRaV-3 infection status among remnant roots collected from the research vineyard for 12 months to May 2008 in the comparison of stump-applied herbicides trial. Gra=Grazon®; R/up= Roundup® Renew; Ans=Answer®; Unt=Untreated control (n=4 samples/treatment/sampling date). Figures in brackets denote weeks since herbicide application. Samples were ELISA tested except in week 51 when two inconclusive results were re-tested using real-time RT-PCR.

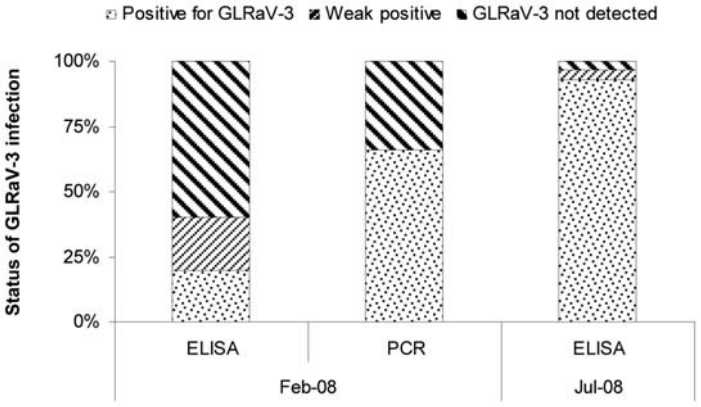

Fig. 2. GLRaV-3 status among remnant roots collected from a commercial vineyard in the Fallow study 1, 26 weeks (February 2008) and 49 weeks (July 2008) after the roots were cut and vines removed. Roots were initially ELISA tested (n=30 root samples per date) and where no virus was detected, a sub-sample was re-analysed by real-time RT-PCR (3 root samples in February only).

mm and a minimum diameter of *ca.* 10 mm, and was tested for the presence of GLRaV-3 as described below.

Foliar-applied herbicide. The third commercial vineyard contained 15-year old cv. Pinot Noir vines on SO4 rootstock. In mid March 2008, the vine foliage was sprayed with glyphosate (boom spray, 1 l/100 l water). In mid July, budwood samples were collected from 30 randomly selected vines spread across the block. ELISA confirmed all samples were positive for GLRaV-3. The vines remained *in situ* until mid August at which time the roots were cut (using the 'L'-shaped blade described above) and the vines removed. There was no targeted programme for vine root removal. In mid September 2008, a 3-tonne digger was used to extract remnant roots from 15 randomly selected plots. The root samples, which were a minimum length of 150-200 mm and a minimum diameter of 8 mm, were tested for the presence of GLRaV-3.

GLRaV-3 testing. ELISA and real-time PCR methods were used to test for GLRaV-3. A sample of 100 mg of cortical root phloem material was taken from three locations per root sample. These were combined and homogenised in 3 ml of extraction buffer as described by Bioreba (http://www.bioreba.ch/files/tecinfo/TI_Buffer_formulation.pdf). Two ml of each sample were then centrifuged for 1 min at 16,000*g* and 200 µl were processed in a DAS-ELISA as per manufacturers' instructions (Bioreba, Switzerland and Sediag, France). Samples with a normalised absorbance reading of 3-fold higher than background levels were considered positive.

Detection of viral RNA was performed using two independent real-time PCR assays. RNA from 150 mg of cortical root material was extracted using published procedures (MacKenzie *et al.*, 1997) and cDNA synthesised using Transcriptor reverse transcriptase (Roche Applied Science, USA), primed with a mix of random primers and oligodT following the manufacturer's instructions. Extraction controls were employed to guard against sample carry-over during the RNA extraction process and no template PCR reactions were used. Real-time PCR analyses were performed with SYBR Green-based methods on a LightCycler 480 instrument (Roche Applied Science, USA). Primers for one of the assays were described by Osman and Rowhani (2006), while the other assay was designed at Linnaeus laboratory (manuscript in preparation). Two assays were employed due to the high sequence variation seen in this virus. Reactions were performed in 96-well format with 10 µl reactions, 2.5 µl of cDNA, 0.3 µM each primer and 1x LightCycler 480 SYBR Green Master (Roche Applied Science, USA). Reactions were cycled using the Linnaeus virus template of 95°C for 5 min initial denaturation followed by 45 cycles consisting of 95°C for 5 sec, 60°C for 5 sec and 72°C for 11 sec. Melting curve analysis was subsequently done to verify resulting amplicon identity. Amplification by either (or both) primer sets was considered a positive result. For real-time PCR testing of mealybugs, individual mealybugs were homogenised with a pipette tip in 500 µl extraction buffer (MacKenzie *et al.*, 1997) and processed and amplified as for root samples.

RESULTS

Comparison of stump-applied herbicides. Roots recovered from the block over the 12-month period remained suitable for virus testing. During the 9 weeks following the application of the three herbicides, GLRaV-3 was detected in 75-100% of all root samples (Fig. 1). Twenty weeks after treating the stumps, no leafroll virus was detected in the Answer® samples but it was detected in the remaining treatments. The Answer® result was not replicated beyond this date; after 51 weeks no herbicide product used in this study was shown to consistently reduce the incidence of GLRaV-3 in remnant roots relative to the untreated control.

At a depth of 100 mm, eight live *P. calceolariae* (crawlers and two adults) were found on one root sample (Answer®) during one sampling date (week 51). The root from which the mealybugs were recovered tested positive for GLRaV-3. The virus status of two crawlers was 'weakly positive' [Ct (cycle threshold) between cycles 35 and 40 with specific GLRaV-3 melting curves], while virus was not detected in the remaining individuals.

Fallow study 1. On both sampling dates, vine roots

were readily recovered from depths ranging from 200–500 mm. The roots were in good condition and suitable for virus testing. The initial ELISA results in February revealed 12 of the 30 root samples (40%) were either positive or weakly positive for GLRaV-3 (Figure 2). Of the remaining 18 samples, three were re-analysed using real-time RT-PCR: leafroll virus was found in two of the samples. Combining both sets of results, leafroll virus was detected in 14 of 30 samples (47%). A total of 20 *P. calceolariae* encompassing juveniles and adults were found on roots recovered from two plots in February. The roots from both plots tested positive for GLRaV-3. Five mealybugs tested positive for GLRaV-3 from one plot, but in the second plot the virus was not detected from any of the six mealybugs tested. On the second sampling date, only ELISA was undertaken and GLRaV-3 was detected in 29 of the 30 samples collected (97%) (Figure 2). No mealybugs were found on this date.

Fallow study 2. Despite a 4-year interval between vine treatment and extraction, remnant roots were readily recovered from each plot. All material was suitable for virus testing. Root samples were initially ELISA tested but no GLRaV-3 was detected. Three samples were re-analysed using real-time RT-PCR; two tested positive. No mealybugs were recovered.

Foliar-applied herbicide. Remnant vine roots were readily recovered from each plot and all material was suitable for virus testing. Roots were ELISA-tested. Six months after the foliar application of glyphosate, 13 of 15 (87%) remnant root samples tested positive for GLRaV-3. No mealybugs were recovered.

DISCUSSION

The vine removal protocols adopted in each vineyard enabled an assessment of the persistence of GLRaV-3 in remnant roots. For many growers the use of herbicide is viewed as a credible strategy for killing grapevines and this action, coupled with the adoption of a suitable fallow period, is thought to effectively prevent GLRaV-3 infection of replanted vines. However, in this study, GLRaV-3 continued to be detected in remnant root samples from all vineyards, regardless of the herbicide used, its use pattern (stump or foliar) or the fallow duration. These data call into question the potential value of herbicides when replanting vineyards, especially the current industry practice based on glyphosate. As far as we are aware, these are the first published data on the persistence of GLRaV-3 in remnant roots.

Unlike stump swabbing, the application of glyphosate to plant foliage is generally the more accepted use pattern for this herbicide. Foliar applications of glyphosate to grapevines are now being used by an increasing number of Hawke's Bay winegrowers. The presence of GLRaV-3 in almost 90% of remnant roots 6 months after treating either stumps or vine foliage suggest that glyphosate use as described here was probably of limited value in killing remnant infected roots and therefore did not reduce the potential for re-infection. It is possible that a fallow period exceeding this timeframe may result in a different outcome and remains to be tested. However, the financial sustainability of an extended fallow period must also be considered. Calls for prolonged delays to re-planting are likely to be financially unattractive to most growers and would probably not be widely supported.

Notwithstanding the financial implications or the use of herbicides, it would appear that the fallow option *per se* may be of limited usefulness. After 4 years of fallow, real-time RT-PCR tests detected GLRaV-3 in remnant vine roots. This result suggests glyphosate applied to vine stumps does not eliminate GLRaV-3, either by killing the roots or more specifically, by destroying phloem structure. This result reinforced the notion that a strategy to eliminate GLRaV-3 appears compromised where there is little or no attempt to remove remnant roots.

The excavation of remnant roots in 'Fallow study 1' where herbicide was not applied, highlighted the disparity between test results undertaken at different times of the year from samples collected in the same block. In February 2008 (southern hemisphere summer), only 40% of ELISA-tested remnant roots were positive for GLRaV-3. ELISA conducted five months later (July, mid winter) showed 97% virus-positive samples. It is not immediately apparent why the results were so different but seasonal changes may have influenced virus replication. Regardless of cause, the results highlighted that like budwood (Teliz *et al*., 1987; Matthews, 1996), the timing of root collection and its testing are important parameters when attempting to detect leafroll viruses.

The discovery of *P. calceolariae* mealybugs on remnant roots at 6 and 12 month intervals after treatment, adds a further dimension to this issue. The results suggest that this species of mealybug was sustained by remnant vine roots for at least 12 months and continued to host the virus. It is therefore likely that these mealybugs are capable of transferring GLRaV-3 to healthy vines in newly replanted vineyards. This combination of factors for root-colonising species of mealybugs such as *P. calceolariae* has the potential to undermine the intent and future success of strategies like the grafted grapevine standard.

These results suggest that mealybugs colonising roots may be of far greater importance than previously thought. We recently observed *P. calceolariae* colonies on the roots of young vines lifted just 12 days after

planting. Washed and bare-rooted before planting, it was unlikely mealybugs were introduced with the young vines. Instead, observations during planting suggest that the source of the infestation was the remnant roots of vines removed four years earlier. In South Africa, the vine mealybug (*Planococcus ficus*) was seen on remnant roots of vines that had been removed two years earlier (Walton and Pringle, 2004). In New Zealand, *P. calceolariae* is widely distributed throughout most of the country, so it is probable that many vineyards host significant populations in the soil and on the roots of many weed species. The movement patterns of mealybugs through the soil both between healthy and infected vines and between vines and weedy hosts should be examined further. These data support the idea first proposed by Pietersen (2004), that the resumption of feeding by viruliferous mealybugs moving from virus-infected remnant roots to the developing roots of virus-free vines may be an important component of GLRaV-3 transmission and spread. Further studies are planned to determine whether mealybugs can acquire GLRaV-3 from remnant vine roots and subsequently infect healthy vines with grapevine leafroll disease.

For the New Zealand wine sector, these issues are crucial to defining the role that remnant vine roots and subterranean mealybugs play in perpetuating the cycle of GLRaV-3. There is certainly increasing evidence that the association between mealybugs and remnant roots has a significant influence on the fate of re-planted vineyards. Should the evidence support this view, we envisage that economic necessity will compel wine sectors around the world to review protocols for vine removal and vector management, both above and below ground.

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