Development of diagnostic protocols for the detection of endemic viruses of Australian grapevines

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Introduction

More than 50 viruses have been reported to infect grapevines worldwide (Martelli 2003) and 11 of those have been reported in Australian grapevines. The 11 grapevine viruses include *Grapevine leafroll associated virus* (GLRaV-) 1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5 and GLRaV-9, *Grapevine virus A* (GVA) , *Grapevine virus B* (GVB) *Grapevine rupestris stem pitting associated virus* (GRSPaV), *Grapevine fleck virus* (GFkV) and *Grapevine fanleaf virus* (GFLV) (Krake et al. 1999; Habili and Rowhani 2002). Although GFLV has been reported in Australia, it was contained in the Rutherglen region and is still considered a quarantineable pathogen (Krake et al. 1999). Viruses can impact the industry at various levels including the quality of planting material, the sustainability and productivity of vineyards, the quality of the end product including wine, table grapes, dried fruit and ornamental grapevines and export capabilities. Grapevine viruses can be transmitted through grapevine planting material and some are transmitted by insect vectors.

The improved productivity and sustainability of the Australian viticulture industry is dependent on the provision of certified high-health planting material, which is routinely pathogen tested for the 11 endemic viruses by biological indexing, enzyme linked immunosorbent assays (ELISA) and reverse transcription polymerase chain reaction (RT-PCR). In Australia, most certified high-health material originates from two nucleus collections based in South Australia and New South Wales. These repositories are used to develop foundation plantings and source blocks for the supply of certified high-health grapevine material to nurseries, via regional vine improvement groups. Certification is supported by, and dependent on, accurate and reliable diagnostic tests for viruses.

Although biological indexing, ELISA and RT-PCR are commonly and globally used for the detection of grapevine viruses, there have been few comprehensive, systematic studies, to determine the reliability of these tests in comparison with eachother in Australia and overseas. Individual studies to determine the reliability of woody indexing on specific indicators have come from routine indexing done by South Australian Vine improvement Scheme during 1974–1987 (Cirami et al. 1988) and the Fruit Variety Foundation in Victoria (Shanmuganathan and Fletcher 1980). The Victorian study showed that Cabernet Franc was more reliable than LN33 (a cross between Courdec 1613 × Thompson Seedless) for leafroll disease detection in Australia. On the other hand, the South Australian indexing data showed that Cabernet Franc was not always reliable for leafroll detection and a second indicator may be required to detect some leafroll strains. A similar study done by Foundation Plant Services (FPS) (UC Davis, USA) compared biological indexing and ELISA for detection of GLRaV 1-4 (Rowhani et al. 1997). This study also showed that Cabernet Franc was not reliable for all strains of GLRaV as ELISA detected some strains that the indicator did not. This result was attributed to the failure of an infected bud chip to survive and/or to the grafting of an uninfected bud due to the uneven distribution of the virus. A study conducted by Krake and Steele Scott (2002) to determine grapevine scion-understock combinations suitable in Australia, found that RT-PCR techniques did not consistently detect virus even when symptoms were observed.

The effectiveness of ELISA and RT-PCR for the detection of some grapevine viruses has been compared in other countries. Most studies show that RT-PCR is more sensitive than ELISA for detection of grapevine viruses, especially on symptomatic plants. Studies indicated that detection of GLRaV 3 by ELISA and RT-PCR in symptomless plants was erratic (Chen et al. 2003) especially when flowers or fruits were used (Ling et al. 2001). ELISA and RT-PCR were shown to be reliable for the detection of GLRaV3 from bark scrapings of field infected grapevines that were symptomatic or not throughout one season (Ling et al. 2001). In contrast, Rowhani et al. (1997) showed that GLRaV3 can be unevenly distributed within the same grapevine and this could lead to inconsistent test results. RSPaV was also detected by RT-PCR in various field grown grapevine varieties throughout the year in all tissues of infected plants, except young buds sampled in summer (Stewart and Nassuth 2001). One study has shown that ELISA may be more sensitive than RT-PCR for detection of GLRaV1 and 3 (Cohen et al. 2003). Significant strain variation is reported for many grapevine viruses and it is possible that no single test will detect all strains of a species.

To date, there have been a lack of studies undertaken to show the reliability of RT-PCR and ELISA for detection of grapevine viruses over time, on replicates of grapevines of different varieties that are inoculated by the same source of virus and maintained in different climatic conditions.

Currently, a four-year research project is being conducted that aims to contribute to the development of grapevine certification protocols for Australia and will:

1. Investigate the specificity and reliability of the molecular (PCR) protocols
2. Investigate the sampling and sensitivity of ELISA and PCR for reliable detection
3. Investigate and document efficient protocols for woody indexing of grapevine viruses
4. Validate serological and molecular protocols by surveying key grapegrowing regions in Australia and update areas of freedom.

The preliminary results of sampling and sensitivity for reliable detection of grapevines viruses by ELISA and RT-PCR are reported here.

Materials and methods

Field sites

To investigate the sampling and sensitivity of ELISA and PCR under Australian conditions, replicate trials were established in a hot climate region (Sunraysia, Victoria) and a cool climate region (Yarra Valley, Victoria) in 2006. Each trial contains two varieties (Shiraz and Chardonnay) and for each variety there are five treatments each consisting of five replicate grapevines. The five treatments include un-inoculated grapevines as a control and grapevines inoculated with GLRaV-2, GLRaV-3, GVA or GFkV. These grapevines were chip bud inoculated, using two buds per grapevine, in October 2006 (Sunraysia) and November 2006 (Yarra Valley).

Sampling

Each grapevine has been sampled monthly and tested monthly by ELISA and PCR since December 2006 (Sunraysia) and January 2007 (Yarra Valley). Early in the season, when there was little shoot growth, 4-8 leaves, with petioles attached, were randomly collected from each grapevine. Later in the season, 3–4 shoots or canes were randomly sampled from each grapevine unless symptomatic material was observed, in which case 3–4 symptomatic shoots were sampled in preference to symptomless material to improve the chance of virus detection. Six hundred milligrams of tissue was taken from each sample, finely chopped and divided equally into two separate grinding bags to be used for ELISA or RT-PCR. Due to the small amount of material available for sampling, virus testing was only done by PCR in July at Sunraysia and August at the Yarra Valley. Testing was not done in September 2007 as the grapevines had been pruned and there were no shoots or canes to sample.

ELISA

The ELISA kits used in this experiment were from Bioreba (GLRaV-2 and GLRaV-3) or AGRITEST (GVA and GFkV) and the tests were done according to the manufacturer's instructions. For each sample, 300 mg of tissue was ground in 3 mL of grapevine extraction buffer (Gugerli 1986). Extracts from infected grapevines were used as a positive control for each virus and buffer controls were also included.

Nucleic Acid extraction

Total RNA was extracted from green grapevine tissue using a modified lysis buffer (MacKenzie et al. 1997) and The X-tractor Gene™ System (Corbett Life Sciences). Briefly, 300 mg of grapevine tissue was ground in 3 mL of the modified lysis buffer and 1 mL of each sample was transferred into separate wells of a 96 well CorProtocol Square-Well Lysis Plate (Corbett Life Sciences), each containing 100 µl of 20% N-lauroylsarcosine. The lysis plate containing the samples was incubated to 65°C for 15 minutes. The plate was centrifuged at 1500 rpm for 5 minutes to clarify the liquid and 500 µl of each sample was transferred to another lysis plate. The lysis plate was placed in the X-tractor and 500 µl of 100% ethanol was added to each sample and the samples were mixed by aspiration. Five hundred microlitres of the mixed samples were then added to the CorProtocol Standard Yield 96-well capture plate (Corbett Life Sciences) and the samples drawn through each well for 5 minutes at a vacuum pressure of 70 Kpa. The capture plate was then washed twice under vacuum with 500 µl propanol wash buffer (Sigma) at 50 Kpa for five minutes and once with 500 µl of 100% ethanol at 45 Kpa for five minutes. A further vacuum step at was done at 40 Kpa for 5 minutes to remove all traces of ethanol. The capture plate was then transferred robotically to a 96 well CorProtocol Elution Plate (Corbett Life Sciences) and 80 µl of RNase, DNase free water was added to each well and allowed to incubate at room temperature for 2 minutes before being drawn through the plate for 5 minutes at 45 Kpa to elute the RNA.

Extracts from phloem scrapes of woody tissue could not be done using the X-tractor due to the precipitation of substances that blocked the capture plate and a different protocol was used. Briefly, 300mg of grapevine tissue was ground in 3ml of the modified lysis buffer and 500 µl of each sample was transferred into separate 1.7 mL centrifuge tubes containing 50 µl of 20% N-lauroylsarcosine. The samples were incubated to 65°C for 15 minutes. To each tube an equal volume of chloroform: isoamyl alcohol (24:1; CH_3:IAA) was added and the samples were mixed using a vortex then centrifuged for 20 minutes at 13000 rpm. The 480 µl of the upper aqueous phase of each sample was transferred to a separate 1.7 mL centrifuge tube and an equal volume of cold isopropanol was added and the samples were mixed by inversion. The samples were centrifuged for 20 minutes at 13000 rpm and the supernatant was discarded. The pellets were washed once with 500 µl of 70% ethanol, allowed to dry and then resuspended in 100 µl of RNase, DNase free water. To each tube, an equal volume of CH_3:IAA (24:1) was added and the samples were mixed using a vortex then centrifuged for 15 minutes at 13000 rpm. The 80 µl of the upper aqueous phase of each sample was transferred to a separate 1.7 ml centrifuge tube and an equal volume of cold isopropanol was added and the samples were mixed by inversion. The samples were centrifuged for 15 minutes at 13000 rpm and the supernatant was discarded. The pellets were washed once with 100 µl of 70% ethanol, allowed to dry and then resuspended in 50 µl of RNase, DNase free water.

Table 1. Primers used in PCR and RT-PCR for detection of housekeeping genes of grapevines and GLRaV-2, GLRaV-3, GVA and GFkV.

Pathogen	Assay	Primer name	Primer sequence (5′-3′)	Expected product size	Reference
RNA house-keeping gene - malate dehydrogenase	RT-PCR	MDH-H968	GCA TCT GTG GTT CTT GCA GG	196 bp	Nassuth et al. 2000.
		MDH–C1163	CCT TTG AGT CCA CAA GCC AA		
Grapevine leafroll associated virus 2 (GLRaV-2)	Specific RT-PCR	V2dCPf2 sense	ACG GTG TGC TAT AGT GCG TG	534 bp	Bertazzon and Angelini 2004
		V2CPr1 antisense	GCA GCT AAG TAC GAA TCT TC		
Grapevine leafroll associated virus 3 (GLRaV-3)	Specific RT-PCR	P3U/	CGC TCA TGG TGA AAG CAG ACG	652 bp	Turturo et al 2005
		P3D	CTT AGA ACA AAA ATA TGG AGC AG		
Grapevine virus A (GVA)	Specific RT-PCR	H587	GAC AAA TGG CAC ACT ACG	429 bp	Minafra and Hadidi. 1994
		C995	AAG CCT GAC CTA GTC ATC TTG G		
Grapevine fleck virus (GFkV)	Specific RT-PCR	GFkV-U279	TGG TCC TCG GCC CAG TGA AAA AGT A	352 bp	Sabanadzovic et al. 2001
		GFkV-L630	GGC CAG GTT GTA GTC GGT GTT GTC		

RT-PCR

Primers for the detection of malate dehydrogenase (MDH) mRNA (Nassuth et al. 2000) were used to determine the quality of the extracted RNA (Table 1). The PCR primers used to detect GLRaV-2, GLRaV-3, GVA and GFkV, the type of assay used and the references are given in Table 1. The SuperScript III One-Step RT-PCR System (Invitrogen) was used for detection viruses and MDH mRNA. One step RT-PCR was conducted according to the manufacturer's instructions except that the total reaction volume was 12 µl for MDH mRNA and 20 µl for each virus and 1 µl of each extract was used in the MDH RT-PCR and 2 µl of each extract was for virus detection. Total RNA extracts from infected grapevines were used as a positive control for each virus RT-PCR. Water controls, without nucleic acid in the RT-PCR mix, were also included. After amplification, 10 µL of each PCR was run on a 2% agarose gel in 0·5 ×Tris-borate-EDTA, stained with ethidium bromide and visualised on a UV transilluminator.

Limit of detection

A preliminary experiment was conducted in March 2008 to determine the limit of detection for each virus at each study site by the RT-PCR and ELISA methods described above. One virus infected grapevine for each virus treatment was selected from each variety at both sites. Material from the five un-inoculated grapevines of each variety at each site was pooled and used to establish the dilution series. For each dilution series, grapevine tissue was finely chopped and divided equally amongst samples used for ELISA or RT-PCR to ensure samples were comparable between the tests. Virus infected or uninfected grapevine tissue was ground in buffer for ELISA or nucleic acid extraction at a ratio of 100 mg tissue to 1 mL buffer. The following dilution series were set up: a) ground infected tissue was diluted in buffer; b) ground infected tissue was diluted in ground uninfected tissue; c) finely chopped infected tissue was diluted with finely chopped uninfected tissue. Each dilution series was set up at the following dilutions: undiluted, 1/5, 1/10, 1/20, 1/50 and 1/100.

Results

Symptom expression

GFkV and GVA symptoms have not been observed in any inoculated grapevine in the 2006/07 or 2007/08 seasons. In the 2006/07 season, leafroll symptoms were observed on 0/5 GLRaV-2 inoculated Shiraz grapevines and 1/5 GLRaV-3 inoculated Shiraz grapevines at Sunraysia and 0/5 GLRaV-2 inoculated Shiraz grapevines and 2/5 GLRaV-3 inoculated Shiraz grapevines at the Yarra Valley. In the 2007/08 season leafroll symptoms were observed on 4/5 GLRaV-2 inoculated Shiraz grapevines and 5/5 GLRaV-3 inoculated Shiraz grapevines at Sunraysia and 4/5 GLRaV-2 inoculated Shiraz grapevines and 5/5 GLRaV-3 inoculated Shiraz grapevines at the Yarra Valley. Leafroll symptoms have not been observed on the GLRaV-2 and GLRaV-3 inoculated Chardonnay grapevines at either site in either season.

Nucleic acid extraction

Nucleic acid was successfully extracted from green tissue, particularly petioles and green shoots, using the automated system. However, when phloem scrapes were used from woody tissue, the addition of ethanol, which was used to precipitate nucleic acid, caused the extracts to become highly viscous and the capture plate filters became blocked. Consequently the CH_3:IAA extraction procedure was used for all lignified tissue. Both methods yield 5–40ng RNA/µl.

Sampling and sensitivity

GLRaV-3 and GFkV were detected 6–7 weeks post-inoculation in both Chardonnay and Shiraz at Sunraysia in December 2006. GLRaV-3 was detected by ELISA and RT-PCR and GFkV was detected by PCR only. By January 2007 positive results were obtained for GLRaV-2,-3 and GFKV at both sites.

The pooled results of the monthly testing by RT-PCR and ELISA of all the inoculated grapevines for each site, regardless of variety or virus treatment for 2006/07 are shown in Figure 1. The June and July/August samples were only tested using PCR as there was not enough material to perform ELISA testing as well. No uninoculated grapevine has tested positive for GLRaV-2, GLRaV-3, GVA or GFkV at each site. GVA was not detected in any of the 10 inoculated grapevines at each site. In most months at both sites, more positive results were obtained by RT-PCR than by ELISA (Figure 1); particularly for GLRaV-2 and GFkV. At each site 20/30

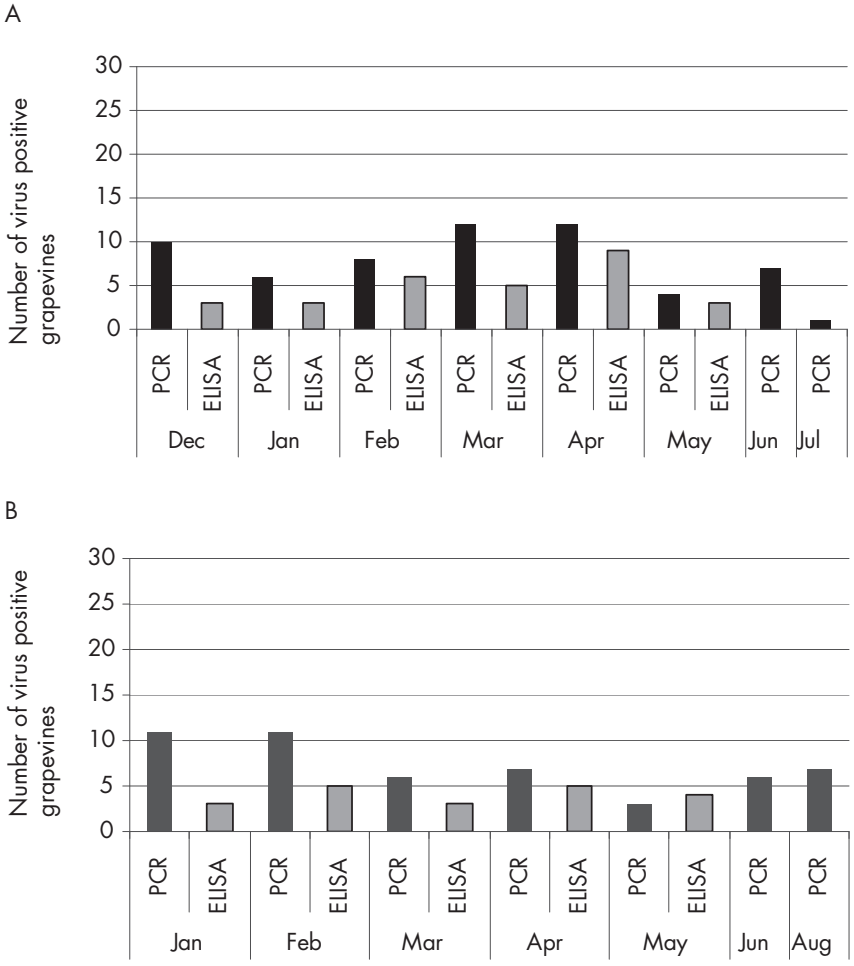

Figure 1. The total number of grapevines that tested positive for virus at A) Sunraysia and B) the Yarra Valley between December 2006 and August 2007. A total of 40 grapevines (20 Shiraz and 20 Chardonnay) were inoculated with GLRaV-2, GLRaV-3, GVA and GFkV at each site, however GVA has not been detected.

grapevines inoculated with GLRaV-2, GLRaV-3 or GFkV tested positive for virus by RT-PCR and ELISA.

The pooled results of the monthly virus testing by RT-PCR and ELISA of all the inoculated grapevines for each site, regardless of variety or virus treatment for 2007/08, are shown in Figure 2. No uninoculated grapevine has tested positive for GLRaV-2, GLRaV-3, GVA or GFkV at each site. GVA was not detected in any of the 10 inoculated grapevines at each site. In most months, at both sites, more positive results for GLRaV-2, GLRaV-3 and GFkV were obtained by RT-PCR than by ELISA. October was the least reliable month for virus testing by ELISA and RT-PCR.

At Sunraysia 27/30 grapevines inoculated with GLRaV-2, GLRaV-3 or GFkV tested positive for virus by RT-PCR and ELISA. More grapevines tested positive for virus by RT-PCR in February, March and May than in any other month by RT-PCR or ELISA. All the Chardonnay and Shiraz grapevines inoculated with GLRaV2 and GLRaV-3 tested positive in most months during the testing period. All Shiraz grapevines and 2/5 Chardonnay grapevines inoculated with GFkV also tested positive in most months.

At the Yarra Valley 29/30 grapevines inoculated with GLRaV-2, GLRaV-3 or GFkV tested positive for virus by RT-PCR and ELISA. More grapevines tested positive for virus by RT-PCR in December and January than in any other month by RT-PCR or ELISA. All GLRaV-2 and GLRaV-3 inoculated Chardonnay and Shiraz grapevines tested positive in most months during the testing period. All Shiraz grapevines and 4/5 Chardonnay grapevines inoculated with GFkV also tested positive in most months. GVA has not been detected.

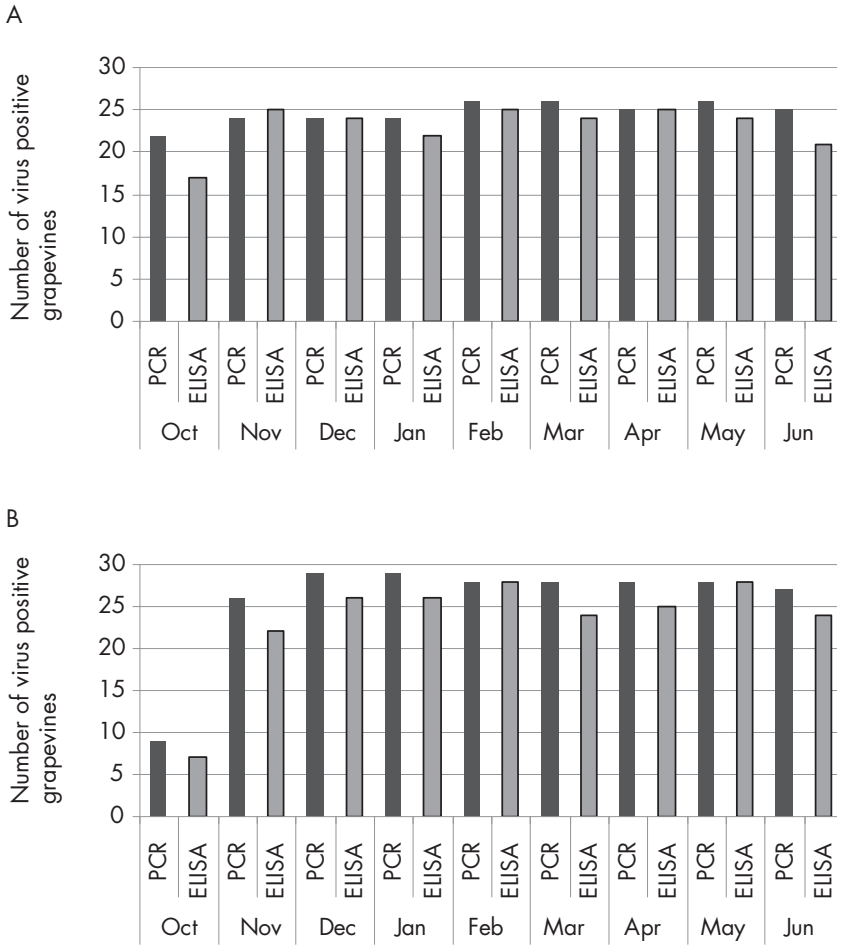

Figure 2. The total number of grapevines that tested positive for virus at A) Sunraysia and B) the Yarra Valley between October 2007 and June 2008. A total of 40 grapevines (20 Shiraz and 20 Chardonnay) were inoculated with GLRaV-2, GLRaV-3, GVA or GFkV at each site, however GVA has not been detected.

It was also observed that the GLRaV-2 and GFkV ELISAs were slow to develop a positive reaction compared with the GLRaV-3 ELISA. For the GLRaV-3 ELISA, clear positive results were observed after three hours of plate development. For the GLRaV-2 ELISA clear positive results were often only obtained after overnight development of the plates. A similar result was also observed for the GFkV ELISA, particularly later in the season from January to June.

Limit of detection

Virus testing by ELISA was reliable down to a dilution of 1/10 for GLRaV-3 and GFkV at each site and in both varieties when virus positive tissue was diluted with uninfected tissue and when the extract of infected material was diluted with the extract of uninfected material. When the extract of infected material was diluted with buffer virus, it could be detected at a dilution of 1/20. ELISA testing for GLRaV-2 was reliable down to 1/5 in each dilution series.

Variable results were associated with virus detection by RT-PCR in the dilutions series experiment for GLRaV-2, GLRaV-3 and GFkV although all housekeeping RT-PCRs indicated the presence of RNA in each sample. In some dilution series made from plant tissue, plant extracts or buffer, virus could be detected up to 1/100 dilution for all viruses. However, not all dilutions were positive within each dilution series. The greatest number of PCR positive results was obtained from the dilution series of GLRaV-3 and negative results were obtained only with dilutions made from plant material and these included 1/100 dilution of Shiraz tissue from Sunraysia, a 1/5 and a 1/20 dilution of Shiraz tissue and a 1/20 and a 1/100 of Chardonnay tissue from the Yarra Valley. The least number of positive results were obtained from GFkV virus from all dilution series and there was no consistent pattern of positive and negative results to indicate if dilution of virus contributed to these results.

Discussion

RT-PCR was found to be more sensitive than ELISA, particularly for GLRaV-2 and GFkV. Based on the results presented here, virus detection is most reliable during late spring (December) to late autumn (May), when green tissue was used. There may be some difference in the reliability of virus testing between climates and RT-PCR detection may be most reliable in December and January in the Yarra Valley (cool climate) and in February and March in Sunraysia (hot climate). The results presented from this study are preliminary and a further year of information will be obtained before final recommendations are made for the timing of virus testing.

The slow development of the GLRaV-2 and GFkV ELISAs may also lead to false negative results if the plates are not observed after an overnight development. The slow reactivity might be associated with low virus titre or low enzyme activity of the conjugated antibodies due to the quality of the antisera or to inhibitors in the grapevine extracts. It may also be associated with low specificity of the antibodies for the virus strains used in this experiment compared to the strains to which they were developed.

Green tissue can be used for virus detection. The use of green tissue will allow certification schemes to test nucleus collection, foundation planting and

mother blocks earlier in the year. Results from this green tissue testing will be useful as they can be obtained and decisions made about the distribution of material to industry well in advance of collecting material for distribution. October was the least reliable month for detection and it is possible that new shoot growth exceeds virus replication at this time of year with lower virus titres leading to false negative results.

Nucleic acid can be reliably extracted from green tissue using the high throughput automated nucleic acid extraction procedure, which may result in a saving to industry due to the reduced time and labour associated with processing samples for the RT-PCR tests. In our hands, the automated nucleic acid extraction procedure can be completed in 1.5–2 days for 96 samples rather than 3–4 days using other methods. An additional advantage is that samples can then be tested for all viruses as opposed to ELISA, for which kits are not available for some viruses such as GRSPaV. Also it will allow the use of RT-PCR tests that can detect all known strains of a virus, whereas we have observed the some of the ELISA kits that are commercially available do not detect all known strains of some viruses in Australia (F. Constable, unpublished).

Although RT-PCR reliability for the June 2007/08 samples appeared to be similar to previous months they could not be extracted using the high throughput automated nucleic acid extraction procedure due to the high viscosity of the samples when ethanol was added to precipitate nucleic acids. The samples of this month consisted of phloem scrapes from woody tissue and it is likely that the samples became viscous due to the co-precipitation of other compounds such as polyphenols and polysaccharides (Iandolino et al. 2004, Reid et al. 2006), which might be in higher concentration in woody tissue compared to green tissue. Consequently the longer method of nucleic acid extraction with CH_3:IAA was used. Although this method was reliable it was more time consuming and in our hands it took 3–4 days to extract nucleic acid from 96 samples In addition, the co-precipitated compounds may form complexes with nucleic acids thus inhibiting transcription of RNA and amplification of cDNA of during RT-PCR (Iandolino et al. 2004) and leading to false negative results. In our experiment it was observed that green tissue was less likely to form viscous extracts after the addition of ethanol, suggesting that there were less polyphenols and polysaccharides present in the tissue. This, in turn, may lead to less inhibition of the RT-PCR reactions when this tissue is used compared to woody tissue and lead to fewer false negative results. Further work will be done to determine whether extracts from woody tissue contain more inhibitors of the RT-PCR than green tissue.

In the first year after inoculation, fewer positive results were obtained compared to the second year, suggesting that new/young infections may be missed by ELISA and RT-PCR. Some grapevine viruses can be transmitted by insect vectors and new infection events can occur in any planting. Consequently it is important to repeat test grapevines, particularly within certification schemes, in case new infection events occur and remain unobserved and undetected in the early stages of infection. It was also observed that some Shiraz grapevines and all Chardonnay grapevines in which virus was detected in the second year did not display typical symptoms associated with grapevine leafroll associated viruses or GFkV. Consequently symptomless infections do occur. This observation indicates that active testing for virus infection is more reliable than symptom expression.

Pooling samples should allow for more samples to be tested at a reduced cost to industry. The dilution series experiment indicated that samples may be pooled up to 1/5 for ELISA and to 1/20 for PCR. These results also indicate that RT-PCR is more sensitive than ELISA, but the presence of false negative results in some samples suggested some inhibition of the viral RT-PCRs, particularly in dilutions derived from plant tissue, even though the housekeeping PCR's indicated the presence of RNA. In this study, samples were used from March collection, when material was starting to lignify and some woody tissue was used, which may have resulted in co-isolation of inhibitory compounds such as polyphenols and polysaccharides. The GLRaV-3 RT-PCR returned more positive results than RT-PCRs for GLRaV-2 and GFkV. It is possible that the negative results for the latter two viruses were a result of a combination of inhibition of the tests by inhibitory compounds co-isolated with the nucleic acid in combination with lower titres of these viruses in grapevine tissue compared to GLRaV-3. Further work needs to be done before pooling of samples is recommended and the experiment will be repeated in summer 2009 using only green tissue.

Conclusions

The results of this study show that RT-PCR is more sensitive and reliable for virus detection compared to ELISA. Grapevine viruses can be detected reliably as early as December in each growing season and green tissue can be used. A high throughput nucleic acid extraction procedure has been developed that should reduce the cost of RT-PCR for grapevine virus detection. The possibility also exists that samples may be pooled to enable testing of more samples at a reduced cost to industry, however further work will be done before final recommendations are made for timing of sampling and pooling of samples for RT-PCR for detection of grapevine viruses.

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