Development and validation of diagnostic protocols for the detection of endemic and exotic pathogens of grapevines



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ABSTRACT

A suite of molecular based diagnostic tests have been developed and validated that will enhance the plant health status and biosecurity of the Australian grapevine industry. Specifically we have developed:

- Reliable sampling strategies for sensitive virus detection
- A high throughput nucleic acid extraction procedure using green tissue that should reduce the cost of RT-PCR for grapevine virus detection. This capability will also improve Australia's responsiveness to an exotic pathogen incursion.
- Validated molecular protocols for ten endemic viruses, 16 exotic viruses and bacterial blight *(Xylophilus ampelinus*).

The survey indicated that Australian grapevines appear to be free of some of the significant grapevine viruses and *X. ampelinus*.

EXECUTIVE SUMMARY

Vine health impacts the viticultural industry at various levels, including the cost and quality of planting material, the sustainability and productivity of vineyards, the quality of the end product (wine, table grapes, dried fruit and ornamental grapevines) and export capabilities. Although Australian viticulture is not affected by some of the serious diseases or pathogens that occur in other countries, some important diseases, and associated pathogens, were introduced to Australian viticulture including leafroll and rugose wood diseases.

In Australia and other countries quarantine and certification programs exist to prevent the introduction and spread of pathogens into viticulture. These programs support the improved productivity and sustainability of the viticulture industry by the provision of high health planting material in which disease-associated pathogens such as viruses and bacteria have not been detected.

Recent literature suggests that there are approximately 60 viruses that can infect grapevine. Many of these are associated with serious diseases of grapevine and are controlled through quarantine and certification programs. There are also 15 significant bacteria, including 10 phytoplasmas, *Xylophilus ampelinus, Xylella fastidiosa, Agrobacterium sp* (*A. rubi and A.vitis*) and *Xanthomonas campestris pv viticola,* that can infect grapevines and which are controlled through quarantine and certification programs.

Of the 60 viruses that infect grapevines worldwide 10 viruses are endemic in Australia and these include *Grapevine virus A (*GVA), *Grapevine virus B* (GVB), *Rupestris stem pitting associated virus* (RSPaV), *Grapevine fleck virus* (GFKV), Grapevine leafroll associated viruses (GLRaV)-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5 and GLRaV-9. Grapevine viruses of quarantineable significance to Australia include: GVB (strains associated with corky bark), *Grapevine virus D* (GVD), *Secoviridae* species (16 in total) and two tombusviruses (*Grapevine Algerian latent virus* and *Petunia asteroid mosaic virus*). Bacteria of quarantine significance to Australia include, *Xylella fastidiosa, Xylophilus ampelinus* and Flavescence dorée and Bois noir phytoplasmas.

There have been some major advances in plant pathogen molecular diagnostics. Improved molecular techniques have been developed overseas which can be used to detect many of the endemic and quarantine pathogens listed for grapevines. In this project an extensive literature review was conducted and international experts were consulted to identify the latest diagnostic protocols available for detection of the ten endemic viruses and GVB (strains associated with corky bark), GVD, *Secoviridae* species, two tombusviruses and *X. ampelinus*. Diagnostic protocols for phytoplasmas and *X. fastidiosa* were previously developed and validated under Australian conditions by DPI Victoria and the University of Adelaide during other projects and were not used in this project.

In this research program, sampling procedures were developed for endemic grapevine viruses and they can be detected reliably as early as December in each growing season. A

high throughput nucleic acid extraction procedure using green tissue (petioles and leaf midribs) was also developed that is reproducible, accurate and sensitive and should reduce the cost of RT-PCR for grapevine virus detection. We recommend that up to five samples can be pooled into the one test sample for grapevine virus detection during spring and summer. This will enable testing of more grapevines at a reduced cost to industry.

The movement of viruses within grapevines can have important implications for virus detection and disease expression. Our results show that it can take more than 12 months for viruses to move from the point of infection to shoots and cordons of the grapevine, which might be sampled for virus detection. This highlights the need for continued and active surveillance of high health planting material within certification schemes, as there is a risk of new infection events, which may go un-detected for some time.

Results of monthly testing showed that RT-PCR is more reliable than ELISA during for virus detection during spring and summer. We also showed that the reliability of the ELISA and PCR tests increased after 12 months and up to three years post-inoculation, possibly because viruses were not sufficiently translocated throughout the grapevines within the first 12 month infection period. Detection of viruses by RT-PCR and ELISA is rarely 100% efficient and re-testing of grapevines within certification programs is recommended to reduce the risk of a false negative result. Based on the results of these experiments we recommend that grapevines to be certified for inclusion into nucleus collections should undergo annual RT-PCR testing for viruses and only be certified and admitted after three years of negative test results.

It was shown that viruses may not be reliably detected by biological indexing and factors affecting this diagnostic method included the success of chip bud inoculation with regard to bud take and transmission of the candidate virus from the chip bud to the grapevine indicator. Environment also impacted on symptom expression and some virus isolates were shown to be symptomless on biological indicators.

Our results in this experiment and throughout this project clearly demonstrate that no single virus detection technique will detect all strains of a virus and that all detection methods can be affected by virus distribution and/or titre. We recommend that biological indexing continue to be used for virus detection in conjunction with molecular tests to increase the chance of detection of viruses, particularly for the varieties and clones that are being introduced into certification programs and during post entry quarantine (PEQ) for new importations. If symptoms are observed on woody indicators it is recommended that virus-specific tests are conducted to identify viruses associated with the observed symptoms.

We surveyed Australian grapevines for endemic and exotic grapevine viruses and *Xylophilus ampelinus.* The purpose of the survey was two-fold: (i) to test the developed protocols under “local” conditions and identify any potential “false positives” or organisms that can make interpretation of results difficult and (ii) to update the disease status for each pathogen in Australia.

We have validated RT-CPR tests for endemic viruses including GLRaV-1, -2, -3, -4, -5, -9, GVA (two tests required), GVB, GFkV and RSPaV (two tests required). Diagnostic tests were also validated for the following exotic pathogens: GLRaV-6, GLRaV-7, *Arabis mosaic virus, Artichoke Italian latent virus*, *Cherry leafroll virus*, *Grapevine Anatolian ringspot virus*, *Grapevine chrome mosaic virus*, *Grapevine deformation virus*, *Grapevine fanleaf virus*, *Peach rosette mosaic virus*, *Raspberry ringspot virus*, *Tobacco ringspot virus, Tomato black ring virus*, *Tomato ringspot virus, Strawberry latent ringspot virus, Grapevine Algerian latent virus*, *Petunia asteroid mosaic virus* and *Xylophilus ampelinus*).

Generic tests for the detection of *Closteroviridae* species, *Ampleovirus* subgroup I species and Ilarvirus species were also validated and will be useful for virus detection in PEQ, especially for GLRaV-6 and -7, GAMV and GLPV. However these will require that sequencing is done on the amplicons that are generated for virus identification.

We have identified three putative viruses of grapevines that need further characterisation and these include a strain of GVD, a previously uncharacterised *Ilarvirus* and an uncharacterised subgroup I *Ampleovirus*. If these putative viruses are real their incidence in Australian vineyards and their impact on vine health needs to be determined and active testing may need to be incorporated into Australian certification programs.

Conclusion

The sampling protocols, extraction procedure and tests developed in this project will provide Australian grapevine certification programs with up to date and improved diagnostic capabilities that are internationally accepted. The recommended tests for endemic grapevine viruses will benefit industry through the production of high health material that has been reliably tested for the pathogens that have a significant negative impact on the yield and quality of fruit. These tests do not replace and should be used in conjunction with biological indexing to improve the chance of virus detection especially for new varieties and clones being incorporated into any nuclear collection.

The development of tests for exotic grapevine pathogens has improved preparedness in case of an incursion and will strengthen pathogen screening protocols in PEQ. These tests can be used during PEQ as active testing for specific pathogens of concern and to support the results of biological indexing and confirm the identity of pathogens. Active testing using the recommended RT-PCR protocols could reduce the cost of importation to growers if a quarantine pathogen is detected early during the PEQ process.

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CHAPTER 1: INTRODUCTION

1.1 Background

The improved productivity and sustainability of the viticulture industry is supported by the provision of high health planting material in which disease-associated pathogens such as viruses and bacteria have not been detected. Vine health impacts the viticultural industry at various levels, including the cost and quality of planting material, the sustainability and productivity of vineyards, the quality of the end product (wine, table grapes, dried fruit and ornamental grapevines) and export capabilities. In Australia and other countries quarantine and certification programs exist to prevent the introduction and spread of pathogens into viticulture.

Recent literature suggests that there are approximately 60 viruses that can infect grapevine (Table1.1; Martelli, 2009). Many of these are associated with serious diseases of grapevine and are controlled through quarantine and certification programs. There are also some diseases of unclear aetiology, but which are thought to be associated with virus or virus-like organisms. Fifteen significant bacteria, including 10 phytoplasmas (Table 1.2), *Xylophilus ampelinus, Xylella fastidfiosa, Agrobacterium sp* (*A. rubi and A. vitis*) and *Xanthomonas campestris pv viticola,* that can infect grapevines are also controlled through quarantine and certification programs.

The selection of “healthy” material from productive clones and varieties and quarantine restrictions have assisted in preventing some of the more serious diseases and pathogens of grapevine entering Australia. Never the less, some serious diseases, including leafroll and diseases of the rugose wood complex, and associated pathogens were introduced to Australian viticulture. The Fruit Variety Foundation (FVF) and the South Australian Vine Improvement Scheme were established in 1972 in Victoria and 1973 in South Australia respectively (Shanmuganathan and Fletcher 1980; Cirami et al, 1988) and aimed to reduce the risk of spread of serious grapevine diseases by providing industry with high-quality, pathogen-tested planting material. Both schemes formed the basis of current Australian grapevine certification programs.

Table1.1. Grapevine viruses (Family, genus and species) currently known to infect grapevines, their associated disease, their insect vector and their prevalence in Australia

Family	Genus	Virus	Disease	Insect vector	Presence in Australia
Closteroviridae	*Ampelovirus* – Subgroup II	*Grapevine leafroll associated virus* (GLRaV)-1, GLRaV-3	Leafroll disease	Mealybug/scale	Yes
	Ampelovirus – Subgroup II	GLRaV-4, GLRaV -5, GLRaV -6, GLRaV -9, Tentative species: GLRaV-10, GLRaV -Pr. GLRaV -De	Leafroll disease	Mealybug/scale	Only GLRaV-4, -5, and -9 have been detected
	Clostervirus	GLRaV-2	Leafroll disease Also associated with graft incompatibilities	Unknown	Yes
	Unassigned	GLRaV-7	Leafroll disease	Unknown	Not detected
Betaflexividae	*Vitivirus*	*Grapevine virus A*(GVA), GVB - strains not associated with Corky bark	Rugose wood – stem grooving Latent	Mealybug/scale	Yes
		GVB strains associated with Corky bark	Rugose wood – Corky bark	Mealybug/scale	No
		GVD, GVE	Corky rugose wood unknown		
	Foveavirus	*Rupestris stem pitting associated virus* (RSPaV)	Rugose wood – stem pitting	Unknown	Yes
	Trichovirus	*Grapevine berry inner necrosis virus (GINV)*	Grapevine berry inner necrosis disease	Unknown/eryophid mite??	Not detected
Alphaflexiviridae	*Potexvirus*	*Potato virus X (PVX)*	Latent	Aphid in other crops	Yes - In other crops
Tymoviridae	*Maculavirus*	*Grapevine Fleck virus (GFkV)*	Fleck on *V.upestris*	Unknown	GFkV – Yes
		Grapevine red globe virus (GRGV)	Latent		GRGV- Not detected
	Marafivirus	*Grapevine rupestris vein feathering virus (GRVFV)*	Vein feathering	Unknown	Not detected

Family	Genus	Virus	Disease	Insect vector	Presence in Australia
		Grapevine asteroid mosaic virus (GAMV)	Mosaic		
		Grapevine syrah virus I (GSyV-I)			
Secoviridae	*Nepovirus*	*Arabis mosaic virus (ArMV)*	Degeneration, Fanleaf	Nematode	Yes – other crops
Subfamily *Comovirinae*	Subgroup A	*Tobacco ringspot virus, (TRSV)*	Decline	Nematode	
		Grapevine deformation virus (GDefV)	Degenertaion	Unknown	Not detected
			Fanleaf, degeneration	Nematode	
		Grapevine fanleaf virus (GFLV)	Fanleaf, degeneration	Nematode	
		Raspberry ringspot virus (RsRSV			
	Nepovirus Subgroup B	*Artichoke Italian latent virus (AILV)*	Fanleaf	Nematode	Not detected
		Grapevine chrome mosaic virus (GCMV)	Degeneration/ yellowing	Nematode	
		Grapevine Anatolian ringspot virus (GARMV)	Mild fanleaf	Unknown	
		Tomato black ring virus (TBRV)	Degeneration/decline	Nematode	
	Nepovirus Subgroup C	*Tomato ringspot virus (ToRSV)*	Decline	Nematode	Yes
		Cherry leafroll virus (CLRV)	Yellow mosaic	Nematode	Not detected
		Peach rosette mosaic virus(PRMV)	Decline	Nematode	
		Blueberry leaf mottle virus (BLMoV)	Decline on *Vitus lambrusca* cv. Concord	Unknown	
		Grapevine Bulgarian latent virus (GBLV)	Latent	Unknown	
		Grapevine Tunisian ringspot virus (GTRSV)	Mild fanleaf	Unknown	

Family	Genus	Virus	Disease	Insect vector	Presence in Australia
	Fabavirus	*Broad bean wilt virus (BBWV)*	Latent	Aphid	Yes – other crops
	Unassigned	*Strawberry latent ringspot virus (SLRSV)*	Fanleaf, degeneration	Nematode	
Tombusviridae	*Tombusvirus*	*Grapevine Algerian latent virus (GALV)* *Petunia asteroid mosaic virus (PeAMV)*	Latent/symptomless	Unknown	Not detected
	Carmovirus	*Carnation mottle virus (CarMV)*	Roditis leaf discolouration (*Carnation mottle virus* in combination with GFLV)		Yes – other crops
	Necrovirus	*Tobacco necrosis virus (TNV)*	Mottle? Yellow spot? On R.St. George	Fungi - *Olpidium brassicae*	
Bromoviridae	*Alfamovirus*	*Alfalfa mosaic virus (AMV)*	Yellow mottle	Aphid	Yes – other crops
	Cucumovirus	*Cucumber mosaic virus (CMV)*		Aphid	Yes – other crops
	Ilarvirus	*Grapevine angular mosaic virus (GAMV)*	Angular mosaic	Unknown	Not detected
		Grapevine line pattern virus (GLPV)	Line pattern	Unknown	
Bunyavidiae	*Tospovirus*	*Tomato spotted wilt virus (TSWV)*	Yellow dwarf	Thrips	Yes – other crops
Luteoviridae	*Luteovirus*	*Grapevine ajinashika disease virus (GADV)*	ajinashika disease – low sugar content in fruit	Unknown	Not detected
Unassigned	*Sobemovirus*	*Sowbane mosaic virus (SOMV)*		Thrips, leafminer fly, beet leafhopper, fleahopper, aphid	Yes - In other crops
	Tobamovirus	*Tobacco mosaic virus (TMV)*		Unknown	Yes - In other crops
		Tomato mosaic virus (ToMV)		Unknown	
	Ideaeovirus	*Raspberry bushy dwarf virus (RBDV)*		Unknown – possible nematode vector	Not detected

Family	Genus	Virus	Disease	Insect vector	Presence in Australia
Unknown		Grapevine labile rod-shaped virus		Unknown	Not detected
		Grapevine Stunt virus	Stunting	Leafhopper (*Arboridia apicalis)*	Not detected
Diseases with a possible virus association			Grapevine enation, Grapevine summer mottle/Grapevine vein mosaic		
		Thought to be RSPaV	Grapevine vein necrosis		
		Possible association with GVB	LN33 stem grooving		

Table 1.2 Grapevine yellows diseases, associated phytoplasmas, their insect vector and their prevalence worldwide (after Boudon-Padieu, 2003; 2005).

Grapevine yellows disease	Phytoplasma name	Ribosomal group (subgroup)	Insect vector	Occurrence
Flavescence dorée	Flavesence dorée (FD; *Candidatus* Phytoplasma vitis*)	16SrV (-C, -D) or Elm Yellows (EY) group	*Scaphoideus titanus* Ball	France, Italy, Spain, Serbia, Slovenia, Switzerland
Palatinate grapevine yellows	Palatinate grapevine yellows (PGY)	16SrV or EY group	*Oncopsis alni* Schrank	Germany
Bois noir, Legno nero, Vergilbungskrankheit Schwarzholzkrankheit	Stolbur (STOL, *Candidatus* Phytoplasma solani*)	16SrXII-A or Stolbur group	*Hyalesthes obsoletus* Sign	Europe, Israel, Lebanon
Australian grapevine yellows	*Candidatus* Phytoplasma australiense	16SrXII-B	Unknown	Australia
Australian grapevine yellows	Tomato big bud (TBB)	16SrII -D	Unknown (*Orosius orientalis* in other crops)	Australia
Buckland Valley grapevine yellows	Buckland Valley grapevine yellows (BVGY)	16SrI-related or Aster Yellows (AY) group	Unknown	Australia
Grapevine yellows	Aster yellows	16SrI (-B, -C) or AY group	Unknown	Italy, Chile, Tunisia
North American grapevine yellows (NAGY)	Virginia grapevine yellows I (NAGY I)	16SrI-A or AY	Unknown	Virginia (US)
	Western X (WX) Virginia grapevine yellows III (NAGYIII)	16SrIII-I or WX	Unknown	New York (US) Virginia (US)
Grapevine yellows	*Candidatus* Phytoplasma fraxini	16SrVII	Unknown	Chile
Grapevine yellows	X-disease	16SrIII	Unknown	Italy, Israel

1.2 Viruses

Table 1 list the viruses that are currently known to infect grapevines and their occurrence in Australia. Grapevines appear to be a sink for viruses from a broad range of families and genera. Many of the viruses are associated with significant diseases of grapevines, however a number are not widespread and are considered of minor importance to viticulture worldwide (Martelli, 1993, Martelli 2009). The viruses which are associated with some of the more serious diseases of grapevines occur in the *Closteroviridae* and include viruses associated with leafroll disease, *Betaflexiviridae* and include viruses associated with the rugose wood complex, *Secoviridae* and many viruses are associated with degeneration and decline of grapevines and the *Tymoviridae* associated with fleck diseases.

Grapevine viruses have been associated with loss in yield and quality of fruit, reduced vine growth and loss in can pruning weight, graft incompatibility, and vine decline and death. For example fruit yield loss has been associated with infection by leafroll viruses and those associated with the rugose wood complex (Abrashevea, 1977; Guidoni et al 2000; Mannini and Credi, 2000; Simon et al 2003; Kovacs et al 2000; Kovacs et al, 2001; Tomazic et al 2000; Tomazic et al 2005; Komar et al 2007). Poor fruit quality as a result of changes in titratable acidity, sugar content, anthocyanin content and aroma associated with reduced terpenoids are often associated with leafroll infections (Borgo and Angelini, 2002; Borgo et al, 2005; Flamini et al 2006; Golino et al 2009a, 2009b Guidoni et al 2000; Kim et al 2003; Komar et al 2007; Kovacs et al 2000; Mannini, 2001; Mannini and Credi, 2000; Malossini et al 2003; Woodham et al 1983;). Reduced growth and cane pruning weight was observed in various rootstocks inoculated with a combination of GLRaV-1, GLRaV-3, GFLV and rugose wood associated viruses (Credi and Babini, 1996). Some strains of GLRav-2 are associated with graft incompatibilities (Bonfiglioli et al 2003, Uyemoto and Rowhani 2003; Uyemoto et al 2000; Uyemoto et al 2000). Viruses in the *Secorviridae*, such as GFLV and ArMV, are associated with significant yield loss, fruit quality and grapevine vigour and vines may decline and die (Auger et al 1992; Martelli 1993; Walter and Martelli, 1996; Golino et al 2003; Legorburu et al 2009; Santini et al 2009) Vine death has been associated with viruses of the rugose wood complex (Tomazic et al 2005).

Conversely some virus species and strains may not cause disease or impact yield and quality parameters especially when they infect grapevines in the absence of other viruses (Clingeleffer and Krake, 2002). For example, GVA infected Marzemino grapevines had reduced bud fertility, but GVA and GLRaV-1 did not significantly alter yield nor soluble solids, total acidity, pH, anthocyanins and polyphenols (Malossini et al 2009a, 2009 b). Interestingly a few studies suggest that there may be benefits from virus infection in grapevines. For example "Crimson Seedless" inoculated with a mixture of GLRaV-3, -5 and -9 and GVA had increased berry weight and desirable berry colour, which was less intense, compared to uninfected grapevines (Brar et al 2008; Cameron 2007).Similarly the mildly leafroll affected cultivar Emperor produced larger and crisper berries compared to symptomless clones (Cameron, 1984).

All grapevine viruses can be transmitted through planting material. Some viruses are also transmitted via insect vectors, including mealybugs and scale, nematodes and aphids.

1.2.1 *Closteroviridae*

Grapevine leafroll associated viruses (GRLaV) are members of the family *Closteroviridae* and belong to the genera: *Ampleovirus* and *Closterovirus*. GLRaV-7 remains unassigned to any genera. GLRaVs are differentiated into these three genera based on their genome structure, nucleic acid and amino acid sequence similarity and biology. Viruses of the *Closteroviridae* are flexuous, rod-shaped virions, 1,250-2,200 nm in length and contain a positive sense, single stranded RNA of 13.7-19.3 kb (Maliogka et al 2008a; Martelli et al., 2002).

Phylogenetic analysis indicates that Ampeloviruses are highly diverse and fall into to two distinct subgroups, I and II (Malioka et al 2008a). Subgroup I includes GLRaV-4,-5,-6,-9, -Pr and –De. Subgroup II Ampeloviruses include GLRaV-1 and GLRaV-3. Full length genome sequences are not available for GLRaV-4, -5, -6, -9 and –De but is available for GLRaV-Pr and sequence analysis indicates that this virus and potentially other GLRaVs in this subgroup have a smaller and simpler genome structure compared to GLRaV-1 and -3 (Maliogka et al 2009). Currently the GLRaV-4,-5,-6 and -9 are considered distinct *Ampelovirus* species. However based on their genome structure, serological relationships and biology (i.e. symptomatology and vectors) there is a suggestion that the taxonomy will be contracted and that these GLRaV species along with GLRaV-Pr and -De will be considered strains of one species (Martelli 2009). Subgroup I Ampeloviruses are thought to be associated with less sever leafroll symptoms compared to those in subgroup II (Martelli 2009). Field spread of leafroll disease associated with GLRaV-1, -3, and -9 has been observed (Habili et al 2003; Habili and Nutter 1997; Martelli 2003) and each of these viruses and GLRaV-5 can be transmitted by mealybugs and scale (Sforza et al 2003; Gugerli, 2003; Sim et al 2003; Zorloni et al 2006). Field spread of GLRaV-4, -5, -6, -Pr and - De has not been reported

GLRaV-2 is a species of the genus *Closterovirus.* It is the only leafroll virus that has been experimentally mechanically transmitted to another herbaceous host, *Nicotiana benthamiana* (Boscia et al 1995; Goszczynski et al 1996). Field spread of GLRaV-2 has not been reported and a vector is unknown. Other *Closterovirus* species however are aphid transmitted (Martelli 2002). Interestingly GVC, at one time considered to be related to the vitiviruses, is now considered to be a strain of GLRaV-2 (Masri et al 2006). Likewise, Grapevine rootstock stem lesion associated virus, is also considered to be a strain of GLRaV-2 (Meng et al 2005). GLRaV-7 is an unassigned species of the *Closteroviridae* although recent research indicates that it is most closely related to *Crinivirus* species, some of which are whitefly transmitted Mikona et al 2009). A vector for GLRaV-7 is unknown and field spread has not been reported.

GLRaV- 8 has only been reported once and its identification was based only on serology and a partial sequence (273bp) of the putative coat protein gene that was amplified by PCR (Monis 2000). It has since been demonstrated that the PCR fragment was amplified from the genome of *Vitis vinifera* and the virus is to be removed from the list of those that infect grapevines (Bertsch et al 2009; Dolja, 2009).

All GLRaV species are successfully transmitted through vegetative propagation and grafting (Martlelli 2003).

GLRaV species are associated with grapevine leafroll disease, although Koch's postulates have not been proven. Grapevine leafroll disease is detected by visual observation of symptoms in sensitive varieties but some varieties are tolerant and may remain symptomless (Krake et al 1999). Differences in symptom severity may also be associated with a specific GLRaV species and stronger symptoms are often attributed GLRaV-1 and -3 (Martelli 2009). However strain variation can occur within each species and different strains may also be

associated with differences in symptom severity. Thus visual observation will not identify the specific GLRaV species that is present. The Cabernet Franc (Table 1.3) is the commonly used biological indicator for grapevine leafroll disease. However, symptom severity on this indicator is also affected by the GLRaV species and strain with which it is inoculated and not all GLRaV strains induce symptoms on the variety (Cirami et al 1988; Rowhani et al 1997; Chapter 2). Biological indexing will also not identify the specific GLRaV species that is present.

Both ELISA and PCR are commonly use for the specific detection of GLRaV species and strains. ELISA kits are commercially available for detection of GLRaV-1-7 and GLRaV-9. Many specific PCR tests exist for each GLRaV species and generic tests for *Closterovirus*es and subgroup I ampeloviruses have also been reported (Chapter 2).

Grapevine leafroll disease affects grapevines in Australia and the associated viruses that are known to occur include GLRaV-1, -2, -3, -4, -5 and -9 (Table1).

1.2.2 Betaflexiviridae

Grapevine viruses of the *Betaflexiviridae* are found in the genera *Vitivirus, Foveavirus* and *Trichovirus.* Viruses of the *Betaflexiviridae* are flexuous, rod-shaped virions, 723-785 nm in length and contain a positive sense, single stranded RNA of 7.4-8.7 kb (Yoshikawa et al 1997; Nolasco et al 2000; Adams et al 2004; Martelli et al 2007).

Vitivirus species include *Grapevine Virus A* (GVA) GVB, GVD and GVE. *Ruspestris stem pitting associated virus* (RSPaV) is the sole Foveavirus species. GVA, GVB, GVD and RSPaV are associated with the rugose wood complex, which includes pitting and grooving symptoms of the wood cylinder and corky bark symptoms (Martelli et al 2007) but Koch's postulate for cause of disease have not been proven. They may also be associated with graft incompatibilities. The association between GVE and rugose wood symptoms is unknown (Nakuane et al 2008).

GVA, GVB and GVE can be transmitted by mealybug and/or scale insects (Nakuane et al 2008; Martelli et al 2007) Field spread of GVA and GVB has been observed (Habili 2006; Bertin et al 2009; Engelbrecht and Kasdorf 1990). Natural spread of RSPaV is not reported and no vector is known, however it occurs with high incidence in many grape growing regions worldwide (Meng et al 1999). GVA, GVB, GVD, GVE and RSPaV are transmitted through propagation and grafting. GVA, GVB and RSPaV occur in Australia, however corky bark disease, which is associated with strains of GVB, is not known to occur in this country.

Grapevine berry inner necrosis virus (GINV) is the only Trichovirus species reported to infect grapevines (Adams et al 2007). The distribution of GINV seems to be limited to Japan where it infects the varieties Kyoho, Pione, Takao and Campbell Early (Yoshikawa et al 1997). In these varieties it has a significant impact on the health of the grapevines, resulting in poor growth and causes necrosis of berries (Yoshikawa et al 1997). Field spread has been observed and it is transmitted by the grape erineum mite *Colomerus vitis* (Kunugi et al 2000).

Biological indexing can be used for detection of rugose wood symptoms associated with GVA, GVB and RSPaV (Table 1.2). This diagnostic test is not used for diseases associated with GVD or GVE. ELISA tests are available for GVA and GVB. Specific RT-PCR tests have been developed for all *Betaflexiviridae* species that infect grapevines (De Meyer et al 2000; Goszczynski and Jooste, 2002, Goszczynski and Jooste, 2003; Minafra et al 1997 MacKenzie 1997 Moreira, et al 2004. Minafra and Hadidi, 1994; MacKenzie 1997 Sciancalepore et al 2006; Chapter 2; Yoshikawa et al 1997; Nakaune et al 2008). A generic test for Vitivirus and Foveavirus species has also been developed (Dovas and Katis 2003a, 2003b).

1.2.3 *Secoviridae*

The *Secoviridae* subfamily *Comovirinae* is well represented amongst the virus species that infect grapevines and include 15 *Nepovirus* species from subgroups A, B and C, one Fabavirus species and Strawberry latent ringspot virus (SLRSV) which is an unassigned species (Table 1.1). Viruses of the *Secoviridae* subfamily *Comovirinae* are icosahedral virions, 25-30 nm in diameter and have a bipartite linear positive sense, single stranded RNA genome composed of RNA1, 6-8 kb and RNA 2, 4-7 kb (Sanafaçon et al 2009).

Many of the *Comovirinae* species cause serious diseases of grapevine often refereed to as “degeneration” in Europe and “decline” in the USA. *Grapevine fanleaf virus* (GFLV) is found in many grape growing regions worldwide. GFLV causes leaf deformation and, depending on the strain, yellow mosaic and/or vein banding of the leaves. Infection by GFLV can lead to significant yield loss and affected grapevines can eventually decline and die (Martelli 1993).

Fanleaf symptoms are also caused by other *Comovirinae* species including *Arabis mosaic virus* (ArMV), *Strawberry latent ringspot virus* (SLRSV), *Tomato black ring virus* (TBRV), *Raspberry ringspot virus* (RsRSV) and *Grapevine chrome mosaic virus* (GCMV) which occur in Europe. In the USA *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV), *Peach rosette mosaic virus* (PRMV) and, although not widespread, *Blueberry leaf mottle virus* (BLMV), are associated with decline of grapevines and can cause symptoms such as stunting, yellow vein and leaf distortion. Both viruses can cause significant yield loss. *Grapevine deformation virus* GDefV is also associated with fanleaf-like symptoms and degeneration of grapevines. However its distribution is limited to Turkey. *Grapevine Anatolian ringspot virus (*GARSV) and *Grapevine Tunisian ringspot virus* (GTRSV) are associated with mild flan leaf symptoms and are limited to Turkey and Tunisia respectively. *Cherry leafroll virus* (CRLV) is associated with yellow mosaic symptoms in Germany and Chile (Ipach et al 2003; Herrera and Madariaga 2001). *Grapevine Bulgarian latent virus* (GBLV) and *Broad bean wilt virus* (BBWV) are latent in grapevine and *Artichoke Italian latent virus* (AILV) is associated with fanleaf-like symptoms and reduced growth (Martelli 1993; Jankulova et al 1978). All three viruses were detected in grapevines in Bulgaria, BBWV is also found in South Africa (Martelli 1993). Bratislava mosaic disease was originally reported in Slovakia in the 1960’s (Pozdena and Vanek 1969) and GCMV is now known to be associated with this disease (Dimou et al 1994).

Field spread of ArMV, GFLV, GCMV, SLSRV, ToRSV, TBRV, TRSV, RsRPV and PRMV in grapevines is reported and all may be disseminated through planting material (Martelli 1993). Many of the nepoviruses and SLSRSV are vectored by *Xiphinema* and *Longidorus* (nematode) species. No nematode vector is known for BLMV, but in blueberries it has been found in pollen on the legs of bees (Childress and Ramdsdell 1987). BBWV is transmitted by several aphid species in other crops.

Fanleaf disease has been reported in Australia, in Victoria and South Australia and *Comovirinae* viruses were found in association with the disease (Taylor 1962, Taylor and Hewitt 1964; Meagher et al 1976; Cirami 1988). In South Australia fanleaf disease affected only a small number of grapevines and occurred in the absence of a vector (Cirami et al 1988). In Victoria both the vector and the disease occurred in the Rutherglen district and quarantine restrictions, due to the presence of Phylloxera, prevented their movement to other regions (Krake et al 1999). In recent years there have been no reports of fanleaf disease in both states and GFLV along with many other *Comovirinae* species that infect grapevines remain quarantine pathogens. Interestingly*, X. vuittenezi*, which vectors CLRV and is implicated in the transmission of GCMV, has been detected in declining Shiraz vineyard near Nuriootpa in South Australia (Walker 2004). The presence of a virus in the grapevines was not reported.

Grapevines may display distinct symptoms that are indicative of *Comovirinae* infections, however they may not differentiate between *Comovirinae* species and strains of each species can induce different symptoms (Martelli et al 1993) Some of the viruses are latent in some varieties and clones. Herbaceous indexing can be used for *Comovirinae* detection as mentioned above. ELISA kits are commercially available for GFLV, ArMV, GCMV, ToRSV, TBRV, TRSV, SLRSV, CRLV, RsRPV, BLMV and BBWV. Specific RT-PCR tests are also available for most *Comovirinae* species (Chapter 2) except BLMV, GBLV and GTRSV but it was reported that these viruses should be detected using generic PCR tests for the *Secoviridae* (formerly *Secoviridae*) family (Maliogka et al 2004), or by *Nepovirus* subgroup PCR tests (Digiaro et al 2007).

1.2.4 *Tymoviridae*

Five viruses of the family *Tymoviridae* infect grapevines and occur in two Genera: *Maculavirus* and *Marafivirus* (Table 1.1, Martelli 2002; Al Rwahnih et al 2009). Viruses in the Tymoviridae have isometric particles *c*. 30 um in diameter which contain positive-sense, single-stranded RNA genomes 6.0 to 7.5 kb in length (Martelli 2002).

Grapevine varieties and clones infected with *Maculavirus* or *Marafivirus* may be symptomless (Martelli 2002; Al Rwahnih et al 2009). However Grapevine fleck virus (GFkV) is associated with fleck symptoms *Grapevine rupestris vein feathering virus (*GRVFV) with vein feathering symptoms and *Grapevine angular mosaic-associated virus* (GAMaV) with mosaic symptoms in *V. rupestris* but are often symptomless in *V. vinifera*. *Grapevine red globe virus* (GRGV) was isolated from grapevines with leafroll symptoms, and *Grapevine Syrah virus-1* (GSyV-1)

from Syrah grapevines exhibiting decline but the association between these two viruses and disease is not known. The impact of these viruses in combination with other viruses is also unknown.

Field spread of GFkV has been reported although no vector is known (Engelbrecht and, Kasdorf, 1990b; Fortunisi et al 1996). There are no reported vectors for GRGV, GRVFV, GAMaV or GSyV-1. However GSyV-1 was detected in the leafhopper *Erythroneura variabili* by RT-PCR (Al Rwahnih et al 2009).

GFkV infects grapevines worldwide, including Australia (Martelli et al 2002b). GRGV is reported from Europe, GSyV-1 is reported from the USA and GRVFV and GAMaV has been detected in the USA and Europe (Sabanadzovic et al 2000, Abou Gahnem-Sabanadzovic et al 2003; Al Rwahnih et al 2009).

1.2.5 *Tombusviridae*

Four viruses from three genera of the family *Tombusviridae* have been reported to infect grapevines (Table 1.1): *Grapevine Algerian latent virus* (GALV) and *Petunia asteroid mosaic virus* (PeAMV) are in the genus *Tombusvirus*, *Carnation mottle virus* (CarMV) is the type member of the genus *Carmovirus* and *Tobacco necrosis virus* (TNV) is in the genus *Necrovirus*. The virus particles are isometric and with a diameter of 28-35nm. Each of the four viruses has a linear, positive-sense, single-stranded RNA and they range between 3.8-4.7kb (Koenig et al 2004).

Spread of *Tombusviridae* species in grapevines has not been reported and they are not widely distributed in grapevines. However in other hosts PeAMV, CarMV and TNV can be spread mechanically on pruning equipment and CarMV is transmitted by contact (Hollings et al 1972 Lommel et al 2005). TNV can be spread by the fungus *Olpidium brassicae* in other hosts (Fry and Campbell 1966). TNV, PeAMV and GALV have been detected in ditches, drainage canals, river water in agricultural areas, including vineyards in Germany and Italy (Koenig et al 1989; Cannizzaro et al 1990; Yi et al 1992, Fuchs et al 1994).

The association of PeAMV and disease is uncertain as it has only been isolated from grapevines infected with other viruses including AMV (Novak and Lanzova 1976). TNV was detected in grapevine leaves of the indicator Rupestris St George displaying symptoms of mottling and yellow spots in South Africa (Cesati and van Regenmortel 1969). CarMV was isolated with GFLV from grapevines showing Roditis leaf discolouration in Greece (Avgelis and Rumbos 1990). Symptoms of the disease include yellowing or reddening of leaf tissue along the veins and the interveinal tissues particularly near the petiole and deformed leaves. Grapevines with Roditis leaf discolouration have reduced yield and fruit quality. (Avgelis and Rumbos 1990, Martelii 1993). GALV was detected in grapevines only in Algeria, but was not associated with symptoms (Galitelli et al 1989).

CarMV, PeAMV and TNV can be detected by commercially available ELISA kits. PCR test are also available for Tombusviruses that should detect all species (Keonig et al 2004) and also

for CarMV (Kong et al 2002; Raikhy et al 2006) and TNV (Zitikaite, and Staniulis 2006, Zitikaite et al 2005).

1.2.6 *Bromoviridae*

Viruses of the *Bromoviridae* that infect grapevines include *Alfalfa mosaic Alfamovirus* (AMV), *Cucumber mosaic Cucumovirus* (CMV) and two members of the Ilarvirus genus: *Grapevine angular mosaic virus (*GAMV) and *Grapevine line pattern virus* (GLPV). AMV has bacilliform particles that are 30- 56 nm in length and 18 nm wide and has a linear positive-sense, single-stranded RNA tripartite genome with a total size of 8.27 kb. CMV has isometric particles that are 29 nm in diameter and has a linear positive-sense, single-stranded RNA tripartite genome with a total size of 8.62Kb. GLPV has polymorphic spherical to bacilliform particles that are 24 to over 100 nm in length and has a tripartite genome. GLPV has isometric particles with a diameter of 24nm and a tripartite, linear, positive-sense, single-stranded RNA genome. (Roossinck et al 2005). All of the viruses have been associated with disease in grapevine

GLPV occurs in Hungary (Lehoczky et al 1989). Initial symptoms associated with GLPV include chlorotic spots, blotches, rings or line patterns on grapevine leaves. During the later stages of infection chlorotic spots or flecking of the leaves may be observed and yield and vigour are reduced (Lehoczky et al 1989; Martelli et al 1993) The virus is transmitted by grafting and propagation. No vector is reported

Grapevine angular mosaic disease was first observed in 1994 and only occurs in Greece (Girgis et al 2009). The disease is caused by GAMV and symptoms include sharp, angular mosaic symptoms on leaves. The leaves may be deformed, flowers abort and grapevines are stunted and gradually decline and die. Yield may be significantly reduced. (Girgis et al 2009). GAMV is only reported in Greece and the virus is transmitted through seed and pollen. No vector is reported.

AMV is associated with yellow mottle disease which includes foliar symptoms of yellow spotting, mosaic and ring patterns. Yield and vigour did not seem to be affected (Martelli 1993). AMV is reported to infect grapevines in Turkey (Akbas and Erdilller 1993) and several European countries including Germany, Switzerland, Czechoslovakia, Hungary and Bulgaria (Martelii 1993; Beczner and Lehoczky, 1981, 1980; Bovey and Brugger 1975; Bercks et al 1973) . Similar symptoms have been observed in Australia but an associated virus was not reported (Krake et al 1999). Field spread of AMV is not reported in grapevine but the virus is transmitted by aphids in other crops.

CMV was originally reported in glasshouse grown grapevines in Denmark (Petersen 1978). More recently is has been reported in field grown grapevines in Turkey (Koklu et al 1998; Paradies et al 2000). CMV is transmitted by aphids in other crops.

Line pattern disease can be diagnosed by visual observation of early symptoms and GLPV can be detected by inoculation onto herbaceous indicators (Martelli et al 1993). AMV and CMV can also be detected by sap inoculation onto herbaceous indicators (Martelli 1993).

GAMV can be detected using PCR (Girgis, 2009). AMV and CMV are detected by commercially available ELISA kits and by PCR (Paradies et al 2000; Xu and Nie 2006).

1.2.7 *Bunyaviridae*

TSWV Tomato spotted wilt virus is the type species of the genus *Tospovirus* in the *Bunyaviridae* TSWV has isometric, enveloped particles that are 85 nm in diameter with a negative sense single stranded tri-partite RNA genome that is 17.2 kb in size (Nichol et al 2005). The virus was reported in Taiwan in grapevines affected by yellow dwarf disease (Chen et al 1981). Symptoms included chlorotic or mottled leaves that were deformed and may have had necrotic spots. Stunting was also observed. No vector was reported but the virus is transmitted by several thrips species in many other crops worldwide (Persely et al 2006). A study in Canada could not transmit the lettuce TSWV strain to eight *V. vinifera* cultivars, 34 *Vitis* hybrids, seven *V. lambusca* cultivars or three *V. rupestris* cultivars (Stobbs and Broadbent 1981). However several strains are reported (Persely et al 2006) and it is possible that the strain infecting grapevines in Taiwan was different to the strain used in Canada. The virus can be detected using PCR techniques and ELISA (Persley, et al 2006)

1.2.8 *Luteoviridae*

Grapevine ajinashika disease is associated with a *Luteovirus* species called *Grapevine ajinashika associated virus* (GAaV) (Namba et al 1979; Namba et al 1991). GAaV has isometric particles that are 28 nm in diameter and contain a linear positive-sense, single-stranded RNA genome which is 6.8kb. The disease is only reported in Japan and affected several *V. vinifera* cultivars (Namba 1979). There are no foliar symptoms but the disease results in significant yield loss because the berries have significantly reduced sugar content and are unpalatable. No PCR or commercial ELISA test are available, but the disease could possibly be diagnosed based on symptoms (fruit quality), electron microscopy and biological indexing onto the cultivar Koshu (Martelli 1993).

1.2.9 *Alphaflexiviridae*

Potato virus X is the type member of the genus *Potexvirus.* It is a flexuous, rod-shaped virus, 515 nm in length and contains a positive sense, single stranded RNA of 6.44 kb (Adams et al 2004; Martelli et al 2007). PVX occurs worldwide in potatoes and it is transmitted by mechanical inoculation. It can be detected by herbaceous indexing, ELISA and PCR (Nie and Singh 2000).

PVX was reported in symptomless grapevines in Tunisia and one grapevine from Italy (Chabbouh et al 1993, Giunched 1973) and therefore may not be economicalay important.

1.2.10 Other viruses

Sowbane mosaic Sobemovirus (SoMV) is not yet assigned to virus family. It has isometric particles 26 nm in diameter containing linear, positive-sense, single-stranded RNA genome which is 4.2Kb (Hull and Fargette 2005). The virus occurs worldwide in many hosts but was

only reported in grapevines in Bulgaria, Germany and Czechoslovakia (Bercks and Querfurth1969; Jankulova, 1972; Pozdena et al 1977). Infection in grapevine is latent (Bercks and Querfurth1969). In other plant hosts SMoV is transmitted by insects, pollen and seed (Table 1.1; Bennett and Costa, 1961).SoMV can be detected by inoculation onto herbaceous indicators and by commercially available ELISA kits.

Two viruses of the genus *Tobamovirus* have been reported to infect grapevine and include *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV). TMV has straight, rigid particles approximately 300 nm in length and short particles 70-100 nm in length may also be present (Lewandowski DJ, 2005). It has a linear, positive-sense, single stranded RNA genome is which is 6.39 Kb in length. It has been reported to infect grapevines in the USA and Europe (Gilmer and Ketls, 1965, 1968; Martelli 1993) but there is little reference to an associated disease and may not be economically important. The virus is transmitted mechanically, by contact and, in grapevines it has been shown to be seed transmitted (Gilmer and Kelts, 1968). There is no evidence of a vector.

ToMV has straight, rigid particles approximately 300 nm in length. It has a linear, positive-sense, single strand of RNA genome is which is 6.38 Kb (Lewandowski 2005). It has appeared on the Australian list of non-quarantineable grapevine viruses and there is reference to two Tobamoviruses infecting grapevines (Martelli 2003) but little other information appears in the scientific literature about this virus infecting grapevine. Thus it is unlikely that this virus plays a significant role in grapevine virus diseases.

Both TMV and ToMV can be detected by inoculation onto herbaceous indicators, commercially available ELISA kits and PCR (Jacobi et al 1998)

Raspberry bushy dwarf Ideaeovirus (RBDV) has isometric particles that are 33nm in diameter. It has a tripartite linear, positive-sense, single-stranded RNA genome and RNA-1 is 5.4kb in length, RNA-2 is 2.2kb in length and RNA-3 is approximately 1kb in length (Jones 2005). It is found grapevines in Slovenia where it is widespread and symptoms in grapevine leaves include curved line patterns and yellowing (Mavric-Plesko et al 2009). The mode of transmission is not known in grapevine although it is not seed transmitted (Mavric-Plesko et al 2009). However the virus was detected in the nematode *Longidorus juvenilis* from a vineyard with RBDV infected grapevines (Mavric-Plesko et al 2009). It can be detected by inoculation onto herbaceous indicators, commercially available ELISA kits and PCR (Mavric-Plesko .et al 2009).

Two viruses have not been characterised and these are Grapevine labile rod-shaped virus (GLRSV; Faggioli et al 1992) and Grapevine stunt virus (GSV; Namba et al 1986). GLRSV is an elongated rod-shaped virus and was found in a symptomless grapevine in Italy Very little other information exists and it is possible that this virus is not widespread and does not play a significant role in grapevine virus associated disease. GSV was found to infect *Vitis vinifera* cv. Campbell Early in Japan with symptoms of stunting and leaf rolling (Namba et al 1986). It is considered an important disease and yield may be affected. The virus particles are

spherical and are approximately 25 nm in diameter *(*Namba et al 1986). GSV was transmitted by grapevine leafhopper (*Arboridia apicalis;* Namba et al 1986). GSV can be detected by electron microscopy or by inoculation onto the variety Campbell Early or *V. thunbergia.*

1.3 Disease of unknown aetiology

Several diseases exist of unknown aetiology but with a possible virus association and these include: Grapevine enation, Grapevine summer mottle/Grapevine vein mosaic, Grapevine vein necrosis and LN33 stem grooving.

Grapevine enation occurs worldwide, including Australia (Martelli 1993; Krake et al 1999) Field symptoms include delayed bud break, slow bushy shoot growth and presence of laminar or cup-shaped outgrowths (enations) on the underside of the basal leaves (Martelli 1993; Krake et al 1999). Leaves with enations are distorted, and shoots may be deformed with cracks near the basal internodes. Symptoms may not occur on the same vines every year. Although it is graft transmissible the transmission rate is low and biological indexing on LN33 is unreliable (Martelli 1993). Thus this disease is bets diagnosed based on visual observation of symptoms on candidate grapevines.

Grapevine summer mottle is a graft transmissible disease reported from Australia in a single grapevine of the cultivar Sidiritis (Krake and Woodham 1978). Although GFLV was not detected, Krake et al (1999) suggested this disease may be the same as Roditis leaf discolouration. Leafroll, fleck, vein mosaic and vein necrosis diseases were diagnosed from the same grapevine by biological indexing. It may be diagnosed by visual inspection and by graft inoculation onto sensitive indicators including Cabernet Sauvignon.

Grapevine vein mosaic is a graft transmissible disease that occurs in Europe and Australia (Martelli 1993; Krake et al 1999). It is considered semi-latent as it is not consistently expressed from year to year. Symptoms include pale green discolorations of leaf tissue adjacent to the main veins, resulting in a feathering or banding effect. Grapevines may be smaller and less vigorous. It may be diagnosed by visual inspection and by graft inoculation onto sensitive indicators including *V. riparia.*

Grapevine vein necrosis occurs worldwide. Typical symptoms include necrosis of the veinlets on *Vitis rupestris* x *Vitis berlandieri* 110 Richter, which can be used as an indictor for the disease. It is often latent in other varieties. Recently it was associated with RSPaV infection (Bouyahia et al 1995).

Symptoms of LN33 stem grooving occur on the stem of LN 33 in a similar manner to corky bark disease (Martelli 1993; Golino 1993). However it differs to corky bark disease as there is no phloem proliferation, internodal swellings or foliar discolorations. It is graft transmissible but no virus has been associated with the disease.

1.4 Bacteria

Fifteen significant bacteria, including 10 phytoplasmas (Table 1.2), *Xylophilus ampelinus, Xylella fastidiosa, Agrobacterium sp* (*A. rubi and A.vitis*) and *Xanthomonas campestris pv viticola,* that can infect grapevines are also controlled through quarantine and certification programs.

Of the ten grapevine phytoplasmas that have been reported to infect grapevines, three occur in Australian and include *Candidatus* Phytoplasma australiense, Tomato big bud phytoplasma and the Buckland Valley grapevine yellows phytoplasmas. The remaining phytoplasmas occur in various countries worldwide (Table 1.2). The epidemiology of these phytoplasmas and associated diseases has been recently reviewed (Constable 2009).

Phytoplasmas are associated with grapevine yellows diseases. The symptoms of the disease include:

- Irregular yellowing in white varieties or reddening in red varieties
- The yellow leaf tissue may become necrotic
- Backward curling of the leaves
- Overlapping of leaves on affected shoots
- Rows of black pustules can develop on the green bark of affected shoots
- Tips of affected shoots may die and shoots may die back
- Affected shoots fail to harden off and remain rubbery
- Flowers on affected shoots may abort or berries may shrivel

Depending on the variety and the phytoplasma, remission of disease is reported. In some situations grapevines may decline and die. The severity of grapevine yellows disease differs depending on the variety, for example Chardonnay and Riesling are more severely affected whilst some rootstocks may be infected by phytoplasmas but do not show disease (Boudon-Padieu 1999). Sensitive varieties may have significant reductions in yields and this has been reported for some grapevine yellows diseases (Caudwell 1964; Magarey and Wachtel 1986).

Grapevine yellows disease can be visually identified on the basis of symptoms but phytoplasma identification requires molecular testing using PCR and confirmation of the phytoplasma identity by sequencing. Molecular techniques for phytoplasmas are generic and should detect all known species and these protocols are validated for Australian viticulture (Gibb et al 1999; Constable et al 2003).

Transmission of phytoplasmas through propagation material can occur and lead to their long distance dispersal and introduction into regions where they have not previously been found. Vectors have not been reported for all phytoplasmas, but is suggested by their epidemiology. Vectors of phytoplasma include leafhoppers, plant hoppers and psyllids.

Of the remaining five bacteria *Xylophilus ampelinus* (syn**.** *Xanthomonas ampelina* and *Erwinia vitivora)* and *Xylella fastidiosa* are considered serious risks to Australia's biosecurity appear on the " Biosecurity Plan for the Viticulture Industry" (Anon 2009).

Xylella fastidiosa causes Pierce's disease of grapevines in the USA. The pathogen and disease have been part of two GWRDC funded research projects to identify establishment potential of the disease in Australian (Merriman et al 2001) and to identify PEQ protocols for active testing of grapevines and other hosts (Luck et al 2002). There is a validated and verified diagnostic protocol in place for *X. fastidiosa* (Luck et al 2002; http://www.padil.gov.au/pbt/index.php?q=node/46&pbtID=109) that has been accepted by the Australian Subcommittee on Plant Health Diagnostic Standards and thus it will not be discussed further here.

Xylophilus ampelinus (syn*. Xanthomonas ampelina* and *Erwinia vitivora*) is a Proteobacteria of the family *Xanthomonadaceae* that causes bacterial blight of grapevine (Anon 1997, Anon 2009). Symptoms of the disease include bud death. In spring shoots may develop dark brown streaks, become chlorotic and stunted and they eventually wilt and die. Stems and petioles develop cankers. Necrotic foliar spots are observed. Bacterial blight can lead to significant yield loss and the life of infected vines may be decreased. The bacteria can colonize grapevine xylem tissue systemically and long distance spread is through infected propagation material. Short distance dispersal may be from shoot to shoot by wind and rain and it can be spread by contaminated tools and machinery. There is no evidence for a vector. The bacterium is present in Europe and South Africa (Anon 1997). It has also been reported but eradicated in Turkey and may occur in Tunisia and Argentina (Anon 1997), although the latter two reports remain unverified. The bacterium can be detected by direct isolation, immunofluorescence, ELISA and PCR. A diagnostic protocol has been developed for the Australian Subcommittee on Plant Health Diagnostic Standards but is not yet validated (http://www.padil.gov.au/pbt/index.php?q=node/46&pbtID=113).

1.5 Disease diagnosis and pathogen detection

Australian quarantine and certification programs are dependent on accurate diagnostic techniques for disease diagnosis and pathogen detection. Methods for grapevine disease diagnosis and virus and bacterial detection that are used in quarantine and certification programs include visual inspection, biological indexing, the enzyme linked immunosorbent assay (ELISA) and molecular methods, particularly polymerase chain reaction (PCR) (Martelli 1993). These methods are primarily used fro virus detection, although visual inspection and PCR testing can be used for the detection of phytoplasmas and other bacteria.

1.5.1 Visual inspection

Many grapevine pathogens are associated with specific symptoms on sensitive varieties and visual inspection can assist in identifying these infected varieties (Martelli 1993; Krake et al 1999). Conversely, symptomless infections can occur in some varieties; these infected grapevines may act as a reservoir for other sensitive varieties. Also, other factors can induce similar symptoms and result in a misdiagnosis of disease. For example magnesium and phosphorous deficiencies often induce symptoms similar to leafroll disease (Krake et al 1999). Consequently visual inspection alone cannot be relied upon for disease diagnosis and

pathogen detection. Active diagnostic testing is required to confirm the presence of viruses and bacteria.

1.5.2 Biological indexing

Biological indexing takes advantage of a sensitive plant response to the presence of pathogens. Indicator plants are inoculated with material of another source and are observed for characteristic symptoms. Two biological indexing methods are used for the detection of grapevine viruses: herbaceous indexing by sap inoculation and chip bud inoculation of woody indicators (Martelli 1993; Krake et al 1999).

Herbaceous indicators are used in Australian post entry quarantine for detection of nepoviruses, BBWV and SLRSV. Herbaceous indicator plant species that are sensitive to nepoviruses include *Chenopodium* spp., in particular *C. quinoa* and *C. amaranticolor*. (Martelli 1993). Grapevine tissue is ground in an appropriate buffer and rubbed onto the leaves of the indicator plants that have been dusted with an abrasive powder. Symptoms can develop within seven days of inoculation, however plants are often observed for up to six weeks post inoculation. Symptoms are dependent of the virus species but can include: chlorotic local lesions, systemic mottling and leaf malformation, tip necrosis and plant death. Some similar symptoms might also be associated with other virus species that have been reported to infect grapevines including SoMV, AMV and CMV.

Woody indictors are currently used for diagnosis of virus associated diseases in Australian PEQ and certification schemes. Chip buds of a candidate plant are grafted onto sensitive indicator grapevines (Figure 1). The indicators used in Australian certification programs and the expected symptoms for leafroll, rugose wood and fleck diseases are listed in table 1.3. This method is time consuming and symptoms may take several years to appear. Indicators are usually planted in the field and symptom development is observed over three growing seasons. The indicator Rupestris St George is observed in spring for symptoms associated with fleck disease and the indicator Cabernet Franc is observed from late summer through autumn for symptoms of leafroll disease. During winter of the third season the bark is stripped from the trunks of the indicators Rupestris St George, Kober 5BB and LN33 to observe rugose wood symptoms. Australian PEQ only uses LN33 to test for corky bark disease.

Table 1.3 The indicators used to detect viruses associated diseases of grapevines and the symptoms that are observed.

Indicator	Disease	Symptoms	Associated virus
Cabernet Franc	Grapevine leafroll	–leaf reddening, often with green veins but sometimes with red veins, and leaf rolling	All GLRaVs, although symptom expression, particularly severity, can vary depending on the virus species.
Rupestris St George	Rugose wood complex - stem pitting	Pitting of the woody cylinder	RSPaV
Rupestris St George	Fleck disease	Vein clearing symptoms along veinlets. Severely affected leaves maybe twisted and curled upwards	GFkV
Kober 5BB	Rugose wood complex - stem grooving	grooving of the woody cylinder	GVA
LN33	Rugose wood complex – corky bark	Grooving and pitting of the stem, internodal swelling with a cracked surface, uneven or no lignification of canes	GVB

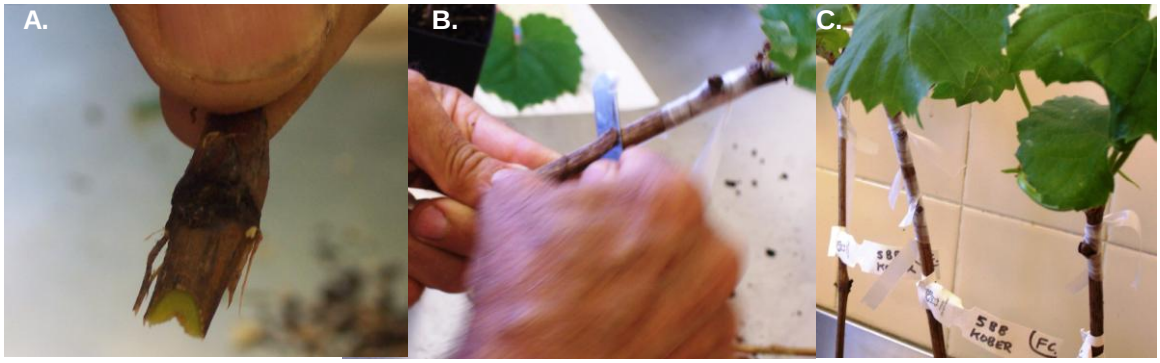


Figure 1.1 Chip bud inoculation of *V. berlandieri* × *V. riparia* cv. Kober 5bb for detection of GVA. A. A chip bud of a candidate grapevine used for inoculation. B. Creating a notch in the indicator vine for insertion of the chip bud. C. Indicator vines with chip buds held in place using budding tape.

Woody indicators should be free of viruses and phytoplasmas, as these can affect symptom expression and they should be isolated from other grapevines to ensure that field infection does not occur and lead to false positive results. Biological indexing onto woody indicators is dependent on the successful inoculation of associated viruses, which are affected by graft take and presence of viruses in the grafted chip buds. Disease expression in the indicators is also affected by strain variation amongst the associated virus species, and some viruses may not induce obvious symptoms on the selected biological indictors. In Australia biological indexing is mainly done in the field and it is possible that environmental factors, such as temperature, light and nutrition, will affect symptom expression. Some indicators develop symptoms that cannot be readily attributed to a known causal agent, or combination of causal agents.

1.5.3 Enzyme-Linked ImmunoSorbent Assay

The Enzyme-Linked ImmunoSorbent Assay (ELISA) is a serological technique using antibodies to detect the presence of an antigen, such as a virus, in a plant sample (Clark and Adams 1977). The technique is simple and is useful for testing large numbers of samples and results can be gained within 24 hours. ELISA assays have been prepared for many grapevine viruses and are commercially available.

Typically the antibodies used in an ELISA assay react to the presence of the protein coat that encapsidates the virus (Clark 1981). The polyclonal antisera may be raised against intact virus particles purified from the host plant or monoclonal antisera can be raised against coat proteins expressed in vitro (Hsu et al 1988).

The reliability of polyclonal antisera is affected by the quality of the purified viruses (Hsu et al 1988). Co-purified plant proteins can cause background colouration, masking weak positive results. If purified virus preparations were obtained from hosts with mixed virus infections, antisera may cross-react with co-purified viruses, leading to false positive results. Monoclonal antisera reduce the risk of cross reaction with contaminating plant proteins and viruses.

The reliability of ELISA tests using polyclonal or monoclonal antisera may be affected the degree of conservation within the coat protein sequence of the virus. Although the nucleotide sequence can be variable the amino acid sequence tends to be conserved amongst virus strains and isolates. Consequently ELISAs tend to detect groups of viruses within a species and specific strains are not distinguished. It is possible that the antibodies will cross react with other virus species that have a similar coat protein (Gugerli 2009). Occasionally significant variation within the coat protein gene leads to significant changes to the structure of the coat protein (Bertazzon and Angelin 2004; Fajardo et al 2007). This can result in non-recognition of the virus particle by the antisera, leading to a false negative result and we have observed this for strains of GLRaV-3 in Australia (F. Constable unpublished).

The reliability of the technique is also dependent on sampling the correct tissue type at the correct time. Traditionally ELISA testing is carried out in autumn when leafroll symptoms are apparent and uses the cortical scrapings from canes.

1.5.4 Molecular testing

In recent years there have been some significant advances in molecular diagnostics for grapevine pathogens using reverse transcription polymerase chain reaction (RT-PCR) and PCR methods. Most grapevine viruses have an RNA genome and reverse transcription (RT) is required to make a DNA copy (cDNA) of the viral RNA before the PCR can proceed. During PCR a thermostable enzyme (e.g. *Taq* DNA polymerase) is used to generate multiple copies of a specific nucleotide sequence from DNA or cDNA. Once the PCR reaction is complete the products are separated on an agarose gel by electrophoresis, stained with a fluorescent dye and viewed under UV illumination. A positive result is recorded when a product of the correct size is observed. More recently real-time (quantitative) PCR (qPCR) techniques have been developed that simultaneously amplify and measure the presence of the target in a PCR reaction (VanGuilder et al 2008). qPCR technology is being adapted for pathogen detection in grapevines (Osman et al 2007 Osman and Rowhani, 2008; Osman et al 2008).

Regardless of the chemistry used, PCR creates multiple copies of the original target nucleic acid and thus is considered much more sensitive than ELISA, which can be limited by the number of virus particles present in a plant sample (Chevalier et al 1995; Sefc et al 2000). However, PCR techniques require sequence information of at least part of the pathogen genome so that primers can be designed. The assays are often targeted to conserved regions of the genome so that multiple strains can be detected. Where there is little sequence information, molecular diagnostic tests have the potential to give false negative results because the genetic variability of strains within the targeted species may reduce the binding capacity of the primers, even in conserved regions of the genome. Consequently PCR assays must be developed and updated as genetic information about grapevine pathogens has become available. False negative results can also be obtained due to inhibition of the reverse transcriptase or DNA polymerase activity by compounds co-extracted with the nucleic acids.

Conversely the potential exists for mis-priming due to similarity of the primer sequences other genetic material, including that of the host plant or other organisms, resulting in the generation of visible PCR products. Often the products that are generated are different to the expected size but occasionally products very similar to the expected size are produced, giving a false positive result. Thus it is important to determine the origin of PCR products that are different to the expected size as mutation or recombination events can lead to detection of smaller or larger genomic fragments of the test organism. Ignoring these products can lead to false negative results. Unexpected positive results, be they bands of the correct size or of a different size, should be confirmed by another test, such as re-extracting from the same grapevine and repeating the PCR test or re-testing by ELISA and/or biological indexing. Sequencing of the PCR product will confirm the origin of the positive PCR result.

1.5.5. Comparisons of diagnostic test reliability

Although biological indexing, ELISA and PCR are commonly used for the detection of grapevine viruses there have been few comprehensive, systematic studies to determine the

reliability of these tests in comparison with each another. 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). Both of these studies were done prior to the emergence of present day ELISA kits and PCR technologies. The Victorian study showed that Cabernet Franc was more reliable than LN33 as an indicator for leafroll disease in Australia. The South Australian study 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 was done by FPS (UCDavis, USA) that 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. These conflicting results were attributed to the failure of an infected bud chip to survive and the grafting of an uninfected bud, due to uneven distribution of the virus, two problems that are regularly encountered in woody indexing programs.

A second study to determine grapevine scion-understock combinations suitable in Australia found that RT-PCR techniques did not consistently detect virus even when symptoms were observed (Scott). This lack of reliability may have been associated with the uneven distribution or strain variation of the viruses or due to the PCR test being too specific to detect the particular virus strain. Significant strain variation is reported for many grapevine viruses and it is possible that no single test will detect all strains of a species. There are no references about the use of ELISA or PCR techniques used to validate symptom expression on biological indicators with time.

The effectiveness of ELISA and RT-PCR for the detection of some grapevine viruses has been compared. 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 are used (Ling et al 2001). ELISA and RT-PCR were shown to be reliable throughout one season for detection of GLRaV-3 from bark scrapings of field infected grapevines that were symptomatic or symptomless (Ling et al 2001). In contrast, one study has shown that ELISA may be more sensitive than PCR for detection of GLRaV-1 and -3 (Cohen et al 2003). Rowhani et al (1997) showed that GLRaV-3 is unevenly distributed within the same vine and could therefore lead to inconsistent test results. These results have implications for certification schemes as they highlight the need to understand sampling and the time of year for testing. To date, no studies have been done to show the reliability of RT-PCR and ELISA for detection of grapevine viruses over time on replicates of vines of two varieties, or more, which have been inoculated by the same source of virus and maintained in different climatic conditions.

1.6 Aims of the project

The Australian viticulture industry has shown a willingness to adopt new techniques for pathogen detection and, as a consequence, PCR methods are currently routinely used by industry. For most viruses multiple primer sets exist for detection by conventional PCR. New strains of grapevine viruses are continually being reported and it is a weakness of PCR technology that the primer sets used in PCR tests may not detect all strains of the target virus. However, RT-PCR techniques are constantly being developed and refined for grapevine virus detection. It is vitally important that bioinformatic analysis supports the selection of primer sets when PCR tests are being developed and they must be properly validated before being used to certify elite planting material for industry.

Development of rapid diagnostics for exotic pathogens will shorten post entry quarantine periods and offer quicker access of new germplasm/varieties to industry. Rapid PEQ diagnostics will improve preparedness in case of an incursion of an exotic grapevine pathogen.

This project aims to contribute to the development of PEQ and grapevine certification protocols by researching the following key areas:

1) Investigate the specificity and reliability of the molecular (PCR) tests for the detection of grapevine viruses.
2) Identify diagnostic protocols for the detection of a range of endemic pathogens. Specifically:
    - understand sampling of grapevines for reliable detection.
    - investigate the sensitivity of the serological and molecular tests that are currently used for virus detection.
3) Identify and adopt best practice molecular protocols for the high risk pathogens that remain on the quarantine list with the aim of reducing the time newly imported clones and varieties spend in PEQ.
4) In collaboration with AQIS, AVIA, SAVII and FPS, investigate and document efficient protocols for woody indexing of grapevine viruses.
5) Validate the above serological and molecular protocols by surveying key grape growing regions in Australia for the identified pathogens. The survey information, including the sampling and diagnostic protocols, will be published in international peer reviewed journals so that they can serve as the standard protocols for Australian diagnostic laboratories and which are accepted worldwide. The diagnostic protocols developed for endemic virus testing will be further validated through screening of the AVIA Nuclear collection.

A direct outcome of this project is a world's best practice diagnostic capability that will support the Australian viticulture industry. The results of this project will improve confidence in the diagnostic tests that are available and facilitate the interpretation of diagnostics testing results. An improved ability to test for endemic pathogens, as a part of a certification scheme for the provision of high health material and exotic pathogens, to assist PEQ, will lower exposure to pest and disease risk for the Australian viticultural industry.

CHAPTER 2: THE ADOPTION AND DEVELOPMENT OF MOLECULAR TESTS FOR THE DETECTION OF ENDEMIC AND EXOTIC PATHOGENS OF AUSTRALIAN GRAPEVINES

2.1 Introduction

There have been significant advances in molecular diagnostics for plant pathogens in recent years. Tests for most of the exotic and endemic viruses and some bacterial pathogens associated with grapevine diseases, which are quarantinable or controlled through certification programs in Australia, have been developed overseas. During the early phase of this project an extensive literature review was conducted and international experts were consulted to identify the latest molecular diagnostic protocols available to detect the identified pathogens of grapevines in Australia. Detailed bioinformatic analyses were done on primer sequences to determine their specificity and ability to detect all known strains of each virus. Several primer pairs for each virus were selected.

The endemic viruses of grapevines for which molecular tests were developed included:

- Six Closterovirids associated with leafroll disease: *Grapevine leafroll associated virus* (GLRaV-) 1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5, GLRaV-9;
- Two Vitiviruses species associated with rugose wood diseases: *Grapevine virus A*(GVA) *Grapevine virus B* (GVB);
- One *Maculavirus* associated with fleck disease: Grapevine fleck virus (GFkV);
- One Foveavirus associated with rugose wood, vein necrosis and syrah decline diseases: *Rupestris stem pitting associated virus* (RSPaV).

The exotic viruses for which RT-PCR tests were to be adopted and developed included:

- Two Closterovirids associated with leafroll disease: GLRaV-6, GLRaV-7;
- Two Vitiviruses species associated with rugose wood diseases: *Grapevine virus D* (GVD); GVB: strains associated with corky bark disease;
- Two Maculaviruses: *Grapevine red globe virus* (GRGV); *Grapevine asteroid mosaic-associated virus* (GAMaV)
- Fifteen nepoviruses: *Arabis mosaic virus* (ArMV), *Artichoke Italian latent virus* (AILV), *Blueberry leaf mottle virus* (BLMV), *Cherry leafroll virus* (CRLV), *Grapevine Anatolian ringspot virus* (GARSV), *Grapevine Bulgarian latent virus* (GBLV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine deformation virus* (GDefV), *Grapevine fanleaf virus* (GFLV), *Grapevine Tunisian ringspot virus* (GTRSV), *Peach rosette mosaic virus* (PRMV), *Raspberry ringspot virus* (RsRSV), *Tobacco ringspot virus* (TRSV), *Tomato black ring virus* (TBRV, includes the strain, Joannes seyve), *Tomato ringspot virus* (ToRSV);

- One Sadwavirus (formerly classified as a *Nepovirus*): *Strawberry latent ringspot virus* (SLRSV);
- Two Tombusviruses; *Grapevine Algerian latent virus* (GALV) and *Petunia asteroid mosaic virus* (PAMV).

Grapevine rootstock stem lesion associated virus and grapevine virus C, which were originally included in the exotic virus list, are now recognised as strains of GLRaV-2 (Meng et al 2005, Masri et al 2006).

We also established the PCR test for *Xylophilus ampelinus* (Botha et al 2001) recommended for the Pest and Disease Image Library (PaDIL) Plant Biosecurity Toolbox (http://www.padil.gov.au/pbt/index.php), which provides best available diagnostic information for rapid identification of exotic pests and diseases for Australia.

Adoption of these tests will improve the efficiency of the Australian post entry quarantine for grapevines and may reduce the existing 18 month waiting period. This will also improve our ability to respond in case of an incursion by providing more accurate and sensitive identification of grapevine pathogens. The tests will also improve the ability of the Australian grapevine certification schemes to determine the pathogen status of selected grapevine varieties and clones prior to inclusion and during regular maintenance and pathogen testing of the high health programs.

2.2 Materials and Methods

2.2.1 Positive controls - endemic viruses

Positive controls for each endemic virus were obtained through the vine improvement associations, the grapevine virus reference collection maintained by Phil Nicholas at SARDI and via commercial diagnostic services (Waite Diagnostics and Victorian DPI Crop Health Services-Crop Hygiene. The positive controls that were used are listed in the results tables for each virus.

2.2.2. Positive controls – exotic viruses and bacteria

Positive controls were obtained from Crop Health Services, international researchers and the Australian Quarantine Inspection Service (Table 2.1). Due to strict quarantine regulations we were unable to obtain positive controls for all the identified viruses and *Xylophilus ampelinus*.

Table 2.1 A list of the origin of positive controls of exotic pathogens acquired, the type of material provided and their origins

Pathogen	Type of material	Origin
CLRV	Total RNA extract	Joanne Mackie DPI Victoria.
Tombusviruses – *Pear latent virus*	partial clone of cDNA encompassing the coat protein ligated into pGEM-T vector	Dr. M. Russo Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italy.
ToRSV	Total RNA extracted from lyophilised infected *Nicotiana benthamiana* from the AGDIA ToRSV ELISA kit	M. Whattam, Australian PEQ, Knoxfield, VIC, Australia
SLRSV	Total RNA extracted from *Narcissus*.	M. Whattam, Australian PEQ, Knoxfield, VIC, Australia
GFLV	Total RNA extracted from grapevine	M. Whattam, Australian PEQ, Knoxfield, VIC, Australia
RSRPV	Partial clone of cDNA of the Coat protein ligated intopCR-4TOPO vector	L. Liefting, MAF, New Zealand
TBRV	Partial clone of cDNA of the Coat protein ligated intopCR-4TOPO vector Total RNA extracted from infected Iris	L. Liefting, MAF, New Zealand, M. Whattam, Australian PEQ, Knoxfield, VIC, Australia
TRSV	Partial clone of cDNA of the Coat protein ligated intopCR-4TOPO vector	L. Liefting, MAF, New Zealand

2.2.3 RNA extraction for detection of viruses

RNA was extracted from the virus infected plant material using the RNeasy® Plant Mini Kit (QIAGEN Pty Ltd, Doncaster, VIC Australia) as described previously (MacKenzie et al 1997).

2.2.4 Primers for RT-PCR and PCR

2.2.4.1 Housekeeping RT-PCR

Primer sequences are listed in Table 2.2. Primers for the detection of NADH dehydrogenase ND2 subunit (ndhB gene, NAD) messenger ribonucleic acid (mRNA; Thompson et al 2003) were used to determine the quality of the extracted RNA. These primers were used to ensure that nucleic acid was present or that there were no inhibitors in the nucleic acid extracts that retard the activity of the RT enzyme or DNA polymerase during the RT-PCR reactions.

2.2.4.2 Pathogen primers

A literature review was conducted and international researchers were consulted to identify tests that are routinely used for virus detection. The primers of the selected tests were

analysed using BlastN (Altschul et al 1997) to determine their suitability to detect all published strains of each virus and the specificity for the virus species. The primers that were selected for development for detection of endemic viruses are listed in Table 2.2 and those that were selected for exotic viruses are listed in Table 2.3.

Primer concentrations were exactly as published for the tests for leafroll virus detection described by Dovas and Katis (2003), Dovas et al (2006) and Maligoka et al (2008), the generic Ilarvirus test described by Maliogka et al (2007), the generic *Secoviridae* test described by Maliogka et al (2004) and the *Nepovirus* subgroup specific tests described by Digiaro et al (2007). For all other one step RT-PCR or PCR tests the final concentration for each primer was 0.2 μM.

2.2.5 RT-PCR and PCR conditions

2.2.5.1 One-step RT-PCR

The SuperScript™ One-Step RT-PCR System (Invitrogen) was used for detection of NAD mRNA and most viruses. One step RT-PCR was done according to the manufacturer's instructions except that 0.4μl of SSIII/Taq enzyme mixture was used per RT-PCR reaction and the total reaction volume was 20 μl.

2.2.5.2 Two step RT-PCR

Two step RT-PCR tests were required for the *Nepovirus* subgroup specific tests described by Digiaro et al (2007), the generic *Maculavirus* tests and specific tests for GFkV, GRGV and GAMaV described by Sabanadzovic et al (2000) and the ToRSV and TRSV tests described by Martin et al (2009). For the *Nepovirus* subgroup specific tests and the generic *Maculavirus* tests and specific tests for GFkV, GRGV and GAMaV, random hexamer primers (Geneworks) were used for reverse transcription (RT) of RNA to cDNA. For ToRSV and TBRV, specific primers were used for reverse transcription (Table 2.3). The total RT reaction volume was 20 μl. Two microlitres of RNA extract, 2 μl of the reverse primer and 7μl sterile distilled water were mixed together, heated to 95^0C for five minutes and then chilled on ice. To this mixture, 4 μl of 5× first strand buffer, 2 μl of 0.1 M DTT, 1 μl of 10 mM dNTP mix and 1 μl of Superscript III RNase H^- Reverse Transcriptase (200 units/μl, Invitrogen) were added. The cDNA synthesis was conducted at 42^0C for 50 min according to the manufacturer's protocol.

2.2.5.3 PCR

Platinum® *Taq* DNA Polymerase (Invitrogen) was used for all PCR reactions according to the manufacturer's instructions except that the total reaction volume was 20 μl and 0.8 units of Platinum® *Taq* DNA Polymerase was used in each reaction. Concentrations of $MgCl_2$ were adjusted for the *Nepovirus* subgroup specific tests as described by Digiaro et al (2007) and generic *Maculavirus* tests and specific tests for GFkV, GRGV and GAMaV as described by Sabanadzovic et al (2000).

For nested PCR 1 µ□l of the first round PCR reaction mixture was added to the PCR mixture containing the second primer pair.

2.2.6 Gel electrophoresis

After amplification, 10 µ□L from each PCR reaction was subjected to electrophoresis in a 1.5% agarose gel using 0·5 ×□ TBE (0·045 M Tris-borate, 1 mM EDTA, pH 8·0) running buffer. Products were stained with ethidium bromide that was incorporated in the gel and visualized by UV transillumination. Water controls, in which no nucleic acid was added to the PCR mix, were also included. DNA markers used were DNA Molecular Weight Marker X (Roche Diagnostics).

2.2.7 Cloning and sequencing

PCR products were purified using the Qiaquick PCR purification kit (Qiagen) if only single PCR products were visible or by the Qiaquick Gel purification kit (Qiagen) if non-specific PCR products were present in addition to the expected PCR product. Purified PCR products generated by specific primers were sequenced directly using an ABI PRISM® BIGDYE™ Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems). PCR products generated using degenerate primers were cloned using the pGEM-T Easy Vector system according to the manufacturer's protocol (Promega). Transformants were screened and selected using standard protocols for blue/white selection (Sambrook et al 1989). To confirm their identity, the cloned products were sequenced using primers SP6 and T7, with the ABI PRISM® BIGDYE™ Terminator Cycle Sequencing Kit Version 3.1

Table 2.2 List of viruses, type of PCR test, primers trialled, annealing temperature, region amplified, expected product size and reference for each test used to detect endemic grapevine viruses in Australia.

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
DNA House-keeping gene	PCR	FD2	AGAGTTTGATCATGGCTCAG	55°C	partial bacterial 16S rRNA gene	1400-1500 bp	Weisberg et al 1991
		RP1	ACG GTT ACC TTG TTA CGA CTT				
RNA House-keeping gene	RT-PCR	AtropaNad2.1a	GGACTCCTGACGTATACGAAGGATC	55ºC	NADH dehydrogenase ND2 subunit) messenger ribonucleic acid	188 bp	Thompson et al 2003
		AtropaNad2.2b	AGCAATGAGATTCCCCAATATCAT				
GVA	Specific RT-PCR	CPdt	TGCCAGAGGTGTTTGAGACAAT	55ºC	Coat protein gene	982bp	De Meyer et al 2000; Goszczynski and Jooste 2002
		MP	TTTTTGTCTTCGTGTGACAACCT				
	Specific RT-PCR	6951F	GAGGTAGATATAGTAGGACCTA	55ºC	Partial coat protein gene	315bp	Goszczynski and Jooste 2003
		6906R	CCTCCTGCAGAGTTAAGGTC				
	Specific RT-PCR	H587	GACAAATGGCACACTACG	52ºC	Partial coat protein gene	430bp	Minafra et al 1997
		C995	AAGCCTGACCTAGTCATCTTGG				
	Specific RT-PCR	H7038	AGGTCCACGTTTGCTAAG	52ºC	Partial p10 gene	235bp	MacKenzie 1997
		C7273	CATCGTCTGAGGTTTCTACTA				
	Specific RT-PCR	Sense	GGCTGAAATGGGCACATACT	53°C	Partial coat protein gene	343bp	Moreira et al 2004.
		anti-sense	CCCTCTTTCACGAACCGTAA				
GVB	Specific RT-PCR	H6980	GTGCTAAGAACGTCTTCACAGC	52ºC	Partial coat protein gene	459bp	Minafra and Hadidi 1994; MacKenzie 1997
		C7439	ATCAGCAAACACGCTTGAACCG				
	Specific RT-PCR	Bh123	ATAGTAGGGGTACCATCAATATCT	52ºC	Partial coat protein gene	373bp	Sciancalepore et al 2006
		Bc637	CTATATCTCGACAGACTGCTCACCC				
		Bc116	TGATGGTACCCCTACTATCTATACC	52ºC	Partial coat protein gene	356bp	
	Specific RT-PCR	Senso	CAAGCAATTCCCCGAGAGT	52ºC	Partial putative movement protein gene and entire coat protein gene	693bp	Moreira et al 2004
		antisenso	TTTAGCCGCACTCCTTGACT				
	Specific RT-PCR	GVB7038	ACTCGTCAGACAACTCTATATC	52ºC	Entire coat protein gene	393bp	Minafra and Hadidi 1994
		GVB6445	ATGGAAAATATATCCCGGATGG				
	Specific RT-PCR	GVBCPF1	CCCATGTACTTCAGGACCC	52ºC	Partial coat protein gene	493bp	Terlizzi and Credi 2006 ICVG
	Specific RT-PCR	CPR3	CTACCACAACAGTAACAACG				
GFkV	Specific RT-PCR	GFkV-U279	TGGTCCTCGGCCCAGTGAAAAAGTA	55 ºC	Replicase gene	352bp	Sabanadzovic et al 2001
		GFkV-L630	GGCCAGGTTGTAGTCGGTGTTGTC				
	Reverse transcription		DNA hexanucleotides		Reverse transcription for cDNA production	Approx 300 bp	Sabanadzovic et al 2000
	Two step Specific RT-PCR	RD1	CYCARCAYAARGTVAACGA	58°C	RNA dependent RNA polymerase		
		FK-AP	GGCCAAATTGGCATTGCATG				

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
RSPaV	Specific RT-PCR	RSP48	AGCTGGGATTATAAGGGAGGT	52°C	Partial coat protein gene	512bp	Nolasco et al 2000
		RSP49	CCAGCCGTTCCACCACTAAT				
	Specific RT-PCR	Sy9F	AGGATTCCAAACTGTAGAGCAA	56°C	replicase gene	628 bp	Habili et al 2006
		Sy8R	TTGGTCGTCATCTTCCAGTT				
	Specific RT-PCR	RSP-H5638	AGGGATTGGCTGTTAGATGTT	50°C	RNA-dependent RNA polymerase	355bp	MacKenzie 1997
		RSP-C5992	CTTCAGGCAACCCCAAAAA				
	Specific RT-PCR	Primers 13	GATGAGGTCCAGTTGTTTCC	56°C	RNA-dependent RNA polymerase	338bp	Meng et al 1999
		Primer 14	ATCCAAAGGACCTTTTGACC				
	Specific RT-PCR	RSP52	TGAAGGCTTTAGGGGTTAG	56°C	RNA-dependent RNA polymerase	904bp	Nolasco et al 2000
		RSP53	CTTAACCCAGCCTTGAAAT				
	Specific RT-PCR	21 Sense	GAGGATTATAGAGAATGCAC	52°C	RNA-dependent RNA polymerase	440bp	Zhang et al 1998
		22 Antisense	GCACTCTCATCTGTGACTCC				
GLRaV 1	Specific RT-PCR	plazleft"	CAGGCGTCGTTTGTACTGTG	55°C	HSP70 gene	540bp	Komínek et al 2005
		plazright	TCGGACAGCGTTTAAGTTCC				
	Specific RT-PCR	pORF9F	GGCTCGAGATGGCGTCACTTATACCTA	55°C	ORF9	633bp	Little and Rezain 2006
		pORF9R	CCTCTAGACACCAAATTGCTAGCGA				
	Specific RT-PCR	GSP3 (GLRA1S)	CGTTCGCGTTACCCACGCTGCCTA	55°C	HSP70 gene	150bp	Good and Monis 2001
		(GSP4) GLRA1A	GCTGGCAAACCTGGTGGACTTTACATC				
GLRaV-2	Specific RT-PCR	LR2-U2 sense	ATAATTCGGCGTACATCCCCACTT	52°C		328bp	Bertazzon and Angelini 2004
		LR2-L2 antisense	GCCCTCCGCGCAACTAATGACAG				
	Specific RT-PCR	LRaV-2(1) sense	AGGCGGATCGACGAATAC	52°C	HSP70 gene	821bp	Abou Ghanem-Sabanadzovic et al 1998
		LRaV-2(2) antisense	ATCCTGTCCGGCGCTGTG				
	Specific RT-PCR	GLR2CP1 sense	ATGGAGTTGATGTCCGAC	52°C	Partial coat protein gene	595bp	Abou Ghanem-Sabanadzovic et al 2000
		GLR2CP2 antisense	TACATAACTTCCCTTCTACC				
	Specific RT-PCR	RGHSP227V sense	GCGACTCCAGCAACTTTAGTGA	52°C	HSP70 gene	550bp	Rowhani et al 2000
		RGHSP777C antisense	GTCTAACGAAAGATCGGGTTCTAAG				
	Specific RT-PCR	V2dCPf2 sense	ACGGTGTGCTATAGTGCGTG	55°C	Partial coat protein gene	589bp	Bertazzon and Angelini 2004
		V2CPr1 antisense	GCAGCTAAGTACGAATCTTC				
	Specific RT-PCR	V2HSPf sense	TTAAAACGTTGGGTGGGTTGC	57°C	HSP70 gene	567bp	
		V2HSPr antisense	ATAGACACGTCGAAGGTCCC				

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
GLRaV-3	Specific RT-PCR	C547	ATTAACTTGACGGATGGCACGC	52°C		340bp	Saldarelli and Hadidi 1994
		H229	ATAAGCATTCGGGATGGACC				
	Specific RT-PCR	LC1/,	CGCTAGGGCTGTGGAAGTATT	52°C	RNA-dependent RNA polymerase	653bp	Turturo et al 2005
		LC2	GTTGTCCCGGGTACCAGATAT				
	Specific RT-PCR	P3U/	CGCTCATGGTGAAAGCAGACG	52	HSP70 gene	546bp	
		P3D	CTTAGAACAAAAATATGGAGCAG				
	Specific RT-PCR	CP3U/	ATGGCATTTGAACTGAAATTAGGGC	57°C	Partial coat protein gene	484bp	
		CP3D	CGGCGCCCATAACCTTCTTACA				
	Specific RT-PCR	c629/	CGGCACGATCGTACTTTCTAA	54°C	RNA-dependent RNA polymerase	300bp	MacKenzie 1997
		h330	GATGCTTTCGCGTATTTCTTG				
GLRaV-4	Specific RT-PCR	LR4F	ACCCTTCATAAGCAGGAACC	56°C	RNA-dependent RNA polymerase	500bp	Fazeli et al 1998
		LR4R	CTTGTGAAACCGACGGCC				
	Specific RT-PCR	HSP4A	CTCAAACCAGCGGCTGTT	52°C	HSP70 gene	441bp	Routh et al 1998
		HSP4B	GTGATACCATATACATACCGACC				
GLRaV-4 and -5	RT-PCR for both viruses	HSP45A	GTATCTYATGTACCAACAGAT	52°C	HSP70 gene	370bp	
		HSP45B	GGTATGAACAARTTCAATGC				
GLRaV-5	Specific RT-PCR	LR5-1F	CCCGTGATACAAGGTAGGACA	62°C	Partial coat protein gene	690bp	Good and Monis 2001
		LR5-1R	CAGACTTCACCTCCTGTTAC				
GLRaV-1,-5	RT-PCR for both viruses	LR5-5F	TTTGGTGAGAATGGGAGTATG	58°C	HSP70 gene	600bp	
		LR5-5R	CTTTCGTCGGCTGACATAGAG				
	RT-PCR for both viruses	GSP1	CGAAGATGGCCGTGTCAATTACTG	66°C	HSP70 gene	200bp	
		GSP9	CGCCGCCGAAGTCGTAGACAACCA				
GLRaV-9	Specific RT-PCR	LR9-F	CGGCATAAGAAAAGATGGCA	55°C	HSP70 gene	390bp	Alkowni et al 2004
		LR9-R	TCATTCACCACTGCTTGAAC				
Closteroviridae	Grapevine leafroll viruses first round	dHSP up1	GGIHTIGAITTYGGIACIACITT	5cycles at 38°C and 35 cycles at 49°C /10s + 44°C /10s +38°C /15S	HSP70 gene	580-620 bp	Dovas and Katis 2003
		dHSP up1G	AGTTYGGGACGACGTT				
		dHSP do2	GTICCICCICCNAARTC				
		dHSP do2C	GTICCICCCCCNAARTC				
	Grapevine leafroll viruses first nested	dHSP nest1	TTYGGGACGACGTTYAGYAC	1 cycles at 48°C and 39 cycles at 54°C		500-535 bp	
		dHSP nest2	TYGGGACGACGTTYTCNAC				
		dHSP nest3	SCIGCIGMISWIGGYTCRTT				
		dHSP nest3G	GCGGMGSWGGGPTCRTT				
GLRaV-1	Specific PCR	LR1-nest4	GGCTTACCYAACCCTTCCAAAG	5cycles at 48°C and	HSP70 gene	490 bp	Dovas et al 2006
GLRaV-2	Specific PCR	LR2-nest5	GCAACCCACCCAACGTTTYAA		HSP70 gene	207 bp	
GLRaV-3	Specific PCR	LR3-nest6	ACCTACCCACCTTTTCGGGTTAAC		HSP70 gene	192 bp	

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
Ampeloviruses closely related to GLRaV-5	PCR for GLRaV-4,5 and -9	LR5clus-nest7	GCATTGAAYTTGTTCATICCRAA	35 cycles at 58°C /10s + 53°C /10s +48°C /15S	HSP70 gene	206 bp	
GLRaV-4,-5,-6,-9	GLRaV-4, -5, -6, -9, -De, -Pr	dHSP-nest2	TYGGGACGACGTTYTCNAC	5cycles at 55°C 35 cycles at 59°C /15s + 55°C /15s	HSP-70	490bp	Maliogka et al 2008
		LR5 clusdoL	GGYTCRTTCACIACIGCYTGIAC				

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Table 2.3 List of pathogens tested for, type of PCR test, primers used and annealing temperature, region amplified, expected product size and reference for each test used to detect exotic grapevine pathogens in Australia.

Pathogen	Test	Primer name	Orien-tation	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
AILV	Specific RT-PCR	AI3	F	GGTCTGGGGTGCCCGTGGCG	52ºC	Partial RNA-2	760bp	Minafra et al 1995
		AI4	R	ATTCACTAGTCCCTATTTAG				
CLRV	Specific RT-PCR	CLRV-5	F	TGGCGACCGTGTAACGGCA	53ºC	RNA1 polyprotein gene	416bp	Werner et al 1997
		CLRV-3	R	GTCGGAAAGATTACGTAAAAGG				
GARSV	Specific RT-PCR	A34-1	R	GAAGATACCCTCACGCATTGCC-	60°C	Partial coat protein	343bp	Gokalp et al 2003
		A34-2	F	CCCAGTGTTTACTCCACACCC				
GCMV and TBRV	Specific RT-PCR	P1	F	ATGGGAGAAGTGCTGG	42°C	Partial RNA-2	GCMV 246bp TBRV 332bp	Le Gall et al 1995
		P2	R	AATCTTTTTGTGTCCAACA				
GDefV	Specific RT-PCR	N66-1	F	GGTCCTATAACATACGG GGCTG	60°C	Partial coat protein	240bp	Cigsar et al 2003
		N66-2	R	CCCCTGATGGTGCTATTGGTCC-				
GFLV and ArMV	Specific RT-PCR	M2	F	(C/T)T(A/G)GATTTTAGGCTCAATGG;:	42°C	movement protein gene	290bp	Wetzel et al 2002
		M3	R	TG(C/T)AA(A/G)CCAGG(A/G)AAGAAAAT;				
GLRaV-6	Specific RT-PCR	LR6up		CGATAGGTTTGGGGGCACT	5cycles at 55°C and 35 cycles at 59°C /15s + 55°C /15S	partial HSP70h sequences	283	Maliogka et al 2008
		LR6do		GGCACAGAACACACCGACAAAAC				
GLRaV-7	Specific RT-PCR	LR7-F		TATATCCCAACGGAGATGGC	55°C	HSP70gene	502bp	Engel et al 2008
		LR7-R		ATGTTCCTCCACCAAAATCG				
Maculavirus – GRGV GAMaV*	Genus specific	Reverse transcription		DNA hexanucleotides		Reverse transcription for cDNA production		
		MTR1		TTCATGCAYGAYGCYMTSATGT	55°C	Methyl transferase region ORF1	575–689bp	Sabanadzovic et al 2000
		MTR2		TCCCAVGCNBHBGVRGTGACCCA				
		RD1		CYCARCAYAARGTVAACGA	58°C	RNA dependent	386bp	

Pathogen	Test	Primer name	Orien-tation	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
		RD2		GCGCATGCABGTSAGRGGG		RNA polymerase ORF1		
	GRGV specific with RD1	RGAP		GGCCAAACTGGGTGCTCACG	58C	RNA dependent RNA polymerase ORF1	386bp	
GVD	Specific RT-PCR	GVDORF2F1		TAA TAG GGC CTA AGT C	50°C	Movement protein gene	371bp	Terlizzi and Credi 2006
		ORF2R1		GGG CGT TGA ATA CAC CTT TAG C				
	Specific RT-PCR	GD1		GTACCTTAGGACGCTCTTCGGG	55°C	RNA binding protein	718bp	Abou-Ghanem et al 1997
		GD2		CGTTGGGTCGAGTGTGAGTACG				
Ilarvirus	Genus specific first PCR	Ilapol up		YTCIAMRTTYGAYAARTC	5 cycles :38°C 35 cyles: 42°C /20s 38°C/10s	polymerase gene on RNA2	381 bp	Maliogka et al 2007
		Ilapol do		GGYTGRTTRTGIGGRAA				
	Genus specific nestedPCR	Ilapolnes up		TCGAMRTTYGAYAARTCICA	5 cycles: 42°C 35 cyles: 47°C /20s 43°C/10s		371bp	
		Ilapolnest d		TGGGGRAAYTTIGYYTCRA				
Nepovirus	Reverse transcription	Reverse transcription		DNA hexanucleotides		Reverse transcription for cDNA production		Digiaro et al 2007
	Group specific subgroup A	NepoA-s		GGHDTBCAKTMYSARRARTGG	50°C	CP-RNA2	255bp	
		NepoA-a		TGDCCASWVARYTCYCCATA				
	Group specific subgroup B	NepoB-s		ATGTGYGCHACYACWGGHATGCA	50°C	CP-RNA2	390bp	
		NepoB-a		TTCTCTDHAAGAAATGCCTAAGA				
	Group specific subgroup C	NepoC-s		TTRKDYTGGYKAAMYYCCA	50°C	UTR-RNA1	640bp	
		NepoC-a		TMATCSWASCRHGTGSKKGCCA				
Secoviridae – Comovirus, Fababvirus and *Nepovirus*,	Family specific RT-PCR	ComF1	F	ACIWSIGARGGITWYCC	5 cycles: 40°C/10 s 37°C/5 s 35 cycles 42°C/30 s	RNA-dependent RNA polymerase	737–789 bp	Maliogka et al 2004
		ComR2	R	AVRTTRTCRTCICCRTA				
	Family specific	ComNeF3	F	TACGWSGGARGGGTWYCC	3 cycles:			

Pathogen	Test	Primer name	Orien-tation	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
	semi-nested PCR	ComNeR4	R	ARRTTRTCRTCGCCRTAIAC	48°C/30 s 37 cycles 50°C/30 s			
		ComNeR5	R	AVRTTRTCRTCGCCRTAIGT				
PRMV	Specific RT-PCR	PRMVV1		♌GTGATGGAATATTTGGAC	60°C	coat protein gene	200bp	Kheder et al 2004
		PRMVC1		♌CCATTGGCAACCGCCAGAGCTAC				
RsRSV	Specific RT-PCR	RpRSVF1	F	TGTGTCTGGTTTTGATGCT	61°C	Partial RNA-2	385bp	Ochoa-Corona et al 2006
		RpRSVR1	R	GAGTGCGATAGGGGCTGTT				
SLRSV	Specific RT-PCR	SLRSV-5D	R	CCCTTGGTTACTTTTACCTCCTCATTGTCC	55°C	Partial coat protein gene	291bp	Faggioli et al 2002
		SLRSV-3D	F	AGGCTCAAGAAAACACAC				
Tombus virus: GALV, PAMV	Specific RT-PCR	TomCPF	F	CCG CCG TAG CAT GAC CAA GTA	55°C	Partial coat protein gene	1000 bp	Russo et al 2002.
		TomCPR	R	CCA TGA ACT GGT CTT GTT CAA				
	Specific RT-PCR	TBSVGralF1	F	AAGGGTAAGGATGGTGAGGA	61°C	polymerase; readthrough protein	590bp	Harris et al 2006
		TBSVGralR1	R	TTTGGTAGGTTGTGGAGTGC				
ToRSV	Specific RT-PCR	U1	F	GAC GAA GTT ATC AAT GGC AGC	55°C	RNA-1 region	450 bp	Griesbach 1995.
		D1	R	TCC GTC CAA TCA CGC GAA TA				
	Specific two-step RT-PCR and nested PCR	ToRSVrt	R	GCGAAAACAACGTCCTTGC	50°C	Reverse transcription		Martin et al 2009
		ToRSVf1	F	CCGTTAGCAGCTTCCAAAAG	56°C	First PCR	512bp	
		ToRSVr1	R	GTCCTCATGGAACCTTTCTC				
		ToRSVf2	F	GGTTATCCAGCCTTAAGCAAG	56°C	Nested PCR	435 bp	
		ToRSVr2	R	CGGTAGGCTATGACAACTAC				
Section .01 TRSV	Specific two-step RT-PCR and nested PCR	TRSVr1	RT primer	GTGTTGGACAAACACGACAC	50°C	Reverse transcription		Martin et al 2009
		TRSVf1	F	GGAAGCTGTATAAACTCAGC	56°C	First PCR	338bp	
		TRSVr1	R	GTGTTGGACAAACACGACAC				
		TRSVf2	F	GAGAGCCCAAGAATGTCTGTC	56°C	Nested PCR	257bp	
		TRSVr2	R	GAACAGTGGGCTCAAACAAC				
Section .02 Xylophilus ampelinus	Single PCR	S3	F	GGTGTTAGGCCGAGTAGTGAG	60°C	ITS	277bp	Botha et al 2001
		s4	R	GGTCTTTCACCTGACGCGTTA				

*GAMaV = Grapevine asteroid mosaic associated virus

2.3 Results and Discussion

RT-PCR assays selected for development were based on BlastN analyses of each primer against published virus isolates available on GenBank. At the time the PCR tests were chosen very few full length sequences were available for each virus and the many partial sequences that were available did not cover the primer annealing sites. Thus, in addition to selecting assays based on bioinformatic analysis, the selection of RT-PCR tests was also based on published information about the success of assays to detect virus isolates of each species in other countries.

For each of the endemic viruses a range Australian isolates were tested. Additionally some grapevine varieties were used in which specific viruses had not been detected or had tested positive using ELISA and/or biological indexing for a virus, but not by RT-PCR.

2.3.1 *Grapevine virus A*

Table 2.4 shows the results of PCR testing of 32 samples for GVA using five RT-PCR assays. The tests chosen for GVA detection were initially trialled using 12 of the 32 samples. Of these 12 samples, GVA had previously been detected in five samples but had not been detected in seven samples. The primers pairs CPdt/MP (De Meyer et al 2000; Goszczynski and Jooste 2002) and 6951F/6906R (Goszczynski and Jooste 2003) were discarded as they were unable to detect as many isolates as the remaining three primer pairs, H587/C995 (Minafra et al 1997), 7273/7038 (MacKenzie 1997) and sense/antisense (Moreira et al 2004).

The latter three primer pairs did not detect all GVA isolates and were further validated against an additional 15 samples: 4/15 samples in which GVA had been previously detected and 11/15 samples in which GVA was not known to occur (Table 2.4). GVA was detected in 11/15 samples by the C995/H587 primer pair, in 7/15 samples by the 7038/7273 primer pair and in 3/15 samples by the sense/antisense primer pair (Table 2.4). GVA was not detected by either of the C995/H587 and 7038/7273 primer pairs when they were trialled against an additional five samples in which GVA had not been previously detected.

Overall the C995/H587 primer pair was the most reliable; however one isolate was not detected by this primer pair but was detected by the 7038/7273 primer pair (Table 2.4). Consequently both primer pairs were chosen for further validation in the national survey and will be recommended for PCR testing of samples for GVA.

GVA had not been detected by biological indexing, ELISA or other PCR assays in the variety/clone Viognier HTK, which was derived from heat-treatment and meristem culture for virus eradication from the Viognier 1968/CX Montpellier clone. To determine if this virus was a recent infection or had survived treatment and gone undetected, we tested an additional four grapevines of Viognier HTK (Table 2.4). We also obtained a sample of the original untreated Viognier 1968/CX Montpellier clone and tested this for GVA using the C995/H587 primer pair. GVA was detected in 3/4 additional Viognier HTK grapevines (Table 2.4) and in the Viognier 1968/CX Montpellier clone.

Table 2.4. A list of grapevines, their virus status for GVA and the results obtained for each grapevine using five RT-PCR assays for GVA detection.

Grapevine variety	Virus status prior to testing	PCR assay				
		H587/C995	7273/7038	Sense/antisense	CPdt/MP	6951F/6906R
Autumn Giant*	GVA	+	-	+	-	-
Bella Verde*	GVA	+	+	+	+	+
Bella Grande*	GVA	-	+	-	+	-
Bella Grande 143	GVA	+	-	-	+	-
Grenache 139	GVA	+	+	-	nt	nt
Grenache 139 HT	GVA	+	+	-	nt	nt
Grenache 139 HT	GVA	+	+	-	nt	nt
Riesling	GVA	+	+	+	nt	nt
Vittoria	GVA	+	+	-	-	-
Autumn Gold	Not detected	+	+	+	-	-
Cab Franc C24-1	Not detected	-	-	nt	nt	nt
Chardonnay 84	Not detected	-	-	-	nt	nt
Chardonnay 84	Not detected	-	-	nt	nt	nt
Chardonnay Penfolds 58	Not detected	-	-	nt	nt	nt
Chenin Blanc	Not detected	+	-	-	nt	nt
Early Sweet	Not detected	+	+	+	+	-
Graciano WA6V6	Not detected	+	+	-	nt	nt
Jade seedless	Not detected	+	-	-	-	-
Montepulciano	Not detected	+	+	+	nt	nt
Pinot Noir	Not detected	+	+	+	nt	nt
Shiraz 1564	Not detected	-	-	nt	nt	nt
Unknown 763	Not detected	-	-	-	-	-
Viognier HTK*	Not detected	+	-	-	-	-
Viognier HTK	Not detected	-	-	-	-	-
Viognier HTK	Not detected	-	-	-	-	-
Viognier HTK	Not detected	+	-	-	nt	nt
Viognier HTK	Not detected	+	-	-	nt	nt
Viognier HTK	Not detected	-	-	-	nt	nt
Viognier HTK	Not detected	+	-	-	nt	nt
Viognier 642	Not detected	-	-	-	nt	nt
Viognier 642	Not detected	-	-	-	nt	nt
5C Teleki 10-48-49 GM	Not detected	-	-	nt	nt	nt
Viognier 1968/CX Montpellier*	Unknown	+	-	nt	nt	nt

* The grapevines that were used for sequence analysis of the p10 gene.

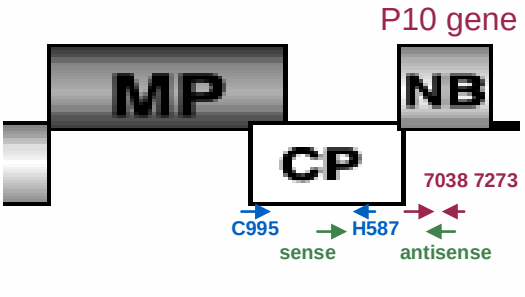


Figure 2.1. Genome organisation of the 3' end of the GVA genome including the movement protein (MP positions 5654-6490) and the coat protein (CP, positions 6414-7010) and the p10 gene (positions 7011-7283). The annealing sites of the primer pair C995/H587, 7038/7273 and sense/antisense are shown

The p10 gene, which may be associated with symptom expression (Galaikparov et al 2003) is the region of the GVA genome that is detected by the 7038/7273 primers (Figure 2.1) while the C995/H587 primer pair were designed to detect the coat protein gene (Figure 2.1). The sense and antisense primers span part of the coat protein and the p10gene (Figure 2.1).

We amplified the p10 gene of the GVA isolate that infects the Viognier HTK (VHTK) clones and the Viognier 1968/CX Montpellier clone (VCXM) using the primers 5'-CGCTGCGCGCATGAATGTGTGTAAG-3' and 5'-GTCATAGTATGACAACCTAGC-3' (Galaikparov et al 2003) and cloned and sequenced the products. We also amplified cloned and sequenced the p10 gene of three additional isolates that infected Autumn Giant (AG), Bella Grande (BG) and Bella Verde (BV) using the same primers. We analysed the sequence data to elucidate the relationship between the different GVA isolates to determine the sequence variability of this region. This may explain why the 7038/7273 primers did not detect the GVA isolate that infects some of the Viognier HTK clones and the original untreated Viognier 1968/CX Montpellier clone.

The five Australian GVA isolates were compared with other published sequences available on Genbank and Table 2.5 shows the % nucleotide sequence similarities between the p10 gene of each of the GVA isolates compared in this study. The results indicate that the p10 gene can be highly variable as nucleotide sequence similarities amongst all the GVA isolates that were compared ranged between 82.3-99.2%. Although the sequence obtained did not cover the regions which the 7038 and 7273 primers bind, it is likely that the variability extends to these regions and would explain why the PCR test to failed to detect GVA isolates in the Viognier HTK clones and the original untreated Viognier 1968/CX Montpellier clone

There was 98.9% sequence similarity between the p10 gene of the GVA isolates from the Viogneir HTK and Viognier 1968/CX Montpellier clone (Table 2.5). The high degree of sequence similarity between the Viognier isolates of GVA suggests that the virus was not eradicated by an intensive heat treatment and meristem culture regime and remained undetected using woody indexing, RT-PCR and ELISA for GVA detection. The p10 gene is required for symptom expression (Galiakporov et al 2003). It is possible that the inability of biological indexing, using the sensitive variety Kober 5BB, to detect the Viognier GVA isolate was due to the genetic variation of the p10 gene in the Viognier isolates.

Table 2.5. The percentage (%) nucleotide sequence similarity of the p10 gene amongst Australian and GVA isolates published on Genebank (accession numbers given in brackets)

GVA Isolate*	PA3 (AF007415)	GVA compete genome (AY244516)	VCXM*	VHTK*	BG*	AG*	GTG11-1 (DQ855084)	GVMPCP (X75433)	BV*	GTR1-2 (DQ855086)	BMo32-1 (DQ855087)	GTR1SD-1 (DQ855081)	KWVMo4-1 (DQ855083)	P163-M5 (DQ855082)
GVA compete genome	99.2													
VCXM	87.7	87												
VHTK	87.5	86.8	98.9											
BG	86.1	85.4	91.6	92.7										
AG	87.4	86.7	92.3	92.7	94.7									
GTG11-1	87.9	87.2	89.8	89.5	90.2	90.5								
GVMPCP	87.2	86.5	89.1	88.8	90.9	91.6	93.2							
BV	86.4	85.7	89.9	90.9	90.2	91.6	87.1	87.8						
GTR1-2	85.8	85.1	88.1	87.8	88.5	89.1	90	88.2	89.5					
BMo32-1	89	88.2	91.6	91.3	89.5	90.9	91.1	91.8	91.6	91.8				
GTR1SD-1	88.2	87.5	89.8	89.5	88.8	90.5	89.7	91.1	91.6	89.3	96.8			
KWVMo4-1	89.7	89	91.6	91.3	91.6	93.3	91.1	91.4	92	90.7	94.6	95		
P163-M5	89.3	88.6	91.2	90.9	90.2	91.9	90.4	90	91.3	90.7	94.6	94.6	97.8	
GTR1-1 (DQ787959)	83	82.3	84.3	84	83	83.3	85.5	83.7	83	84.4	84.8	84.8	87.9	87.6

* The GVA isolates that were sequence for this study: VCXM= Viognier 1968/CX Montpellier; VHTK = Viognier HTK; AG = Autumn Giant; BG = Bella Grande; BV = Bella Verde

2.3.2 *Grapevine virus B*

Table 2.6 shows the results of PCR testing of 8 samples for GVB using five RT-PCR assays. GVB had been previously detected in five of the eight samples, of which two had tested positive for corky bark disease in quarantine using biological indexing. The remaining three GVB isolates were not associated with corky bark disease. Both GVBC6980/GVBH7439 (Minafra & Hadidi, 1994; MacKenzie 1997) and Bh123/Bc637 (Sciancalepore et al 2006) detected GVB in all known positive samples and also detected GVB in one sample where GVB infection was suspected. Thus both primer pairs were considered reliable for GVB detection. However the GVBC6980/H7439 primer pair was chosen for further validation as this primer pair was more frequently reported in the literature for GVB detection. Also BlastN analysis indicated that the Bc637 primer (Sciancalepore et al 2006) had significant similarity with some GVA isolates and may result in annealing and subsequent non-specific detection of this closely related virus.

Table 2.6. A list of grapevines, their virus status for GVB and the results obtained for each grapevine using five RT-PCR assays for GVB detection

Grapevine variety	Virus status prior to testing	PCR assay				
		GVBC6980/H7439	Bh123/Bc637	Senso/antisenso	GVB6445/7038	CPF1/CPr3
AQ2206	GVB	+	+	nt	nt	nt
AQ2207	GVB	+	+	nt	nt	nt
Durif	GVB	+	+	nt	nt	nt
Jade Seedless	GVB	+	+	nt*	nt	nt
Ruspestris St George	GVB	+	+	-	-	-
AQ2208	Not detected	+	+	nt	nt	nt
Cabernet Franc	Not detected	-	-	-	-	-
Jade Seedess	Not detected	-	-	-	-	-

*nt = not tested

2.3.3 *Grapevine fleck virus*

The results of PCR testing of 10 samples for GFkV using three RT-PCR assays are presented in Table 2.7. GFkV had been previously detected in five of these samples. The U279/L630 primer pair (Shi et al 2003) detected GFkV in all known positive samples and also detected the virus in one sample where GFkV infection was not previously detected.

The RD1/RD2 PCR assay is a generic two step method that uses random hexamers in the first step to transcribe RNA to cDNA. The RD1/RD2 primer pair are used in the PCR step and are designed to detect all *Maculavirus* species known to infect grapevines (Sabanadzovic et al. 2000). Although these primers generated a PCR product of the expected size for some of our GFkV isolates, they did not detect all GFkV isolates used in our comparison. Furthermore this assay is more complicated and costly than the one-step PCR assay and may not be suitable for commercial high-throughput diagnostic testing. However it may be useful when

screening quarantine samples for the presence of Maculaviruses that have not yet been detected in Australia, including GRGV and GAMaV.

The third assay that was tried is based on the generic two step assay described above, except that it used the GFkV specific primer FK-AP in place of the RD2 degenerate primer for specific GFkV detection (Sabanadzovic et al. 2000). This assay did not detect GFkV in any of the 10 samples in this study and it is possible the GFkV specific primer does not match Australian GFkV isolates.

Based on these results the U279/L630 RT-PCR assay for GFkV detection was chosen for further validation during the National survey. The RD1/RD2 test was not included in the national survey even though they were likely to detect other *Maculavirus* species, because the detection of GFkV may have confounded the interpretation of the results. Instead the RD1 forward primer was combined with the RGAP primer for specific detection of GRGV.

Table 2.7. A list of grapevines, their virus status for GFkV and the results obtained for each grapevine using three RT-PCR assays for GFkV detection

Grapevine variety	Virus status prior to testing	PCR assay		
		U279/L360	RD1/RD2	RD1/FK-AP
Bella Grande	GFkV	+	+	-
Bella Verde	GFkV	+	+	-
Cabernet Franc	GFkV	+	-	-
Ruspetsris St George	GFkV	+	+	-
Vittoria	GFkV	+	+	-
Autumn Giant	Not detected	-	-	-
Autumn Gold	Not detected	-	-	-
Early sweet	Not detected	+	-	-
Jade seedless	Not detected	-	-	-
Unknown 763	unknown	-	-	-

2.3.4 *Grapevine Rupestris stem pitting associated virus*

Table 2. 8 shows the results of PCR testing of 14 samples for RPSaV using five RT-PCR assays. RSPaV had been previously detected in five of the 14 samples. The RSP48/49 primer pair (Nolasco et al., 2000) detected RSPaV in all the known RSPaV positive samples. The remaining PCR tests did not detect RSPaV in all the known RSPaV positive samples. The RSP48/49 primer pair also detected RSPaV in six samples of unknown RSPaV status and RSP52/53 (Nolasco et al., 2000) was the only other primer pair to detect RSPaV in the same samples as the RSP48/49 primer pair. The 13/14 primer pair (Meng et al 1999) produced two PCR products very close to the expected product size and sequence analysis indicated these bands were not associated with virus (data not shown). This doublet band could confound the analysis of the PCR tests for RSPaV, causing a false positive result and consequently these primers were not chosen for further validation.

Based on these results the RSP48/49 primer pair were considered more reliable that the other four assays and were chosen for further validation. This finding supports previous research done by Habili et al (2006). The Sy9F/8R was also included for validation in the national survey and screening the AVIA collection because of its potential to detect and

differentiate strains of RSPaV that are symptomless on the biological indicator Rupestris St George but may be associated with Shiraz disease in Australia (Habili et al 2006).

Table 2.8. A list of grapevines, their virus status for RSPaV and the results obtained for each grapevine using five RT-PCR assays for RSPaV detection

Grapevine variety	Virus status prior to testing	PCR assay				
		RSP48/49	Sy9F/8R	RSPH/C	13/14	RSP52/53
Bella Grande	RSPaV	+	+	+	+	+
Bella Verde	RSPaV	+	+	+	+	+
Early sweet	RSPaV	+	-	-	doublet	-
Jade seedless	RSPaV	+	-	+	+	-
Vittoria	RSPaV	+	+	+	+	+
Autumn Giant	Unknown	-	-	-	-	-
Autumn Gold	Unknown	-	-	-	doublet	-
Unknown 763	Unknown	-	-	-	doublet	-
Cabernet Franc	Unknown	+	+	+	+	+
Chardonnay I10V5	Unknown	+	+	+	+	+
Kober5BB	Unknown	+	+	-	doublet	+
LN33	Unknown	+	+	+	+	+
Rupsetris St George	Unknown	+	-	-	+	+
Shiraz	Unknown	+	+	+	+	+

2.3.5 *Grapevine leafroll associated virus 1*

GLRaV-1 had been previously detected in six of the 16 samples (Table 2.9). The GSP3/GSP4 primers detected GLRaV-1 in five of the six known positive samples and in three samples in which GLRaV-1 had not been previously detected. None of the remaining RT-PCR tests produced as many positive results as the GSP3/GSP4 primers; however the GSP1/9 primer pair produced an additional positive result in the Autumn Gold that was not detected by any other primer pair. It is possible that the band was related to a GLRaV-5 strain as this primer pair was designed to detect both GLRaV-1 and GLRaV-5 (Good and Monis 2001) but this was not determined.

Table 2.9. A list of grapevines, their virus status for GLRaV-1 and the results obtained for each grapevine using five RT-PCR assays for GLRaV-1 detection

Grapevine variety	Virus status prior to testing	PCR assay				
		GSP3/ GSP4	plazleft/right	GSP1/9*	pORF9F/R	GLRaV 1f/r
Shiraz	GLRaV-1	+	+	-	+	
Bella Grande	GLRaV-1	+	+	+	-	+
LR1	GLRaV-1	+	+	-	-	-
Rootstock SO4	GLRaV-1	-	-	-	-	-
Bella Grande	GLRaV-1	+	+	+	-	
Montepulciano	GLRaV-1	+	-	-	+	+
Autumn Giant	unknown	-	-	-	-	-
Autumn Gold	unknown	-	-	+	-	-
Bella Verde	unknown	+	-	+	-	-
Cabernet Franc	unknown	-	-	-	-	-
Early sweet	unknown	+	-	-	-	-
Furmint	unknown	-	-	-	-	-
Jade seedless	unknown	-	-	-	-	-
Unknown 763	unknown	-	-	-	-	-
Vittoria	unknown	+	-	-	-	-
Verdelho	unknown	-	-	-	-	-

*detects GLRaV-1 and GLRaV-5

The SO4 rootstock had previously been reported as GLRaV-1 infected but the virus was not detected by any of the tests used in this study. It is possible that the lack of detection was due to uneven distribution and/or titre of the virus in grapevines and the sample that was taken did

not contain virus or virus concentrations were below the level of detection by PCR tests, or that the lack of GLRaV-1 detection in this sample was due to inhibition caused by co-purified compounds that interfere with the RT and/or PCR enzymes. It is also possible that the previous detection was a false positive result: the tests used in previous detection are not known

The annealing temperature recommended for the GSP3/GSP4 primer pair was 58°C (Good and Monis 2001), however in this study the bands observed post PCR analysis were faint, indicating that this temperature may be too stringent and reduced the annealing capacity of the primers. When the PCR was repeated at a lower annealing temperature of 55°C the bands observed were brighter. This effect is possibly due to variation at the annealing sites one or both primers – further work would is required to confirm this.

Based on these results the GSP3/GSP4 PCR test, at an annealing temperature of 55°C, was further validated during the survey and screening of the AVIA collection. The GSP1/9 primers would also be useful for detection as it may be that they detect strains of GLRaV-1 that are not detected by the GSP3/GSP4 primer pair.

2.3.6 *Grapevine leafroll associated virus* 2

Fifteen samples were used to assess the five GLRaV-2 primer pairs (Table 2.10). The Flame Seedless samples and Malbec CW14 samples were chosen because a GLRaV-2 infection was suspected. GLRaV-2 had been previously detected in only one of the15 samples and the virus was detected in a sample from the same grapevine using 4/5 of the PCR tests that were tried. The V2dCPf2 sense/V2CPr1 antisense, LR2-U2 sense/ L2 antisense and the V2HSPf sense/V2HSPr antisense primer pairs (Bertazzon and Angelini 2004) detected GLRaV-2 in a second sample in which GLRaV-2 had not been previously detected.

Table 2.10. A list of grapevines, their virus status for GLRaV-2 and the results obtained for each grapevine using five RT-PCR assays for GLRaV-2 detection

Grapevine variety	Virus status prior to testing	PCR assay				
		V2dCPf2 sense/ V2CPr1 antisense	LR2-U2 sense/ L2 antisense	V2HSPf sense/ V2HSPr antisense	LRaV-2(1) sense/ LRaV -2(2) antisense	RGHSP227V sense/ RGHSP777C antisense
Rupestris st George	GLRaV-2	+	+	+F	+F	-
Unknown 763	unknown	-	-	-	-	-
Autumn Giant	unknown	-	-	-	-	-
Autumn Gold	unknown	-	-	-	-	-
Bella Grande	unknown	-	-	-	-	-
Bella Verde	unknown	-	-	-	-	-
Early sweet	unknown	-	-	-	-	-
Flame seedless	unknown	-	-	-	-	-
Flame seedless	unknown	-	-	-	-	-
Flame seedless	unknown	-	-	-	-	-
Jade seedless	unknown	-	-	-	-	-
Jade Seedless	unknown	+	+	+F	-	-
Malbec CW 14	unknown			-	-	-
Vittoria	unknown	-	-	-	-	-
Rootstock SO4	unknown	-	-	-	-	-

Faint bands were observed during post-PCR analysis with the V2HSPf sense/V2HSPr antisense test, suggesting a reduced annealing capacity of the primers for both GLRaV-2

strains and this primer pair were not considered for further validation. Although the results using the LR2-U2 sense/ L2 antisense primer pair appeared to be equally capable of detecting GLRaV-2, isolates the V2dCPf2 sense/V2CPr1 antisense test were chosen for further validation as other studies showed that these primers were capable of detecting Rootstock stem lesion associated strains of GLRaV-2 that were not detected by the LR2-U2 sense/ L2 9 primer pair (Bertazzon and Angelini 2004).

2.3.7 *Grapevine leafroll associated virus 3*

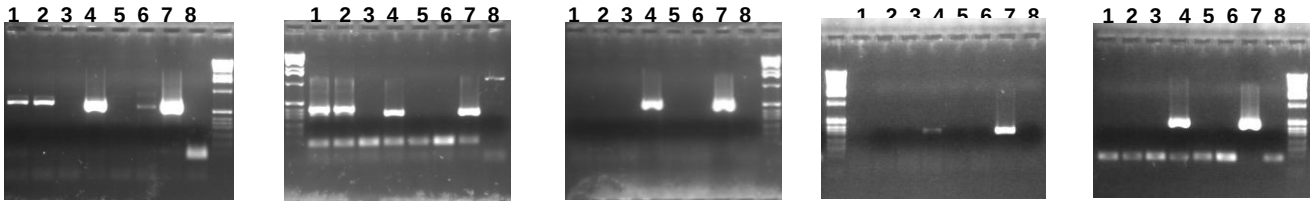
Table 2.11 shows the results of PCR testing of 14 samples for GLRaV-3 using five RT-PCR assays and an additional 28 samples using two RT-PCR tests. GLRaV-3 had been previously detected in two of the14 samples (Bella Verde and Malbec CW 14) that were screened in this study. All five RT-PCR tests were able to detect these GLRaV-3 isolates (Table 2.11, Figure 2.2). The P3U/P3D primer pair detected an additional three GLRaV-3 isolates and the C547/H229 primer pairs detected an additional two isolates that were not detected by the other tests (Table 2.11, Figure 2.2).

The two Flame seedless grapevines and the rootstock SO4 in which GLRaV-3 was detected by PCR in this study had previously tested positive for the virus only by ELISA and never by previous PCR tests done in other laboratories (Julian Connellan pers.comm). Malbec CW 14 is reported to be symptomatic for leafroll disease in Australia but GLRaV-3 had not been previously detected in this variety using PCR, ELISA and biological indexing (P. Nicholas pers. comm.). The H330/C629 primer pair only generated a faint positive result for GLRaV-3 in the Malbec CW 14 sample in our hands (Figure 2.3) suggesting a reduced annealing capacity of the primers with this strain. It is possible that all three grapevines had been previously tested using the H330/C629 primer pair, which were most commonly used by many laboratories for GLRav-3 detection at that time and obtained a false negative result.

An additional 28 samples were screened using the P3U/P3D and C547/H229 primer pairs. GLRaV-3 had been detected previously in 4/28 of the additional samples and both the P3U/P3D and C547/H229 primer pairs detected these isolates. Both primer pairs also detected GLRaV-3 in an additional five samples. The C547/H229 occasionally generated larger products (Figure 2.3). Sequence analysis indicated that these strains are GLRaV-3 but are significantly different to the GLRaV-3 type strain (Genbank accession number AF037268) and most closely related to a divergent GLRaV-3 strain from Italy (Genbank DQ314610 Angelini et al 2006). The P3U/P3D PCR test was further validated during the survey and screening of the AVIA collection.

Table 2.11 A list of grapevines, their virus status for GLRaV-3 and the results obtained for each grapevine using five RT-PCR assays for GLRaV-3 detection

Grapevine variety	Virus status prior to testing	PCR assay				
		P3U/P3D	C547 H229	LC1 LC2	CP3U/CP3D	H330/c629
Bella Verde	GLRaV-3	+	+	+	+	+
Malbec CW 14	GLRaV-3	+	+	+	+	+F
Autumn Giant	Unknown	-	-	-	-	-
Autumn Gold	Unknown	-	-	-	-	-
Bella Grande	Unknown	-	-	-	-	-
Early sweet	Unknown	-	-	-	-	-
Flame seedless V1	Unknown	+	+	-	-	-
Flame seedless V2	Unknown	+	+	-	-	-
Flame seedless SA	Unknown	-	-	-	-	-
Jade seedless	Unknown	-	-	-	-	-
Jade Seedless	Unknown	-	-	-	-	-
Rootstock SO4	Unknown	+	-	-	-	-
Vittoria	Unknown	-	-	-	-	-
Unknown 763	Unknown	-	-	-	-	-
Crimson Seedless 217	GLRaV-3	+	+	nt	nt	nt
Crimson Seedless 76	GLRaV-3	+	+	nt	nt	nt
Graciano WA6V6	GLRaV-3	+	+	nt	nt	nt
Graciano WA6V6	GLRaV-3	+	+	nt	nt	nt
Lagrein H9V9	GLRaV-3	+	+	nt	nt	nt
Cabernet Sauvignon Lc6	Unknown	-	-	nt	nt	nt
Chardonnay 84	Unknown	-	-	nt	nt	nt
Chardonnay Q390-09	Unknown	-	-	nt	nt	nt
Chenin Blanc FGV13	Unknown	+	+	nt	nt	nt
Chenin Blanc Steen	Unknown	-	-	nt	nt	nt
FurmintE3V11	Unknown	+	+	nt	nt	nt
Grenache139K4	Unknown	-	-	nt	nt	nt
Grenache LCE11-2	Unknown	-	-	nt	nt	nt
MerlotD3V14	Unknown	-	-	nt	nt	nt
Mondeuse F3V16	Unknown	+	+	nt	nt	nt
Montepulciano CSIRO	Unknown	+	+	nt	nt	nt
Pinot Noir 115TC	Unknown	-	-	nt	nt	nt
Ramsey A11V2	Unknown	-	-	nt	nt	nt
Riesling FLV 544-6	Unknown	-	-	nt	nt	nt
Riesling 239GM	Unknown	-	-	nt	nt	nt
Riesling I10V14	Unknown	-	-	nt	nt	nt
Rubired C5V14	Unknown	-	-	nt	nt	nt
Sauvignon BlancF4V6	Unknown	-	-	nt	nt	nt
Sultana H5	Unknown	-	-	nt	nt	nt
TemperanilloD8V12	Unknown	-	-	nt	nt	nt
Touriga E6V12	Unknown	+	+	nt	nt	nt
Verdelho 168	Unknown	-	-	nt	nt	nt
101-14 100HT	Unknown	-	-	nt	nt	nt
140 RuggeriQ45-3A	Unknown	-	-	nt	nt	nt



a. **b.** **c.** **d.** **e.**

Figure 2.2. Detection of GLRaV-3 by the five different primer pairs: a) P3U/P3D; b) C547/H229; c) LC1/LC2; d) C629/H330; e) CP3U/CP3. Samples are: lane 1 Flame seedless V1; lane 2 Flame seedless V2; lane 3 Flame seedless SA; lane 4, Malbec cw14, lane 5, Jade seedless; lane 6, SO4 rootstock; lane 7 Graciano WA6V6; lane 8, no template control.

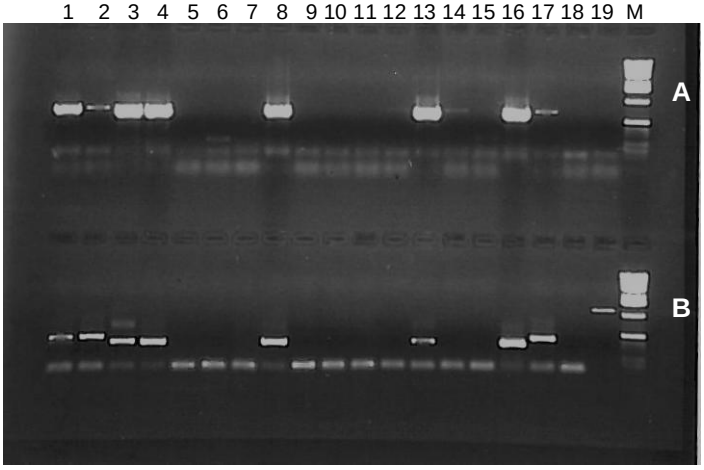


Figure 2.3. Detection of GLRaV-3 by the two different primer pairs: a) P3U/P3D and b) C547/H229. Samples are: lane 1, Crimson seedless 1; lane 2 Crimson seedless 2; lane 3 Graciano WA6V6; lane 4, Lagrein, lane 5, Grenache; lane 6, Temperanillo D8V12; lane 7 Sultana H5; lane 8, Furmint E3V11; lane 9 140 Ruggeri Q45-3A; lane 10, Chardonnay 84; lane 11, Sauvignon BlancF4V6 ; lane 12, Rubired C5V14; lane 13, Graciano WA6V6; lane 14, Verdelho 168; lane 15, Riesling; lane 16, Crimson seedless 3; lane 17, Crimson seedless 4; lane 18, Lagrein H9V9; lane 19, no template control; M, Marker 10.

2.3.8 *Grapevine leafroll associated virus* 4, -5 and -9

Seven RT-PCR assays for detection of GLRaV-4, 5 and /or -9 were assessed in this study (Table 2.12). At least one of GLRaV-4, -5 and -9 had previously been detected in eight of the 11 samples that were screened. The LR4F/LR4R (Fazeli et al 1998) and HSP4A/HSP4B (Routh et al 1998) primer pairs were designed to specifically detect GLRaV-4. The HSP4A/HSP4B primer pair detected GLRaV-4 in both GLRaV-4 infected samples and also in one additional sample, Chenin blanc, which was not known to be infected with GLRaV-4. The LR4F/LR4R primer pair detected the virus in only one sample.

Table 2.12 A list of grapevines, their virus status for GLRaV-4, -5 and -9 and the results obtained for each grapevine using seven RT-PCR assays for the detection of each virus.

Grapevine variety	Virus status prior to testing	PCR assay					
		LR4F LR4R	HSP4A HSP4B	HSP45A HSP45B	LR5-1F LR5-1R	LR5-5F LR-5R	LR9-F LR9-R
TemperanilloD8V12	GLRaV-5	-	-	-	-	-	-
Sultana H5	GLRaV-4	+	+	-	-	-	-
Sauvignon BlancF4V6	GLRaV-4	-	+	-	-	-	-
Graciano WA6V6	unknown	-	-	-	-	-	-
Chenin Blanc FGV13	unknown	-	+	-	-	-	-
Grenache LCE11-2	GLRaV-9	-	-	-	-	-	+
Cabernet Sauvignon Lc6	GLRaV-9	-	-	-	-	-	+
Shiraz	GLRaV-9	-	-	-	-	-	+
Crimson Seedless 89	GLRaV-3,-5,-9	nt	nt	-	+	+	+
Crimson Seedless 89EB	GLRaV-3,-5,-9	nt	nt	-	+	+	+
Verdehlo 168	unknown	nt	nt	-	nt	?	nt

The HSP45A/ HSP45B primer pair (Routh et al 1998) were designed to detect both GLRaV-4 and -5 but did not detect either virus in any sample.

The primers LR5-1F/LR5-1R (Good and Monis 2001) were specifically designed to detect GLRaV-5 and detected the virus in 2/3 samples previously reported to be infected with this virus.

The primers LR5-5F /LR5-5R (Good and Monis 2001) were designed to detect GLRaV-5 and -9 and did detect virus in two samples known to be infected with both viruses, but did not detect GLRaV-5 or-9 in any other sample that was reported to be infected with either virus. However they did produce to band of approximately the correct size in the Verdelho 168, for which a GLRaV infection had been suspected but none of the species had been detected. The identity of this virus could not be confirmed.

The LR9-F/LR9-R primers were designed to specifically detect GLRaV-9 and detected the virus in 5/5 infected samples.

Based on these results we chose the primers HSP4A/HSP4B, LR5-1F/LR5-1R and LR9-F/LR9-R which were specific for GLRaV-4, -5 and -9 respectively, for further validation during the survey and screening of the AVIA collection.

GLRaV-5 had been detected by RT-PCR in another laboratory (P. Nicholas, pers.comm.) but information about the test that was used is not available. This grapevine did test positive for a GLRaV species in this study (Table 2.13) and during the survey (Chapter 6) using a *Closteroviridae* generic test and an *Ampelovirus* subgroup I test. The Subgorup I test detects GLRaV-4, -5, -6, and -9 and it is likely that the Temperanillo grapevine used in this study is infected with a Amplelovrius subgroup I species or strain that is not detected with any of the tests used in this study. More work is required to determine the identity of the virus detected in this grapevine.

2.3.9 Generic and specific *Grapevine leafroll associated virus* detection

PCR testing of two samples in which GLRaV species had not been detected and 12 samples in known to be infected with a variety of GLRaV species were screened using the generic and specific PCR tests developed by Dovas and Karis (2003) and Dovas et al 2006. GLRaV-1, -2 (Table 2.13).The generic nested PCR test detected GLRaVs in 11/12 samples in which virus had been previously detected but not in the two samples which had tested negative using other GLRaV diagnostic tests.

Table 2.13 A list of grapevines, their virus status for different GLRaV species and the results obtained for each grapevine using a generic *Closteroviridae* PCR test (All GLRaVs), specific tests for the detection of GLRaV-1 (LR1-nest4), -2 (LR2-nest5) and -3 (LR3-nest6) and subgroup I ampleloviruses (LR5clus-nest7)

Grapevine variety	Virus status prior to testing	PCR assay				
		All GLRaVs	LR1-nest4	LR2-nest5	LR3-nest6	LR5clus-nest7
Montepulciano	GLRaV-1	+	+	-	-	-
Rootstock SO4	GLRaV-1	-	-	-	-	-
Rupestris St George	GLRaV-2	+	-	+	-	-
Rupestris St George	GLRaV-2	+	+	+	-	-
Chenin Blanc	GLRaV-3	+	-	-	+	-
Mondeuse	GLRaV-3	+	-	+	+	-
Sauvignon Blanc	GLRaV-4	+	+	-	-	+
Rubired	GLRaV-4	+	-	-	-	+
Temperanillo	GLRaV-5	+	+	-	-	+
Cabernet Sauvignon	GLRaV-9	+	-	-	+	+
Shiraz Waite	GLRaV-9	+	-	-	-	+
Crimson seedless 358	GLRaV-3, -5, -9	+	-	-	+	+
HT - no virus	ND	-	-	-	-	-
HT - no virus	ND	-	-	-	-	-

GLRaV-1 was detected in four samples. Three of these samples were thought to be infected with different GLRaV species.

GLRaV-2 was detected in three samples, two which were know to be infected with GLRaV-2 and one in which GLRaV-3 had previously been detected. GLRaV-3 was detected in four samples, three of which were known to be infected with the virus and one in which GLRaV-9 had previously been detected.

The LR5clus-nest7 test was designed to detect Ampeloviruses closely related to GLRaV-5, including GLRaV-4, -5 and -9. The PCR assay detected virus in all samples infected with these GLRaV species.

Each of the specific PCRs for GLRaV-1 (LR1-nest4), -2 (LR2-nest5) and -3 (LR3-nest6) and the subgroup I *Ampelovirus* (LR5clus-nest7) PCR test generated strong non-specific bands in many of the samples and were not further validated. However the *Closteroviridae* nested PCR did not produce non-specific bands close to the expected size and was therefore chosen for further validation during the national survey, especially as the use of this nested generic GLRaV assay is potentially more sensitive than the specific assays and could reduce the cost of testing to industry by reducing the number of PCR test required. During the survey we also established and validated a second generic PCR test for detection of subgroup I Ampeloviruses comprising of GLRaV-4, -5, -6 and -9 and two new and tentative species called GLRaV-Pr and GLRaV –De using the primer pair dHSP-nest2/ LR5 clusdoL (Maliogka et al 2008). The reliability of this test is discusses in Chapter 6.

2.3.10 PCR development for Exotic pathogens

Because of strict AQIS regulations we were unable to obtain nucleic acid extracts for all of the exotic viruses and *X. ampelinus*. The permit that was finally obtained in June 2009 only allowed nucleic acid extracted from micro-organisms, not from plant material, and this limited

us to obtaining PCR products or partial genomes that were cloned, which many laboratories could not provide without additional work and cost to them. Table 2.1 lists the positive controls that were obtained during the project.

As a consequence of the difficulties in obtaining an appropriate permit, some tests were established in our laboratory without positive controls and trialled during the national survey. Even without positive controls we could still assess their specificity, in particular the risk of false positive results, but they are not considered appropriately validated at this stage. These tests included specific tests for:

- GLRaV-6 (Maliogka et al 2008)
- GLRaV-7 (Engel et al 2008),
- AILV (Minafra et al 1995),
- GARSV (Gokalp et al 2003),
- GDefV (Cigsar et al 2003),
- GRGV (Sabanadzovic et al 2000),
- GVD (Abou-Ghanem et al 1997; Terlizzi and Credi, 2006)
- PRMV (Kheder et al 2004).

We are still in the process negotiating with AQIS to obtain appropriate positive controls. Although we can now obtain extracted nucleic acid for *X. ampelinus* (Botha et al 2001) the laboratory that was contacted did not respond to our request.

Some of the tests developed in this study were generic and could detect several viruses or groups of viruses and in some instances we had at least one positive control for these tests. Thus we do consider these validated for the viruses that they detect. These included generic tests for GCMV and TBRV (LeGall et al 1995), GFLV and ArMV (Wetzel et al 2002), *Maculavirus* detection for GFkV, GRGV and GAMV using RD1/RD2 primers (Sabandadzovic et al 2000), *Closteroviridae* detection for GLRaVs (Dovas and Katis, 2003), a generic PCR test for detection of GLRaV-4, -5, -6, -9, -Pr and –De using the primer pair dHSP-nest2/ LR5 clusdoL (Maliogka et al 2008); and Tombusvirus detection (Russo et al 2002; Harris et al 2006). Each of these tests was trialled during the national survey.

No specific PCR tests have been published for BLMV, GBLV and GTRSV but it was reported that these viruses should be detected using generic PCR tests for the *Secoviridae* family (Maliogka et al 2004), or by *Nepovirus* subgroup PCR tests (Digiaro et al 2007). These tests also had the potential to reduce the number of tests used for detection of the grapevine infecting nepoviruses.

The *Secoviridae* semi-nested PCR (Maliogka et al 2004) was designed to detect known and unknown species within the family. The primers were based on the aligned sequences of 17

Secoviridae members including several nepoviruses that infect grapevines: TRSV, TBRV, GCMV, ToRSV, PRMV and GFLV. The authors showed that this semi-nested RT-PCR assay could detect TRSV as well as ArMV and CLRV. We trialled this assay against several GFLV isolates, ToRSV isolate, CLRV and the Sadwavirus SLRSV using the conditions described by Maliogka et al (2004). Positive results were obtained for GFLV but multiple bands were obtained for SLRSV. ToRSV and CLRV were not detected, although it is possible that this was due to the quality of the extracts, which were degraded and gave very low signals in specific PCR tests. We did not continue to validate this test.

The *Nepovirus* subgroup specific RT-PCR tests developed by Digiaro et al (2007) were also trialled with the sub-group A primers against GFLV and the subgroup C primers against CLRV and ToRSV using the conditions described but they did not work in our hands.

Currently the most recently published assays for *Nepovirus* subgroups A and B described by Wie and Clover (2008) are being trialled in another DPI project. So far these assays have not worked in our hands but trials are ongoing. The *Secoviridae* PCR (Maliogka et al 2004) will also be retried within this DPI project as soon as additional positive controls can be obtained. Should any of these assays be successful we will do further work to validate them for *Nepovirus* detection in grapevine.

The PCR developed for detection of endemic strains of GVB using the GVBC6980/H7439 primer pair also detects strains associated with corky bark disease and was validated during the National survey. Dovas at Katis (2003a) also reported the development of a generic test for viruses of the Rugose wood complex that should detect GVA, GVB, GVD and RSPaV. The test worked well in our hands however was not able to differentiate between RSPaV and the three *Vitivirus* species. There is a need to differentiate between RSPaV and the *Vitivirus* species because the presence of RSPaV is tolerated in Australian certification schemes due to its high incidence in Australian viticulture while vitiviruses are not tolerated, therefore we did not develop this generic test for further use.

2.4 Conclusions

We have established one step RT-PCR tests for ten endemic grapevine viruses: GVA, GVB, GFkV, RSPaV, and GLRaV-1, 2,-3, 4, 5 and 9, which were easy to use and reliable for the detection of these viruses. Each test further validated during the national survey (Chapter 6). GVA and RSPaV may require the use of two RT-PCR tests to detect all strains.

Although we could not obtain all the positive controls that were required, we have established specific molecular tests and some generic tests for most exotic viruses. Specific tests were developed for GLRaV-6, GLRaV-7, GVB GVD, GRGV, GAMaV, AILV, CRLV, GARSV, GDefV, PRMV, TRSV, ToRSV and SLRSV. Generic tests that were designed to detect two or more viruses included a test for ArMV and GFLV, GCMV and TBRV, Maculaviruses including GFkV GRGV and GAMaV; Tombusviruses including GALV and PAMV.

Generic tests which could detect all *Closteroviridae* viruses and Ampeloviruses closely related to GLRaV-5, including GLRaV-4, -5, -6 and -9, -De and –Pr were also established. These tests can detect both exotic and endemic GLRaVs and maybe useful for initial screening of local and imported grapevine material to identify potential GLRaV infection. These generic tests are more complicated and time consuming because they are a nested PCR assay; however in our hands they were easy to use and are likely to be more sensitive. An added advantage is that the nested step may reduce the risk of false negative results due to inhibition of reverse transcriptase or *Taq* polymerase by compounds such as polyphenols and polysaccharides that may be co-precipitated during the extraction procedure. A disadvantage is that nested PCR assays are prone to PCR contamination and may lead to false positive results. If these tests are useful, they could reduce the cost of PCR testing for PEQ and the local certification schemes.

Specific tests were not available for BLMV, GBLV or GTRSV and the generic *Secoviridae* and *Nepovirus* subgroup PCR tests did not work well enough in our hands to encourage us to continue with their development for the detection of grapevine nepoviruses during the project. However generic PCR detection for nepoviruses is being developed within another DPI project and if successful the test will be validated for grapevines. In the meantime herbaceous indexing for BLMV, GBLV, and GTRSV will be required during PEQ.

We also established the PCR test for *Xylophilus ampelinus* (Botha et al 2001). Detailed protocols for each of the above mentioned pathogens are included in appendix 1

New strains of viruses are regularly discovered and it will be important to ensure the tests adapted and developed during this project can detect them. As new strains of viruses continue to be discovered, new tests continue to be developed by other researchers. It is possible that some of these tests will be more reliable than those adapted and developed during this project and it will be important to assess new tests as they become available and validate these if they seem to be more reliable.

Since the identification of the protocols recommended in this chapter, many new tests have been developed that involve the use of RT-quantitative PCR (RT-qPCR) assays using intercalating dyes and probe based technology (Osman et al 2007; Osman and Rowhani, 2008; Osman et al 2008). This technology requires significant understanding and information about strain variation of grapevine viruses that we did not have time or resources to investigate. The use of probe based assays, such as Taqman, was not considered for this project as they can add a high degree of specificity to the test and could lead to false negative results.

The list of PCR assays recommended for Grapevine virus detection is included in Chapter 7 along with other recommendations based on the outcomes of this research project.

CHAPTER 3: A COMPARISON OF ELISA AND PCR FOR THE DETECTION OF ENDEMIC VIRUSES IN TWO CLIMATES DURING THREE GROWING SEASONS

3.1 Introduction

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 10 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.

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 GLRaV-3 from bark scrapings of field infected symptomatic or symptomless grapevines throughout one season (Ling et al 2001). Another study showed that GLRaV-3 maybe unevenly distributed within the same grapevine and could lead to inconsistent test results (Rowhani et al 1997). 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).

In contrast, one study has shown that ELISA may be more sensitive than RT-PCR for detection of GLRaV-1 and -3 (Cohen et al 2003). More recently it was shown that grapevine viruses could be detected in various tissues, including buds, leaf petioles, expanded leaves, green phloem tissue and cortical scrapings at different times of the year and it was found that phloem from lignified canes gave the most reliable results for both PCR and ELISA (Fiore et al 2009). However ELISA was shown to be more sensitive in this study than RT-PCR for detection of viruses in leaf petioles, completely expanded leaves and green phloem tissues during summer and early autumn.

There have only been a few studies that were conducted to show the reliability of RT-PCR and ELISA for detection of grapevine viruses over time, and in most cases these were only conducted over one growing season (Ling et al 2001; Chen et al 2003; Fiore et al 2009). No studies have compared the influence of different varieties that are inoculated by the same source of virus and maintained in different climatic conditions. In this study we established replicate trials in a hot climate region (Sunraysia, Victoria) and a cool climate region (Yarra Valley, Victoria) each consisting of two varieties (Shiraz and Chardonnay) and for each

variety there were five treatments each consisting of five replicate grapevines. The five treatments included un-inoculated grapevines as a control and grapevines inoculated with GLRaV-2, GLRaV-3, GVA or GFkV. The preliminary results of sampling and sensitivity for reliable detection of grapevines viruses by ELISA and RT-PCR in two Australian climates are reported.

3.2 Materials and Methods

3.2.1 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 contained two varieties (Shiraz and Chardonnay) and for each variety there were five treatments each consisting of five replicate grapevines. The five treatments included un-inoculated grapevines as a control and grapevines inoculated with one of GLRaV-2, GLRaV-3, GVA or GFkV. These grapevines were chip bud inoculated, using two virus-infected buds per grapevine, in October 2006 (Sunraysia) and November 2006 (Yarra Valley).

3.2.2 Sampling

Each grapevine was sampled monthly and tested monthly by ELISA and PCR from December 2006 (Sunraysia) and January 2007 (Yarra Valley) until June 2009. 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 shoots of symptomatic material was 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 2007 at Sunraysia and August 2007 at the Yarra Valley. Testing was not done in September 2007 or in August and September in 2008 as the grapevines had been pruned and there were no shoots or canes to sample. ELISA testing was not done in June 2009 as antisera was unavailable.

3.2.3 Enzyme linked immunosorbent assay

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 300mg of tissue was ground in 3ml 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. A sample was considered positive if the OD reading was two times that of the healthy control.

3.2.4 Nucleic Acid extraction

Total nucleic acid was extracted from green grapevine tissue using a modified lysis buffer (MacKenzie et al 1997) and the QIAxtractor (Qiagen). Briefly, 300mg of grapevine tissue was ground in 3ml of the modified lysis buffer and 1ml of each hoogenate was transferred into separate wells of a 96 Square-Well,1.2 ml, lysis plate (Pathtech), 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 second lysis plate was placed in the QIAxtractor 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 96-well 800 µl long drip Unifilter capture plate (Whatman) 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 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 elution plate (Qiagen) and 200 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 theRNA.

Extracts from phloem scrapes of woody tissue collected in May, June and July could not be processed using the QIAxtractor 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 13,000 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 13,000 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 13,000 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.

If the CH_3:IAA protocol did not produce RNA that could be used in PCR then total RNA was extracted from samples using the RNeasy® Plant Mini Kit (QIAGEN) with a modified lysis

buffer (MacKenzie et al 1997). In June 2009 the RNeasy protocol, rather than CH_3:IAA protocol, was used to obtain nucleic acid from samples that were not successfully extracted using the QIAxtractor due to time constraints.

The yield of RNA was determined using a Nanodrop ND1000 spectrophotometer (Thermo Scientific) according to the manufacturer's instructions.

3.2.5 RT-PCR

Primers for the detection of NADH dehydrogenase ND2 subunit (ndhB gene, NAD) messenger ribonucleic acid (mRNA; Thompson et al 2003) were used to determine the quality of the extracted RNA (Table 3.1). The PCR primers used to detect GLRaV-2, GLRaV-3, GVA and GFkV were developed during this project (Chapter 2) and are given in Table 3.1. The SuperScript One-Step RT-PCR System (Invitrogen) was used for detection viruses and NAD mRNA. One step RT-PCR was conducted according to the manufacturer's instructions except that the total reaction volume was 12.5 µl for NAD mRNA containing 1 µl of each extract and 20 µl for each virus and used 2 µl of each extract. 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 1.5 % agarose gel in 0·5 × Tris-borate-EDTA, stained with ethidium bromide and visualised on a UV transilluminator.

3.2.6 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 100mg tissue to 1ml 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 established at the following dilutions: undiluted, 1/5, 1/10, 1/20, 1/50 and 1/100. The experiment was repeated again in January 2009 using material from Chardonnay and Shiraz collected at the Yarra Valley trial site only. Each dilution series for both varieties was established at the following dilutions: undiluted, 1/2, 1/5, 1/10, 1/20, 1/50 and 1/100.

3.3 Results

3.3.1 Symptom expression

GFkV and GVA symptoms were not observed in any inoculated Chardonnay or Shiraz grapevine in during the three year experiment from November 2006 until June 2009. Leafroll symptoms were not observed on the GLRaV-2 and GLRaV-3 inoculated Chardonnay grapevines at either site in any season. 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. In 2008/09 leafroll symptoms were not observed in GLRaV-2 or GLRaV-3 inoculated Shiraz grapevines at either site.

3.3.2 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, 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.

3.3.3 Sampling and sensitivity

The results of monthly testing of all the inoculated grapevines for each site from December 2006 until June 2009 are shown in Figure 3.1. The number of grapevines in which virus was detected in given in Table 3.2. The total number and proportion (%) of positive results for each inoculated grapevine in each season, obtained by PCR and ELISA testing, is given in Table 3. GVA was not detected in any inoculated grapevine at either site during the three seasons. When the combined results for GLRaV-2, GLRaV-3 and GFkV are considered, the lowest proportion of positive results by RT-PCR and ELISA was obtained in season one (2006/07) at each site (13-33%, Table 3.2). The proportion of positive results increased in season 2 (74-84%, Table 3.2) and the greatest proportion of positive results was observed in season 3 (79-96%, Table 3.2). Viruses were detected by RT-PCR in a greater proportion of samples at each site during each season when compared to ELISA (Table 3.2).

Season 1, December 2006- June 2007: The samples collected at each site for June 2007 and July/August 2007 were only tested using PCR as there was not enough material to perform ELISA testing as well. No un-inoculated grapevine tested positive for GLRaV-2, GLRaV-3, GVA or GFkV at either site in season 1. GVA was not detected in any of the inoculated grapevines at either site. When the combined results for all four viruses are considered, more positive results were obtained by RT-PCR than by ELISA for most months at both sites in season 1 (Figure 3.1, Table 3.2), particularly for GLRaV-2 and GFkV. GLRaV-

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

The frequency of detection of virus in each grapevine at each site was variable amongst the replicate grapevines for each virus during the six month period and viruses were detected only once in some vines while in a few grapevines, viruses were detected in 5/6 sampling periods in Sunraysia and 5/5 sampling periods in the Yarra Valley (Table 3.2). During the 2006/07 season one Shiraz grapevine inoculated with GLRaV-2 and one Shiraz grapevine inoculated with GFkV tested positive by RT-PCR every time they were tested during January until May in the Yarra Valley. Two Shiraz and two Chardonnay grapevines inoculated with GLRaV-3 tested positive by RT-PCR in five of the six months they were tested during December until May in Sunraysia.

At Sunraysia during season 1 GLRaV-2, GLRaV-3 and/or GFkV were detected in 20/30 the inoculated Chardonnay and Shiraz grapevines and 19/30 tested positive for virus by RT-PCR and 11/30 tested positive using ELISA (Table 3.3). In Sunraysia during May, GLRaV-2 was detected by ELISA in one Chardonnay grapevine which was not detected by RT-PCR.

In the Yarra Valley during season 1, virus was detected in a total of 17/30 Chardonnay and Shiraz grapevines inoculated with GLRaV-2, GLRaV-3 or GFkV using ELISA and/or RT-PCR. RT-PCR detected viruses in 16/30 inoculated grapevines and ELISA detected viruses in 11/30 inoculated grapevines (Table 3.3). In the Yarra Valley, GLRaV-2 was also detected during May in one Chardonnay grapevine by ELISA that was not detected by RT-PCR. However, GLRaV-2 was detected in 4/5 Chardonnay grapevines, including this particular grapevine, during August when ELISA testing was not done (Figure 3.1).

Season 2, October 2007- August 2008: No un-inoculated grapevine tested positive for GLRaV-2, GLRaV-3, GVA or GFkV at each site during October 2007- August 2008. GVA was not detected in any of the 10 inoculated grapevines at each site over the same time period. In most months, at both sites, more positive results for GLRaV-2, GLRaV-3 and GFkV were obtained by RT-PCR than by ELISA. When the combined results for all four viruses are considered, the least number of positive results was obtained by ELISA and RT-PCR in October 2007 (Figure 3.1). The frequency of detection of virus in each grapevine at each site was less variable compared to season 1 (Figure 3.1) and viruses were detected in most months in many inoculated grapevines during the 10 month period using RT-PCR.

At Sunraysia 27/30 Chardonnay and Shiraz grapevines inoculated with GLRaV-2, GLRaV-3 or GFkV tested positive for virus by RT-PCR and ELISA during the 2007/08 season (Table 3.3). When the combined results for all three viruses are considered, more grapevines (26/30) tested positive for virus by RT-PCR in February, March, May and July than in any other month by RT-PCR or ELISA (Figure 3.1), however the maximum number of positive results (27/30) was not achieved using RT-PCR or ELISA in any month. All the Chardonnay and Shiraz

grapevines inoculated with GLRaV-2 and GLRaV-3 tested positive in most months during the testing period (Table 3.2). All Shiraz grapevines and 2/5 Chardonnay grapevines inoculated with GFkV also tested positive in most months (Figure 3.1, Tables 3.2 and 3.3).

At the Yarra Valley 29/30 Chardonnay and Shiraz grapevines inoculated with GLRaV-2, GLRaV-3 or GFkV tested positive for virus by RT-PCR and ELISA during the 2007/08 season (Table 3.3). When the combined results for all three viruses are considered, the maximum number of positive results (29/29) were obtained by RT-PCR in December and January (Figure3.1). The maximum number of positive results was not achieved using ELISA in any month. All GLRaV-2 and GLRaV-3 inoculated Chardonnay and Shiraz grapevines tested positive at in most months during the testing period (Table 3.2). All Shiraz grapevines and 4/5 Chardonnay grapevines inoculated with GFkV also tested positive in most months (Tables 3.2 and 3.3).

Season 3, October 2008- June 2009: No un-inoculated grapevine tested positive for GLRaV-2, GLRaV-3, GVA or GFkV at each site during October 2008- June 2009. GVA was not detected in any of the 10 inoculated grapevines at each site over the same time period. ELISA was not used for virus detection in June 2009. In most months, at both sites, more positive results for GLRaV-2 and GFkV were obtained by RT-PCR than by ELISA (Figure3.1, Table 3.2). However, when the combined results for the entire sampling period were considered, more positive results were obtained by ELISA than RT-PCR for GLRaV-3 inoculated Chardonnay and Shiraz grapevines at Sunraysia and the Yarra Valley in season 3 (Table 3.2). More positive results were obtained by RT-PCR and ELISA in GFkV inoculated Chardonnay at both sites compared to previous seasons and the virus was detected in all inoculated Chardonnay grapevines at least once throughout the season using both tests (Figure 3.1, Tables 3.2 and 3.3)

At Sunraysia during season 3, 30/30 Chardonnay and Shiraz grapevines inoculated with GLRaV-2, GLRaV-3 or GFkV tested positive for virus and virus was detected in 30/30 grapevines by RT-PCR and in 29/30 grapevines by ELISA (Table 3.3). When the combined results for all three viruses are considered, more grapevines (29/30) tested positive for virus by RT-PCR in February and April than in any other month by RT-PCR or ELISA (Figure 3.1), however the maximum number of positive results (30/30) was not achieved using RT-PCR or ELISA in any month. Detection of GLRaV-3 by RT-PCR and ELISA was more variable from month to month compared to season 2 and GLRaV-3 was less frequently detected by RT-PCR in season 3 compared to season 2 (Table 3.2). One inoculated Shiraz grapevine did not test positive in any month for GFkV by ELISA but did test positive twice using RT-PCR (Tables 3.2 and 3.3).

At the Yarra Valley 30/30 Chardonnay and Shiraz grapevines inoculated with GLRaV-2, GLRaV-3 or GFkV tested positive for virus by RT-PCR and ELISA during the 2008/08 season and virus was detected in 30/30 grapevines by RT-PCR and ELISA (Table 3.3). When the combined results for all three viruses are considered, the maximum number of positive results

(30/30) were obtained by RT-PCR in January, March and May and by ELISA in November and January (Figure 3.1). All GLRaV-2 and GLRaV-3 inoculated Chardonnay and Shiraz grapevines tested positive at in most months during the testing period (Table 3.2). All Shiraz grapevines and 4/5 Chardonnay grapevines inoculated with GFkV also tested positive in most months (Tables 3.2 and 3.3).

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.

3.3.4 Limit of detection.

March 2008: 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 GLRaV-3 infected material was diluted with buffer virus 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 that 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.

January 2009: GLRaV-2: Positive results using ELISA, at 1.5-2 X the OD reading of the buffer and healthy controls were observed for the undiluted tissue only at 2 hours after substrate addition for all dilution series made from the GLRaV-2 inoculated Chardonnay and Shiraz material. After 18 hours (i.e. overnight) of ELISA development clear positive results (>2 X the OD reading for the healthy control) were obtained for the undiluted and 1/5 dilution for Shiraz and the dilution 1/10 for Chardonnay when the dilution series was made from plant tissue. The sensitivity of the ELISA improved when the dilution series was made from extract of GLRaV-2 infected tissue and diluted with extract of uninfected material and positive results were achived down to the 1/5 dilution for Shiraz and 1/10 dilution for Chardonnay. The sensitivity improved further when the infected extract was diluted in buffer and GLRaV-2 was detected in the 1/10 dilution for Shiraz and 1/20 dilution for Chardonnay.

RT-PCR detected GLRaV-2 down to 1/10 and also in the 1/100 dilutions of the series made from Shiraz plant tissue and down to 1/20 for the dilution series made from Chardonnay plant tissue. GLRaV-2 was detected by RT-PCR down to the 1/100 dilution in both varieties when the dilutions series were made with tissue extracts and diluted out with buffer.

GLRaV-3: Clear positive results were observed for GLRaV-3 30 minutes after substrate addition down to 1/10 for dilution series made from Chardonnay plant tissue. A similar result was obtained for the dilution series made from Shiraz plant tissue at 1/2, 1/5, 1/10 and 1/50 but not for the 1/20 dilution. Clear positive results were obtained down to 1/100 in both varieties when the extract of infected material was diluted with the extract of uninfected material. When the ELISA plates were allowed to develop for 2h, virus testing for GLRaV-3 by ELISA was reliable down to a dilution of 1/100 for all dilutions series of both varieties, except the 1/20 dilution of the Shiraz plant tissue. After 18 hours development the ELISA positive results were at least 5X and up to 10 X greater than the OD reading for the uninfected and buffer controls for both varieties except the 1/20 dilution of the Shiraz plant tissue. RT-PCR detected GLRaV-3 in all dilution series down to the 1/100 dilution for both varieties except the 1/20 dilution of the Shiraz plant tissue.

GFkV: Clear positive results were observed for GFkV in Chardonnay 30 minutes after substrate addition down to 1/2 for dilution series made from plant tissue and extracts and down to 1/100 in the dilution series made with buffer. Clear positive results were observed for GFkV in Shiraz 30 minutes after substrate addition down to 1/5 for dilution series made from plant tissue, down to 1/2 for dilution series made with plant extracts and down to 1/10 in the dilution series made with buffer. After 2h of ELISA development clear positive results in Chardonnay were obtained down to 1/50 in plant tissue dilutions, 1/10 in plant extract dilutions and 1/100 in buffer dilutions. For Shiraz clear positive results were obtained after 2h down to 1/20 in plant tissue dilutions, 1/5 in plant extract dilutions and 1/100 in buffer dilutions. When the ELISAs were allowed to develop over night clear positive results in Chardonnay were obtained down to 1/50 in plant tissue dilutions, 1/20 in plant extract dilutions and 1/100 in buffer dilutions. For Shiraz clear positive results were obtained after overnight development down to 1/20 in plant tissue dilutions, 1/10 in plant extract dilutions and 1/100 in buffer dilutions. RT-PCR detected GFkV in all dilution series of both varieties down to the 1/100 dilution.

3.4 Discussion

Although 100% efficiency for virus detection was not always observed, GLRaV-2, GLRaV-3 and GFkV could be reliably detected in green tissue, including petioles and stems, during early summer (December) to autumn (May) by ELISA and RT-PCR once the grapevines had been infected for more than one year. October was the least reliable month for virus detection and it is possible that growth of the actively growing shoot exceeds virus replication at this time of year with lower virus titres leading to false negative results.

Interestingly our results are in contrast to those of a recent and similar study reported by Fiore et al (2009), who found that green tissue of petioles, shoots and expanded leaves, tested during December until April in Chile were not as reliable for virus detection as buds, shoot tips and unfolded leaves in September or phloem tissue from mature canes sampled between December until April. It is possible that there were differences between the study in Chile and this study, such as climate, horticultural practices, virus isolates and grapevine cultivar that contributed to the differences between the results. It is also possible that the disparities were associated with experimental differences such as the sampling, extraction and PCR protocols, including enzymes and primers for RT-PCR and ELISA kits that may affect the efficiency of the tests. Both studies used different ELISA kits and PCR tests for GLRaV-2 and GLRaV-3 detection and different RNA extraction procedures for PCR detection, and a few of these parametrs could affect detection efficiency. In this study there was no apparent difference in the reliability of virus testing by RT-PCR or ELISA between the Yarra Valley and Sunraysia climates.

Based on the proportion of positive results obtained during the three years at each site and on the dilution experiments to determine the limit of detection for GLRaV-2, GLRaV-3 and GFkV, RT-PCR was found to be more sensitive than ELISA indicating that this test is more reliable for grapevine virus detection in Australia. This is in contrast to other studies (Cohen et al 2003, Fiore et al 2009) which showed that ELISA can be more reliable for grapevine virus detection than RT-PCR.

However, in our study occasionally ELISA detected virus in more samples than RT-PCR in the seasonal detection during three years and in some samples in the dilution series experiments. This indicated that the RT-PCR tests were occasionally giving false negative results even when the housekeeping gene (HKG) RT-PCR indicated the presence of RNA in our nucleic acid extracts. This effect was more evident when material was starting to lignify. For example when the dilution series was done in March 2008, using lignifying material, a larger proportion of false negative results were obtained with RT-PCR compared to the results obtained for the same experiment done in January 2009 using green tissue.

Compounds that may be co-extracted with and bind RNA from grapevines such as polyphenols and polysaccharides (Iandolino et al 2004; Reid et al 2006; Tesniere and Vayda, 1991) can inhibit RT-PCR and cause false negative results (Baker et al 1990; Lopez-Gomez and Gomez-Lim, 1992; Schneiderbauer et al 1991). Although we use a HKG RT-PCR to determine nucleic acid “quality” (i.e. the RNA that is present and can be detected) it is possible that a positive HKG test could lead to a false interpretation of a negative result for virus, i.e. a false negative result. It may be that detection of HKG mRNA but not viral RNA is because more of the highly abundant HKG mRNA is available for reverse transcription and amplification compared to low abundant targets such as viruses in woody perennial plants. Although ELISAs are less sensitive than RT-PCR for virus detection, they may also be less susceptible to the effect of inhibitors and give positive results when RT-PCR does not. Thus it

is possible that the RNA that was extracted in this study was not always of sufficient “quality” to allow detection of viruses by RT-PCR.

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 may 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 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 polyphenels 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. In June 2008/09 we modified the RNA extraction protocol for the high throughput automated nucleic acid extraction procedure and added half the volume of extract to the capture plate. This resulted in a greater number of samples to be extracted by this process but many samples had to be extracted using the RNeasy method developed by MacKenzie et al (1997), which is the most reliable of the three extraction protocols for woody tissue. The RNeasy method is more expensive than the high throughput automated nucleic acid extraction procedure, thus we recommend the testing of green tissue during December until March to reduce the cost of testing whilst still producing reliable RT-PCR results.

The results of the dilution series experiments, particularly with green tissue in January 2009, also showed that ELISA was generally less sensitive for virus detection compared to RT-PCR, and low virus titre can lead to false negative results. 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. Our results suggested that ELISA can be susceptible to the effects of inhibitors in the grapevine extracts. This effect was observed when the dilution series of the virus positive plant extract diluted in uninfected extract was compared to the extract diluted in buffer for GLRaV-2 and GFkV. Positive results were often obtained in more dilute samples and the OD readings, particularly for GFkV, were often higher for the buffer dilution series compared to the extract dilution series (data not shown). It is also possible that the low reactivity of the GFkV and GLRaV-2 ELISAs was associated with low specificity of the antibodies for the virus strains used in this experiment compared to the strains to which they were developed.

During this experiment 3-4 shoots per vine were collected each month and petioles and vascular tissue from stems were sub-sampled from each shoot. This process allowed the same vines to be sampled each month without significantly affecting the growth and canopy structure of the grapevine. The greatest proportion of positive results achieved was 100% and this occurred in the Yarra Valley during season 3 by RT-PCR in November 2008 and January, March and May in 2009 and by ELISA in January 2009. These results indicate that the viruses may not be distributed evenly and /or in high titre throughout a grapevine and can lead to false negative results. Because detection of viruses by RT-PCR and ELISA was rarely 100% efficient, it is important to re-test grapevines routinely to increase the chance of detection.

Although some virus infection was observed 6-7 weeks post-inoculation at each site, fewer positive results were obtained in season 1 compared to the seasons 2 and 3 and the largest proportion of positive results was obtained in season 3. These results suggest that new/recent infections may be missed by ELISA and RT-PCR and that virus titre may not reach consistently detectable levels until more that two years after inoculation. This effect was more evident in grapevine inoculated with GFkV than for grapevines inoculated with GLRaV-2 and GLRaV-3, which were consistently positive after 12 months. Thus there is a risk of false negative results associated with recent infection events, particularly within 6-12 months of infection occurring. This result affirms our recommendations in Chapter 6 that grapevines to be certified for inclusion into nucleus collections should undergo annual testing and only be certified and admitted after three years of negative results.

To determine if some spread of virus might have occurred during the three year experiment and confounded our results all inoculated and un-inoculated vines at both field sites were tested twice for all four viruses. None of the un-inoculated grapevines tested positive for GLRaV-2, GLRaV-3, GVA or GFkV. The inoculated grapevines did not test positive for the viruses with which they were not inoculated indicating that the virus that was detected in each grapevine was associated with our deliberate inoculation and that the inoculated viruses had not spread during the trial (data not shown). However, viruses, particularly GLRaV-1, -3, -4, -5 and -9, GVB and GVA are spread by mealybug and scale insects (Martelli 1993) and the field spread of GLRaV-3 and GLRaV-9 have been reported in Australia (Habili et al 2003; Habili and Nutter 1997). Some of the mealybug and scale vectors for theses viruses are also known to occur in Australian vineyards (Constable and Drew, 2004). It is possible therefore that field spread of viruses could occur, and our results show that new/ recent virus infections may go undetected for several years during routine testing. We therefore recommend that grapevines within certification schemes, at the nucleus level, foundation plants and mother blocks, be routinely and repeatedly tested by RT-PCR for viruses during the life of the vines.

Virus detection in certified Australian mother blocks is often dependent on visual inspection alone, especially as it is not viable to test every grapevine in a mother planting. We observed that Chardonnay grapevines inoculated with GLRaV-2, GLRaV-3 and GFkV at both sites did

not display obvious symptoms of virus infection and GFkV associated symptoms were not observed in Shiraz at both sites. Leafroll symptoms were observed in some Shiraz grapevines at both sites in seasons 1 and 2. However we also observed that not all of the GLRaV-2 and GLRaV-3 inoculated and infected Shiraz grapevines displayed symptoms in the season 2 and none displayed symptoms in the season 3. The reason for the variable symptom expression in the Shiraz is not known. It is possible that the environmental conditions do influence plant response to virus infection and virus titre, which in turn affects symptom expression. In particular, for season 3 we hypothesise that the extreme heat experienced during several days, at times greater than 40°C, across Victoria in early February 2009 may have affected virus titre and/or the plant response to virus infection and remained symptomless in this season, even though some Shiraz vines had displayed leafroll symptoms in previous seasons. These observations have important implications for certification schemes at every level because symptom expression is variable from one season to the next and virus infection may be missed by visual inspection alone. Thus we recommend active testing, especially by RT-PCR, of material which is to be certified as virus tested.

The dilution series experiments indicated that samples can be pooled which 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 1/10 for PCR. However to reduce the risk of false negative results we recommend pooling up to five samples for both tests. The GLRaV-3 RT-PCR and ELISA tests returned more positive results than RT-PCR and ELISA tests 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 in combination with lower titres of these viruses in grapevine tissue compared to GLRaV-3.

Nucleic acid can be reliably extracted from green tissue using the high throughput automated nucleic acid extraction procedure developed for this study, 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 done in 1.5-2 days for 96 samples rather than 3-4 days using other methods.

An additional advantage of the molecular methods 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. The RT-PCR tests that we identified in Chapter 2 detect all known strains of endemic viruses in Australia, whereas we have observed the some of the ELISA kits that are commercially available do not detect all known Australian virus strains (F. Constable, unpublished).

3.5 Conclusions

The results of this study show that RT-PCR is more 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. The use of green tissue should reduce the effect of inhibitors and therefore the risk of a false negative result. The use of green tissue will allow

certification schemes to test nucleus collection, foundation planting and mother blocks earlier in the year, which will be useful as results can be obtained and decisions made about the distribution of budwood material to industry well in advance of collecting material for distribution. Detection of viruses by RT-PCR and ELISA is unlikely to be 100% efficient and re-testing of grapevines within certification programs is recommended to also reduce the risk of a false negative result. We also recommend that grapevines to be certified for inclusion into nucleus collections should undergo annual testing and only be certified and admitted after three years of negative results.

A high throughput nucleic acid extraction procedure has been developed that should reduce the cost of RT-PCR for grapevine virus detection. We recommend that up to five samples can be pooled for grapevine virus detection during spring and summer to enable testing of more samples at a reduced cost to industry.

Table 3.1 Primers used in RT-PCR for detection of housekeeping genes and GLRaV-2, GLRaV-3, GVA and GFkV of grapevines.

Pathogen	Primer name	Primer sequence (5'-3')	Tm	Expected product size	Reference
RNA House-keeping gene	AtropaNad2.1a	GGACTCCTGACGTATACGAAGGATC	55°C	188 bp	Thompson et al 2003
	AtropaNad2.2b	AGCAATGAGATTCCCCAATATCAT			
Grapevine leafroll assocated virus 2 (GLRaV-2)	V2dCPf2 sense	ACG GTG TGC TAT AGT GCG TG	55°C	534 bp	Bertazzon and Angelini 2004
	V2CPr1 antisense	GCA GCT AAG TAC GAA TCT TC			
Grapevine leafroll assocated virus 3 (GLRaV-3)	P3U/	CGC TCA TGG TGA AAG CAG ACG	52°C	652 bp	Turturo et al 2005
	P3D	CTT AGA ACA AAA ATA TGG AGC AG			
Grapevine virus A (GVA)	H587	GAC AAA TGG CAC ACT ACG	52°C	429 bp	Minafra and Hadidi. 1994
	C995	AAG CCT GAC CTA GTC ATC TTG G			
Grapevine fleck virus (GFkV)	GFkV-U279	TGG TCC TCG GCC CAG TGA AAA AGT A	55°C	352 bp	Sabanadzovic et al 2001
	GFkV-L630	GGC CAG GTT GTA GTC GGT GTT GTC			

Table 3.2. The number of times virus was detected in each inoculated grapevine by PCR and ELISA for GLRaV-2, GLRaV-3 and GFkV at each site and in each season for the months in which both tests were used*.

Sunraysia	Shiraz								Chardonnay							
	2006/07		2007/08		2008/09		Three year total		2006/07		2007/08		2008/09		Three year total	
	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA
GLRaV-2 r1	1/6	0/6	9/10	9/10	8/8	7/8	18/24	16/24	0/6	1/6	10/10	10/10	6/8	4/8	16/24	15/24
GLRaV-2 r2	0/6	0/6	3/10	1/10	8/8	4/8	11/24	5/24	2/6	1/6	10/10	10/10	8/8	8/8	20/24	19/24
GLRaV-2 r3	0/6	0/6	8/10	3/10	8/8	8/8	16/24	11/24	1/6	0/6	9/10	10/10	8/8	7/8	18/24	17/24
GLRaV-2 r4	4/6	1/6	9/10	9/10	8/8	8/8	21/24	18/24	3/6	1/6	10/10	9/10	8/8	8/8	21/24	18/24
GLRaV-2 r5	2/6	1/6	8/10	8/10	7/8	7/8	17/24	16/24	0/6	0/6	10/10	8/10	6/8	4/8	16/24	12/24
Total GLRaV-2	7/30 (23%)	2/30 (7%)	37/50 (74%)	30/50 (60%)	39/40 (98%)	34/40 (85%)	83/120 (69%)	66/120 (55%)	6/30 (20%)	3/30 (10%)	49/50 (98%)	47/50 (94%)	36/40 (90%)	31/40 (78%)	91/120 (76%)	81/120 (68%)
GLRaV-3 r1	0/6	0/6	10/10	10/10	8/8	8/8	18/24	18/24	0/6	0/6	10/10	10/10	8/8	8/8	18/24	18/24
GLRaV-3 r2	2/6	0/6	10/10	10/10	7/8	8/8	19/24	18/24	0/6	0/6	10/10	10/10	8/8	8/8	18/24	18/24
GLRaV-3 r3	5/6	4/6	10/10	10/10	6/8	8/8	21/24	22/24	2/6	0/6	10/10	8/10	7/8	7/8	19/24	15/24
GLRaV-3 r4	5/6	5/6	10/10	9/10	7/8	8/8	22/24	23/24	5/6	4/6	10/10	10/10	8/8	8/8	23/24	22/24
GLRaV-3 r5	0/6	0/6	10/10	9/10	7/8	7/8	17/24	17/24	5/6	5/6	10/10	10/10	7/8	8/8	22/24	23/24
Total GLRaV-3	12/30 (40%)	9/30 (30%)	50/50 (100%)	48/50 (96%)	35/40 (88%)	39/40 (98%)	97/120 (81%)	96/120 (80%)	12/30 (40%)	9/30 (30%)	50/50 (100%)	48/50 (96%)	36/40 (90%)	39/40 (98%)	100/120 (83%)	96/120 (80%)
GFkV r1	0/6	0/6	10/10	9/10	8/8	6/8	18/24	15/24	1/6	0/6	9/10	9/10	7/8	6/8	17/24	15/24
GFkV r2	1/6	0/6	10/10	8/10	7/8	6/8	18/24	14/24	0/6	0/6	0/10	1/10	8/8	6/8	8/24	7/24
GFkV r3	3/6	0/6	4/10	1/10	2/8	0/8	9/24	1/24	1/6	0/6	0/10	0/10	2/8	1/8	3/24	1/24
GFkV r4	3/6	2/6	10/10	8/10	8/8	5/8	21/24	15/24	0/6	0/6	1/10	0/10	8/8	6/8	9/24	6/24
GFkV r5	2/6	0/6	10/10	7/10	8/8	7/8	20/24	14/24	4/6	3/6	8/10	8/10	7/8	6/8	19/24	17/24
Total GFkV	9/30 (30%)	2/30 (7%)	34/50 (68%)	33/50 (66%)	33/40 (83%)	24/40 (60%)	76/120 (63%)	59/120 (49%)	6/30 (20%)	3/30 (10%)	18/50 (36%)	18/50 (36%)	32/40 (80%)	25/40 (63%)	56/120 (47%)	46/120 (38%)
Combined virus total	28/90 (31%)	13/90 (14%)	121/150 (81%)	111/150 (74%)	107/120 (89%)	97/120 (81%)	256/360 (71%)	221/360 (61%)	24/90 (27%)	15/90 (17%)	117 (78%)	113/150 (75%)	104/120 (87%)	95/120 (79%)	247/360 (67%)	223/367 (62%)

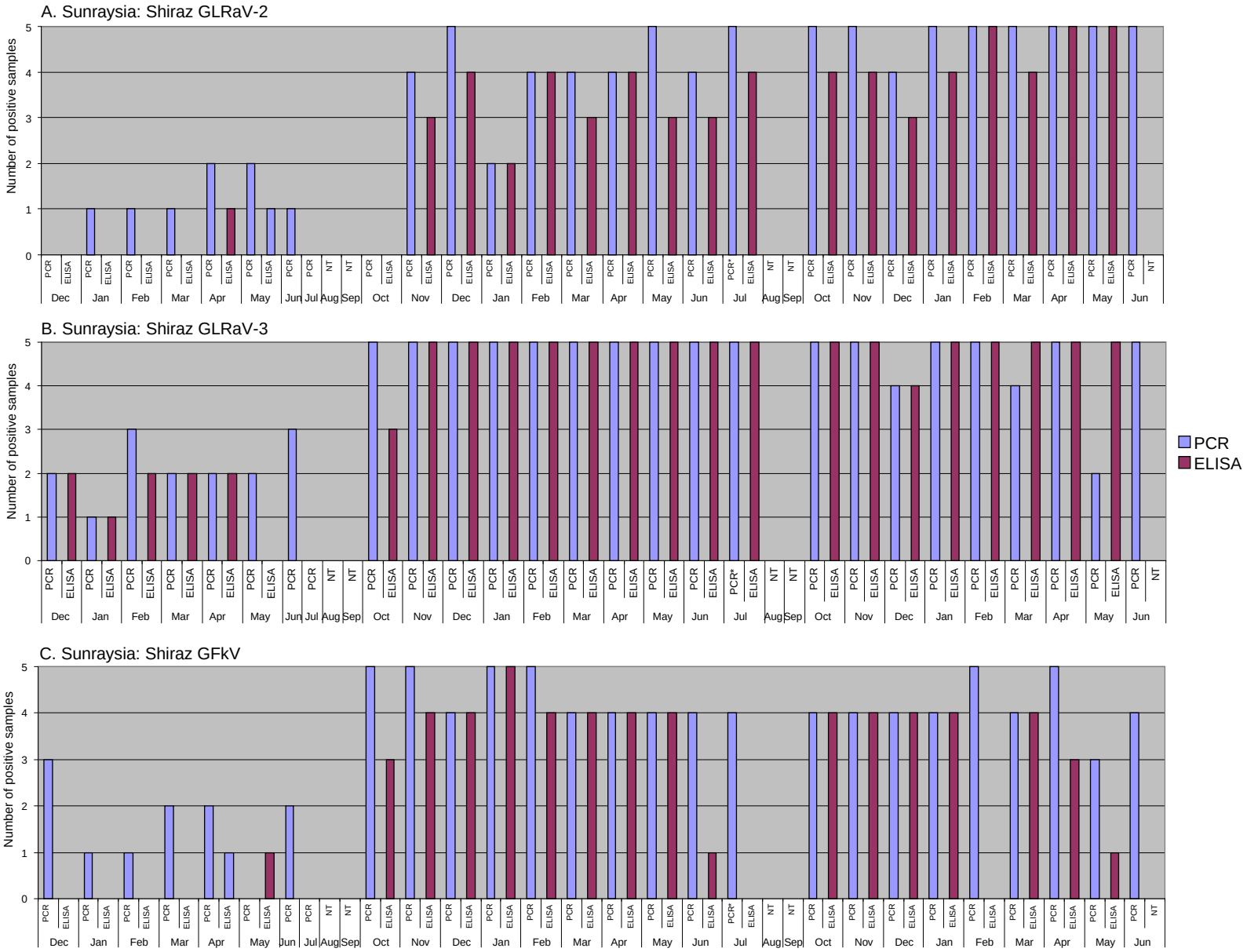
Yarra Valley	Shiraz								Chardonnay							
	2006/07		2007/08		2000/09		Three year total		2006/07		2007/08		2000/09		Three year total	
	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA
GLRaV-2 r1	0/5	0/5	9/10	8/10	6/8	7/8	15/24	15/24	0/5	0/5	10/10	10/10	8/8	8/8	18/24	18/24
GLRaV-2 r2	5/5	2/5	9/10	8/10	7/8	7/8	21/24	17/24	0/5	0/5	8/10	8/10	7/8	5/8	15/24	13/24
GLRaV-2 r3	2/5	0/5	9/10	6/10	8/8	6/8	19/24	12/24	0/5	1/5	6/10	2/10	8/8	8/8	14/24	11/24
GLRaV-2 r4	0/5	0/5	9/10	4/10	8/8	8/8	17/24	12/24	1/5	0/5	10/10	9/10	8/8	8/8	19/24	17/24
GLRaV-2 r5	0/5	0/5	9/10	8/10	8/8	8/8	17/24	16/24	1/5	0/5	9/10	8/10	8/8	8/8	18/24	16/24
Total GLRaV-2	7/25 (28%)	2/25 (8%)	37/50 (74%)	30/50 (60%)	39/40 (98%)	34/40 (85%)	83/120 (69%)	66/120 (55%)	2/25 (8%)	1/25 (4%)	43/50 (86%)	37/50 (74%)	39/40 (98%)	37/40 (93%)	84/120 (70%)	75/120 (63%)
GLRaV-3 r1	0/5	0/5	9/10	9/10	7/8	8/8	16/24	17/24	4/5	4/5	8/10	9/10	8/8	8/8	20/24	21/24
GLRaV-3 r2	1/5	0/5	9/10	8/10	7/8	8/8	17/24	16/24	1/5	0/5	10/10	9/10	8/8	8/8	19/24	17/24
GLRaV-3 r3	3/5	2/5	9/10	9/10	7/8	8/8	19/24	19/24	2/5	1/5	9/10	9/10	7/8	8/8	18/24	18/24
GLRaV-3 r4	4/5	4/5	9/10	9/10	8/8	8/8	21/24	21/24	1/5	1/5	10/10	9/10	8/8	8/8	19/24	18/24
GLRaV-3 r5	1/5	0/5	9/10	9/10	8/8	8/8	18/24	17/24	0/5	0/5	9/10	8/10	8/8	8/8	17/24	16/24
Total GLRaV-3	9/25 (36%)	6/25 (24%)	45/50 (90%)	44/50 (88%)	37/40 (93%)	40/40 (100%)	91/120 (76%)	90/120 (75%)	8/25 (32%)	6/25 (24%)	46/50 (92%)	44/50 (88%)	39/40 (98%)	40/40 (100%)	93/120 (78%)	90/120 (75%)
GFkV r1	5/5	0/5	9/10	6/10	8/8	6/8	22/24	12/24	3/5	2/5	7/10	6/10	7/8	6/8	17/24	14/24
GFkV r2	3/5	1/5	8/10	8/10	7/8	6/8	18/24	15/24	0/5	0/5	10/10	9/10	8/8	7/8	18/24	16/24
GFkV r3	0/5	0/5	9/10	8/10	6/8	6/8	15/24	14/24	0/5	0/5	9/10	8/10	6/8	6/8	15/24	14/24
GFkV r4	0/5	1/5	9/10	8/10	8/8	7/8	17/24	16/24	0/5	0/5	1/10	0/10	8/8	5/8	9/24	5/24
GFkV r5	1/5	0/5	9/10	8/10	7/8	6/8	17/24	14/24	0/5	1/5	10/10	9/10	8/8	6/8	18/24	16/24
Total GFkV	9/25 (36%)	2/25 (8%)	44/50 (88%)	38/50 (76%)	36/40 (90%)	31/40 (78%)	89/120 (74%)	71/120 (59%)	3/25 (12%)	3/25 (12%)	37/50 (74%)	32/50 (64%)	37/40 (93%)	30/40 (75%)	77/120 (64%)	65/120 (54%)
Combined virus total	25/75 (33%)	10/75 (13%)	126/150 (84%)	112/150 (75%)	112/120 (93%)	105/120 (88%)	263/360 (73%)	227/360 (63%)	13/75 (17%)	10/75 (13%)	126/150 (84%)	113/150 (75%)	115/120 (96%)	107/120 (89%)	245/360 (71%)	230/360 (64%)

* The table only included data from the months in which both tests were used for detection. Grapevines were tested six times in 2006/07, ten times in 2007/08 and eight times in 2008/09. GVA was not detected and the results were not included in this table.

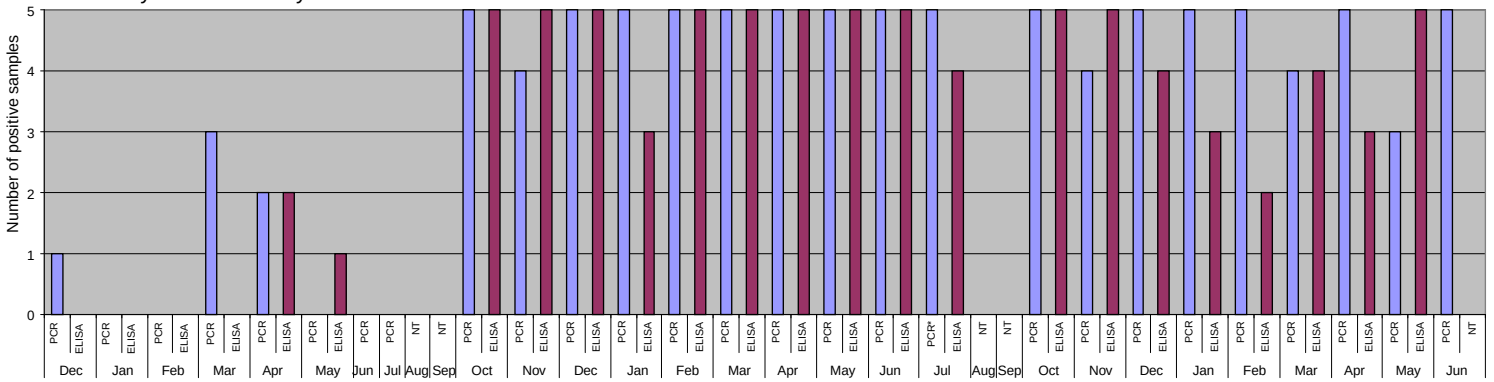
Table 3.3. The number of Shiraz and Chardonnay grapevines in which GLRaV-2, GLRaV-3, GVA and GFkV were detected by PCR and ELISA in each season, for the months in which both tests were used.

Sunraysia	Shiraz						Chardonnay					
	2006/07		2007/08		2008/09		2006/07		2007/08		2008/09	
	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA
GLRaV-2	3/5	2/5	5/5	5/5	5/5	5/5	3/5*	3/5*	5/5	5/5	5/5	5/5
GLRaV-3	3/5	2/5	5/5	5/5	5/5	5/5	3/5	2/5	5/5	5/5	5/5	5/5
GVA	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
GFkV	4/5	1/5	5/5	5/5	5/5	4/5	3/5	1/5	3/5*	3/5*	5/5	5/5
Total	10/20	5/20	15/20	15/20	15/20	14/20	9/20	6/20	13/20	13/20	15/20	15/20
Total combined PCR and ELISA	10/20		15/20		15/20		10/20		14/20		15/20	
Yarra Valley												
GLRaV-2	2/5	1/5	5/5	5/5	5/5	5/5	2/5*	1/5*	5/5	5/5	5/5	5/5
GLRaV-3	4/5	2/5	5/5	5/5	5/5	5/5	4/5	3/5	5/5	5/5	5/5	5/5
GVA	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
GFkV	3/5	2/5	5/5	5/5	5/5	5/5	1/5	2/5	5/5	4/5	5/5	5/5
Total	9/20	5/20	15/20	15/20	15/20	15/20	7/20	6/20	15/20	14/20	15/20	15/20
Total combined PCR and ELISA	10/20		15/20		15/20		7/20		15/20		15/20	

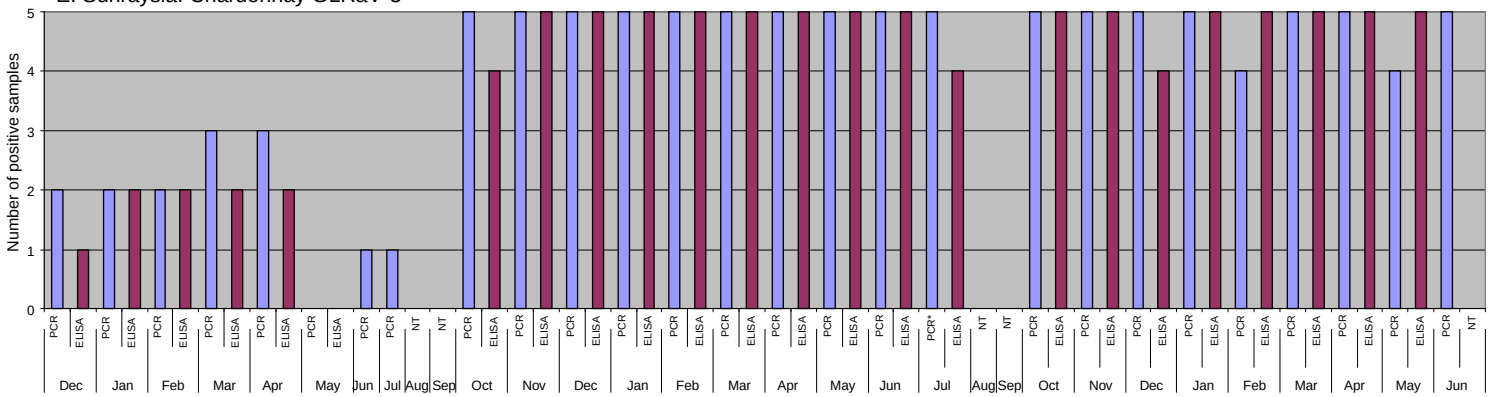
*In each case one grapevine tested positive for virus by ELISA that was not detected by PCR.


A. Sunraysia: Shiraz GLRaV-2
B. Sunraysia: Shiraz GLRaV-3
C. Sunraysia: Shiraz GFkV
Number of positive samples
0
1
2
3
4
5
PCR
ELISA
NT
PCR*
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun

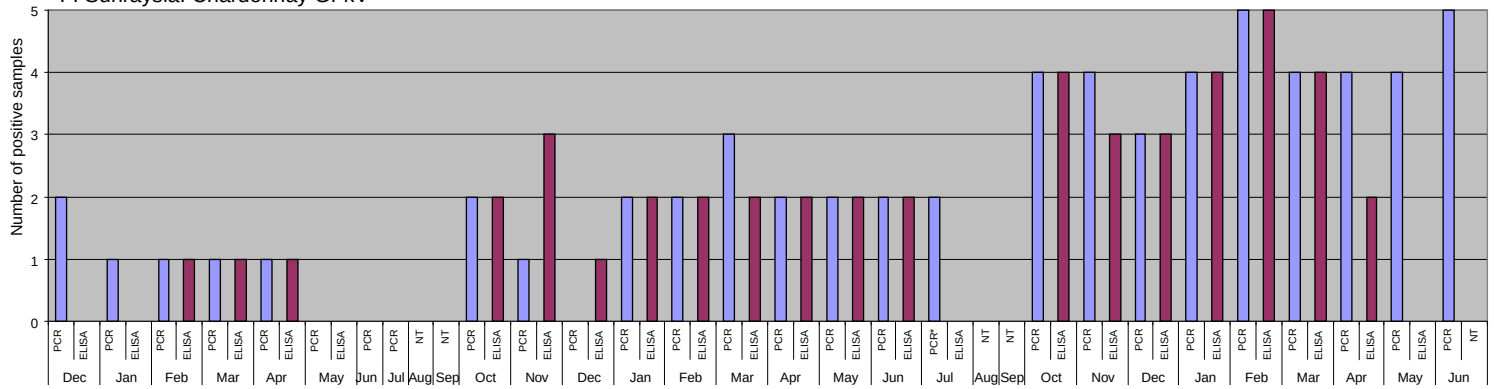
D. Sunraysia: Chardonnay GLRaV-2



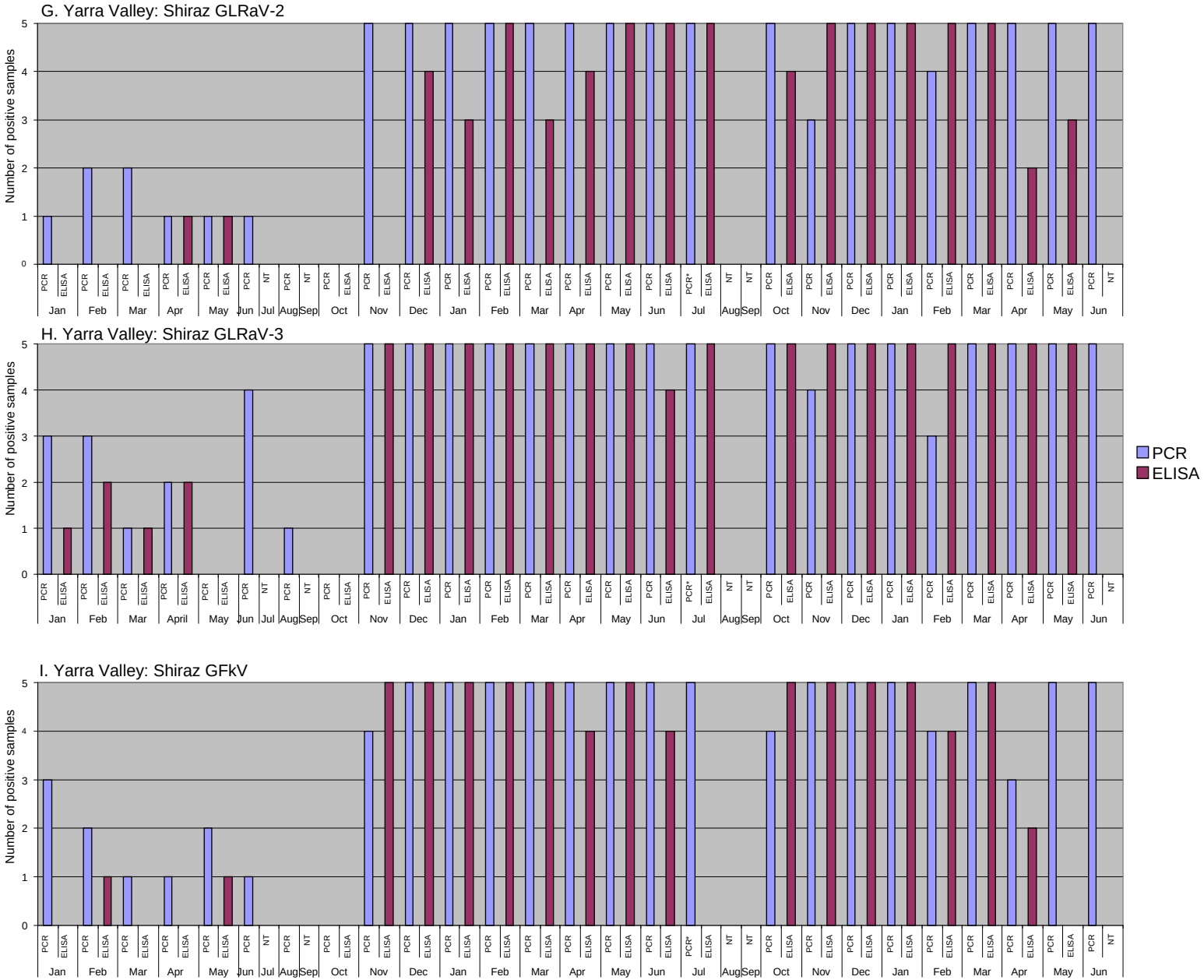
E. Sunraysia: Chardonnay GLRaV-3



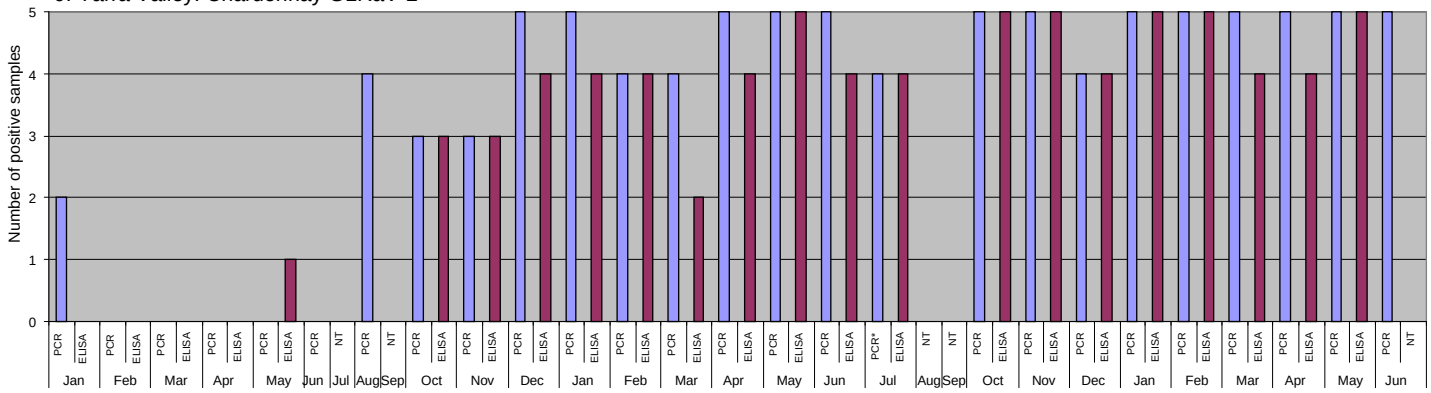
F. Sunraysia: Chardonnay GFkV



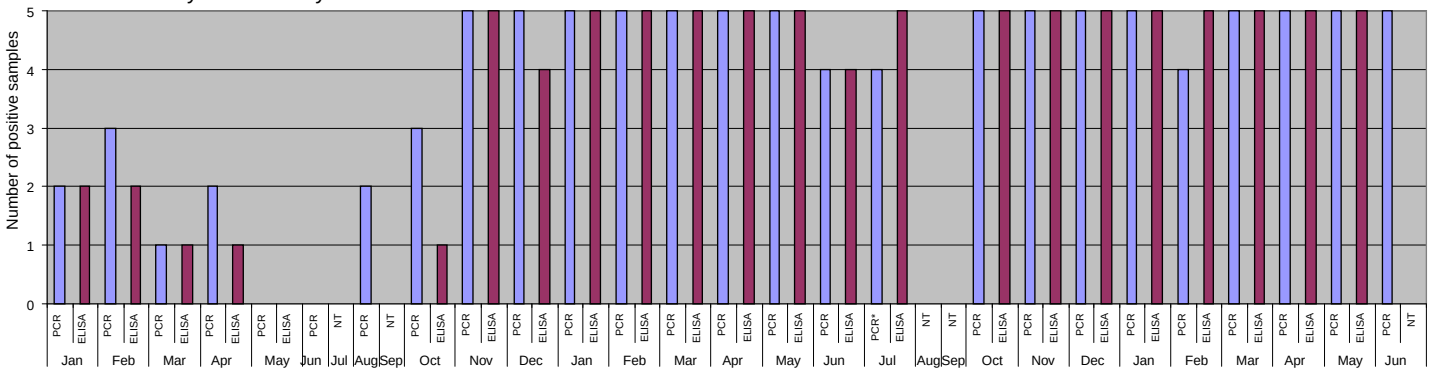
PCR
ELISA


G. Yarra Valley: Shiraz GLRaV-2
H. Yarra Valley: Shiraz GLRaV-3
I. Yarra Valley: Shiraz GFkV
Number of positive samples
0
1
2
3
4
5
PCR
ELISA
PCR*
NT
Jan
Feb
Mar
Apr
April
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
PCR
ELISA

J. Yarra Valley: Chardonnay GLRaV-2



K. Yarra Valley: Chardonnay GLRaV-3



L. Yarra Valley: Chardonnay GFkV

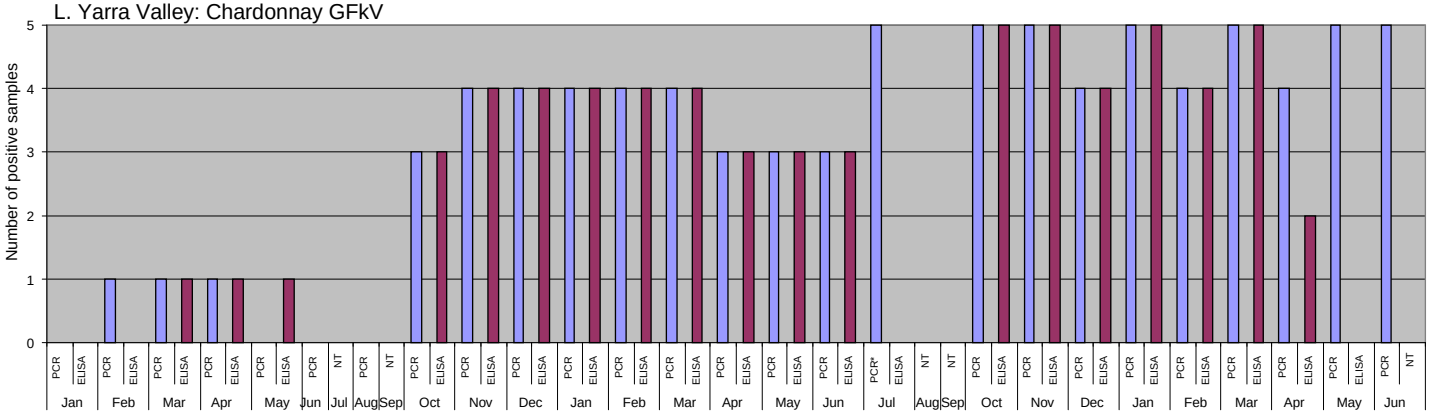


Figure 3.1. The total number of grapevines that tested positive for each site during 2006-2009. A: GLRaV-2 in Shiraz at Sunraysia; B: GLRaV-3 in Shiraz at Sunraysia; C: GFkV in Shiraz at Sunraysia; D: GLRaV-2 in Chardonnay at Sunraysia; E GLRaV-3 in Chardonnay at Sunraysia; F: GFkV in Chardonnay at Sunraysia; G: GLRaV-2 in Shiraz at the Yarra Valley; H GLRaV-3 in Shiraz at the Yarra Valley; I: GFkV in Shiraz at the Yarra Valley; J: GLRaV-2 in Chardonnay at the Yarra Valley; K: GLRaV-3 in Chardonnay at the Yarra Valley; L: GFkV in Chardonnay at the Yarra Valley. 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

CHAPTER 4 THE MOVEMENT OF VIRUSES WITHIN A GRAPEVINE OVER TIME

4.1 Introduction

The detection of viruses by RT-PCR is unlikely to be 100% efficient (Chapter3), and this may be affected by the timing of the infection event and the timing of sampling. We showed that there is a risk of false negative results associated with recent infection events, particularly within 6-12 months of infection occurring and that the virus may not be consistently detected until more that two years after inoculation.

To better understand the reliability of the PCR tests with time we established an experiment to determine the variation in distribution of virus within a grapevine in two different climates. Replicates of one variety (Chardonnay) were graft inoculated with a combination of viruses and maintained in the field in a cool climate (Yarra Valley) and a hot climate (DPI Sunraysia). Replicate grapevines from each region were destructively sampled every 20 cm from roots, trunk cordon and shoots in spring and autumn. Each sample was tested for the presence of virus to determine: 1) if there is change in virus location within grapevines with the season and 2) if there is a difference in the virus location within grapevines between regions. This experiment will have important implications for sampling procedures, particularly the frequency of testing individual grapevines over time.

4.2 Materials and Methods

4.2.1 Field sites

To investigate how viruses are distributed throughout a grapevine with time under Australian conditions, replicate trials were established in a hot climate region (Sunraysia, Victoria) and a cool climate region (Yarra Valley, Victoria) in 2006. The variety Chardonnay was used. Each trial had four treatments which included grapevines chip bud inoculated with GLRaV-2, GLRaV-3, GVA or GFkV. Each treatment consisted of 18 replicate grapevines. These grapevines were chip bud inoculated, using two buds per grapevine, in October 2006 (Sunraysia) and November 2006 (Yarra Valley). In November 2006, shortly after inoculation many grapevines in this trial died due to severe frost. These grapevines were replaced in December 2006.

4.2.2 Sampling

At Sunraysia 3 replicate grapevines of each treatment were removed and destructively sampled at six monthly intervals from November 2006 until April 2009. At The Yarra Valley, three replicate grapevines were also removed and destructively sampled but sampling did not begin until April 2007 due to the replanting that occurred in November 2006.

Grapevines were sampled at 20 cm intervals from the base of the trunk and along the shoots or along the cordons with composite shoot samples taken from each cordon sample section. In November 2006, one sample was taken from the “main” root section, which would have initially formed the lower part of the cutting used for propagation and if the root system was

large enough, two samples were taken form the “lateral” roots that had emerged from the main root: the first sample was taken from parts that were closer to the “main root and the second closer to the root tips. From April 2007, two root samples were collected from each grapevine: a sample was collected from the “main” root; the second sample was taken from the “lateral” roots that had emerged from the main root.

4.2.3 Nucleic Acid extraction

Three hundred milligrams of tissue was taken from each sample nucleic acid extraction. Total nucleic acid was extracted from green grapevine tissue using the QIAxtractor and extracts from phloem scrapes of woody tissue were prepared using the chloroform: isoamyl alcohol procedure also described in chapter 3. Where quality extracts could not be obtained using either method, RNA was extracted using the RNeasy® Plant Mini Kit (QIAGEN) with a modified lysis buffer (MacKenzie et al., 1997).

4.2.4 RT-PCR

Primers for the detection of NADH dehydrogenase ND2 subunit (ndhB gene, NAD) messenger ribonucleic acid (mRNA; Thompson et al 2003) were used to determine the quality of the extracted RNA. The following RT-PCR tests were used for virus detection:

- GLRaV-2 using the primer pair V2dCPf2 sense/V2CPr1 antisense (Bertazzon and Angelini 2004),
- GLRaV -3 using the primer pair P3U/P3D (Turturo et al. 2005),
- GVA using the primer pair C995/H587 (Minafra et al 1997)
- GFkV using the primer pair GFkV-U279/GFkV-L630 (Shi et al 2003).

4.3 Results

The results of the destructive testing are given in Table 4.1 for Sunraysia and Table 4.2 for the Yarra Valley.

November 2006

In November 2006 at Sunraysia, within six weeks of inoculation GLRaV-2, GLRaV -3 and GFkV were detected in 2/3, 3/3 and 2/3 grapevines respectively (Table 4.1). GLRaV-2, GLRaV -3 and GFkV had infected the main roots of 1/2, 3/3 and 2/2 grapevines and GLRaV-3 and GFkV had also moved into the lateral roots of 2/3 and 1/2 grapevines. Each virus was also detected in the trunk in most samples near the chip buds and occasionally above and/or below the inoculation points. GFkV was detected in one shoot sample, most distant from the trunk of one grapevine.

April 2007

In April 2007, 5-6 months post inoculation at Sunraysia, viruses were more frequently detected in the shoot samples of the GLRaV-2, GLRaV-3 and GFkV inoculated grapevines compared to November 2007 (Table 4.1). At Sunraysia GLRaV-2, GLRaV-3 and GFkV were observed in shoot samples of 3/3, 2/3 and 3/3 inoculated grapevines respectively. At the

Yarra Valley field site GLRaV-2, GLRaV-3 and GFkV were observed in root and trunk samples of 3/3, 2/3 and 3/3 inoculated grapevines respectively and in shoot samples of 2/3, 2/3 and 3/3 inoculated grapevines respectively (Table 4.2). In April 2007 GVA was detected in the roots of one grapevine in the Yarra Valley and in one grapevine in Sunraysia.

November 2007

In November 2007 in Sunraysia GLRaV-2, GLRaV-3 and GFkV were detected in 3/3, 3/3 and 2/3 inoculated grapevines respectively (Table4.1). GLRaV-2 was detected primarily in the root, trunk and cordon samples of two grapevines and in one shoot sample only of one of these grapevines. In the third grapevine only one cordon sample tested positive for GLRaV-2. GLRaV-3 was detected in trunk, cordon and shoots samples of all thee grapevines and the main root sample of one grapevines. GFkV was detected in the root, cordon and shoot samples of both grapevines and in the trunk sample of one grapevine. All shoot, cordon and trunks samples tested positive in 2/3 GLRaV-3 inoculated grapevines and in 1/3 GFkV inoculated grapevine.

Table 4.1 The number of root, trunk, cordon and shoot samples in which GLRaV-2, GLRaV-3, GFkV and GVA were detected at Sunraysia in November, 2006 and in April and November 2007.

Virus	Tissue type	November 2006			April 2007			November 2007		
		Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
GLRaV-2	Roots Main	0/1[1]	1/1	0/1	1/1	1/1	0/1	0/1	1/1	1/1
	Roots Lateral	0/2	0/1	0/2	0/1	1/1	0/1	0/1	1/1	1/1
	Trunk	1/2 (+buds) [2]	1/2 (+buds)	0/3	0/1	1/2 (+buds)	1/2	0/4	1/1	3/4
	Cordon	NA[3]	NA	NA	NA	NA	NA	1/12	4/4	10/10
	Shoot	0/4	0/3	0/3	4/5	2/3	3/4	0/0	0/1	1/10
GLRaV-3	Roots Main	1/1	1/1	1/1	0/1	0/1	0/1	0/1	1/1	0/1
	Roots Lateral	1/2	1/1	0/2	0/1	0/1	0/1	0/1	0/1	0/1
	Trunk	2/3	2/3	2/2	1/2	0/1	1/1	2/3	3/3	1/1
	Cordon	NA	NA	NA	NA	NA	NA	7/11	10/10	2/2
	Shoot	0/3	0/3	0/2	2/3	1/11	0/1	3/11	8/8	1/1
GFkV	Roots Main	1/1	0/1	1/1	0/1	1/1	0/1	0/1	1/1	1/1
	Roots Lateral	0/1	0/2	2/2	0/1	0/1	0/1	0/1	1/1	1/1
	Trunk	3/3	0/3	2/2	0/1	0/3	0/4	0/1	0/1	3/3
	Cordon	NA	NA	NA	NA	NA	NA	0/2	4/4	9/9
	Shoot	0/3	0/3	1/3	2/2	2/2	2/6	0/3	1/3	8/8
GVA	Roots Main	0/1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1
	Roots Lateral	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1	0/1
	Trunk	0/2	0/3	0/2	0/3	0/3	0/3	0/3	0/44	0/3
	Cordon	NA	NA	NA	NA	NA	NA	0/8	0/6	0/7
	Shoot	0/2	0/2	0/2	0/10	0/8	0/14	0/8	0/6	0/7

[1] The number of samples that were positive/the number of samples that were tested
[2] Sample where viruses were inoculated with chip buds
[3] NA – not available, no cordon formed.

In November 2007 in the Yarra Valley GLRaV-2, GLRaV-3 and GFkV were detected in 3/3, 3/3 and 2/3 inoculated grapevines respectively (Table 4.2). GLRaV-2 was detected in the root samples of 3/3 grapevines, the trunk samples of 2/3 grapevines and the cordon and shoot samples of 1/3 grapevines. GLRaV-3 was detected in the root samples of 1/3 grapevines, the trunk samples of 1/3 grapevines and all cordon and shoot samples of 3/3 grapevines. GFkV was detected in all root, cordon and shoot samples of 1/3 grapevines and in most cordon and shoot samples of 1/3 grapevines.

Table 4.2 The number of root, trunk, cordon and shoot samples in which GLRaV-2, GLRaV-3, GFkV and GVA were detected at The Yarra Valley in April and November 2007.

Virus	Tissue type	April 2007			November 2007		
		Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
GLRaV-2	Roots Main	0/1[1]	1/1	1/1	1/1	1/1	1/1
	Roots Lateral	1/1	1/1	1/1	1/1	1/1	0/1
	Trunk	3/3	1/1	1/1	0/1	1/1	2/2
	Cordon	NA[2]	NA	NA	0/4	0/2	3/4
	Shoot	2/5	1/8	0/5	0/4	0/2	4/4
GLRaV-3	Roots Main	0/1	1/1	0/1	0/1	0/1	0/1
	Roots Lateral	0/1	1/1	1/1	0/1	0/1	1/1
	Trunk	2/3 (+buds)[3]	0/4	1/1	3/3	0/1	0/2
	Cordon	NA	NA	NA	2/2	3/3	2/4
	Shoot	2/9	1/16	0/1	2/2	2/2	2/4
GFkV	Roots Main	1/1	1/1	1/1	0/1	1/1	0/1
	Roots Lateral	0/1	1/1	1/1	0/1	1/1	0/1
	Trunk	3/3	1/1	1/1	0/1	1/1	0/2
	Cordon	NA	NA	NA	4/5	2/2	NA
	Shoot	14/22	5/7	3/4	5/5	2/3	0/2
GVA	Roots Main	1/1	0/1	0/1	0/1	0/1	0/1
	Roots Lateral	1/1	0/1	0/1	0/1	0/1	0/1
	Trunk	0/1	0/1	0/3	0/3	0/1	0/1
	Cordon	NA	NA	NA	0/3	0/4	0/2
	Shoot	0/5	0/9	0/5	0/3	0/4	0/1

[1] The number of samples that were positive/the number of samples that were tested
[2] NA – not available, no cordon formed.
[3] Sample where viruses were inoculated with chip buds

4.4 Discussion

Testing was done in November 2006, April 2007 and November 2007 at Sunraysia and in April 2007 and November 2007 in the Yarra Valley After this time the grapevines continued to be collected and tissue samples taken and frozen. There remain approximately 1500 samples that could be extracted and tested. The difficulty of working with woody samples using the QIAxtractor (Chapter 2) meant that the samples needed to be extracted using a longer and more time consuming method. The chloroform: isoamyl alcohol procedure was not used because it was too time consuming. The RNeasy method (McKenzie et al 1997; Chapter 2)

could not be used because of cost. The results that were obtained indicate how viruses might move throughout a grapevine with time during 12 months.

Due to the inconsistency of RNA quality on the woody extracts sampled at different times of the year it was decided not to develop RT-quantitative PCR (RT-qPCR) assays to determine virus quantity in these grapevines. RT-qPCR assays have a strict requirement for high quality RNA extracts for accurate measurement of the target (Udvardi et al 2008), which could not be consistently, quickly or cost effectively generated for this experiment. The extracts that were produced were not of sufficient quality due to co-extracted proteins, polyphenols and polysaccharides which can inhibit both the reverse transciptase and DNA polymerase activity in the PCR reactions.

November 2006

The results of November 2006 suggest that grapevine viruses tend to move down from the point of inoculation towards the roots during early stages of infection. Similar results were reported by Cohen et al (2003) for GLRaV-3 inoculated grapevines. The mechanism for long distance transport of viruses in many plant hosts including grapevines is not well understood, although it is widely accepted that long distance transport occurs through the phloem and may be influenced by the direction of flow of metabolites. GLRaV-2, GLRaV-3 and GFkV are phloem limited (Martelli, 1993) and it is likely that their initial movement from the point of inoculation basipetally towards the roots was influenced by the transport of photoassimilates from leaves acting as a source to roots acting as a sink. .This initial basipetal movement of *Citrus tristeza closterovirus* (CTV), which is also phloem-limited, from the point of infection to the roots was also observed in 44 days post-inoculation (PDI) in citrus seedlings (Gafny et al. 1995).

GFkV was detected in one shoot sample of one grapevine and it is possible that the virus moved quickly to this point within six weeks post inoculation. However natural field spread of GFkV has been reported, although a vector is unknown (Hevin et al 1973; Engelbrecht and Kasdorf 1990; Fortusini et al, 1996) and it is possible the distal detection of GFkV in one shoot sample of one grapevine was a result of field spread rather than movement within the grapevine. GLRaV-2 and GLRaV-3 were not detected in the shoots. However, we did not observe field spread of GFkV virus in any other trial for this project.

April 2007

The results of April 2007 indicate the GLRaV-2, GLRaV-3 and GFkV can spread systemically throughout a grapevine within 6 months and that there is acropetal movement of the viruses towards the shoots after the initial basipetal movement that was observed six weeks post-inoculation. The movement of viruses towards the shoots was also observed in the CTV infected citrus seedling at 51-58 DPI depending on the CTV isolate (Gafny et al 995). The means by which viruses are transported in the phloem is not well understood, however it is generally accepted that the viruses are transported by the phloem with the flow of metabolites

to various plant tissues (Santa Cruz 1999). Phloem transportation is bidirectional and moves metabolites to where they are required by the plant (Peel 1974). Thus it is not surprising that phloem limited viruses are also moved in both directions and become distributed throughout the plant.

It could have been interesting to destructively sample inoculated grapevines more frequently during the first 6 months of this experiment to determine how quickly viruses could move from the point of inoculation down to the roots and up into the shoots of inoculated grapevines. Our results do indicate that viruses could move into the shoots as quickly as six weeks post inoculation, especially as GFkV was detected in the shoots of inoculated grapevine after six weeks during this experiment and GLRaV-2, GLRaV-3 and GFkV could be detected in the some shoots of the grapevines that were used to develop sapling strategies six weeks post-inoculation (Chapter 3). However, the results indicate that viruses are less frequently detected at this time and infection of the shoots by six weeks may not occur or may occur erratically. The lower level of detection of viruses in the trunk and roots in April 2007 may have bee due to lower virus numbers, however, it may also be that false negative results were obtained due to inhibition of the RT-PCR test by co-purified compounds (Chapter 2).

In April 2007 GVA was detected in the roots of one grapevine in the Yarra Valley and in one grapevine in Sunraysia. GVA was detected in a total 3/53 inoculated grapevines during each of the experiments of this project suggesting that the rate of transmission of this virus through grafting is low. However, it is also possible, but less likely, that GVA moves slowly within the grapevine and/or remains below detectable levels.

November 2007

The November 2007 results from both sites show that viruses can spread systemically throughout an entire grapevine and reach detectable levels within 12 months. However viruses were not detected in all root, trunk, cordon and shoots samples of each inoculated grapevine. For example in Sunraysia in one inoculated grapevine GLRaV-3 was detected in 1/1 main root sample, 3/3 trunk samples, 10/10 cordon samples and 8/8 shoot samples while in a secondr inoculated grapevine GLRaV-3 was detected in 2/3 trunk samples, 7/11 cordon samples and 3/11 shoot samples. The variation in detection between the grapevines for each virus might be associated with uneven distribution of viruses within a grapevine. It may also be that virus titres were below delectable levels and/or inhibitors reduced the efficiency of the PCR tests to detect virus in all samples and some false negative results were obtained (Chapter 2).

4.5 Conclusions

The movement of viruses throughout grapevines may have important implications for virus detection and disease expression. Our results show that it can take more than 12 months for viruses to move from the point of infection to parts of the grapevine, such as shoots and cordons, which might be sampled for virus detection. This highlights the need for continued

and active surveillance using diagnostic testing of grapevines within certification schemes for grapevine viruses, as there is a risk of new infection events, which may go un-detected for some time. Our results suggest that there was no significant difference in detection between sites during the 12 months.

These results also explain the variability in detection of viruses in the first year of the monthly sampling experiments presented in Chapter 3. It is likely that viruses were not sufficiently translocated throughout the grapevines within the first 12 months to ensure reliable detection. Testing the remaining samples could verify the improved reliability of the PCR tests after 12 months and up to three years that was observed in the monthly sampling experiments (Chapter 3). However the results presented here indicate that viruses may systemically spread throughout some grapevines within 12 months, which would explain the increased reliability of the monthly testing after this time. It is likely that the increased reliability is also a result of virus replication within the grapevines, leading to elevated virus titre.

GVA was not frequently detected during this study. If GVA virus moves slowly within the grapevine and/or remains below detectable levels then there is a risk of obtaining false negative results for GVA infection. Studies over a longer time period may determine the rate of movement of GVA. However it is possible that we failed to transmit GVA to the grapevines, especially as GVA was never detected in any other experiment even at three years post inoculation (Chapter 3, Chapter 5).

CHAPTER 5: A comparison of biological indexing for grapevine virus-associated diseases in three in different climatic conditions in Australia.

5.1 Introduction

For many years biological indexing has been used to support the provision of high health grapevine planting material in Australia. All imported varieties and clones undergo biological indexing onto the indicator *Vitis berlandieri* × Conderc 1613 cv. LN33 during PEQ for corky bark disease. Varieties and clones being introduced into the nucleus collections of certification programs have traditionally been tested by biological indexing onto *Vitis vinifera* cv. Cabernet Franc for leafroll disease, *V. rupestris* cv. Rupestris St George for fleck, vein necrosis and rupestris stem pitting diseases, LN33 for corky bark and leafroll diseases and onto *V. berlandieri* × *V. riparia* cv. Kober 5BB for stem grooving disease.

In Australia individual studies to determine the reliability of woody indexing on specific indicators were conducted during 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). Both of these studies were done prior to the emergence of present day ELISA kits and PCR technologies. The Victorian study showed that Cabernet Franc was more reliable than LN33 as an indicator for leafroll disease in Australia. The South Australian study 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 was done by Foundation Planting Services (FPS, UCDavis, USA) that 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. It was shown that these conflicting results may be attributed to the failure of an infected bud chip to survive or the grafting of an uninfected bud, due to uneven distribution of the virus. These two problems are regularly encountered in woody indexing programs. No studies have been reported that compare replicated woody indexing trials in different climatic conditions.

The aim of this study was to determine the reliability, accuracy and speed of woody indexing (WI) for virus detection in different climatic conditions. Identical experiments were conducted in the field or in the screenhouse at three different sites: 1) in a hot climate (field, SARDI, Loxton); 2) in a cool climate (field, Yarra Valley); 3) under similar conditions to those imposed by PEQ (screenhouse, DPI Knoxfield). A pictorial protocol of symptom expression was developed and can be used by industry and scientists as future reference point for symptom expression.

5.2 Materials and Methods

5.2.1 Field sites

To determine the reliability, accuracy and speed of woody indexing (WI) for virus detection in different climatic conditions, identical experiments were established in the field at three different sites: 1) in a hot climate (field, SARDI, Loxton, SA); 2) in a cool climate (field Yarra Valley, Victoria); 3) under similar conditions to those imposed by PEQ (screenhouse, DPI Knoxfield). At each site there were 12 virus-WI treatments, totalling 48 grapevines (12 treatments x [3 replicates of virus inoculated indicator + one negative control, un-inoculated indicator]). The treatments are listed in Table 5.1. These grapevines were chip bud inoculated with each virus source, using two buds per grapevine, in October 2006 (Loxton and Yarra Valley) or January 2007 (Knoxfield). Prior to indexing the woody indicator material and the virus sources were tested to determine their virus status. The viruses detected in the candidate grapevines are listed in Table 1. RSPaV was detected in the woody indicators used for this trial.

Table 5.1 List of woody indicators, the disease and associated virus that should be detected virus, the virus source used for inoculation and the viruses that were detected in the source used in the woody indexing trial.

Woody indicator	Disease detected	Virus associated with the disease	Virus source for inoculation	Viruses detected in the candidate grapevines*
Rupestris St George	Stem pitting	RSPaV	Shiraz 1654	RSPaV
Rupestris St George	Stem pitting	RSPaV	Cabernet Franc C24-1	RSPaV
Rupestris St George	Fleck	GFkV	5C Teleki 10-48-99 GM	GFkV
Rupestris St George	Fleck	GFkV	Chardonnay 84	GFkV and RSPaV
LN33	Corky bark	GVB	Jade seedless R35V2	GVB and RSPaV
LN33	Corky Bark	GVB	Rupestris St George A5V11	GVB, GLRaV-2, GFkV and RSPaV
Kober 5BB	Stem grooving	GVA	Viognier HTK	GVA and RSPaV
Kober 5BB	Stem grooving	GVA	Grenache 139HT SAN23-22-83	GVA and RSPaV
Cabernet Franc	leafroll	GLRaV-9	Shiraz BVRC 12	GLRaV-9 and RSPaV
Cabernet Franc	leafroll	GLRaV-3	Graciano WA6V6	GLRaV-3 and RSPaV
Cabernet Franc	leafroll	GLRaV-2	Chardonnay Pen 58	GLRaV-2, GFkV and RSPaV
Cabernet Franc	leafroll	GLRaV-1	Shiraz RVC14	GLRaV-1 and RSPaV

* Presence of the viruses in the candidate grapevines was was confrmed by RT-PCR

At Loxton grapevines were grown upright without cordons and trained to 1.8 metres. In the Yarra Valley the grapevines were grown on a two wire trellis with vertical shoot positioning. At

Knoxfield grapevines were grown in 20cm pots without support and were pruned back to the trunk in winter.

5.2.2 Symptom observation

Rupestris St George inoculated with GFkV grapevines were observed in spring 2007, 2008 and 2009 for fleck symptoms, Cabernet Franc grapevines inoculated with GLRaV-1, GLRaV -2, GLRaV -3 and GLRaV -9 were observed during late summer to autumn in 2007, 2008 and 2009 for development of leafroll symptoms. During autumn of 2007, 2008 and 2009, the periderm of each trunk was peeled back to observe development of rugose wood symptoms on Rupsetris St George, LN33 and Kober 5BB that might be associated with RSPaV, GVB and GVA respectively.

In winter 2009, after two years and eight months for the Yarra Valley and Loxton and two years and five months for Knoxfield, the trunks of Rupestris St George, LN33 and Kober 5BB inoculated with RSPaV, GVB and GVA respectively were collected, boiled for 60 minutes or autoclaved at 121°C for 30 minutes. As soon as they were cool enough to handle the outer bark, periderm and the phloem were removed to observe rugose wood symptoms affecting the xylem tissue, such as corkiness, pitting and grooving.

5.2.3 RT-PCR

In April 2008 and November 2008 each grapevine was tested for the presence of the specific virus with which it was inoculated using the high throughput extraction procedure and RT-PCR techniques developed in Chapter 2. Primers for the detection of NADH dehydrogenase ND2 subunit (ndhB gene, NAD) messenger ribonucleic acid (mRNA; Thompson et al 2003) were used to determine the quality of the extracted RNA. The following RT-PCR tests were used for virus detection:

- GLRaV-1 using the primer pair GSP3 /GSP4 (Good and Monis 2001),
- GLRaV-2 using the primer pair V2dCPf2 sense/V2CPr1 antisense (Bertazzon and Angelini 2004),
- GLRaV -3 using the primer pair P3U/P3D (Turturo et al. 2005),
- GLRaV -9 using the primer pair LR9-F/LR9-R (Alkowni et al 2005),
- GVA using the primer pair C995/H587 (Minafra et al 1997)
- GVB using the primer pair H6980/C7439 (Minafra & Hadidi, 1994; MacKenzie 1997)
- GFkV using the primer pair GFkV-U279/GFkV-L630 (Shi et al 2003).
- RSPaV using both the RSP48/RSP49 (Zhang et al 1998; Nolasco et al 2000) and Sy9F/Sy8R (Habili et al 2006) primer pairs.

5.3 Results

The presence of symptoms associated with the expected disease during each year and at each site is listed in Table 5.2. RT-PCR was used to verify transmission of viruses to the indicators and the pooled results of testing from May 2009 and November 2009.are given in Table 5.2.

During the three year experiment Rugose wood symptoms on Rupestris St George, LN33 and Kober 5BB woody indicators inoculated with RSPaV, GVB and GVA were not obvious in the field by simply peeling back the outer bark and periderm.

RSPaV was detected in all inoculated Rupestris St George at each site and in the un-inoculated controls using the RSP48/RSP49 primer pair (Table 5.2). RSPaV was detected in 11/18 inoculated controls and not in the un-inoculated controls using the Sy9F/Sy8R primer pair (Table 5.2). After three years when the outer bark, periderm and phloem were removed by boiling or autoclaving, stem pitting symptoms associated with both RSPaV isolates were observed on the trunks of the inoculated Rupestris St George indicators grown at Loxton (Figure 5.1) but not at Knoxfield or in the Yarra Valley. Stem pitting symptoms were not observed on any un-inoculated indicator.

GVA was detected in only one Kober 5BB indicator that was chip bud inoculated with Grenache 139HT at Loxton. After three years when the outer bark, periderm and phloem were removed by boiling or autoclaving. Stem grooving symptoms were not observed on the GVA infected indicator at Loxton or any in other indicator plant inoculated with GVA at Loxton, Knoxfield and the Yarra Valley.

GVB was detected in 12/18 inoculated LN33 indicators. After three years when the outer bark, periderm and phloem were removed by boiling or autoclaving, Corky bark symptoms were not observed on any LN33 indicator inoculated with GVB at Knoxfield and the Yarra Valley. However at Loxton one LN33 vine showed very mild rugose wood symptoms that were possible associated with GVB infection in the variety Rupestris St George (Figure 5.2).

Rupestris St George indicators inoculated with GFkV-B from 5C Teleki 10-48-99 GM did not show symptoms in any year at any site. Fleck symptoms, associated with GFkV-A from Chardonnay 84, were not observed in any year at Knoxfield. In the Yarra Valley suspected GFkV symptoms were observed in spring 2009 on one Rupestris St George indicator inoculated with GFkV-A from Chardonnay 84. Fleck symptoms were observed on Rupestris St George indicators inoculated with GFkV-A from Chardonnay 84 at Loxton during spring 2008 and spring 2009 (Figure 5.3). GFkV was detected in all inoculated Rupsetris St George at each site.

GLRaV-1 was detected in 5/9 inoculated Cabernet Franc indicators. GLRaV-2 was detected in 8/9 inoculated Cabernet Franc indicators. GLRaV-3 was detected in 7/9 inoculated Cabernet Franc indicators. GLRaV-9 was detected in 5/9 inoculated Cabernet Franc indicators. Clear leafroll symptoms were not observed on any Cabernet Franc indicators at

Knoxfield under screenhouse conditions. In autumn 2007 leafroll symptoms associated with GLRaV-1 (Figure 5.4), GLRav-2 (Figure 5.5), GLRaV-3 (Figure 5.6) and GLRaV-9 (Figure 5.7) were observed on a few Cabernet Franc indicators in the Yarra Valley, but not at Loxton (Table 5.2). In autumn 2008, leafroll symptoms associated with GLRaV-1 (Figure 5.8; Figure 5.9), GLRaV -2 (Figure 5.10; Figure 5.11), GLRaV-3 (Figure 5.12a; Figure 5.13) and GLRaV -9 (Figure 5.14; Figure 5.15) were observed on all inoculated Cabernet Franc indicators at Loxton and the Yarra Valley. The leafroll symptoms associated with GLRaV-3 and GLRaV-9 inoculations were not strong at Loxton in 2008. In 2009 leafroll symptoms were observed on all inoculated Cabernet Franc indicators at Loxton, although the symptoms on vines inoculated with GLRaV-3 were not strong (Figure 5.12b). In the Yarra Valley, suspected leafroll symptoms were observed as early as January 2009 on a few basal leaves of 2/3 Cabernet Franc indicators inoculated with GLRaV-3 and 1/3 Cabernet Franc indicators inoculated with GLRaV-9 with but these were not observed from February 2009. No leafroll symptoms were observed from February 2009 in the Yarra Valley on any Cabernet Franc indicator inoculated with GLRaV-1 or GLRaV -2.

Leafroll symptoms were observed on1/3 Cabernet Franc indicators inoculated with GLRaV-1 at Loxton and the Yarra Valley in which GLRaV-1 was not detected. Similarly Leafroll symptoms were observed on1/3 Cabernet Franc indicators inoculated with GLRaV-9 at Loxton and the Yarra Valley in which GLRaV-9 was not detected.

5.4 Discussion

To the best of our knowledge this study is the first comprehensive comparison of the reliability of biological indexing at three different climatic conditions. The results indicate that biological indexing is not always reliable for the detection of grapevine diseases that have been associated with specific grapevine viruses. Several factors appear to affect the reliability of this diagnostic test including success of transmission and environment.

The PCR results indicate that some virus species, for example GVA, are less efficiently transmitted to biological indicators compared to other viruses such as GLRaV-3. If the negative PCR results indicate that GVA was not transmitted to the indicators then this would explain why symptoms were not observed. Candidate buds of GVA infected Grenache and Viognier grapevines did appear to take on most vines, although the buds did not produce green shoots. However, it is possible that the candidate buds did not take, but were held in place due the development of host callus tissue around the wound.

The low rate of GVA transmission during this experiment, and in other experiments of this project, suggest that some strains of GVA may not be frequently transmitted by grafting. It may be that the strains that were used in our experiments of the project induced a hypersensitive defence response in the inoculated grapevines that lead to an incompatibility between the chip bud and the grapevine and the virus was prevented from moving into the new hosts. If this is the case then it is possible that biological indexing will miss GVA infections and lead to false negative results. Other researchers have also found that the rate

of transmission of some viruses is low in woody indexing and that to increase the likelihood of transmission at least five candidate buds per vine should be used (Jerry Uyemoto, UC Davis, pers.comm).

GVB was detected by PCR in some inoculated indicators at each site but, corky bark symptoms were not observed. However in one LN33 indicator inoculated with GVB from Rupestris St George A5V11 at Loxton some subtle rugose wood symptoms were suspected (Figure 5.2). By definition this symptom was not corky bark, which must have externally visible bark swelling and phloem proliferation (Golino1993; Martelli, 1993). It is possible that the symptom observed fit the definition of LN33 stem grooving in which pitting and grooving symptoms are observed once the bark is stripped back to reveal the wood cylinder (Golino, 1993; Martelli, 1993). However, the symptom observed in this trial was milder than that which has been published for LN33 stem grooving (Martelli, 1993) and it may be that what was observed was not associated with any virus infection, but with some other physical damage to the vine. The association between LN33 stem grooving and viruses is unknown.

The PCR results for RSPaV in Rupestris St George identified a common problem in biological indexing using woody indicators, which is that the woody indicators may be infected with a latent virus. In this study RSPaV was detected using the RSP48/RSP49 primer pair in material of the indicator Rupestris St George that was used for propagation and it was detected in the inoculated and un-inoculated indicators after inoculation with different RSPaV isolates. These results indicate that the RSPaV strains detected using RSP48/RSP49 primer pair may not be associated with rugose wood diseases, particularly as the un-inoculated controls did not show symptoms and because some of the inoculated vines at Knoxfield and the Yarra Valley did not show symptoms.

All RSPaV inoculated grapevines at Loxton displayed stem pitting symptoms, which were not observed on the un-inoculated controls. These inoculated indicators also tested positive for RSPaV using the Sy9F/Sy8R primer pair and the un-inoculated controls did not. It is possible that this primer pair is better at discriminating between disease-associated strains of RSPaV than the RSP48/RSP49 primer pair as suggested by Habili et al (2006). However RSPaV symptoms were not observed in the Sy9F/Sy8R positive indicators at Knoxfield or the Yarra Valley. It is therefore possible that this primer pair also detect strains of RSPaV that are not associated with disease, or symptoms did not develop on the indicators due to other factors that might influence disease expression. Further work is required to understand what influences different strains of RSPaV have on disease expression in grapevines. Improved information about the genetic differences between strains and the factors involved in inducing disease could assist to develop a better diagnostic tool for disease associated strains of this virus.

Biological indexing relies on the interpretation of symptoms to diagnose a disease and does not necessarily identify specific viruses. In some situations mixed virus infections may have a significant impact on symptom expression. For example vein banding symptoms are

observed when grapevine fanleaf virus is mixed with grapevine yellow speckle viroid (Krake et al 1999) and Roditis leaf discolouration may be associated with a mixture of GFLV and CarMV (Avangelis and Rumbos, 1990). It is possible that latent infections of the indicator or that candidate grapevine by viruses like RSPaV are important in triggering a host response to other viruses so that symptoms are expressed in biological indicators. Conversely the presence of latent viruses may act to “cross-protect” against other virus infection and result in a false negative result. Cross protection of mild strains against severe strains to prevent disease is a strategy used in other crops, such as citrus with *Citrus tristeza virus* (Fulton, 1986). More work is required to understand how combinations of viruses influence disease in grapevines.

This study suggests that environment can have a significant impact on disease expression in grapevines. For example leafroll symptoms were observed as early as 6 months post-inoculation in some Cabernet Franc indicators in the Yarra Valley, but these were symptoms not observed in the field at Loxton or in the screenhouse at Knoxfield.

Strong GLRaV-3 symptoms were not observed at Loxton. The inoculated indictors did not grow well at this site and it may be that these vines did not display strong leafroll symptoms due to the poor growth. The reason for the poor growth is not known but it seems unlikely that GLRaV-3 alone caused the vines to grow poorly, since the inoculated grapevines in the Yarra Valley grew well. However it is difficult to exclude the possibility that GLRaV-3 contributed to the poor growth in combination with other factors. Alternatively the poor growth of these grapevines may have resulted in weak leafroll symptoms.

Symptoms were never observed in the screenhouse at Knoxfield during the three year trial for any virus indicator combination. However each virus was detected in at least one indicator at Knoxfield and this suggests that leafroll symptom expression was masked or suppressed in the screenhouse environment that was provided. Cabernet franc grapevines maintained in the screenhouse did not grow vigorously and leaves were often pale in colour, suggesting that they may have been stressed. It is possible that continued growth of grapevines in smaller pots contributed to the stress exhibited by the grapevines, which may have affected symptom expression, but pot size was chosen due to restrictions on space for this trial. Although GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-9 were not detected in all inoculated indicators at Knoxfield all buds seemed to have taken on each indicator (data not shown) and the lack of symptom expression in some indicators may have been associated with a lack of virus transmission,

The differential expression of leafroll symptoms between Loxton and the Yarra Valley may be due to environmental differences, such as temperature. Higher temperatures can affect virus replication (Dawson et al, 1978) and symptom expression (Tanaka, 1988). On average, the mean maximum monthly temperatures in the Yarra Valley are 3-5°C cooler than Loxton throughout the year and mean minimum temperature that are 0-3°C cooler during the year and the largest differences for both are observed during the summer months when

grapevines are actively growing. The hotter temperatures at Loxton during the first growing season may have reduced the rate of replication of viruses and resulted in a lack of symptom expression due to low titre and/or low virus movement. In the second year leafroll symptoms were observed at both sites and it is likely that virus titres had reached high enough numbers to induce symptoms at both sites. In Chapter 4 we observed the movement of viruses basipetally from the point of inoculation and that it can take more than more than 12 months for viruses to move from the point of infection to the shoots and cordons. It may be that viruses must be located in the shoots to cause symptom expression and that GLRaVs did not reach the shoots of inoculated grapevines at Loxton until the following season and thus remained symptomless in autumn 2007.

Some GLRaV inoculated indicators displayed possible leafroll symptoms on a few basal leaves in the Yarra Valley in January 2009, However from February 2009 onwards no symptoms were observed in the Yarra Valley in the same indicator plants. At Loxton leafroll symptoms were not observed until April 2009. The reason for this differential expression between the two sites is unknown. It is possible that environmental factors, such as the sudden onset of unusually high temperatures experienced in the Yarra Valley during January and February 2009, could have contributed to the suppression of leafroll symptoms, especially as the average temperatures leading up to these hot days were cooler than those experienced in Loxton.

Assuming that GLRaV species were the cause of leafroll symptoms in this trial, the PCR results of this experiment suggested that PCR testing may also not have been a reliable indicator of the transmission of GLRaV viruses. Leafroll symptoms were observed on all indicators inoculated with GLRaV-1 and GLRaV-9 at Loxton and the Yarra Valley, but virus was not detected in 1/3 indicators for each virus at each site. It is possible that this was due to inhibitors that affected the efficiency of the PCR test to detect viruses, or that the samples that were taken did not contain sufficient virus. This suggests that, at times, biological indexing can be more sensitive than RT-PCR tests. It is also possible that the symptoms observed were associated with other problems and misdiagnosed as leafroll symptoms.

Differential expression of symptoms associated with GFkV was observed between the two sites. That is, symptoms were not observed at the Yarra Valley or at Knoxfield, even though GFkV could be detected, but symptoms were observed at Loxton. GFkV symptoms do not persist throughout the growing season, possibly due to the onset of hot temperatures (Tanaka, 1988). However, if cooler temperatures do favour fleck symptom expression, then it seems that temperature alone may not explain the lack of symptoms observed in the Yarra Valley. The average temperature for the Yarra Valley is 19.3°C -24.7°C /6.4°C -9.3°C (day/night) during October-December compared to Loxton, where symptoms were observed and which is warmer and has average day/night temperatures of 24°C -29.6°C /8.3°C -13°C during the same period.

One study has shown that under greenhouse conditions higher temperatures, (28°C day and 23°C night) can reduce the expression of leafroll and fleck diseases in grapevines compared to lower temperatures (20°C day and 17°C night). It was also shown that shading can reduce symptom expression of both diseases, which may explain why these diseases were not observed on indicators in the screenhouse. It is possible that lack of experience in the interpretation of symptoms, particularly for subtle symptoms like those associated with GFkV, could result in false negative results.

5.5 Conclusions

The results of this experiment show that viruses may not be reliably detected by biological indexing. Factors that effect biological indexing include the success of chip bud inoculation with regard to bud-take and transmission of the candidate virus from the candidate bud to the grapevine indicator. Environment also impacts on symptom expression and a screenhouse or glasshouse environment may not be reliable for biological indexing for some viruses, particularly GLRaVs. Variable expression of symptoms was observed with time at each trial site and between both trial sites: the Yarra Valley GLRaV inoculated grapevines displayed symptoms during the first two seasons and Loxton GLRaV inoculated grapevines displayed symptoms during the last two seasons. These variations may also be associated with fluctuations in the environment. The specific environmental factors that influence symptom expressions are unknown, but we hypothesize that temperature may have a significant role. It is also possible that shading and day length could influence symptom expression.

Our results in this experiment and throughout this project clearly demonstrate that no single virus detection technique will detect all strains of a virus and that all detection methods can be affected by virus distribution and/or titre. We recommend that biological indexing continue to be used for virus detection to increase the chance of detection of viruses, particularly for the varieties and clones that are being introduced into certification programs and during PEQ for new importations. If symptoms are observed on woody indicators it is recommended that virus-specific tests e conducted to identify viruses associated with the observed symptoms.

Based on this trial we recommend that biological indexing be carried out for a minimum of three years. This recommendation is supported by the results of Chapters 3 and 4, which suggested that viruses were not sufficiently translocated throughout the grapevines within the first 12 months to ensure reliable detection and that the reliability of the ELISA and PCR tests increased after 12 months and up to three years.

One of the aims of this work was to produce a pictorial protocol of symptom expression that will be developed for industry and scientists. The images produced from this woody indexing trial are included with the RT-PCR protocols contained in Appendix 5. These images are of indicators have a known virus profile and are inoculated with candidate buds of grapevines also with a known virus profile, which historically is not the case for routine woody indexing for virus detection in grapevines. These images will supplement to book entitled “Graft Transmitted Diseases of Grapevines” by Krake, et al (1999).

Table 5.2. The results of PCR testing on the inoculated indicators at Knoxfield (K), Loxton (L) and the Yarra Valley (Y) two year post-inoculation and presence or absence of symptoms during the first three years post-inoculation

Indicator	Virus	Replicate	Results of PCR testing			Results of woody indexing								
			Knoxfield	Loxton	Yarra Valley	2006/7			2007/8			2008/9[1]		
						K	L	Y	K	L	Y	K	L	Y
Rupestris St George un-inoculated control			+/-[2]	+/-	+/-	-[4]	-	-	-	-	-	-	-	-
Rupestris St George/Shiraz 1654	RSPaV	rep1	+/+[3]	+/+	+/+	-	-	-	-	-	-	-	+[5]	-
		rep2	+/+	+/+	+/-	-	-	-	-	-	-	-	+	-
		rep3	+/+	+/+	+/-	-	-	-	-	-	-	-	+	-
Rupestris St George un-inoculated control			+/-	+/-	+/-	-	-	-	-	-	-	-	-	-
Rupestris St George/Cabernet Franc C24-1	RSPaV	rep1	+/+	+/+	+/-	-	-	-	-	-	-	-	+	-[6]
		rep2	+/-	+/+	+/-	-	-	-	-	-	-	-	+	-
		rep3	+/-	+/+	+/+	-	-	-	-	-	-	-	+	-
Rupestris St George un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
Rupestris St George/5C Teleki 10-48-99 GM	GFkV	rep1	+	+	+	-	-	-	-	-	-	-	-	-
		rep2	-	-	+	-	-	-	-	-	-	-	-	-
		rep3	-	-	+	-	-	-[6]	-	-	-	-	-	-
Rupestris St George un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
Rupestris St George/Chardonnay 84	GFkV	rep1	+	+	+	-	-	-	-	+	-	-	+	-
		rep2	+	+	+	-	-	-	-	+	-	-	+	-
		rep3	+	+	+	-	-	-	-	+	-	-	+	-
LN33 un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
LN33/Jade seedless R35V2	GVB	rep1	+	+	-	-	-	-	-	-	-	-	-	-
		rep2	-	+	-	-	-	-	-	-	-	-	-	-
		rep3	+	+	-	-	-	-	-	-	-	-	-	-
LN33 un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
LN33/Rupestris St George A5V11	GVB	rep1	+	+	+	-	-	-	-	-	-	-	-	-
		rep2	-	+	+	-	-	-	-	-	-	-	-	-
		rep3	-	+	+	-	-	-	-	-	-	-	-[6]	-
Kober 5BB un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
Kober 5BB /Viognier HTK	GVA	rep1	-	-	-	-	-	-	-	-	-	-	-	-
		rep2	-	-	-	-	-	-	-	-	-	-	-	-
		rep3	-	-	-	-	-	-	-	-	-	-	-	-
Kober 5BB un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
Kober 5BB /Grenache 139HT	GVA	rep1	-	-	-	-	-	-	-	-	-	-	-	-
		rep2	-	-	-	-	-	-	-	-	-	-	-	-

Indicator	Virus	Replicate	Results of PCR testing			Results of woody indexing								
			Knoxfield	Loxton	Yarra Valley	2006/7			2007/8			2008/9[1]		
						K	L	Y	K	L	Y	K	L	Y
		rep3	-	+	-	-	-	-	-	-	-	-	-	-
Cabernet Franc un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
Cabernet Franc/Shiraz BVRC 12	GLRaV-9	rep1	-	+	-	-	-	-	-	+	+	-	+	-
		rep2	-	+	+	-	-	-	-		+	-	+	-[4]
		rep3	+	-	+	-	-	+	-	+	+	-	+	+
Cabernet Franc un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
Cabernet Franc/Graciano WA6V6	GLRaV-3	rep1	+	+	+	-	-	+	-	+	+	-	+	+
		rep2	-	+	+	-	-	+	-	+	+	-	+	+
		rep3	-	+	+	-	-	-	-	+	+	-	+	+
Cabernet Franc un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
Cabernet Franc/Chardonnay Pen 58	GLRaV-2	rep1	+	+	+	-	-	-	-	+	+	-	+	-
		rep2	+	+	+	-	-	-	-	+	+	-	+	-
		rep3	-	+	+	-	-	-	-	-	+	-	+	-
Cabernet Franc un-inoculated control			-	-	-	-	-	-	-	-	-	-	-	-
Cabernet Franc/Shiraz RVC14	GLRaV-1	rep1	+	+	+	-	-	-	-	+	+	-	+	-[4]
		rep2	-	+	+	-	-	-	-	+	+	-	+	-
		rep3	-	-	-	-	-	+	-	+	+	-	+	-

[1]In 2008/9 only the presence of rugose wood symptoms associated with RSPaV, GVA and GVB was determined after the outer bark and phloem tissue had been removed.

+/-[2] = Positive for RSPV using the RSP48/RSP49 primer pair but negative using the S9F/Sy8R primer pair

+/+[2] = Positive for RSPV using the RSP48/RSP49 primer pair and the S9F/Sy8R primer pair

-[4] = Symptoms were not observed

+[5] = Symptoms were observed

-[6] symptoms suspected but were not clear.



Figure 5.1. A. Stem pitting symptoms (top, arrow) on *Vitis rupestris* (Rupestris St George) inoculated with a strain of RSPaV from Cabernet Franc C24-1 compared to un-inoculated *V. rupestris* (Rupestris St George, bottom).
B. Stem pitting symptoms (Vines 1, 3 and 4, arrows) on *Vitis rupestris* (Rupestris St George) inoculated with a strain of RSPaV from Cabernet Franc C24-1 compared to un-inoculated *V.rupestris* (Rupestris St George, Vine 2).



Figure 6.2. Possible mild rugose wood symptoms (top, arrow) on LN33 inoculated with GVB compared to un-inoculated LN33 (bottom).

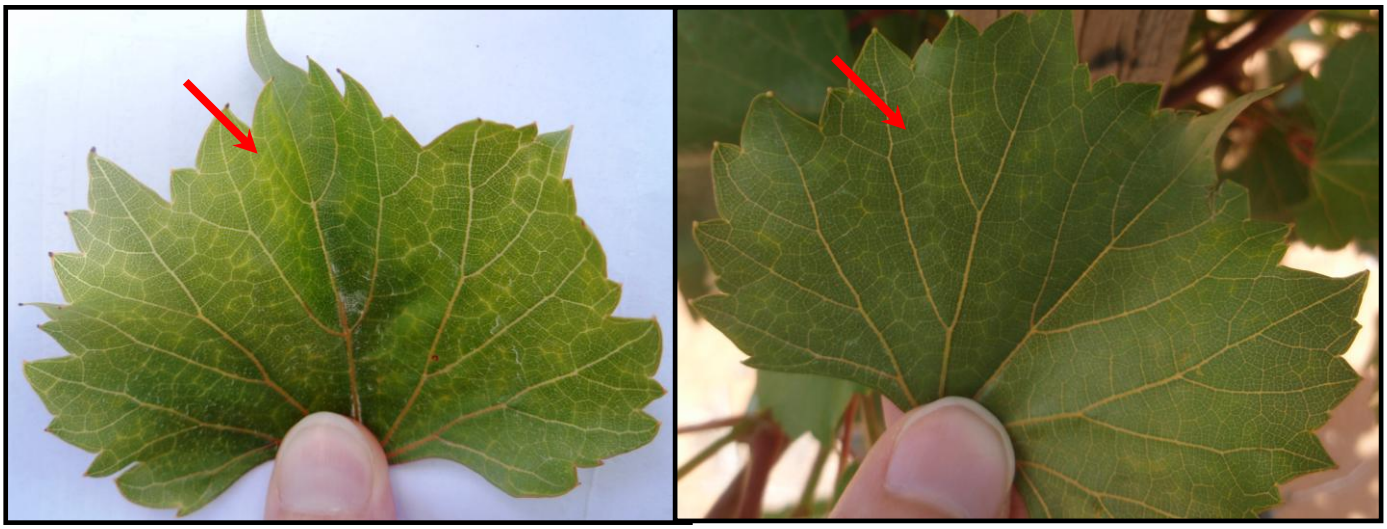


Figure 5.3. Fleck symptoms on *Vitis rupestris* (Rupestris St George) inoculated with GFkV from Chardonnay 84. Note the vein clearing symptoms on minor veins (arrow). Symptoms observed in spring 2009 at Loxton.

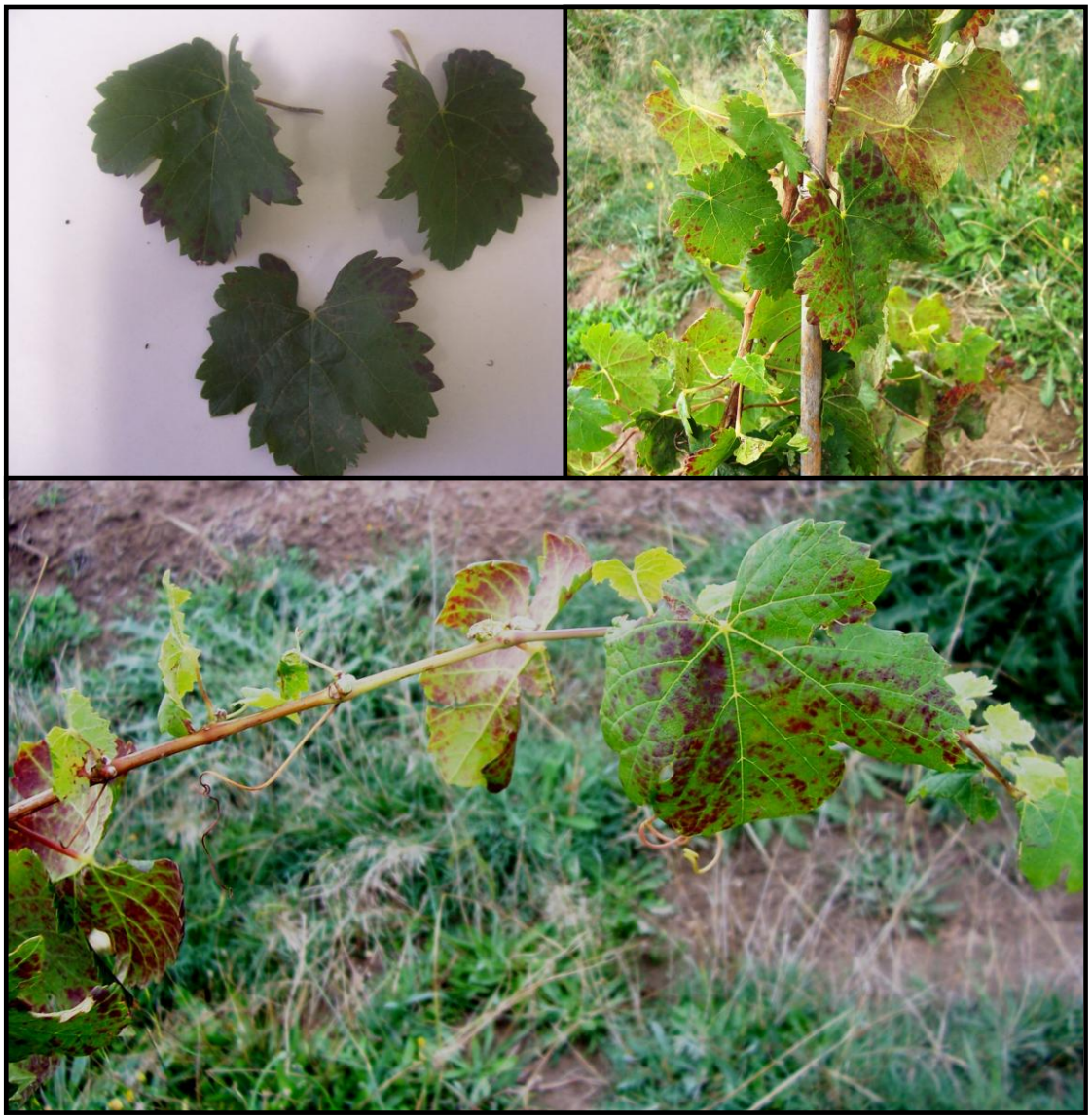


Figure 5.4. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-1 from the variety Shiraz RVC14. Symptoms were observed in autumn 2007 at the Yarra Valley.



Figure 5.5. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-2 from the variety Chardonnay Pen58. Symptoms were observed in autumn 2007 at the Yarra Valley.



Figure 5.6. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-3 from the variety Graciano WA6V6. Symptoms were observed in autumn 2007 at the Yarra Valley.



Figure 5.7. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-9 from the variety Shiraz BVRC 12. Symptoms were observed in autumn 2007 at the Yarra Valley.



Figure 5.8. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-1 from the variety Shiraz RVC14. Symptoms were observed in autumn 2008 at Loxton.



Figure 5.9. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-1 from the variety Shiraz RVC14. Symptoms were observed in autumn 2008 at he Yarra Valley.



Figure 5.10. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-2 from the variety Chardonnay Pen 58. Symptoms were observed in autumn 2008 at Loxton. Note the variation in symptom severity between leaves and vines.



Figure 5.11. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-2 from the variety Chardonnay Pen 58. Symptoms were observed in autumn 2008 at the Yarra Valley. Note the variation in symptom severity between leaves and vines.



A. **B.**

Figure 5.12. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-3 from the variety Graciano WA6V6. A. Symptoms were observed in autumn 2008 at Loxton. B. Symptoms were observed in autumn 2009 at Loxton



Figure 5.13. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-3 from the variety Graciano WA6V6. Symptoms were observed in autumn 2008 at the Yarra Valley.



Figure 5.14. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-9 from the variety Shiraz BVRC 12. Symptoms were observed in autumn 2008 at Loxton.



Figure 5.15. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with GLRaV-9 from the variety Shiraz BVRC 12. Symptoms were observed in autumn 2008 at the Yarra Valley.

CHAPTER 6: A SURVEY OF AUSTRALIAN GRAPEVINES FOR ENDEMIC AND EXOTIC VIRUSES AND *XYLOPHILUS AMPELINUS*

6.1 Introduction

In 2008/09 field surveys were conducted in viticultural growing regions of mainland Australia to validate the molecular tests developed in this project under local conditions. This validation process was required to ensure that the recommended PCR tests did not detect nucleic acids of plant hosts or other micro-organisms that may occur in Australia.

The field survey will also update the disease status and areas of freedom for each pathogen in Australia. It is possible that some of the pathogens of quarantine significance are present in Australia, but remain symptomless within their hosts. For example, GVB occurs in Australia but is not associated with corky bark disease and the disease is still considered quarantineable. It remains unclear what the distribution of GVB is in Australia. Although GFLV has been reported in Australia in Victoria and South Australia it was contained in the Rutherglen region and is still considered a quarantineable pathogen (Taylor 1962; Taylor and Hewitt 1964; Meagher et al 1976; Cirami 1988; Krake et al 1999). It is possible that, in combination with selection of healthy plant material from the country of origin, our post entry quarantine facilities have been successful in preventing entry of these pathogens. The results of our survey are reported below.

The diagnostic protocols developed for ten endemic virus testing were also used to screen 140 clones and varieties of the AVIA nuclear collection which was established through GWRDC funds. The collection is an important industry resource and it represents the highest health planting material available in Australia and inclusion of material in this survey provided an opportunity to further validate the health status of this nuclear collection.

6.2 Methods

6.2.1 Survey sampling

Samples from 218 grapevines were collected from vineyards and home gardens in grape growing regions from New South Wales (NSW, 35), Queensland (QLD, 15), South Australia (SA, 77), Victoria (VIC, 51) and Western Australia (WA, 40). Sampling began in November 2008 and was completed in May 2009. The samples included wine grape varieties, rootstocks and table grapes. Older grapevines were selected in preference to more recent importations. Diseased grapevines were selected in preference to healthy grapevines, and preferably not from certified sources. Green shoots with leaves attached were collected where possible but if they were unavailable lignified canes, with leaves attached were taken. Three to four shoots ca. 50 cm long were randomly sampled from each grapevine. If the shoots or canes were much longer than 50cm then the lower (basal) 50cm of the shoot was collected.

6.2.2 AVIA sampling.

Samples from 140 cultivars and clones were collected from the AVIA nuclear collection during two seasons. In April 2007, 70 clones and cultivars were selected and sampled. The same 70 plants were sampled again in November 2007. In April 2008 a further 70 clones and cultivars were selected and sampled and these were sampled again in December 2008. For each clone or cultivar there were up to four grapevines and each grapevine was sampled separately. Three to four shoots ca. 20 cm long were randomly sampled from each grapevine. If the shoots or canes were much longer than 50cm then the lower (basal) 50cm of the shoot was collected. Green shoots with leaves attached were collected where possible but if they were unavailable lignified canes, with leaves attached were accepted. Tissue from each shoot of a grapevine was sub-sampled and pooled into one sample for nucleic acid extraction so that each nucleic extract represented up to four vines per sample. The amount of tissue used for each nucleic extract was 500mg.

6.2.3 Nucleic acid extraction

Total nucleic acid was extracted from each sample using the protocol developed for use on the QIAxtractor (Qiagen) as described in Chapter 3.

6.2.4 RT-PCR and PCR amplification

Primers for the detection of NADH dehydrogenase ND2 subunit (ndhB gene, NAD) messenger ribonucleic acid (mRNA; Thompson et al 2003) were used to determine the quality of the extracted RNA (Table 6.1). Primers developed for bacterial phylogenetic studies (Weisberg et al 1991) were used in PCR to determine the quality of DNA extracts from plants (Table 6.2).The PCR primers used to detect each exotic and endemic pathogen, the type of assay used and the references containing specific cycling conditions for each pathogen are given Table 6.1 for the endemic viruses and Table 6.2 for the exotic pathogens. The positive controls that were used are listed in Table 6.3.

Some additional GLRaV species-specific tests for GLRaV-4, GLRaV-5 and GLRaV-6 (Maliogka et al 2008), not compared in the original primer selection, were also trialled against the survey samples. Primer concentrations and cycling conditions for the specific RT-PCR tests for GLRaV-4,-5, and- 6 were as described by Maliogka et al (2008).

The AVIA samples were tested for ten endemic viruses using the specific RT-PCR tests listed in Table 6.1. The AVIA samples were not tested using the generic *Closteroviridae*, the *Ampelovirus* subgroup I or the specific GLRaV-4 and -5 tests listed in Table 6.1 or any of the exotic pathogen tests listed in Table 6.2.

Table 6.2 List of pathogens tested for, type of PCR test, primers used and annealing temperature, region amplified, expected product size and reference for each test used to detect endemic grapevine pathogens in Australia.

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
DNA House-keeping gene	PCR	FD2	AGAGTTTGATCATGGCTCAG	55°C	partial bacterial 16S rRNA gene	1400-1500 bp	Weisberg et al 1991
		RP1	ACG GTT ACC TTG TTA CGA CTT				
RNA House-keeping gene	RT-PCR	AtropaNad2.1a	GGACTCCTGACGTATACGAAGGATC	55ºC	NADH dehydrogenase ND2 subunit) messenger ribonucleic acid	188 bp	Thompson et al 2003
		AtropaNad2.2b	AGCAATGAGATTCCCCAATATCAT				
Closteroviridae	Grapevine leafroll viruses first round	dHSP up1	GGIHTIGAITTYGGIACIACITT	5 cycles of: 43ºC for 10 s 38ºC for 5s 35 cycles of: 43ºC for 30 s OR for the Ampelovirus Subgroup I nested PCR: 10 cycles of: 38ºC for 30s 30 cycles of: 49ºC for 10 s 44ºC for 10 s 38ºC for 15 s	HSP-70	580-620 bp	Dovas & Katis 2003
		dHSP up1G	AGTTYGGGACGACGTT				
		dHSP do2	GTICCICCICCNAARTC				
		dHSP do2C	GTICCICCCCCNAARTC				
	Grapevine leafroll viruses first nested	dHSP nest1	TTYGGGACGACGTTYAGYAC	1 cycle of: 48ºC for 15 s 39 cycles of: 54ºC for 30 s 72ºC for 10 s + 1 s after each cycle		500-535 bp	
		dHSP nest2	TYGGGACGACGTTYTCNAC				
		dHSP nest3	SCIGCIGMISWIGGYTCRTT				
		dHSP nest3G	GCGGMGSWGGGPTCRTT				
Ampelovirus Subgroup I	GLRaV-4, -5, -6, -9, -De, -Pr	dHSP-nest2	TYGGGACGACGTTYTCNAC	5cycles of 55°C/30s and 35 cycles of: 59°C /15s 55°C /15S	HSP-70	490bp	Maliogka et al 2008
		LR5 clusdoL	GGYTCRTTCACIACIGCYTGIAC				
GLRaV-4	Specific RT-PCR	LR4up	CCAACTGTCGTGGGTATAAGGAAT		HSP-70	204bp	
		LR4do	CCCAGACACCGGTCCTATACTIA				
GLRaV-5	Specific RT-PCR	LR5up	CTCTGCTTTTCTGCTGGCA		HSP-70	161bp	
		LR5do	TATCTTTTATCTCCCGATAAACGAG				
GLRaV 1	Specific RT-PCR	GSP3 (GLRA1S)	CGTTCGCGTTACCCACGCTGCCTA	55°C	HSP70 gene	150bp	Good and Monis 2001

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
		(GSP4) GLRA1A	GCTGGCAAACCTGGTGGACTTTACATC				
GLRaV-2	Specific RT-PCR	V2dCPf2 sense	ACGGTGTGCTATAGTGCGTG	55°C	Partial coat protein gene	589bp	Bertazzon and Angelini 2004
		V2CPr1 antisense	GCAGCTAAGTACGAATCTTC				
GLRaV-3	Specific RT-PCR	P3U/	CGCTCATGGTGAAAGCAGACG	52°C	HSP70 gene	546bp	Turturo et al 2005
		P3D	CTTAGAACAAAAATATGGAGCAG				
GLRaV-4	Specific RT-PCR	HSP4A	CTCAAACCAGCGGCTGTT	52°C	HSP70 gene	441bp	Routh et al 1998
		HSP4B	GTGATACCATATACATACCGAC				
GLRaV-5	Specific RT-PCR	LR5-1F	CCCGTGATACAAGGTAGGACA	62°C	Partial coat protein gene	690bp	Good and Monis 2001
		LR5-1R	CAGACTTCACCTCCTGTTAC				
GLRaV-9	Specific RT-PCR	LR9-F	CGGCATAAGAAAAGATGGCA	55°C	HSP70 gene	390bp	Alkowni et al 2005
		LR9-R	TCATTCACCACTGCTTGAAC				
GVA	Specific RT-PCR	H587	GACAAATGGCACACTACG	52ºC	Partial coat protein gene	430bp	Minafra et al 1997
		C995	AAGCCTGACCTAGTCATCTTGG				
	Specific RT-PCR	H7038	AGGTCCACGTTTGCTAAG	52ºC	Partial p10 gene	235bp	MacKenzie 1997
		C7273	CATCGTCTGAGGTTTCTACTA				
GVB	Specific RT-PCR	H6980	GTGCTAAGAACGTCTTCACAGC	52ºC	Partial coat protein gene	469 bp	Minafra & Hadidi, 1994; MacKenzie 1997
		C7439	ATCAGCAAACACGCTTGAACCG				
GFkV	Specific RT-PCR	GFkV-U279	TGGTCCTCGGCCCAGTGAAAAAGTA	55ºC	RNA replicase gene	315bp or 415 bp	Shi et al 2003
		GFkV-L630	GGCCAGGTTGTAGTCGGTGTTGTC				
RSPaV	Specific RT-PCR	RSP48	AGCTGGGATTATAAGGGAGGT	52°C	Partial coat protein gene	512bp	Nolasco et al 2000
		RSP49	CCAGCCGTTCCACCACTAAT				
	Specific RT-PCR	Sy9F	AGGATTCCAAACTGTAGAGCAA	56°C	replicase gene	628 bp	Habili et al 2006
		Sy8R	TTGGTCGTCATCTTCCAGTT				
		AtropaNad2.2b	AGCAATGAGATTCCCCAATATCAT				

Table 6.3 List of pathogens tested for, type of PCR test, primers used and annealing temperature, region amplified, expected product size and reference for each test used to detect exotic grapevine pathogens in Australia.

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
DNA House-keeping gene	PCR	FD2 RP1	AGAGTTTGATCATGGCTCAG ACG GTT ACC TTG TTA CGA CTT	55°C	partial bacterial 16S rRNA gene	1400-1500 bp	Weisberg et al 1991
AILV	Specific RT-PCR	AI3 AI4	GGTCTGGGGTGCCCGTGGCG ATTCACTAGTCCCTATTTAG	52ºC	Partial RNA-2	760bp	Minafra et al 1995
CLRV	Specific RT-PCR	CLRV-5 CLRV-3	TGGCGACCGTGTAACGGCA GTCGGAAAGATTACGTAAAAGG	53°C	RNA1 polyprotein gene	416bp	Werner et al 1997
GARSV	Specific RT-PCR	A34-1 A34-2	GAAGATACCCTCACGCATTGCC- CCCAGTGTTTACTCCACACCC	60°C	Partial coat protein	343bp	Gokalp et al 2003
GCMV and TBRV	Specific RT-PCR	P1 P2	ATGGGAGAAGTGCTGG AATCTTTTTGTGTCCAACA	42°C	Partial RNA-2	GCMV 246bp TBRV 332bp	Le Gall et al 1995
GDefV	Specific RT-PCR	N66-1 N66-2	GGTCCTATAACATACGG GGCTG CCCCTGATGGTGCTATTGGTCC-	60°C	Partial coat protein	240bp	Cigsar et al 2003
GFLV and ArMV	Specific RT-PCR	M2 M3	(C/T)T(A/G)GATTTTAGGCTCAATGG;: TG(C/T)AA(A/G)CCAGG(A/G)AAGAAAAT;	42°C	movement protein gene	290bp	Wetzel et al 2002
GLRaV-6	Specific RT-PCR	LR6up LR6do	CGATAGGTTTGGGGGCACT GGCACAGAACACACCGACAAAAC	5cycles at 55°C and 35 cycles at 59°C /15s + 55°C /15S	partial HSP70h sequences	283	Maliogka et al 2008
GLRaV-7	Specific RT-PCR	LR7-F LR7-R	TATATCCCAACGGAGATGGC ATGTTCCTCCACCAAAATCG	55°C	HSP70gene	502bp	Engel et al 2008
Maculavirus – GRGV GAMaV*	Genus specific	Reverse transcription	DNA hexanucleotides		Reverse transcription for cDNA production		Sabanadzovic et al 2000
		RD1 RD2	CYCARCAYAARGTVAACGA GCGCATGCABGTSAGRGGG	58°C	RNA dependent RNA polymerase ORF1	386bp	
	GRGV specific with RD1	RGAP	GGCCAAACTGGGTGCTCACG	58°C	RNA dependent RNA polymerase ORF1	386bp	
	Genus specific	MTR1 MTR2	TTCATGCAYGAYGCYMTSATGT TCCCAVGCNBHBGVRGTGACCCA	55°C	Methyl transferase region ORF1	575–689bp	
GVD	Specific RT-PCR	GVDORF2F1	TAA TAG GGC CTA AGT C	50°C	Movement protein gene	371bp	Terlizzi and Credi 2006

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
		ORF2R1	GGG CGT TGA ATA CAC CTT TAG C				
	Specific RT-PCR	GD1	GTACCTTAGGACGCTCTTCGGG	55°C	RNA binding protein	718bp	Abou-Ghanem et al 1997
		GD2	CGTTGGGTCGAGTGTGAGTACG				
Ilarvirus	Genus specific first PCR	Ilapol up	YTCIAMRTTYGAYAARTC	5 cycles 38°C 35 cyles + 42°C /20s 38°C/10s	polymerase gene on RNA2	381 bp	Maliogka et al 2007
		Ilapol do	GGYTGRTTRTGIGGRAA				
	Genus specific nestedPCR	Ilapolnes up	TCGAMRTTYGAYAARTCICA	5 cycles 42°C 35 cyles + 47°C /20s 43°C/10s		371bp	
		Ilapolnest d	TGGGGRAAYTTIGYYTCRA				
PRMV	Specific RT-PCR	PRMVV1	ÓGTGATGGAATATTTGGAC	60°C	coat protein gene	200bp	Kheder et al 2004
		PRMVC1	ÓCCATTGGCAACCGCCAGAGCTAC				
RsRSV	Specific RT-PCR	RpRSVF1	TGTGTCTGGTTTTGATGCT	61°C	Partial RNA-2	385bp	Ochoa-Corona et al 2006
		RpRSVR1	GAGTGCGATAGGGGCTGTT				
SLRSV	Specific RT-PCR	SLRSV-5D	CCCTTGGTTACTTTTACCTCCTCATTGTCC	55°C	Partial coat protein gene	291bp	Faggioli et al 2002
		SLRSV-3D	AGGCTCAAGAAAACACAC				
Tombus virus: GALV, PAMV	Specific RT-PCR	TomCPF	CCG CCG TAG CAT GAC CAA GTA	55°C	Partial coat protein gene	1000 bp	Russo et al 2002.
		TomCPR	CCA TGA ACT GGT CTT GTT CAA				
	RT-PCR	TBSVGralF1	AAGGGTAAGGATGGTGAGGA	61°C	polymerase; readthrough protein	590bp	Harris et al 2006
		TBSVGralR1	TTTGGTAGGTTGTGGAGTGC				
ToRSV	Specific RT-PCR	U1	GAC GAA GTT ATC AAT GGC AGC	55°C	RNA-1 region	450 bp	Griesbach 1995.
		D1	TCC GTC CAA TCA CGC GAA TA				
	Specific two-step RT-PCR and nested PCR	ToRSVrt	GCGAAAACAACGTCCTTGC	50°C	Reverse transcription		Martin et al 2008
		ToRSVf1	CCGTTAGCAGCTTCCAAAAG	56°C	First PCR	512bp	
		ToRSVr1	GTCCTCATGGAACCTTTCTC				
		ToRSVf2	GGTTATCCAGCCTTAAGCAAG	56°C	Nested PCR	435 bp	
		ToRSVr2	CGGTAGGCTATGACAACTAC				
TRSV	Specific two-step RT-PCR and nested PCR	TRSVr1	GTGTTGGACAAACACGACAC	50°C	Reverse transcription		Martin et al 2008
		TRSVf1	GGAAGCTGTATAAACTCAGC	56°C	First PCR	338bp	
		TRSVr1	GTGTTGGACAAACACGACAC				
		TRSVf2	GAGAGCCCAAGAATGTCTGTC	56°C	Nested PCR	257bp	
		TRSVr2	GAACAGTGGGCTCAAACAAC				

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
Xylophilus ampelinus	Single PCR	S3	GGTGTTAGGCCGAGTAGTGAG	60°C	ITS	277bp	Botha et al 2001
		s4	GGTCTTTCACCTGACGCGTTA				

*GAMaV = Grapevine asteroid mosaic associated virus

The endemic viruses of grapevines for which molecular tests were validated against the survey samples included:

- Six *Closteroviridae* viruses associated with leafroll disease: *Grapevine leafroll associated virus* (GLRaV-) 1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5, GLRaV-9;
- Two Vitivirus species associated with rugose wood diseases: *Grapevine virus A*(GVA) *Grapevine virus B* (GVB);
- One Maculavirus species: Grapevine fleck virus (GFkV);
- One Foveavirus species associated with rugose wood, vein necrosis and Syrah decline diseases: *Rupestris stem pitting associated virus* (RSPaV).

The exotic viruses for which RT-PCR tests were validated included:

- Two *Closteroviridae* viruses associated with leafroll disease: GLRaV-6, GLRaV-7;
- Two Vitivirus species associated with rugose wood diseases: *Grapevine virus B* (GVB): strains associated with corky bark disease; GVD.
- One Maculavirus species: *Grapevine red globe virus* (GRGV);
- 15 nepoviruses: *Arabis mosaic virus* (ArMV), *Artichoke Italian latent virus* (AILV), *Cherry leafroll virus* (CLRV), *Grapevine Anatolian ringspot virus* (GARSV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine deformation virus* (GDefV), *Grapevine fanleaf virus* (GFLV), *Peach rosette mosaic virus* (PRMV), *Raspberry ringspot virus* (RsRSV), *Tobacco ringspot virus* (TRSV), *Tomato black ring virus* (TBRV, includes the strain, Joannes seyve), *Tomato ringspot virus* (ToRSV);
- One Sadwavirus (formerly classified as a *Nepovirus*): *Strawberry latent ringspot virus* (SLRSV);
- Two Tombusviruses; *Grapevine Algerian latent virus* (GALV) and *Petunia asteroid mosaic virus* (PAMV).

Grapevine rootstock stem lesion associated virus and grapevine virus C, which were originally included in the exotic virus list, are now recognised as strains of GLRaV-2 (Meng et al 2005, Masri et al 2006).

We also validated the PCR test for *Xylophilus ampelinus*

Water controls, without nucleic acid in the RT-PCR or PCR mix, were included in each test. After amplification, 10 µ□L 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.

6.2.5 Cloning and sequencing

PCR products that were amplified during the survey and were equal to, or of similar size to, the expected PCR product were either sequenced directly or by cloning, and analysed using BlastN(Altschul et al 1997) to determine their identity (Joyce et al 2006).

Table 6.3 A list of positive control acquired from other researchers, the type of material provided and their origins

Pathogen	Type of material	Origin
GFkV	Total RNA extract of grapevine 5C Teleki 10-48-49 Gm.	Mr P Nicholas SARDI Nuriootpa.
GLRaV-1	Total RNA extract of grapevine Shiraz,	Mr J. Connellan VAMVVIA, Irymple.
GLRaV-2	Total RNA extract of grapevine cv. Chardonnay Penfold 58.	Mr.P Nicholas SARDI Loxton, SA
GLRaV-3	Total RNA extract of grapevine cv. Graciano WA6V7	Mr.P Nicholas SARDI Loxton, SA.
GLRaV-4	Total RNA extract of grapevine cv. Sultana H5	Mr.P Nicholas SARDI Loxton, SA
GLRaV-5	Total RNA extract of grapevine cv. Crimson Seedless	Mr. I. Cameron Department of Agriculture WA.
GLRaV-9	Total RNA extract of grapevine cv. Shiraz,	Waite Campus, University of Adelaide, SA. Dr. N. Habili
RSPaV	Total RNA extract of grapevine cv. Shiraz 1654	Mr.P Nicholas SARDI Loxton, SA
GVA	Total RNA extract of grapevine cv. Grenache 139HT	Mr.P Nicholas SARDI Loxton, SA
GVB	Total RNA extract of grapevine cv. Rupestris St George	Mr J. Connellan VAMVVIA, Irymple.
CLRV	Total RNA extract of grapevine	Mrs. J. Mackie DPI, Knoxfield, Victoria.
Tombusviruses – *Pear latent virus*	partial clone of cDNA encompassing the coat protein ligated into pGEM-T vector	Dr. M. Russo Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italy.
ToRSV	Total RNA extracted from lyophilised infected *Nicotiana benthamiana* from the AGDIA ToRSV ELISA kit	M. Whattam, Australian PEQ, Knoxfield, VIC, Australia
SLRSV	Total RNA extracted from *Narcissus*.	M. Whattam, Australian PEQ, Knoxfield, VIC, Australia
GFLV	Total RNA extracted from grapevine	M. Whattam, Australian PEQ, Knoxfield, VIC, Australia
RSRPV	Partial clone of cDNA of the Coat protein ligated into pCR-4TOPO vector	L. Liefting, MAF, New Zealand
TBRV	Partial clone of cDNA of the Coat protein ligated intopCR-4TOPO vector Total RNA extracted from infected Iris	L. Liefting, MAF, New Zealand, M. Whattam, Australian PEQ, Knoxfield, VIC, Australia
TRSV	Partial clone of cDNA of the Coat protein ligated intopCR-4TOPO vector	L. Liefting, MAF, New Zealand

6.3 Results

During the survey we screened 218 grapevine samples for 33 grapevine viruses and one bacterium. A total of 42 tests were used including the RNA and DNA housekeeping tests to determine nucleic acid quality. The list of samples and the test results are presented in appendix 5. The age of the grapevines that were sampled was between 5 and 141 years old.

6.3.1 Survey results for endemic viruses

Our results indicate that the specific RT-PCR tests for GLRaV-1 (Good and Monis 2001), GLRaV-2 (Bertazzon and Angelini 2004), GLRaV-3 (Turturo et al 2005), GLRaV-4 (Routh et al 1998), GLRaV-5 (Good and Monis 2001), GLRaV -9 (Alkowni et al 2005), GVB (Minafra & Hadidi, 1994; MacKenzie 1997) and GFkV (Shi et al 2003) were reliable and generally specific for the target pathogen as very little non-specific detection of non-target nucleic acid was observed. In our hands, the specific nested PCR tests for GLRaV-4 and GLRaV-5 developed by Maliogka et al (2008) produced many non-specific bands of various size that made interpretation of the PCR results difficult.

The PCR tests for GVA and RSPaV were also generally specific for the target pathogens and very low levels of non-specific amplification of non-viral nucleic acid was observed. Two PCR tests for GVA were assessed during the survey. GVA-specific primer pairs H587/C995 and H7038/C7273 detected GVA in 38% and 29% of samples respectively (Table 6.4). The H7038/C7273 primer pair did not detect GVA in any samples that the H587/C995 did not. Two primer pairs, RSP48/RSP49 and Sy9F/Sy8R, were assessed for RSPaV detection. Both primer pairs detected strains of RSPaV that the other pair did not. The RSP48/RSP49 detected RSPaV in 92% of samples and the Sy9F/Sy8R detected RSPaV in 83% of samples (Table 6.4).

Grapevine leafroll associated viruses were detected in 47% (103/218) of the grapevines that were sampled for this survey (Table 6.4). The specific tests detected GLRaV species in 95/103 (92%) of GLRaV positive samples (Table 6.5). The *Closteroviridae* generic test, which should detect all GLRaV species, generated PCR products of the correct size in 79% (81/103) of GLRaV positive samples (Table 6.5). The *Ampelovirus* subgroup I test, that should detect viruses closely related to GLRaV-4,-5,-6,-9, -Pr and –De, generated PCR products of the correct size in 50% (52/103) of the GLRaV positive samples (Table 6.5). PCR products of the correct size were generated using the *Closteroviridae* generic test and/or the *Ampelovirus* subgroup I test in 8/103 (8%) samples that did not test positive using GLRaV species specific tests (Table 6.5).

Sequencing confirmed the positive results obtained using the generic *Closteroviridae* primers with two samples that also tested positive with specific tests for GLRaV-1 (83-92% sequence similarity) and GLRaV-3 (92% sequence similarity). However the *Closteroviridae* generic test and the *Ampelovirus* subgroup I test can generate products that are not the correct size and

sequencing of several products indicated that these amplicons may be associated with grapevine nucleic acid (data not shown).

Table 6.4. The number (%) of grapevines in which each grapevine virus was detected in each state

State	NSW (35)[1]	QLD (15)	SA (77)	VIC (51)	WA (40)	Total (218)
No Virus	0/35	0/15	0/77	2/51 (4%)	2/40 (5%)	4/218 (2%)
Virus detected	35/35 (100%)	15/15 (100%)	77/77 (100%)	49/51 (96%)	38/40 (95%)	214/218 (98%)
RSPaV – total	35/35 (100%)	15/15 (100%)	73/77 (95%)	49/51 (96%)	34/40 (85%)	206/218 (94%)
RSPaV – RSP48/RSP49	*33/35 (94%)*	*15/15 (100%)*	*71/77 (92%)*	*49/51 (96%)*	*32/40 (80%)*	*200/218 (92%)*
RSPaV – Sy9F/Sy8R	*32/35 (91%)*	*14/15 (93%)*	*62/77 (81%)*	*43/51 (84%)*	*31/40 (78%)*	*182/218 (83%)*
GVA	11/35 (31%)	1/15 (7%)	38/77 (49%)	21/51 (41%)	11/40 (28%)	82/218 (38%)
GVA-H587/C995	*11/35 (31%)*	*1/15 (7%)*	*38/77 (49%)*	*21/51 (41%)*	*11/40 (28%)*	*82/218 (38%)*
GVA - H7038/C7273	*8/35 (23%)*	*0/15*	*26/77 (34%)*	*19/51 (37%)*	*10/40 (25%)*	*63/218 (29%)*
GVB	0/35	0/15	1/77 (1.3%)	1/51 (2%)	0/40	2/218 (0.9%)
GFkV	4/35 (11%)	4/15 (27%)	34/77 (44%)	22/51 (43%)	5/40 (13%)	69/218 (32%)
The total number of grapevines in which GLRaVs were detected[2]	13/35 (37%)	3/15 (20%)	36/77 (47%)	33/51 (65%)	18/40 (45%)	103/218 (47%)
The total number of grapevines in which GLRaVs were detected using specific RT-PCR tests [3]	12/35 (34%)	3/15 (20%)	33/77 (43%)	29/51 (57%)	18/40 (45%)	95/218 (44%)
GLRaV-1	3/35 (9%)	2/15 (13%)	10/77 (13%)	13/51 (25%)	3/40 (8%)	29/218 (13%)
GLRaV-2	2/35 (6%)	1/15 (7%)	7/77 (9%)	4/51 (8%)	2/40 (5%)	14/218 (6%)
GLRaV-3	3/35 (9%)	1/15 (7%)	11/77 (14%)	10/51 (20%)	7/40 (18%)	32/218 (14%)
GLRaV-4	0/35	0/15	1/77 (1.3%)	2/51 (4%)	0/40	3/218 (1.3%)
GLRaV-5	2/35 (6%)	0/15	6/77 (8%)	9/51 (18%)	2/40 (5%)	19/218 (9%)
GLRaV-9	5/35 (14%)	0/15	6/77 (8%)	6/51 (12%)	8/40 (20%)	25/218 (11%)
The number of grapevines in which unknown GLRaV sp. Were detected[4]	1/35 (3%)	0/15	3/77 (4%)	4/51 (8%)	0/40	8/218 (4%)

[1]The number of samples tested in each state
[2]Includes grapevines that tested positive using any GLRaV specific, *Closteroviridae* group or subgroup I test.
[3]The number of grapevines in which GLRaVs were detected using specific primers
[4]Detected using the *Closteroviridae* group or *Ampelovirus* subgroup I tests only.

Closteroviruses were detected in eight samples that were not positive using GLRaV species specific tests. the *Closteroviridae* generic and/or the *Ampelovirus* subgroup I tests, Four of the eight samples tested positive with both the *Closteroviridae* generic test and the *Ampelovirus* subgroup I test, two of the samples tested positive with the *Closteroviridae* generic test only and two samples tested positive with the *Ampelovirus* subgroup I test only (Table 6.5).

Table 6.5. The number (%) of grapevines in each state in which grapevine leafroll viruses were detected using specific and generic RT-PCR.

Virus	NSW	QLD	SA	Vic	WA	Total
Total number of samples in which GLRaVs were detected	13 (13%)	3 (3%)	36 (35%)	33 (32%)	18 (17%)	103
The number of samples in which GLRaV species were detected using specific primers	12/13 (92%)	3/3 (100%)	33/36 (92%)	29/33 (88%)	18/18 (100%)	95/103 (92%)
The number of samples that tested positive with specific primers only	*4/13*	*1/3*	*7/36*	*1/33*	*1/18*	*14/103*
The number of samples in which only one GLRaV sp. was detected	10/13 (77%)	2/3 (67%)	25/36 (69%)	21/33 (64%)	15/18 (83%)	73 (71%)
LR1	*2/10*	*1/2*	*6/25*	*6/21*	*2/15*	17/73
LR2	*1/10*	*0/2*	*4/25*	*2/21*	*2/15*	9/73
LR3	*2/10*	*1/2*	*9/25*	*5/21*	*4/15*	21/73
LR4	*0/10*	*0/2*	*1/25*	*0/21*	*0/15*	1/73
LR5	*1/10*	*0/2*	*3/25*	*4/21*	*0/15*	8/73
LR9	*4/10*	*0/2*	*2/25*	*4/21*	*7/15*	17/73
The number of samples in which two GLRaV sp. were detected	1/13 (8%)	1/3 (33%)	7/36 (19%)	4/33 (12%)	2/18 (11%)	15 (15%)
Combinationsof GLRaV species observed	*No[1]. Sp[2].* *1/1 1+5*	*No. Sp.* *1/1 1+5*	*No. Sp.* *2/7 1+2* *2/7 1+9* *1/7 2+9* *1/7 3+5* *1/7 5+9*	*No. Sp.* *1/4 1+9* *2/4 1+5* *1/4 2+3*	*No. Sp.* *1/2 1+3* *1/2 3+5*	
The number of samples in which three GLRaV sp. were detected.	1/13 (8%)	0/3	1/36 (3%)	1/33 (3%)	1/18 (6%)	4 (4%)
Combinationsof GLRaV species observed	*No. Sp.* *1/1 2+3+9*	*No. Sp.*	*No. Sp.* *1/1 3+5+9*	*No. Sp.* *1/1 1+2+3*	*No. Sp.* *1/1 3+5+9*	
The number of samples in which four GLRaV sp. were detected				3/33		3 (3%)
Combinationsof GLRaV species observed	*No. Sp.*	*No. Sp..*	*No. Sp.*	*No. Sp.* *2/3 1+3+4+5* *1/3 1+3+5+9*	*No. Sp.*	
The number of samples in which GLRaV species were detected using the generic *Closteroviridae* and *Ampelovirus* subgroup I tests	9/13 (69%)	2/3 (67%)	29/36 (81%)	32/33 (97%)	17/18 (94%)	89/103 (86%)
The number of samples in which GLRaV sp were detected using the *Closteroviridae* test	9/13 (69%)	2/3 (67%)	28/36 (78%)	28/33 (85%)	17/18 (94%)	81/103 (37%)
The number of samples in which GLRaV sp were detected using the *Ampelovirus* subgroup I test	8/13 (62%)	0/15	15/36 (42%)	21/33 (41%)	9/18 (23%)	52/103 (24%)
The number of samples in which GLRaV sp were detected using the Closteroviridae group and Ampelovirus subgroup I *only*	*1/13 (8%)*	*0/3*	*2/36 (6%)*	*1/33 (3%)*	*0/18*	*4/103 (4%)*
The number of samples in which GLRaV sp were detected using the Closteroviridae group test only	*0/13*	*0/3*	*0/36*	*2/33 (9%)*	*0/18*	*2/103 (2%)*
The number of samples in which GLRaV sp were detected using the Ampelovirus subgroup I *test only*	*0/13*	*0/3*	*1/36 (3%)*	*1/33 (3%)*	*0/18*	*2/103 (2%)*

1 No = number of samples 2 Sp =grapevine leafroll virus species detected in the samples

PCR products of samples 36 and 139 (Appendix 5), which tested positive with the both *Closteroviridae* and/or Subgroup I generic tests, and of sample 191 (Appendix 5), which tested positive with the *Ampelovirus* subgroup I test only, were cloned and sequenced to determine their identity. Sequence analysis indicated that these three GLRaV strains were most closely related to two strains of GLRaV-De and a GLRaV-5 strain (Table 6.6). Preliminary analysis suggested that samples 191 and 36 had approximately 85% similarity with each other. Sample 139 had 77% similarity with sample 191 and 81% similarity with sample 36. Further work needs to be done to determine the identity of these GLRaV strains.

Table 6.6. Sequence similarity between the GLRaV species detected in samples 36, 139 and 191 and GLRaV-de and GLRaV-5 species that are published on GenBank

Sample ID	GLRaV-De (AM494935)	GLRaV-De (AM745349)	GLRaV-5 (EU815935)	Additional GLRaV-5 strains
139	87%	84%	86%	79-80%
191	80%	79%	83%	less than 77%
36	81%	80%	83%	less than 75%

Sequence analysis of the PCR products of the two additional samples that tested positive with both *Closteroviridae* and or Subgroup I generic tests indicated that one was most closely related to GLRaV-5 (92%) and the isolate most closely related to other GLRaV-9 (92%).

Ampelovirus subgroup I species were detected in 44/103 (43%) of GLRaV positive samples using specific tests for GLRaV-4,-5 and -9 (Table 6.7). The Subgroup 1 generic test detected 52/103 (50%) of samples (Table 6.7). Five of the 44 samples that were positive using specific tests did not test positive with the subgroup I generic test. Sixteen of the 52 samples that tested positive with the subgroup I test did not test positive with the specific tests for GLRaV-4,-5 and -9.

Table 6.7. The number (%) of grapevines in which *Ampelovirus* Subgroup 1 Grapevine leafroll viruses were detected in each state using specific and generic RT-PCR tests

Virus	NSW	QLD	SA	Vic	WA	Total
Total number of samples in which GLRaVs were detected	13 (13%)	3 (3%)	36 (35%)	33 (32%)	18 (17%)	103
The total number of samples in which GLRaVs were detected using the generic *Closteroviridae* and GLRaV I subgroup tests	9/13 (69%)	2/3 (67%)	29/36 (81%)	32/33 (97%)	17/18 (94%)	89/103 (86%)
The number of samples in which GLRaVs were detected using the *Closteroviridae* test	9/13 (69%)	2/3 (67%)	28/36 (78%)	28/33 (85%)	17/18 (94%)	81/103 (37%)
The number of samples in which GLRaVs were detected using the Closteroviridae group primers only	*1/13 (8%)*	*2/3*	*2/36 (6%)*	*3/33 (9%)*	*0/18*	*6/103 (6%)*
The number of samples in which GLRaVs were detected using the GLRaV Subgroup I test	8/13 (62%)	0/15	15/36 (42%)	21/33 (41%)	9/18 (23%)	52/103 (24%)
The number of samples in which GLRaVs were detected using the GLRaV Subgroup I test only	*0/13*	*0/3*	*1/36*	*1/33*	*0/18*	*2/103 (2%)*
The number of samples in which Sub-group 1 viruses were detected using specific tests	7/13 (54%)	0/3	12/36 (33%)	14/33 (42%)	9/17 (53%)	44/103 (43%)
The number of samples in which Sub-group 1 viruses were detected using specific tests only *LR4* *LR5* *LR9*	*2/7* *2*		*2/12* *2*	*1/14* *1*	*0/9*	*5/103*
Subgroup testing positive with Closteroviridae	*8*	*0*	*14*	*17*	*9*	
Total number of samples positive for Subgroup I viruses using specific RT-PCR for GLRaV-4,-5 or -9 or the subgroup I test	10/13 (77%)	0/3	19/36 (53%)	22/33 (67%)	9/17 (53%)	60/103 (58%)

6.3.4 The incidence of mixed and single infections of endemic viruses in Australian grapevines

Viruses were detected in 98% (214/218) of the samples (Table 6.3). Endemic viruses were not detected in 4/218 (2%) of grapevines that were tested. RSPaV was most frequently detected (96%; 206/214) and 72/206 (35%) of the RSPaV infected grapevines were single infections, i.e. they were infected by RSPaV in the absence of the other endemic viruses. Single virus infections were only observed in two additional grapevines, one with GVA and one with GLRaV-1. GVA was detected in 36% of samples (79/218), GFkV was detected in 32% (69/218) of samples, GVB was detected in 0.9% (2/218) of samples and leafroll viruses were detected in 47% (103/218) of samples. GLRaV-3 was the most frequently detected leafroll virus (14%; 32/218 samples) and GLRaV-4 was the least frequently detected leafroll virus (1.3%; 3/218 samples) (Tables 6.4 and 6.5).

A combination of GVA, GVB, GFkV and/or GLRaVs was found in 43% (93/218) of grapevines (Table 6.8). There was no obvious pattern of association in the occurrence of each virus species. Single GLRaV species infections were observed in 71% (73/103) of GLRaV infected grapevines (Table 6.5). Mixed infections with two GLRaV species were observed in 15% (15/103) grapevines, with three GLRaV species in 4% (4/104) of grapevines and with four GLRaV species in 3% (3/103) of grapevines (Table 6.5). There was no obvious pattern of association in GLRaV species occurrence amongst the mixed infections.

Table 6.8. The number of grapevines in which single and mixed infections of viruses were observed.

Virus	NSW (35)*	QLD (15)	SA (77)	VIC (51)	WA (40)	Total (218)
No virus	0/35	0/15	0/77	2/51 (4%)	2/40 (5%)	4/218 (2%)
RSPaV	19/35 (54%)	9/15 (60%)	16/77 (21%)	10/51 (20%)	18/40 (45%)	72/218 (33%)
GVA	0/35	0/15	1/77 (1.3%)	0/51	0/40	1/218 (0.5%)
LR	0/35	0/15	0/77	0/51	1/40 (2.5%)	1/218 (0.5%)
RSPaV+GVA	1/35 (3%)	0/15	7/77 (9%)	3/51 (6%)	1/40 (2.5%)	12/218 (6%)
RSPaV_GFkV	2/35 (6%)	3/15 (20%)	12/77 (16%)	3/51 (6%)	1/40 (2.5%)	22/218 (10%)
RSPaV+ LR*	2/35 (6%)	1/15 (7%)	3/77 (4%)	5/51 (10%)	3/40 (7.5%)	14/218 (6%)
RSPaV+GVA+GFkV	0/35	0/15	5/77 (6%)	0/51	0/40	5/218 (2%)
RSPaV+GVA+LR	9/35 (26%)	1/15 (7%)	12/77 (16%)	10/51 (20%)	10/40 (25%)	42/218 (19%)
RSPaV+LR+GFkV	1/35 (3%)	1/15 (7%)	7/77 (9%)	10/51 (20%)	4/40 (10%)	23/218 (11%)
RSPaV+GVA+GFkV +LR	1/35 (3%)	0/15	10/77 (13%)	7/51 (14%)	0/40	18/218 (8%)
RSPaV+GVA+GVB +LR	0/35	0/15	1/77 (1.3%)	0/51	0/40	1/218 (0.5%)
RSPaV+GVA+ GVB+LR+GFkV	0/35	0/15	0/77	1/51 (2%)	0/40	1/218 (0.5%)
GVA+LR	0/35	0/15	1/77 (1.3%)	0/51	0/40	1/218 (0.5%)
LR+GFkV	0/35	0/15	1/77 (1.3%)	0/51	0/40	1/218 (0.5%)
GVA+GFkV +LR	0/35	0/15	1/77 (1.3%)	0/51	0/40	1/218 (0.5%)

*LR= for one or more leafroll viruses were detected, the breakdown of single and multiple GLRaVs infections is listed in Table 6.4.

6.3.2 AVIA testing

The results of the AVIA testing are given in Table 6.9 and represent testing of two thirds of the AVIA collection for endemic viruses. Seventy samples varieties, representing approximately one third of the nucleus collection were sampled and tested in May and November 2008. An additional 70 varieties were sampled and tested in May and November 2009.

Table 6.9. The number of grapevines from the AVIA collection in which viruses were detected during Autumn and Spring of 2008 and 2009.

State	April 2008	November 2008	May 2009	November 2009
No Virus Virus detected	21	9	7	2
RSPaV – total	49	61	61	68
RSPaV – RSP48/RSP49	*49*	*55*	*60*	*61*
RSPaV – Sy9F/Sy8R	*NT*	*53*	*37*	*53*
GVA	0		0	
GVB	0		0	
GFkV	1	3	0	1
GLRaV-1	0		0	
GLRaV-2	0	1	0	
GLRaV-3	0		0	1
GLRaV-4	0		0	
GLRaV-5	0		0	
GLRaV-9	0		0	

In both years RSPaV was more frequently detected in November (late spring) compared to April or May (autumn). In November 2008 and in May and November 2009, the RSP48/RSP49 primers returned more positive results for RSPaV than the Sy9F/Sy8R primers. However, both primers detected strains of RSPaV that the other pair did not each time they were used.

GFkV was detected in one sample in April 2008 and the same variety tested positive for GFkV in November 2008. The detection of GFKV in this sample had confirmed a questionable result from previous PCR testing conducted in previous years but which had not tested positive for GFkV using all other GFkV-specific tests available at that time. GFkV was detected in two additional samples in November 2008.

In November 2008 GLRaV-2 and GFkV were detected in a sample which had previously tested negative during April 2008 and in previous testing, prior to this project. Each individual grapevine was re-sampled and tested in January 2008 and GLRaV-2 was detected in all four samples. Sequence analysis confirmed that the virus detected was most closely related to GLRaV-2 and it had 96-99% sequence similarity with the rootstock stem lesion strains of GLRaV-2 and 71-77% similarity with other GLRaV-2 strains.

In November 2009 GLRaV-3 was detected in one sample. The sub-samples were individually extracted and re-tested and GLRaV-3 was detected in only one of the four samples. This cultivar had not tested positive for GLRaV-3 in previous testing in May 2009 or prior to this project. GFkV was also detected in one sample during November 2009.

6.3.3 Survey results for exotic viruses

The following specific RT-PCR tests for exotic pathogens were reliable and generally specific for the target organism as very little non-specific detection of non-target nucleic acid were observed for CLRV, GARSV (Gokalp et al 2003), GDefV (Cigsar et al 2003), GLRaV-7 (Engel et al 2008) GVB (Minafra and Hadidi, 1994; MacKenzie 1997), GVD using the primer pair GVDORF2F1 /ORF2R1 (Terlizzi and Credi 2006), RsRSV (Ochoa-Corona et al 2006), SLRSV (Faggioli et al 2002), PRMV (Kheder et al 2004), ToRSV using the primer pair D1/U1 (Griesbach 1995) and *Xylophilus ampelinus* (Botha et al 2001). If bands were observed they were usually faint and not the correct size. The following generic tests for two or more viruses were also reliable and generally specific: Tombusviruses detected using the TomCPF/TomCPR primers (Russo et al 2002) and the TBSVGralF1/ TBSVGralR1 (Harris et al 2006) and GFLV and ArMV detected using the M2/M3 primers (Wetzel et al 2002). The TBSVGralF1/ TBSVGralR1 occasionally generated non-specific PCR products that were not of the correct size and sequence analysis of one of these products indicated that this was associated with grapevine nucleic acid.

The specific RT-PCR for AILV (Minafra et al 1995) and the semi-specific test for GCMV and TBRV (Le Gall et al 1995) generated some faint non-specific bands that were slightly smaller than or similar to the correct size. Sequence analysis indicated that these products were not associated with plant virus nucleic acid and some were associated with grapevine nucleic acid.

The specific nested PCR tests for GLRaV-6 (Maliogka et al 2008), ToRSV and TRSV (Martin et al 2008) produced multiple non-specific bands of various size in many of the individual samples including products similar to the expected size. Multiple non-specific bands were also observed in the first round reactions of the ToRSV and TRSV RT-PCR tests. Additionally the ToRSV PCR test did not detect the positive control that was used.

The specific RT-PCR for GVD detection using the primers GD1/GD2 produced bands of the correct size in some samples that were not positive using the GVDORF2F1 /ORF2R1 primer pair. The PCR products of two samples were sequenced and the analysis indicated that these amplicons had 75% and 69-86% sequence similarity with strains of GVA. There were no significant matches with the equivalent region of GVD. The GVDORF2F1 /ORF2R1 also generated PCR products of the correct size in one sample but a positive result was not obtained with the GD1/GD2 primer pair. Sequence analysis indicated that the PCR product generated by the GVDORF2F1 /ORF2R1 primers had greatest nucleotide sequence similarity (92%) with the coat protein gene of GVD (GenBank accession No. Y07764).

Before testing every sample in the survey with the RD1/RD2 primers these Maculavirus generic primers were tried against six samples that were known to be infected with GFkV. We also assessed the MTR1/MTR2 primers against the same samples. The MTR1/2 primers and the RD1/2 primers detected virus in 3/6 and 1/6 samples respectively indicating that neither pair were reliable for Maculavirus detection. Because there were a large proportion of

grapevines with GFkV (71/218) we did not use the generic Maculavirus primers for Maculavirus detection. Instead we applied the specific primers RD1/RGAP for GRGV detection. No positive results were observed, nor were any non-specific products.

Ilarvirus species that infect grapevines and are associated with specific symptoms are *Grapevine angular mosaic virus* (GAMV) and *Grapevine line pattern virus* (GLPV): the latter is only a tentative member of the *Ilarvirus* genus. To further develop a test for detection of Ilarviruses in grapevine a generic test that should detect all *Ilarvirus* species (Maliogka et al 2007) was applied to all the survey samples. Faint bands of approximately the correct size were generated in 45/218 samples. A subset of these, 12/45 were cloned and sequenced. When the sequence was analysed 5/12 had 80% sequence similarity with *Parietaria mottle Ilarvirus* and 73% sequence similarity with GAMV. There is no sequence available for comparison with GLPV. Sequence analysis of the remaining 7/12 products indicated that the primers were detecting non-specific nucleic acid associated with grapevine.

6.4 Discussion

The principle aim of this study was to determine the reliability and specificity of the tests developed in our laboratory for detection of endemic and exotic pathogens of grapevine. It is critical that the virus specific tests do not genertae non-specific amplicons of non-target nucleic acid. Our results of the survey and from screening part of the AVIA collection indicate that many of tests were reliable and generally specific for the target pathogen as very little non-specific detection of non-target nucleic acid was observed.

Specific RT-PCR tests that were validated and proved to be reliable and specific for endemic viruses include:

- GLRaV-1 using the primer pair GSP3 /GSP4 (Good and Monis 2001),
- GLRaV -2 using the primer pair V2dCPf2 sense/V2CPr1 antisense (Bertazzon and Angelini 2004),
- GLRaV -3 using the primer pair P3U/P3D (Turturo et al 2005),
- GLRaV -4 using the primer pair HSP4A/HSP4B (Routh et al 1998),
- GLRaV -5 using the primer pair LR5-1F/LR5-1R (Good and Monis 2001),
- GLRaV -9 using the primer pair LR9-F/LR9-R (Alkowni et al 2005),
- GVB using the primer pair H6980/C7439 (Minafra & Hadidi, 1994; MacKenzie 1997)
- GFkV using the primer pair GFkV-U279/GFkV-L630 (Shi et al 2003).

Both RSP48/RSP49 (Nolasco et al, 2000) and Sy9F/Sy8R (Habili et al 2006) detected RSpaV strains that the other test did not during the survey and during screening of the AVIA collection. Thus we recommend both PCR tests are used to ensure detection of all known Australian isolates of RSPaV.

The C995/H587 primer pair (Minafra et al 1997) detected 17 strains of GVA that were not detected by the 7038/7273 primer pair (MacKenzie 1997). However, during RT-PCR development, the 7038/7273 primer pair occasionally detected GVA isolates that were not detected by the C995/H587 primer pair (Chapter 2). Therefore we recommend the use of both RT-PCR assays for GVA detection in Australian grapevines.

The *Closteroviridae* generic test was designed to detect all *Closteroviridae* species (Dovas and Katis 2003) and the *Ampelovirus* subgroup I test was designed to detect GLRaV-4,-5,-6,-9, -Pr, -De and other closely related Subgroup I ampeloviruses (Maliogka et al 2008). Our results indicated that the *Closteroviridae* generic and/or the *Ampelovirus* subgroup I tests may not detect all strains of the GLRaV species to which they are designed. However, the *Closteroviridae* generic test detected GLRaV species and strains in 79% (75/95) of the samples in which GLRaV species were detected using specific tests. Sixteen of the 52 samples that tested positive with the *Ampelovirus* subgroup I test did not test positive with the specific tests for GLRaV-4,-5 and -9, however GLRaV-1,-2 and -3 were detected in 13 of these samples. It is possible that the *Ampelovirus* subgroup I primers are cross re-acting with subgroup II (GLRaV-1 and -3) Ampeloviruses or with GLRaV-2 or it may be that these grapevines are infected with subgroup I Ampeloviruses that were not detected using the specific tests.

Sequencing indicated that some Australian grapevines are infected by a previously uncharacterised subgroup I ampelovirus strain or species that was most closely related to GLRaV-De (Maliogka et al 2008a). *Ampelovirus* classification is primarily based on biological traits such as vector, host and symptoms and molecular characteristics such as genome structure and organisation. Amino acid and nucleic acid sequence similarity of the HSP70 and coat protein genes has been used to define an *Ampelovirus* species (Martelli et al 2002; Faquet et al 2005). Where possible, information about the virus particle size and serological specificity are also used. Ampeloviruses within subgroup I show a high degree of variability amongst species and between species (Martelli, 2006; Maliogka et al 2008b) and it has been proposed that the classification be contracted so that each currently recognised and putative species within the group is regarded as a strain of the one species (Martelli 2009). Sequence analysis of the uncharacterised Ampeloviruses identified in this study was based on a small portion of the HSP70 gene and it is possible that the strains that were detected are in fact more distant strains of currently recognised subgroup I ampelovirus species, in particular GLRaV-5 and -9. Further work is required to characterise these strains to confirm their identity and determine their incidence in Australian viticulture.

Because both the Ampelovirus subgroup I and *Closteroviridae* generic tests can detect GLRaV strains that the virus specific tests do not, it is recommended that these assays be used for diagnostic testing of Australian grapevines. However they cannot replace the specific tests because they do not detect all known GLRaV species and strains that are know to infect Australian grapevines. Because of their potential to detect GLRaV-6 and -7, which are not

reported in Australia, we also recommend their use for testing of imported material in post entry quarantine (PEQ). The identity of viruses detected using these tests must be confirmed through sequencing.

Based on our results of this survey of 218 grapevines we recommend the following molecular tests for exotic grapevine pathogens:

- CLRV using the primer pair CLRV-5/CLRV-3 (Werner et al 1997),
- GARSV using the primer pair A34-1/ A34-2 (Gokalp et al 2003),
- GDefV using the primer pair N66-1/ N66-2 (Cigsar et al 2003),
- GFLV and ArMV detected using the M2/M3 primers (Wetzel et al 2002).
- RsRSV using the primer pair (Ochoa-Corona et al 2006),
- SLRSV using the primer pair (Faggioli et al 2002),
- PRMV using the primer pair (Kheder et al 2004),
- ToRSV using the primer pair D1/U1 (Griesbach 1995) and
- GLRaV-7 using the primer pair LR7-F/ LR7-R (Engel et al 2008)
- GRGV using the primer pair RD1/RGAP (Sabanadzovic et al 2000)
- GVB using the primer pair H6980/ C7439 (Minafra & Hadidi, 1994; MacKenzie 1997),
- GVD using the primer pair GVDORF2F1 /ORF2R1 (Terlizzi & Credi 2006),
- Tombusviruses detected using the pairs TomCPF/TomCPR (Russo et al 2002) or TBSVGralF1/ TBSVGralR1 (Harris et al 2006)
- *Xylophilus ampelinus* using the primer pair (Botha et al 2001).

The specific RT-PCR for AILV (Minafra et al 1995) and the semi-specific test for GCMV and TBRV (Le Gall et al 1995) can occasionally generate PCR products that may confuse the interpretation of the results. However, if combined with sequencing to determine the origin of the product these tests could be used with caution for PEQ.

We do not recommend the use of the specific nested PCR tests for GLRaV-6 (Maliogka et al 2008), ToRSV and TRSV (Martin et al 2008) because they produced multiple non-specific bands of various sizes in many of the individual survey samples. As mentioned above the use of the subgroup I and *Closteroviridae* generic tests, could be used with sequencing to identify the virus species for GLRaV-6 detection.

The specific RT-PCR for GVD detection using the primers GD1/GD2 produced bands of the correct size in some samples that were not positive using the GVDORF2F1 /ORF2R1 primer pair. Analysis of the nucleotide sequences of the two PCR products indicated that these two amplicons were 75% and 69-86% similar with strains of GVA. There were no significant

matches with the equivalent region of GVD. The GVDORF2F1 /ORF2R1 also generated PCR products of the correct size in one sample but this was not detected using the GD1/GD2 primer pair. Sequence analysis indicated that this amplicon was 92% similar with the coat protein of one GVD isolate (Genbank accession No. Y07764). This result suggests that GVD is present in Australia. However the identification of this virus was based on a small (376 bp) product that was compared to the only published GVD isolate containing sequence of the coat protein gene and it is possible that variation elsewhere along the genome could indicate that this is a different Vitivirus species. Further work is required to verify that this virus is in fact GVD and to determine the incidence of this virus throughout Australia.

Before testing every sample in the survey the Maculavirus generic primers RD1/RD2 and MTR1/MTR2 primer pairs these were tried against six samples that were known to be infected with GFkV. The MTR1/MTR2 primers and the RD1/RD2 primers detected Maculaviruses in 3/6 and 1/6 samples respectively indicating that neither pair were reliable for Maculavirus detection. Because there were a large proportion of grapevines with GFkV (71/218) we did not use the generic Maculavirus primers for Maculavirus detection. Instead we applied the specific primers RD1/RGAP for GRGV detection. No positive results were observed, nor were any non-specific products.

Although we recommend the use of some molecular tests for Comovirinae species (*Nepovirus* and SLRSV) detection, we cannot recommend a specific test for each Comovirinae species that can infect grapevine, as not all of the tests that were used were reliable and there are no published specific PCR tests for BLMV, GBLV and GTRSV. As mentioned in Chapter 2, there are three published generic test that should detect many Comovirinae species (Digiaro et al 2007; Maliogka et al 2004; Wie and Clover (2008). Unfortunately, in our hands, these primer sets did not work well and we do not recommend the use of these primer sets for generic Comovirinae species detection. However, the Comovirinae PCR (Maliogka et al 2004) and the assays for *Nepovirus* subgroups A and B (Wie and Clover (2008) are being assessed through another DPI project and if successful they will be validated for grapevine. Many Comovirinae species can be detected using commercially available antisera in the form of ELISA kits. But ELISA kits do not exist for all grapevine-infecting Comovirinae species (e.g. GBLV, GTRV, GARMV, GDefV), furthermore the cost of antisera is increasing making these tests more expensive for PEQ use. Therefore ELISA may not be a viable option for Comovirinae species detection in grapevine.

In the meantime most Comovirinae species can be detected using a range of herbaceous indicators that include *Chenopodium quinoa* (BBLMV, PRMV, CLRV, GARMV, GDefV, GFLV, GBLV, GCMV, TBRV, ArMV, ToRSV, TBRV, TRSV, GTRSV), *Chenopodium armaranticolor* (GDeF, GARMV, CLRV, GFLV, RsRSV ToRSV, TBRV), *Cucumis sativus* (AILV, SLRV ToRSV, TBRV) and *Gomphena globosa* (AILV, GFLV, GCMV, GBLV). We recommend a combination of these indicator plants be used for Comovirinae species detection. The PCR tests that we recommend could be used as a support tool to confirm the identity of

Comovirinae species detected using herbaceous indicators or in combination with herbaceous indicators if specific viruses need to be identified quickly. For those Comovirinae species which do not have a specific PCR test, electron microscopy could be used to confirm the presence of spherical particles typical of Comovirinae species if symptoms are observed.

The GVB test that was used in this project detects symptomless and corky bark associated strains of GVB. Currently the only means to identify the corky bark disease is to use the biological indicator LN33. We recommend that biological indexing for corky bark disease continue and that the RT-PCR test be used to confirm the presence or absence of GVB, particularly as chip bud inoculation to biological indicators may not be 100% efficient (Chapter 4).

None of the 218 samples screened in the national survey tested positive for AILV, ArMV, CLRV, GARSV, GCMV, GDefV, GFLV, GLRaV-6, GLRaV-7, GRGV, RsRSV, SLRSV, PRMV, TBRV, ToRSV, Tombusviruses or *Xylophilus ampelinus* indicating that mainland Australia remains free of some of the most significant viral and bacterial grapevine pathogens. Generic tests to detect species within groups, genera or families including a generic test to detect viruses within the family *Closteroviridae*, which includes GLRaV species, and a generic test to detect GLRaVs within subgroup I of the *Ampelovirus* genus of the family *Closteroviridae* were used to screen the 218 samples. As mentioned above, the generic *Closteroviridae* test and the *Ampelovirus* subgroup I test detected some *Closteroviridae* species that need further characterisation and initial sequence analysis suggested these were were not GLRaV-6 or-7.

A generic *Ilarvirus* test was also used to screen the 218 samples. The *Ilarvirus* test detected a putative virus in several samples that may be previously uncharacterised. The putative virus that was detected was most closely related to *Parietaria mottle Ilarvirus* and less closely related to GAMV. GLPV is an *Ilarvirus* that infects grapevines in Hungary and infects very few cultivars (Lehoczky et al 1989, Lehoczky et al 1992,). Thus it seems unlikely that the virus we detected is GLPV however there is no sequence information available for comparison. Further work needs to be done to characterise the virus that was detected and determine its incidence in Australian viticulture. Because some of the PCR products generated by the generic *Ilarvirus* test were approximately the correct size but were associated with grapevine nucleic acid this test cannot be routinely applied for generic detection of Ilarviruses in grapevine unless the ampicons are sequenced for verification of *Ilarvirus* presence.

The results show that some endemic viruses are prevalent in Australian viticulture. RSPaV infected most (94%) of grapevines that were tested. Similar observations have been previously reported (Habili et al 2000). The reason for the high incidence of this virus in Australian viticulture is unknown, but several possibilities for transmission and spread do exist.

Rupestris stem pitting disease was first described in the 1970's and biological indexing for the disease identification was introduced in the 1980's (Goheen, 1988; Assam et al 1991). More recently molecular techniques for RSPaV detection have been adopted (Zhang et al 1998;

Nolasco et al 2000; Habili et al 2006). The virus is transmitted through propagation, grafting and seed (Meng et al 1999; Lima et al 2006).

It may be that RSPaV has been present in Australia for many years in many varieties, prior to the introduction of detection techniques, and it has been successfully and efficiently transmitted by propagation and grafting over time. It might also be that prior to the introduction of molecular detection, many RSPaV strains went undetected because they are symptomless on the biological indicator Rupestris St George or transmission to the indictor failed. Although RSPaV can be detected in pollen, pollen transmission is not proven (Lima et al 2006) but if possible then this might also explain the high incidence observed. No vector is known for RSPaV, but it is possible that an efficient vector exists in Australia for this virus.

It is important to note that RSPaV is difficult to eliminate from a grapevine through heat therapy and meristem tip culture for virus eradication (Gribaudo et al 2006). This observation is likely to explain the high incidence of this virus in the AVIA collection, even in heat treated varieties and clones.

Leafroll viruses, GVA and GFkV were also prevalent in grapevines tested in this survey and were detected in 47%, 36% and 32% of samples tested respectively. The majority of samples for this survey came from uncertified sources, and could explain the high incidence of the viruses. This is supported by the low incidence, except for RSPaV, of viruses detected in the AVIA material.

One of the aims of certification programs in most countries is to control the spread of viruses in planting material. GLRaV -1, -3, -4, -5 and -9, GVA and GVB are spread naturally by mealy bug and scale in other countries and natural spread of GLRaV-1, -3 and -9 has been observed in Australia (Habili et al 2003; Habili and Nutter 1997). Some of the vectors that occur overseas also occur in Australian vineyards and could account for this natural spread. A vector for GFkV has not been reported although some evidence of natural field spread exists (Engelbrecht and Kasdorf 1990; Fortusini et al 1996). It is possible that the incidence of GLRaVs, GVA and GFkV in Australian vineyards is also associated with natural field spread.

Grapevine leafroll associated virus 2, GLRaV-3 and GFkV were detected in a few AVIA grapevines and GVA was detected in Viognier HTK mother vines of the VAMVVIA collection during the development of the GVA RT-PCR assay (Chapter 2). Planting material for these collections was selected because viruses had not been detected by biological indexing, ELISA and PCR techniques. It is possible that these were recent infection events since the development of the collections. However it is possible that these viruses are symptomless or mild strains which remained undetected during previous testing, including biological indexing, ELISA and PCR, even when best available detection techniques were used at the time. This hypothesis is supported by the high sequence similarity between the GVA strain infecting the Viognier HTK clone and the GVA detected in the original source material that had not been treated (Chapter 2). This observation highlights the need to update diagnostic protocols as new information about species variants and new tests become available.

Our findings, as presented in Chapter 3, have shown that testing of material in autumn to be less reliable than earlier testing with green tissue and a slightly different extraction procedure. Screening of the AVIA collection provided us with a unique opportunity to further validate our extraction and sampling protocols and verified that late spring and early summer are appropriate for grapevine virus detection. The results, particularly the late spring detection of GFkV, GLRaV-2 and GLRaV-3 in some samples and RSPaV in a larger number of grapevines compared to autumn, supported our suggestion that testing in autumn (April and May) can lead to false negative results and that testing was more reliable earlier in the season. The detection of GLRaV-3 in one grapevine in a pooled sample of four verified that grapevine samples can be pooled and virus can still be accurately detected even if only one grapevine is infected, using our extraction techniques.

Virus combinations can be associated with more severe effects on grapevine performance than single infections in grapevines (Komar et al 2006). Interestingly leafroll viruses were more commonly detected as single species infections rather than two or more GLRaVs found together and this may be due to a “natural” selection for less diseased grapevine material by growers. We observed that combined infections between GVA, GFkV and/or GLRaVs occurred more frequently (38%) than those viruses occurring individually (23%) with or without RSPaV. GVA and GLRaV species occurred together more frequently than either virus type with GFkV, but there was no pattern of association between the type of GLRaV species and GVA (data not shown). It has been suggested that GVA can act as a helper virus in the insect transmission of GLRaV-1 and -3 (Fortunisini et la 1997; Zorloni et al 2006) and this could explain the higher incidence of GLRaVs and GVA together compared to the individual infection of each virus species.

As no samples were obtained from Tasmania, we cannot speculate on the disease status of exotic or endemic viruses and area freedom of this state.

6.5 Conclusions

The purpose of the survey was two-fold: (i) to test protocols under “local” conditions and identify any potential “false positives” or organisms that can make interpretation of results difficult and (ii) to update the disease status for each pathogen.

We have validated RT-CPR tests for ten endemic viruses including GLRaV-1, -2, -3, -4, -5, -9, GVA, GVB, GFkV and RSPaV. We have also validated sampling procedures that improve the timing of testing of these viruses by RT-PCR to reduce the risk of false negative results. Our research will provide industry with greater ability to interpret virus RT-PCR testing results and improve confidence in the molecular testing regimes for grapevine virus detection. The sampling protocols, extraction procedure and tests developed in this project will provide Australian certification programs with up-to-date and improved diagnostic capabilities that are internationally accepted. The tests will benefit industry through the production of high health material that has been reliably tested for the pathogens that have a significant negative impact on the quality of fruit for wine production. These tests do not replace and should be

used in conjunction with biological indexing to improve the chance of virus detection especially for new varieties and clones being incorporated into any nuclear collection.

It is possible that strains of viruses exist in Australian grapevines that could not be detected with any of the tests we developed. As such it is vitally important that new tests continue to be assessed as they come to hand and that current molecular tests are validated against new strains of target pathogens as more sequence information becomes available.

Since the inception of this project, and particularly since 2007, many new PCR assays have been developed that may be better than the tests suggested here. However, the validation of new tests could take an additional 1-2 years and is therefore out of the scope of this project.

Diagnostic tests were validated for AILV, ArMV, CLRV, GARSV, GCMV, GDefV, GFLV, GLRaV-6, GLRaV-7, GRGV, RsRSV, SLRSV, PRMV, TBRV, ToRSV, *Tombusvirus* species and *Xylophilus ampelinus*. We also validated generic tests for the detection of *Closteroviridae* species and *Ilarvirus* species that could also be used for virus detection in PEQ, especially for GLRaV-6 and -7 and for GAMV and GLPV. The development of these tests has improved preparedness in case of an incursion of any of these grapevine pathogens. These tests will not replace biological and herbaceous indexing for grapevine viruses in PEQ but could be used during PEQ as active testing for specific pathogens of concern or to support the results of biological indexing and confirm the identity of pathogens. Active testing could reduce the cost of importation to growers if a quarantine pathogen is detected early during the PEQ process.

We have identified three putative viruses of grapevines that need further characterisation and these include a strain of GVD, a previously uncharacterised *Ilarvirus* and an uncharacterised subgroup I *Ampelovirus*. If these putative viruses are real their incidence in Australian vineyards and their impact on vine health needs to be determined and active testing may need to be incorporated into Australian certification programs.

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

The aim of this project was to provide the Australian viticulture industry with a world's best practice diagnostic capability for the detection of endemic and exotic grapevine viruses and bacteria by researching the following key areas:

1) Investigate the specificity and reliability of the molecular (PCR) protocols for the detection of grapevine viruses.

2) Identify diagnostic protocols for the detection of a range of endemic pathogens. Specifically:

   - understand sampling of grapevines for reliable detection.
   - investigate the sensitivity of the serological and molecular tests that are currently used for virus detection.

3) Identify and adopt best practice molecular protocols for the high risk pathogens that remain on the quarantine list with the aim of reducing the time newly imported clones and varieties spend in PEQ.

4) In collaboration with AQIS, AVIA, SAVII and FPS, investigate and document efficient protocols for woody indexing of grapevine viruses.

5) Validate the above serological and molecular protocols by surveying key grape growing regions in Australia for the identified pathogens. The survey information, including the sampling and diagnostic protocols, will be published in international peer reviewed journals so that they can serve as the standard protocols for Australian diagnostic laboratories and which are accepted worldwide. The diagnostic protocols developed for endemic virus testing will be further validated through screening of the AVIA Nuclear collection.

We have identified and validated RT-PCR tests for endemic viruses including GLRaV-1, -2, -3, -4, -5, -9, GVA (two tests required), GVB, GFkV and RSPaV (two tests required) (Table7.1). Diagnostic tests were also validated for the following exotic pathogens: GLRaV-6, GLRaV-7, *Arabis mosaic virus* (ArMV), *Artichoke Italian latent virus* (AILV), *Cherry leafroll virus* (CLRV), *Grapevine Anatolian ringspot virus* (GARSV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine deformation virus* (GDefV), *Grapevine fanleaf virus* (GFLV), *Peach rosette mosaic virus* (PRMV), *Raspberry ringspot virus* (RsRPV), *Tomato black ring virus* (TBRV), *Tomato ringspot virus* (ToRSV), *Strawberry latent ringspot virus* (SLRSV), *Grapevine Algerian latent virus* (GALV), *Petunia asteroid mosaic virus* (PAMV) and *Xylophilus ampelinus* (Table 7.2).

We recommend the use of generic tests for the detection of *Closteroviridae* species and *Ampleovirus* subgroup I species (Table 7.1) to support the detection of Grapevine leafroll associated virus species and strains that may not be detected using the specific RT-PCR

tests. These tests should also detected GLRaV-6, which does not have a validated specific test. A generic test for *Ilarvirus* species was also validated and could also be used for detection of GAMV and GLPV. Amplicons generated using the *Closteroviridae* species, *Ampleovirus* subgroup I species and *Ilarvirus* tests will require sequencing for virus identification.

A high throughput nucleic acid extraction procedure using green tissue (petioles and leaf midribs) was developed and validated for grapevines and should reduce the cost of RT-PCR for grapevine virus detection. This method was developed for the QIAxtractor, which can simultaneously extract up to 96 samples. If this equipment is not available we recommend the use of the protocol described by MacKenzie et al (1997). The nucleic acid from the QIAextractor is more dilute than that extracted using the RNeasy procedure.

- **We recommend using 2μl of 200μl extract from the QIAxtractor or 1μl of 50μl RNA from the RNeasy procedure in a 20 μl RT-PCR.**

The QIAxtractor generates total nucleic acid (DNA and RNA) and if a different method is required for DNA for detection of bacteria including *X. ampleinus*, *Xylella fastidiosa* and phytoplasmas then we recommend the method described by Green et al (1999) and 1μl of DNA from this procedure can be used in a 20 μl PCR reaction. This method is well validated for grapevines and other plant species. It can be used for detection of phytoplasmas and *X. fastidiosa* (Constable et al 2003; Constable et al 2007).

Sampling procedures were also developed for detection of endemic grapevine viruses by RT-PCR and ELISA and we recommend:

- **Sampling of four shoots per vine, including leaves with petioles**
- **Sampling between December and March in each growing season.**
- **Up to five samples can be pooled for grapevine virus detection using RT-PCR during spring and summer to enable testing of more samples at a reduced cost to industry.**

It can take more than 12 months for viruses to move from the point of infection to other parts of the grapevine that are used for virus detection such as shoots and cordons.

- **We recommend continued and active surveillance using RT-PCR for diagnostic testing of grapevines within certification schemes to improve the probability of detection of grapevine viruses.**
- **We recommend that biological indexing for virus on woody indicators be carried out for a minimum of three years.**

The results of this project suggest that biological indexing, ELISA and PCR techniques may not detect all strains of a virus and that all detection methods can be affected by virus distribution and/or titre and environment:

- **We recommend that biological indexing onto woody and herbaceous indicators continue to be used in combination with RT-PCR techniques to increase the chance of detection of grapevine viruses, particularly for the varieties and clones that are being introduced into certification programs and during PEQ for new importations.**
- **If symptoms are observed on biological indicators it is recommended that virus-specific tests are conducted to identify viruses associated with the observed symptoms.**

We have provided the Australian viticulture industry with reliable diagnostic protocols for pathogens that affect vineyard sustainability and productivity. The results of this project will improve industry confidence in the diagnostic testing for grapevine pathogens and facilitate the interpretation of diagnostics testing results. An improved ability to test for endemic pathogens, as a part of a certification scheme for the provision of high health material and exotic pathogens, to assist PEQ, will lower exposure to pest and disease risk for the viticultural industry. They will also benefit industry through the production of high health material that has been reliably tested for the pathogens that have a significant negative impact on the quality of fruit for wine production. The development of tests for exotic pathogens has improved preparedness in case of an incursion.

We were able to update the disease status for a number of exotic grapevine viruses including GLRaV-6, GLRaV-7, *ArMV, AILV*, *CLRV*, *GARSV*, *GCMV*, *GDefV*, *GFLV*, *PAMV, PRMV*, *RsRSV*, *TBRV*, *ToRSV, SLRSV, GALV* and *X. ampelinus*.

- **Australian grapevines appear to be free of some of the significant grapevine viruses and *X. ampelinus*, which can impact of production and quality.**

We have identified three putative viruses of Australian grapevines that need further characterisation and include a strain of GVD, a previously uncharacterised *Ilarvirus* and an uncharacterised subgroup I *Ampleovirus*. If these putative viruses are real their incidence in Australian vineyards and their impact on vine health needs to be determined and active testing may need to be incorporated into Australian certification programs.

Table 7.1 List of recommended PCR tests for endemic grapevine viruses in Australia including primers, annealing temperature, region amplified, expected product size and reference for each test.

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
DNA House-keeping gene	PCR	FD2	AGAGTTTGATCATGGCTCAG	55°C	partial bacterial 16S rRNA gene	1400-1500 bp	Weisberg *et al.*, 1991
		RP1	ACG GTT ACC TTG TTA CGA CTT				
RNA House-keeping gene	RT-PCR	AtropaNad2.1a	GGACTCCTGACGTATACGAAGGATC	55ºC	NADH dehydrogenase ND2 subunit) messenger ribonucleic acid	188 bp	Thompson et al 2003
		AtropaNad2.2b	AGCAATGAGATTCCCCAATATCAT				

Pathogen		Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
Section .03	*Closteroviridae*	Grapevine leafroll viruses first round	dHSP up1	GGIHTIGAITTYGGIACIACITT	5 cycles of: 43ºC for 10 s 38ºC for 5s 35 cycles of: 43ºC for 30 s OR for the Ampelovirus Subgroup I nested PCR: 10 cycles of: 38ºC for 30s 30 cycles of: 49ºC for 10 s 44ºC for 10 s 38ºC for 15 s	HSP-70	580-620 bp	Dovas & Katis 2003
			dHSP up1G	AGTTYGGGACGACGTT				
			dHSP do2	GTICCICCICCNAARTC				
			dHSP do2C	GTICCICCCCCNAARTC				
		Grapevine leafroll viruses first nested	dHSP nest1	TTYGGGACGACGTTYAGYAC	1 cycle of: 48ºC for 15 s 39 cycles of: 54ºC for 30 s 72ºC for 10 s + 1 s after each cycle		500-535 bp	
			dHSP nest2	TYGGGACGACGTTYTCNAC				
			dHSP nest3	SCIGCIGMISWIGGYTCRTT				
			dHSP nest3G	GCGGMGSWGGGPTCRTT				
Section .04 Subgroup I	*Ampelovirus*	GLRaV-4, -5, -6, -9, -De, -Pr	dHSP-nest2	TYGGGACGACGTTYTCNAC	5cycles of 55°C/30s and 35 cycles of: 59°C /15s 55°C /15S	HSP-70	490bp	Maliogka et al 2008
			LR5 clusdoL	GGYTCRTTCACIACIGCYTGIAC				

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
Section .05 GLRaV 1	Specific RT-PCR	GSP3 (GLRA1S)	CGTTCGCGTTACCCACGCTGCCTA	55°C	HSP70 gene	150bp	Good and Monis 2001
		(GSP4) GLRA1A	GCTGGCAAACCTGGTGGACTTTACATC				
Section .06 GLRaV-2	Specific RT-PCR	V2dCPf2 sense	ACGGTGTGCTATAGTGCGTG	55°C	Partial coat protein gene	589bp	Bertazzon and Angelini 2004
		V2CPr1 antisense	GCAGCTAAGTACGAATCTTC				
Section .07 GLRaV-3	Specific RT-PCR	P3U/	CGCTCATGGTGAAAGCAGACG	52°C	HSP70 gene	546bp	Turturo et al 2005
		P3D	CTTAGAACAAAAATATGGAGCAG				
Section .08 GLRaV-4	Specific RT-PCR	HSP4A	CTCAAACCAGCGGCTGTT	52°C	HSP70 gene	441bp	Routh et al 1998
		HSP4B	GTGATACCATATACATACCGAC				
Section .09 GLRaV-5	Specific RT-PCR	LR5-1F	CCCGTGATACAAGGTAGGACA	62°C	Partial coat protein gene	690bp	Good and Monis 2001
		LR5-1R	CAGACTTCACCTCCTGTTAC				
Section .10 GLRaV-9	Specific RT-PCR	LR9-F	CGGCATAAGAAAAGATGGCA	55°C	HSP70 gene	390bp	Alkowni et al 2005
		LR9-R	TCATTCACCACTGCTTGAAC				
GVA	Specific RT-PCR	H587	GACAAATGGCACACTACG	52ºC	Partial coat protein gene	430bp	Minafra et al 1997
		C995	AAGCCTGACCTAGTCATCTTGG				
	Specific RT-PCR	H7038	AGGTCCACGTTTGCTAAG	52ºC	Partial p10 gene	235bp	MacKenzie 1997
		C7273	CATCGTCTGAGGTTTCTACTA				
GVB	Specific RT-PCR	H6980	GTGCTAAGAACGTCTTCACAGC	52ºC	Partial coat protein gene	469 bp	Minafra & Hadidi, 1994; MacKenzie 1997
		C7439	ATCAGCAAACACGCTTGAACCG				
GFkV	Specific RT-PCR	GFkV-U279	TGGTCCTCGGCCCAGTGAAAAAGTA	55ºC	RNA replicase gene	315bp or 415 bp	Shi et al 2003
		GFkV-L630	GGCCAGGTTGTAGTCGGTGTTGTC				

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
Section .11 RSPaV	Specific RT-PCR	RSP48	AGCTGGGATTATAAGGGAGGT	52°C	Partial coat protein gene	512bp	Nolasco et al 2000
		RSP49	CCAGCCGTTCCACCACTAAT				
	Specific RT-PCR	Sy9F	AGGATTCCAAACTGTAGAGCAA	56°C	replicase gene	628 bp	Habili et al 2006
		Sy8R	TTGGTCGTCATCTTCCAGTT				
		AtropaNad2.2b	AGCAATGAGATTCCCCAATATCAT				

Table 7.2 List of recommended tests for detection of exotic grapevine pathogens in Australia including the type of PCR test, primers used, annealing temperature, region amplified, expected product size and reference for each test.

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
AILV	Specific one step RT-PCR	AI3	GGTCTGGGGTGCCCGTGGCG	52ºC	Partial RNA-2	760bp	Minafra et al 1995
		AI4	ATTCACTAGTCCCTATTTAG				
CLRV	Specific one step RT-PCR	CLRV-5	TGGCGACCGTGTAACGGCA	53°C	RNA1 polyprotein gene	416bp	Werner et al 1997
		CLRV-3	GTCGGAAAGATTACGTAAAAGG				
GARSV	Specific one step RT-PCR	A34-1	GAAGATACCCTCACGCATTGCC-	60°C	Partial coat protein	343bp	Gokalp et al 2003
		A34-2	CCCAGTGTTTACTCCACACCC				
GCMV and TBRV	Specific one step RT-PCR	P1	ATGGGAGAAGTGCTGG	42°C	Partial RNA-2	GCMV 246bp TBRV 332bp	Le Gall et al 1995
		P2	AATCTTTTTGTGTCCAACA				
GDefV	Specific one step RT-PCR	N66-1	GGTCCTATAACATACGG GGCTG	60°C	Partial coat protein	240bp	Cigsar et al 2003
		N66-2	CCCCTGATGGTGCTATTGGTCC-				

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
Section .12 GFLV and ArMV	Specific one step RT-PCR	M2	(C/T)T(A/G)GATTTTAGGCTCAATGG;:	42°C	movement protein gene	290bp	Wetzel et al 2002
		M3	TG(C/T)AA(A/G)CCAGG(A/G)AAGAAAAT;				
GLRaV-7	Specific one step RT-PCR	LR7-F	TATATCCCAACGGAGATGGC	55°C	HSP70gene	502bp	Engel et al 2008
		LR7-R	ATGTTCCTCCACCAAAATCG				
GRGV	Specific two step RT-PCR	Reverse transcription	DNA hexanucleotides		Reverse transcription for cDNA production	386bp 386bp	Sabanadzovic et al 2000
		RD1	CYCARCAYAARGTVAACGA	58°C	RNA dependent RNA polymerase ORF1		
		RGAP	GGCCAAACTGGGTGCTCACG	58°C			
GVD	Specific one step RT-PCR	GVDORF2F1	TAA TAG GGC CTA AGT C	50°C	Movement protein gene	371bp	Terlizzi and Credi 2006
		ORF2R1	GGG CGT TGA ATA CAC CTT TAG C				
Ilarvirus	Genus specific first PCR	Ilapol up	YTCIAMRTTYGAYAARTC	5 cycles of 38°C 35 cylesof: 42°C /20s 38°C/10s	polymerase gene on RNA2	381 bp	Maliogka et al 2007
		Ilapol do	GGYTGRTTRTGIGGRAA				
	Genus specific nestedPCR	Ilapolnes up	TCGAMRTTYGAYAARTCICA	5 cycles of 42°C 35 cyles of: 47°C /20s 43°C/10s		371bp	
		Ilapolnest d	TGGGGRAAYTTIGYYTCRA				
PRMV	Specific RT-PCR	PRMVV1	óGTGATGGAATATTTGGAC	60°C	coat protein gene	200bp	Kheder et al 2004
		PRMVC1	óCCATTGGCAACCGCCAGAGCTAC				
RsRSV	Specific RT-PCR	RpRSVF1	TGTGTCTGGTTTTGATGCT	61°C	Partial RNA-2	385bp	Ochoa-Corona et al 2006
		RpRSVR1	GAGTGCGATAGGGGCTGTT				

Pathogen	Test	Primer name	Primer sequence (5'-3')	Tm	Region amplified	Expected product size	Reference
SLRSV	Specific RT-PCR	SLRSV-5D	CCCTTGGTTACTTTTACCTCCTCATTGTCC	55°C	Partial coat protein gene	291bp	Faggioli et al 2002
		SLRSV-3D	AGGCTCAAGAAAACACAC				
Tombus virus: GALV, PAMV	Specific RT-PCR	TomCPF	CCG CCG TAG CAT GAC CAA GTA	55°C	Partial coat protein gene	1000 bp	Russo et al 2002.
		TomCPR	CCA TGA ACT GGT CTT GTT CAA				
	RT-PCR	TBSVGralF1	AAGGGTAAGGATGGTGAGGA	61°C	polymerase; readthrough protein	590bp	Harris et al 2006
		TBSVGralR1	TTTGGTAGGTTGTGGAGTGC				
ToRSV	Specific RT-PCR	U1	GAC GAA GTT ATC AAT GGC AGC	55°C	RNA-1 region	450 bp	Griesbach 1995.
		D1	TCC GTC CAA TCA CGC GAA TA				
Section .13 Xylophilus ampelinus	Single PCR	S3	GGTGTTAGGCCGAGTAGTGAG	60°C	ITS	277bp	Botha et al 2001
		S4	GGTCTTTCACCTGACGCGTTA				

Appendix 1: Communication:

Presentations:

Dr Constable attended the Australian Vine Improvement Association annual general meetings in 2006, 2007 and 2008 and presented the ongoing results of this project.

Constable, F.E., Nicholas, P., Nancarrow, N. and Rodoni B.C. 2009. A survey for endemic and exotic viruses of Australian grapevines. *16th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Dijon, France 30 August-4 September 2009*

Constable, F.E., Nicholas, P., Nancarrow, N. and Rodoni B.C. 2009. Development and validation of sampling strategies for the detection of endemic viruses of Australian grapevines. *16th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine Dijon, France 30 August-4 September 2009*

Constable, F.E., Nicholas, P. and Rodoni B.C. 2008. Development and validation of diagnostic protocols for the detection of endemic and exotic pathogens of grapevines.*8th Australasian Plant Virology Workshop, Rotorua, New Zealand*

Constable, F.E., Nicholas, P., Connellan, J., Bass, T., Habili, N and Rodoni B.C. 2007.Characterisation of Australian vitiviruses not associated with diseases of the rugose wood complex. *9th International Congress of Plant Pathology, Torino, Italy, August 2008.*

Constable, F.E., Nicholas, P., Connellan, J., and Rodoni B.C. 2008. Development and validation of diagnostic protocols for the detection of endemic pathogens of Australian grapevines. *Australian Society for Viticulture and Oenology Seminar series: Breaking the Mould – a pest and disease update, Mildura, Victoria. 2008.*

Constable, F.E., Nicholas, P., Connellan, J., Bass, T., Habili, N and Rodoni B.C. 2007. Development and validation of diagnostic protocols for the detection of endemic pathogens of Australian grapevines. *1st National Viticulture Research Conference, Davis, California, USA, 18th -20th July 2007.*

Published papers:

Constable FE. 2009. Phytoplasma epidemiology: Grapevines as a model. In. PG Weintraub and P. Jones (Eds), Phytoplasmas genomes, plant hosts and vectors, CABI, Wallingford, UK.

Constable, F.E., Nicholas, P., Connellan, J., and Rodoni B.C. 2008. Development and validation of diagnostic protocols for the detection of endemic pathogens of Australian grapevines. In: Australian Society for Viticulture and Oenology Seminar proceedings: Breaking the Mould – a pest and disease update, Mildura, Victoria. 2008

Papers in preparation:

- A Review of Grapevine Viruses and Bacteria and their Relation to Australian Viticulture
- Sampling Stratgies for Reliable Grapevine Virus Detection in Australia.
- The Reliability of Biological Indexing for Virus Associated Diseases of Grapevine in a Cool Climate and a Warm Climate in Australia
- Survey for Exotic and Endemic Pathogens of Grapevines in Australia

Appendix 2: Intellectual Property:

No Intellectual Property has arisen from this project. All results from this project will be made public to ensure transparency and understanding of the protocols developed

Appendix 3 References

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Appendix 4: Staff List all staff that have been engaged on the Project.

Name	Title	Position	Qualification	Agency	% of time to be spent on the project
1. Brendan Rodoni	Dr.	Project Supervisor	PhD	DPI	20%
2.Fiona Constable	Dr.	Chief Investigator	PhD	DPI	100%
3.Phil Nicholas	Mr	Other staff		SARDI	10%
4. Tony Bass	Mr.	Other staff		SARDI	10%

APPENDIX 5: Molecular protocols for the detection of grapevine pathogens

RNA Extraction Protocol Using MacKenzie Buffer and the QIAxtractor (Qiagen)

RNA Extraction Protocol Using MacKenzie Buffer and the RNeasy® Plant Mini Kit (Qiagen)

DNA extraction procedure using the QIAGEN DNeasy® Plant mini kit

Molecular detection of NADH dehydrogenase ND2 subunit (ndhB gene) mRNA using one-step RT-PCR

Molecular detection of 16S ribosomal DNA using PCR

Molecular detection of *Ampleovirus* subgroup I species using nested PCR

Molecular detection of *Artichoke Italian latent virus* using one step RT-PCR

Molecular detection of *Cherry leafroll virus* using one step RT-PCR

Molecular detection of generic *Grapevine leafroll associated virus* species using *Closteroviridae* nested PCR

Molecular detection of *Grapevine leafroll associated virus 1* using one step RT-PCR

Molecular detection of *Grapevine leafroll associated virus 2* using one step RT-PCR

Molecular detection of *Grapevine leafroll associated virus 3* using one step RT-PCR

Molecular detection of *Grapevine leafroll associated virus 4* using one step RT-PCR

Molecular detection of *Grapevine leafroll associated virus 5* using one step RT-PCR

Molecular detection of *Grapevine leafroll associated virus 7* using one step RT-PCR

Molecular detection of *Grapevine leafroll associated virus 9* using one step RT-PCR

Molecular detection of *Grapevine Anatolian ringspot virus* using one step RT-PCR

Molecular detection of *Grapevine chrome mosaic virus* and *Tomato black ring virus* using one step RT-PCR

Molecular detection of *Grapevine deformation virus* using one step RT-PCR

Molecular detection of *Grapevine fanleaf virus* and *Arabis mosaic virus* using one step RT-PCR

Molecular detection of *Grapevine fleck virus* using one step RT-PCR

Molecular detection of *Grapevine virus A* p10 gene using one step RT-PCR

Molecular detection of *Grapevine virus A* coat protein gene using one step RT-PCR

Molecular detection of *Grapevine virus B* using one step RT-PCR

Molecular detection of *Grapevine virus D* using one step RT-PCR

Molecular detection of *Grapevine red globe virus* using one step RT-PCR

Molecular detection of *Ilarvirus* species using nested PCR

Molecular detection of *Peach rosette mosaic virus* using one step RT-PCR

Molecular detection of *Raspberry ringspot virus* using one step RT-PCR

Molecular detection of *Rupestris stem pitting associated virus* replicase gene using one step RT-PCR

Molecular detection of *Rupestris stem pitting associated virus* cat protein gene using one step RT-PCR

Molecular detection of *Strawberry latent ringspot virus* using one step RT-PCR

Molecular detection of *Tomato ringspot virus* using one step RT-PCR

Molecular detection of *Tombusvirus* species read through protein gene using one-step RT-PCR

Molecular detection of *Tombusvirus* species coat protein gene using one-step RT-PCR

Molecular detection of *Xylophilus ampelinus* DNA using PCR

RNA Extraction Protocol Using MacKenzie Buffer and the QIAxtractor (Qiagen)

Materials and equipment

1. QIAxtractor
2. 96 Square-Well,1.2 ml, lysis plate
3. Adhesive plastic film to cover Lysis plate and unused wells of the capture plate
4. 3mL transfer pipettes
5. 96-well 800 µl long drip Unifilter capture plate (Whatman)
6. Caps or adhesive foil to cover the elution plate
7. 96 well elution plate (Qiagen)
8. 20-200 µl and 200-1000 µl pipettes
9. 20-200 µl and 200-1000 µl sterile filter pipette tips
10. Autoclave
11. Balance
12. Bench top centrifuge
13. Distilled water
14. Freezer
15. Homex grinder and bags or mortar and pestle
16. Scalpel handle
17. Sterile scalpel blades
18. Vortex
19. Water bath at 70°C
20. Buffers
    - **MacKenzie buffer for RNA Extraction**

Reagent	**Final concentration**	**Amount needed for 1L**
Guanidine Thiocyanate	4M	472.64 g
3M Sodium Acetate	0.2M	66.67 ml
0.5M EDTA pH 8.0	25mM	50 ml
PVP-40	2.5%(w/v)	25 g

Add 1% (v/v) β- mecaptoethanol to the MacKenzie buffer just before use

- **Propanol Wash buffer for the Corbett X-Tractor**

Reagent	**Final concentration**	**Amount needed for 700ml**
Ethanol	25%	175ml
2-Propanol	25%	175ml
1M Tris-Cl, pH 8	10mM	7ml
NaCl	100 mM	4.09g

Make up to 700mL with dH_2O (Dissolve salts in water first before adding alcohols)

- **Ethanol (absolute)**

Method

Using the Homex homogeniser or mortar and pestle

1. With a sterile scalpel, scrape 300 mg of vascular tissue from the stem of the plant sample and place in a grinding bag (containing mesh to assist homogenisation) or mortar.
2. Add 30 ml of MacKenzie extraction buffer to the bag or mortar.
3. In a fume hood, add 30 µl of β-mercaptoethanol to the bag or mortar.
4. Place bag in Homex homogeniser and grind sample. If using a mortar, grind in the fume hood with a pestle until a slurry has formed.

5. In a fume hood, transfer 1 ml of the sample to one well of containing 100 µl of 20% N-lauroylsarcosine sodium salt (Sarkosyl).
6. Aspirate the sample to mix to mix.
7. Place the tube in a 70˚C water bath and incubate the samples for 15 minutes. Agitation in water bath is preferable but not necessary.
8. Close the tube and centrifuge at maximum speed for 2 minutes.
9. Transfer a maximum of 500 µl of the plant extract into a well of a 96 Square-Well,1.2 ml, lysis plate.
10. Place the second lysis plate in the QIAxtractor.
11. Add ethanol, propenol wash buffer and RNase-free sterile water tot e appropriate tubs and program the QIAxtractor to do the following:
    - 11.1. Add 500 µl of 100% ethanol to each sample
    - 11.2. Mix the samples by aspiration.
    - 11.3. Transfer 500 µl of the mixed samples to the 96-well 800 µl long drip Unifilter capture plate (Whatman)
    - 11.4. Apply a vacuum pressure of 70 Kpa for 5 minutes to draw samples through the filter.
    - 11.5. Transfer 500 µl of the 500 µl propanol wash buffer to the capture plate
    - 11.6. Apply a vacuum pressure of 50 Kpa for 5 minutes to draw through wash buffer
    - 11.7. Repeat 11.5 and 11.6
    - 11.8. The capture plate was then washed twice under vacuum with (Sigma) at 50 Kpa for 5 minutes
    - 11.9. Transfer 500 µl of 100% ethanol to the capture plate
    - 11.10. Apply a vacuum pressure of 45 Kpa for five minutes to draw through ethanol wash.
    - 11.11. Apply a vacuum pressure of 40 Kpa for 5 minutes to remove all traces of ethanol.
    - 11.12. Transfer the capture plate to a 96 well elution plate (Qiagen) and
    - 11.13. Transfer 200 µl of RNase, DNase free water to each well of the capture plate
    - 11.14. Incubate at room temperature for 2 minutes
    - 11.15. Apply a vacuum pressure of 45 Kpa for 5 minutes to elute the RNA.
    - 11.16. Transfer the capture plate to it's original position
12. Remove the elution plate and seal tubes with caps or adhesive foil
13. Store at -80°C

MacKenzie, DJ, McLean, MA, Murkerji, S and Green, M. 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. *Plant Disease* 81, 222-226.

RNA Extraction Protocol Using MacKenzie Buffer and the RNeasy® Plant Mini Kit (Qiagen)

Materials and equipment

1. QIAGEN RNeasy® Plant mini kit
2. 1.5 ml centrifuge tubes or 2 ml screw cap centrifuge tubes
3. 20-200 μl and 200-1000 μl pipettes
4. 20-200 μl and 200-1000 μl sterile filter pipette tips
5. Autoclave
6. Balance
7. Bench top centrifuge
8. Distilled water
9. Ice machine
10. Freezer
11. Fast Prep Machine or Homex grinder and bags or mortar and pestle
12. Scalpel handle
13. Sterile scalpel blades
14. Vortex
15. Water bath at 60°C
16. Buffers

- MacKenzie buffer for RNA Extraction

Reagent	Final concentration	Amount needed for 1L
Guanidine Thiocyanate	4M	472.64 g
3M Sodium Acetate	0.2M	66.67 ml
0.5M EDTA pH 8.0	25mM	50 ml
PVP-40	2.5%(w/v)	25 g

Add 1% (v/v) β- mecaptoethanol to the MacKenzie buffer just before use

Method:

Using a tissue lysator (Fast Prep) Machine

1. With a sterile scalpel, scrape 100mg of vascular tissue from the stem of the plant sample and place in a screw cap tube with three glass beads. The tube should be almost full (including balls) but not solidly packed.
2. Add 990 µL of MacKenzie buffer to tube.
3. In a fume hood, add 10 µl of β-mercaptoethanol to tube and shake to mix.
4. Place tube in Fast Prep machine.
5. Agitate for 30 seconds at a speed of 4.5. Repeat three times. Sample may need more agitation if leaf material is tough.
6. In a fume hood, add 100 µl of 20% Sarkosyl (lauryl sarcosine sodium salt) to the tube and shake to mix.
7. Place the tube in a 70˚C water bath and incubate the samples for 15 minutes. Agitation in water bath is preferable but not necessary.
8. Centrifuge the tube containing the sample for 30 seconds at 14,000 rpm, in a bench top centrifuge, to pellet the beads and other large pieces of plant material.
9. Transfer a maximum of 750 µl of the plant extract onto the lilac QIAshredder™ column. Close the tube and centrifuge at maximum speed for 2 minutes.

10. Mix 450 μl of the flowthrough with 225 μl of ethanol (96-100%), mix by pipetting and transfer all the mixture to the pink RNeasy® mini spin column, which has been placed in a 2 ml collection tube. Close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
11. Discard flow-through and replace column back into the same 2 ml collection tube.
12. Add 700ul of QIAGEN buffer RW1 to the pink RNeasy® spin mini column, close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
13. Discard flow-through and place column into a new 2 ml collection tube
14. Add 500ul of QIAGEN buffer RPE (wash buffer) to the pink RNeasy® mini spin column, close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
15. Discard flow-through and replace column back into the same 2 ml collection tube.
16. Add another 500ul of QIAGEN buffer RPE (wash buffer) to the pink RNeasy® mini spin column, close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
17. Discard flow-through and replace column back into the same 2 ml collection tube. Centrifuge at maximum speed for 1 minute.
18. Place the pink RNeasy® mini spin column in an appropriately labelled 1.5 ml centrifuge tube. Add 50 μl of RNase-free sterile water directly to the filter (don't apply down the side of the tube), close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 1 minute to elute the RNA.

Using the Homex homogeniser or mortar and pestle

1. With a sterile scalpel, scrape 200 mg of vascular tissue from the stem of the plant sample and place in a grinding bag (containing mesh to assist homogenisation) or mortar.
2. Add 1980 µl of MacKenzie extraction buffer to the bag or mortar.
3. In a fume hood, add 20 µl of β-mercaptoethanol to the bag or mortar.
4. Place bag in Homex homogeniser and grind sample. If using a mortar, grind in the fume hood with a pestle until a slurry has formed.
5. In a fume hood, transfer 1 ml of the sample to a 1.5 ml centrifuge tube.
6. In a fume hood, add 100 µl of 20% Sarkosyl (lauryl sarcosine sodium salt) to the tube and shake to mix.
7. Place the tube in a 70˚C water bath and incubate the samples for 15 minutes. Agitation in water bath is preferable but not necessary.
8. Transfer a maximum of 750 µl of the plant extract onto the lilac QIAshredder™ column. Close the tube and centrifuge at maximum speed for 2 minutes.
9. Mix 450 µl of the flowthrough with 225 µl of ethanol (96-100%), mix by pipetting and transfer all the mixture to the pink RNeasy® mini spin column, which has been placed in a 2 ml collection tube. Close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
10. Discard flow-through and replace column back into the same 2 ml collection tube.
11. Add 700ul of QIAGEN buffer RW1 to the pink RNeasy® mini spin column, close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
12. Discard flow-through and place column into a new 2 ml collection tube
13. Add 500ul of QIAGEN buffer RPE (wash buffer) to the pink RNeasy® mini spin column, close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
14. Discard flow-through and replace column back into the same 2 ml collection tube.
15. Add another 500 µl of QIAGEN buffer RPE (wash buffer) to the pink RNeasy® mini spin column, close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 15 s.
16. Discard flow-through and replace column back into the same 2 ml collection tube. Centrifuge at maximum speed for 1 minute.
17. Place the pink RNeasy® mini spin column in an appropriately labelled 1.5 ml centrifuge tube. Add 50 µl of RNase-free sterile water directly to the filter (don't apply down the side of the tube), close the tube and centrifuge at ≈ 8000 × G (≈ 10 000 rpm) for 1 minute to elute the RNA.

MacKenzie, DJ, McLean, MA, Murkerji, S and Green, M. 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. *Plant Disease* 81, 222-226.

DNA extraction procedure using the QIAGEN DNeasy® Plant mini kit

Materials and equipment

1. QIAGEN DNeasy® Plant mini kit
2. 1.5 ml centrifuge tubes
3. 20-200 µl and 200-1000 µl pipettes
4. 20-200 µl and 200-1000 µl sterile filter pipette tips
5. Autoclave
6. Balance
7. Bench top centrifuge
8. Distilled water
9. Ice machine
10. Freezer
11. Mortars and pestles or Homex grinder and bags
12. Scalpel handle
13. Sterile scalpel blades
14. Vortex
15. Water bath at 60°C
16. Buffers
17. Reference: Green MJ, Thompson DA and MacKenzie DJ, 1999. Easy and efficient DNA extraction from woody plants for the detection of phytoplasmas by polymerase chain reaction. *Plant Disease* **83,** 482-485

- **CTAB extraction buffer**

	Final Concentration
CTAB (cetylmethylammonium bromide)	2.5%
1M Tris, pH 8.0	100mM
NaCl	1.4M
5M EDTA, pH8.0	50mM
polyvinylpyrrolidone (PVP-40)	1%

Reagent	**Final concentration**	**Amount needed for 1L**
CTAB (cetylmethylammonium bromide)	2.5%	25 g
Sodium Chloride	1.4 M	56 g
1M Tris, pH 8.0 (sterile)	100 mM	100 ml
0.5M EDTA, pH8.0 (sterile)	20 mM	40 ml

Make up to volume with sterile distilled water. Store at room temperature. Just before use, add 0.2% 2-mercaptoethanol (v/v) to the required volume of buffer.

- **95% Ethanol**

Method

1) Pre-heat Qiagen buffer AE buffer to 65°C
2) Weigh approximately 0.5 g of leaf midribs and petioles.
3)
   a) Place the material in a sterile mortar with 5 ml of CTAB extraction buffer containing 0.2% 2 – mercaptoethanol and grind thoroughly with the sterile pestle.

or

   b) Place the material in a grinding bag (containing mesh to assist homogenisation) with 5 ml of CTAB extraction buffer containing 0.2% 2 – mercaptoethanol. Place the bag in Homex homogeniser and grind the sample.

4) With a sterile plastic transfer pipette transfer 500ul of extract to a 1.5 ml centrifuge tube and add 4 μl of RNase A (Supplied with the DNeasy® kit), cap tube and incubate at 65°C for 25-35 minutes, mixing gently several times.

5) Add 162 μl of QIAGEN buffer AP2 to extract. Invert 3 times to mix and place on ice for 5 minutes.

6) Pour extract into a QIAshredder™ column and centrifuge at maximum speed for 2 minutes.

7) Transfer 450 μl of flowthrough from QIAshredder™ column to a 1.5 ml centrifuge tube containing 675μl QIAGEN buffer AP3. Mix by pipetting.

8) Transfer 650 μl of extract onto a DNeasy® spin column and centrifuge at 10000rpm for 1 minute

9) Discard flow-through and add the rest of the sample to the column and centrifuge at 8000 rpm for 1 minute

10) Place DNeasy® column in a new 2 ml collection tube and add 500 μl of QIAGEN buffer AW (wash buffer) and centrifuge at 8000 rpm for one minute.

11) Discard flowthrough and add another 500 μl of QIAGEN buffer AW and centrifuge at maximum speed for 2 minutes.

12) Discard flowthrough and collection tube. Ensure that the base of the column is dry (re-centrifuge the column if it remains a little wet) and place in an appropriately labelled 1.5 ml centrifuge tube. Add 100 μl of pre-warmed AE buffer directly to the filter (don't apply down the side of the tube) and centrifuge at 8000 rpm for 1 minute. Discard column and store DNA in at -20°C.

Molecular detection of NADH dehydrogenase ND2 subunit (ndhB gene) mRNA using one-step RT-PCR

Introduction
Reverse transcriptase polymerase chain reaction (RT-PCR) of NADH dehydrogenase ND2 subunit (ndhB gene, NAD) messenger ribonucleic acid (mRNA) as described by Thompson *et al* 2003 and is commonly referred to as a "house keeping gene" RT-PCR. The RT-PCR amplifies NADH mRNA in a variety of plant species to determine the quality of the extracted RNA. This standard RT-PCR ensures that RNA is present or that there are no inhibitors in the RNA extracts that retard the activity of the either the reverse transcriptase or the DNA polymerase.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).
Controls
Positive control: RNA of known good quality.
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers Thompson *et al* 2003.

AtropaNad2.1a	GGACTCCTGACGTATACGAAGGATC
AtropaNad2.2b	AGCAATGAGATTCCCCAATATCAT

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

12.5 μl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor
Set up all reactions on ice.

Reagent	Volume
Sterile (RNase, Dnase free) water	4.5μl
10 μM primer AtropaNad2.1a	0.25μl
10 μM primer AtropaNad2.2b	0.25μl
2 × reaction mix (Contains buffer, dNTPs and $MgCl_2$)	6.25μl
SuperScript™ III RT/Platinum® Taq mix	0.25μl
RNA template	1μl
Total reaction volume	12.5 μl

12.5 μl RT-PCR reaction master mix for nucleic acid prepared using RNeasy columns
Set up all reactions on ice.

Reagent	Volume*
Sterile (RNase, Dnase free) water	5μl
10 μM primer AtropaNad2.1a	0.25μl
10 μM primer AtropaNad2.2b	0.25μl
2 × reaction mix (Contains buffer, dNTPs and $MgCl_2$)	6.25μl
SuperScript™ III RT/Platinum® Taq mix	0.25μl
RNA template	0.5μl
Total reaction volume	12.5 μl

RT-PCR cycling conditions*

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
55ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
23ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X from Roche was used to determine the size of the products. The expected size of the PCR product is 188 bp.

Thompson JR, Wetzel S, Klerks MM, Vaskova D, Schoen CD, Spak J and Jelkmann W. 2003. Multiplex RT-PCR detection of four aphid-borne strawberry viruses in Fragaria spp. in combination with a plant mRNA specific internal control. *Journal of Virological Methods* 111, 85-93

Molecular detection of 16S ribosomal DNA using PCR

Introduction

Polymerase chain reaction (PCR) was used by Weisberg et al 1991 for bacterial phylogenetic studies. This test will non-specifically amplify the 16S rDNA of any bacteria. This generic bacterial PCR can be used to check the quality of DNA extracts from plants or most bacteria. The PCR ensures that DNA is present or that there are no inhibitors in the DNA extracts that retard the activity of the DNA polymerase.

DNA extraction

Total nucleic acid extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or DNA extraction was done according to the DNA extraction protocol using the CTAB buffer and the Dneasy® Plant Mini Kit (Qiagen).

Controls

Positive control: DNA of known good quality
No template control: Sterile distilled water

PCR

Primers: Published Primers (Weisberg et al 1991)
FD2 5'-AGA GTT TGA TCA TGG CTC AG-3'
RP1 5'-ACG GTT ACC TTG TTA CGA CTT-3'

PCR

Invitrogen Platinum® Taq DNA Polymerase was used.

One 25 μl RT-PCR reaction master mix – using a normal PCR mix

Reagent	Volume – 1 reaction
Sterile (RNase, Dnase free) water	18.15μl
10 × reaction buffer	2.5μl
50 mM $MgCl_2$	0.75μl
dNTP mixture 10 mM	0.5μl
10 μM primer FD2	1μl
10 μM primer RP1	1μl
Platinum® Taq (5 units/μl)	0.1μl
DNA template or control	1μl
Total reaction volume	25 μl

Pipette 24μl of reaction mix into each tube then add 1μl of DNA template

RT-PCR cycling conditions

One cycle of:
95ºC for 1 min

30 cycles of:
95ºC for 45 s
55ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 10 min
25ºC hold

Electrophorese PCR products (10μl) on a 1% agarose gel containing ethidium bromide and visualise using an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected PCR product size is approx. 1400-1500 bp.

Weisberg, WG, Barns, S, Pelletier, DA and Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173, 697-703.

Molecular detection of *Ampelovirus* subgroup I species using nested PCR

Introduction
Nested PCR was used by Maliogka et al (2008) to detect Grapevine leafroll associated virus species *Ampleovirus* subgroup I in grapevine. The RT-PCR amplifies part of the HSP70 gene of *Ampleovirus* subgroup I species including GLRaV-4, -5, -6 and -9.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GLRaV-4,-5 or -9 infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Maliogka et al 2008)

dHSP up1	GGIHTIGAITTYGGIACIACITT
dHSP up1G	AGTTYGGGACGACGTT
dHSP do2	GTICCICCICCNAARTC
dHSP do2C	GTICCICCCCCNAARTC

Nested PCR
Primers: Published Primers (Maliogka et al 2008)

dHSP-nest2	TYGGGACGACGTTYTCNAC
LR5 clusdoL	GGYTCRTTCACIACIGCYTGIAC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	0.2µl
10 µM primer dHSP up1	2µl
10 µM primer dHSP do2	0.4 µl
10 µM primer dHSP do2C	3µl
10 µM primer dHSP up1G	3µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 μl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	0.2μl
10 μM primer dHSP up1	2μl
10 μM primer dHSP up1G	0.4 μl
10 μM primer dHSP do2	3μl
10 μM primer dHSP do2C	3μl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10μl
SuperScript™ III RT/Platinum® Taq mix	0.4μl
RNA template or control	2μl
Total reaction volume	20 μl

RT-PCR cycling conditions

One cycle of:
42ºC for 55 min
50ºC for 2 min
94ºC for 4 min

10 cycles of:
94ºC for 30 s
38ºC for 30s
72ºC for 20 s

30 cycles of:
94ºC for 30 s
49ºC for 10 s
44ºC for 10 s
38ºC for 15 s
72ºC for 20 s

One cycle of:
72ºC for 2 min
24ºC hold

Nested PCR
Invitrogen Platinum® *Taq* DNA Polymerase kit was used.

25 μl RT-PCR reaction master mix

Reagent	Volume – 1 reaction
Sterile (RNase, Dnase free) water	15.15μl
10 × reaction buffer	2.5μl
50 mM $MgCl_2$	0.75μl
dNTP mixture 10 mM	0.5μl
10 μM primer dHSP nest3	2.5μl
10 μM primer dHSP nest3G	2.5μl
Platinum® Taq (5 units/μl)	0.1μl
DNA template of RT-PCR product	1μl
Total reaction volume	25 μl

PCR cycling conditions

1 cycle of:
94ºC for 3 min

5 cycles of:
94ºC for 30 s
55ºC for 30 s
72ºC for 20 s

39 cycles of:
94ºC for 30 s
59ºC for 15 s
55ºC for 15 s
72ºC for 20 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size of the one-step RT-PCR product is 580-620bp and the expected size of the nested PCR product is 490bp.

Maliogka VI, Dovas CI and Katis NI. 2008. Generic and species-specific detection of viruses belonging to an evolutionary distinct lineage within the *Ampelovirus* genus *Journal of Virological Methods* 109: 217-226

Molecular detection of *Artichoke Italian latent virus* using one step RT-PCR

Introduction
RT-PCR was used by Minafra et al (1995) to detect *Artichoke Italian latent virus* (AILV) in grapevine. The diagnostic test amplifies a partial fragment of the coat protein in RNA-2 of AILV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: AILV in artichoke or grapevine.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Minafra et al 1995)

AI3 GGTCTGGGGTGCCCGTGGCG
AI4 ATTCACTAGTCCCTATTTAG

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer AI3	0.4µl
10 µM primer AI4	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer AI3	0.4µl
10 µM primer AI4	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
52ºC for 30 s
72ºC for 45 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 760 bp.

Minfra A, Grieco, F, Gallitelli D and Martelli GP. 1994. Improved PCR procedures for multiple identification of some artichoke and grapevine viruses. *Bulletin OEPP/EPPO Bulletin* 25: 283-287.

Molecular detection of *Cherry leafroll virus* using one step RT-PCR

Introduction
RT-PCR was used by Werner et al (1997) to simultaneously detect in *Cherry leafroll virus* (CLRV) in birch, beech and petunia. The test amplifies part of the RNA1 polyprotein gene of CLRV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: Total RNA extracted from CLRV infected Cherry.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Werner et al 1997)

CLRV-5 TGGCGACCGTGTAACGGCA
CLRV-3 GTCGGAAAGATTACGTAAAAGG

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer CLRV-5	0.4µl
10 µM primer CLRV-3	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer CLRV-5	0.4µl
10 µM primer CLRV-3	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
53ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 290bp for GFLV and ArMV.

Werner R, Mühlbach HP, abd Büttner C, 1997. Detection of *Cherry leafroll nepovirus* (CLRV) in birch, beech and petunia by immuno-capture RT-PCR using a conserved primer pair. *Europen Journal of Forest pathology* 27, 309-318

Molecular detection of generic *Grapevine leafroll associated virus* species using *Closteroviridae* nested PCR

Introduction
Nested PCR was used by Dovas and Katis (2003) to detect Grapevine leafroll associated virus species in grapevine. The RT-PCR amplifies part of the HSP70 gene of *Closteroviridae* species.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GLRaV infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Dovas and Katis 2003)

dHSP up1	GGIHTIGAITTYGGIACIACITT
dHSP up1G	AGTTYGGGACGACGTT
dHSP do2	GTICCICCICCNAARTC
dHSP do2C	GTICCICCCCCNAARTC

Nested PCR
Primers: Published Primers (Dovas and Katis 2003)

dHSP nest1	TTYGGGACGACGTTYAGYAC
dHSP nest2	TYGGGACGACGTTYTCNAC
dHSP nest3	SCIGCIGMISWIGGYTCRTT
dHSP nest3G	GCGGMGSWGGGPTCRTT

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 μl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	3.8μl
10 μM primer dHSP up1	2μl
10 μM primer dHSP up1G	0.8 μl
10 μM primer dHSP do2	1μl
10 μM primer dHSP do2C	1μl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10μl
SuperScript™ III RT/Platinum® Taq mix	0.4μl
RNA template or control	2μl
Total reaction volume	20 μl

20 μl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	3.8μl
10 μM primer dHSP up1	2μl
10 μM primer dHSP up1G	0.8 μl
10 μM primer dHSP do2	1μl
10 μM primer dHSP do2C	1μl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10μl
SuperScript™ III RT/Platinum® Taq mix	0.4μl
RNA template or control	2μl
Total reaction volume	20 μl

RT-PCR cycling conditions

One cycle of:
48ºC for 30 min
50ºC for 2 min
94ºC for 4 min

5 cycles of:
94ºC for 30 s
43ºC for 10 s
38ºC for 5s
72ºC for 15 s

35 cycles of:
94ºC for 30 s
43ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
24ºC hold

Nested PCR
Invitrogen Platinum® *Taq* DNA Polymerase kit was used.

25 μl RT-PCR reaction master mix

Reagent	Volume – 1 reaction
Sterile (RNase, Dnase free) water	12.15μl
10 × reaction buffer	2.5μl
50 mM $MgCl_2$	0.75μl
dNTP mixture 10 mM	0.5μl
10 μM primer dHSP nest1	2μl
10 μM primer dHSP nest2	2μl
10 μM primer dHSP nest3	2μl
10 μM primer dHSP nest3G	2μl
Platinum® Taq (5 units/μl)	0.1μl
DNA template of RT-PCR product	1μl
Total reaction volume	25 μl

PCR cycling conditions

1 cycle of:
94ºC for 3 min
48ºC for 15 s
72ºC for 15 s

39 cycles of:
94ºC for 30 s
54ºC for 30 s
72ºC for 10 s + 1 s after each cycle

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size of the one-step RT-PCR product is 580-620bp and the expected size of the nested PCR product is 500-535bp.

Dovas CI and Katis NI. 2003. A spot multiplex nested RT-PCR for the simultaneous and generic detection of viruses involved in the aetiology of grapevine leafroll and rugose wood of grapevine. *Journal of Virological Methods* 109, 217-226

Molecular detection of *Grapevine leafroll associated virus* 1 using one step RT-PCR

Introduction
RT-PCR was used by Good and Monis (2001) to detect *Grapevine leafroll associated virus* 1 (GLRaV-1) in grapevine. The RT-PCR amplifies part of the HSP70 gene of GLRaV-1.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GLRaV-1 infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Good and Monis 2001)

GSP3 (GLRA1S) CGTTCGCGTTACCCACGCTGCCTA
(GSP4) GLRA1A GCTGGCAAACCTGGTGGACTTTACATC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer GSP3	0.4µl
10 µM primer GSP4	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer GSP3	0.4µl
10 µM primer GSP4	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
55ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 150bp.

Good X, and Monis J. 2001. Partial genome organization, identification of the coat protein gene, and detection of *Grapevine leafroll-associated virus-5*. Phytopathology 91, 274-281.

Molecular detection of *Grapevine leafroll associated virus* 2 using one step RT-PCR

Introduction
RT-PCR was used by Bertazzon and Angelini (2004) to detect *Grapevine leafroll associated virus* 2 (GLRaV-2) in grapevine. The RT-PCR amplifies part of the coat protein gene of GLRaV-2.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GLRaV-2 infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Bertazzon and Angelini 2004)

V2dCPf2 sense	ACGGTGTGCTATAGTGCGTG
V2CPr1 antisense	GCAGCTAAGTACGAATCTTC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer V2dCPf2 sense	0.4µl
10 µM primer V2CPr1 antisense	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer V2dCPf2 sense	0.4µl
10 µM primer V2CPr1 antisense	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
55ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 589bp.

Bertazzon N and Angelini E. 2004. Advances in the detection of *Grapevine leafroll-associated virus* 2 variants. *Journal of Plant Pathology* 86, 283-290

Molecular detection of *Grapevine leafroll associated virus* 3 using one step RT-PCR

Introduction
RT-PCR was used by Turturo et al (2005) to detect *Grapevine leafroll associated virus* 3 (GLRaV-3) in grapevine. The RT-PCR amplifies part of the coat protein gene of GLRaV-3.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GLRaV-3 infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Turturo et al 2005)

P3U CGCTCATGGTGAAAGCAGACG
P3D CTTAGAACAAAAATATGGAGCAG

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer P3U	0.4µl
10 µM primer P3D	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer P3U	0.4µl
10 µM primer P3D	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
52ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. Primer P3U anneals at positions 7424-7444 of the GLRaV-3 *GenBank* accession AF037268 (HSP70 gene) and amplification occurs in "forward" (5'-3') direction. Primer P3D anneals at positions 8076-8054 of the GLRaV-3 *GenBank* accession AF037268 (HSP70 gene) and amplification occurs in "reverse" (3'-5') direction. The expected product size is approx. 546bp.

Turturo C, Saldarelli P, Yafeng D, Digiaro M, Minafra A, Savino V and Martelli GP. 2005. Genetic variability and population structure of Grapevine leafroll-associated virus 3 isolates. *Journal of General Virology* 86, 217–224

Molecular detection of *Grapevine leafroll associated virus* 4 using one step RT-PCR

Introduction
RT-PCR was used by Routh et al (1998) to detect *Grapevine leafroll associated virus* 4 (GLRaV-4) in grapevine. The RT-PCR amplifies part of the coat protein gene of GLRaV-4

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GLRaV-4 infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Routh et al 1998)

HSP4A CTCAAACCAGCGGCTGTT
HSP4B GTGATACCATATACATACCGAC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer HSP4A	0.4µl
10 µM primer HSP4B	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer HSP4A	0.4µl
10 µM primer HSP4B	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
52ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 441bp.

Routh G, Zhang YP., Saldarelli P, and Rowhani A. 1998. Use of degenerate primers for partial sequencing and RT-PCR–based assays of Grapevine leafroll–associated viruses 4 and 5. *Phytopathology* 88,1238-1243.

Molecular detection of *Grapevine leafroll associated virus* 5 using one step RT-PCR

Introduction
RT-PCR was used by Good and Monis (2001) to detect *Grapevine leafroll associated virus* 5 (GLRaV-5) in grapevine. The RT-PCR amplifies part of the coat protein gene of GLRaV-5.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GLRaV-5 infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Good and Monis 2001)

LR5-1F CCCGTGATACAAGGTAGGACA
LR5-1F CAGACTTCACCTCCTGTTAC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer LR5-1F	0.4µl
10 µM primer LR5-1F	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer LR5-1F	0.4µl
10 µM primer LR5-1F	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
52ºC for 30 min
72ºC for 40 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 690bp.

Good X and Monis J. 2001. Partial genome organization, identification of the coat protein gene, and detection of *Grapevine leafroll-associated virus-5*. Phytopathology 91, 274-281.

Molecular detection of *Grapevine leafroll associated virus* 7 using one step RT-PCR

Introduction
RT-PCR was used by Engel et al (2008) to detect *Grapevine leafroll associated virus* 7 (GLRaV-7) in grapevine. The RT-PCR amplifies part of the HSP70 gene of GLRaV-7

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GLRaV-7 infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Engel et al 2008)

LR7-F TATATCCCAACGGAGATGGC
LR7-R ATGTTCCTCCACCAAAATCG

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer LR7-F	0.4µl
10 µM primer LR7-R	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer LR7-F	0.4µl
10 µM primer LR7-R	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
55ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 502bp.

Engel EA, Escobar P, Montt C, Gomez-Talquenca S and Valenzuela PDT. 2008. First report on the occurrence of *Grapevine leafroll-associated virus 7* and *9* in Chilean grapevines. *Plant Disease* 92, 1252.

Molecular detection of *Grapevine leafroll associated virus* 9 using one step RT-PCR

Introduction
RT-PCR was used by Alkowni et al (2004) to detect *Grapevine leafroll associated virus* 9 (GLRaV-9) in grapevine. The RT-PCR amplifies part of the coat protein gene of GLRaV-9

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GLRaV-9 infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Alkowni et al 2004)

LR9-F CGGCATAAGAAAAGATGGCA
LR9-R TCATTCACCACTGCTTGAAC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer LR9-F	0.4µl
10 µM primer LR9-R	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer LR9-F	0.4µl
10 µM primer LR9-R	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
55ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 390bp.

Alkowni R, Rowhani A, Daubert S and Golino D. 2004. Partial charaterization of a new *Ampelovirus* with grapevine leafroll disease. *Journal of Plant Pathology* 86, 123-133

Molecular detection of *Grapevine Anatolian ringspot virus* using one step RT-PCR

Introduction
RT-PCR was used by Gokalp et al (2003) to detect *Grapevine Anatolian ringspot virus* (GARSV) in grapevine. The diagnostic test amplifies a partial fragment of the coat protein in RNA-2 of AILV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: GARSV in artichoke or grapevine.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Gokalp et al 2003)

A34-1 GAAGATACCCTCACGCATTGCC-
A34-2 CCCAGTGTTTACTCCACACCC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer A34-1	0.4µl
10 µM primer A34-2	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer A34-1	0.4µl
10 µM primer A34-2	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
60ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 343 bp.

Gokalp K, Digiaro M, Cigsar I, Abou Ghanem-Sabanadzovic N, De Stradis A, Boscia D and Martelli GP. 2003. Properties of a previously undescribed nepovirus from south east Anatolia. *Journal of Plant Pathology* 85, 35–41.

Molecular detection of *Grapevine chrome mosaic virus* and *Tomato black ring virus* using one step RT-PCR

Introduction
RT-PCR was used by Le Gall et al (1995) to study recombination in *Grapevine chrome mosaic virus* (GCMV) and *Tomato black ring virus* (TBRV). The test amplifies a partial fragment of RNA-2 of GCMV and TBRV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: Partial clone of cDNA of the TBRV Coat protein ligated intopCR-4TOPO vector or total RNA extracted from TBRV infected Iris.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (La Gall et al 1995)

P1 ATGGGAGAAGTGCTGG
P2 AATCTTTTTGTGTCCAACA

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer P1	0.4µl
10 µM primer P2	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer P1	0.4µl
10 µM primer P2	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
42ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 246bp for GCMV and 332bp for TBRV

Le Gall O, Candresse T. and Dunez J. 1995. Transfer of the 31 non-translated region of grapevine chrome mosaic virus RNA-1 by recombination to tomato black ring virus RNA-2 in pseudorecombinant isolates *Journal of General Virology* 76, 1285-1289.

Molecular detection of *Grapevine deformation virus* using one step RT-PCR

Introduction
RT-PCR was used by Cigsar et al (2003) to detect *Grapevine deformation virus* (GDefV) in grapevine. The diagnostic test amplifies a partial fragment of the coat protein in RNA-2 of GDefV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GDefV infected grapevine.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Cigsar et al 2003)

N66-1 GGTCCTATAACATACGG GGCTG
N66-2 CCCCTGATGGTGCTATTGGTCC-

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer N66-1	0.4µl
10 µM primer N66-2	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer N66-1	0.4µl
10 µM primer N66-2	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
60ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 240 bp.

Cigsar I, Digiaro M, Gokalp K, Abou Ghanem-Sabanadzovic N, De Stradis A, Boscia D, and Martelli GP. 2003. Grapevine deformation virus, a novel Nepovirus from Turkey. *Journal of Plant Pathology* 85, 183–191.

Molecular detection of *Grapevine fanleaf virus* and *Arabis mosiac virus* using one step RT-PCR

Introduction
RT-PCR was used by Wetzel et al (2002) to simultaneously detect in *Grapevine fanleaf virus* (GFLV) and *Arabis mosiac virus* (ArMV) in grapevine. The test amplifies a partial movement protein gene of GFLV and ArMV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: Total RNA extracted from GFLV infected grapevine.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Wetzel et al 2002)

M2 (C/T)T(A/G)GATTTTAGGCTCAATGG;:
M3 TG(C/T)AA(A/G)CCAGG(A/G)AAGAAAAT;

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer M2	0.4µl
10 µM primer M3	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer M2	0.4µl
10 µM primer M3	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 20 s
42ºC for 20 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 290bp for GFLV and ArMV.

Wetzel T, Jardak R, Meunier L, Ghorbel A, Reustle GM and Krczal G. 2002. Simultaneous RT/PCR detection and differentiation of arabis mosaic and grapevine fanleaf nepoviruses in grapevines with a single pair of primers. *Journal of Virological Methods* 101 63–69

Molecular detection of *Grapevine fleck virus* using one step RT-PCR

Introduction
RT-PCR was used by Shi et al (2003) to detect *Grapevine fleck virus* (GFkV) in grapevine. The diagnostic test amplifies a partial fragment of the RNA replicase gene of GFkV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GFkV infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Shi et al 2003)

GFkV-U279 TGGTCCTCGGCCCAGTGAAAAAGTA
GFkV-L630 GGCCAGGTTGTAGTCGGTGTTGTC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer U270	0.4µl
10 µM primer L630	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer U279	0.4µl
10 µM primer L630	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	2o µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
55ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. Shi et al (2003) observed variants that had an insertion within domain VI of the replicase gene. Thus there are two expected product sizes of approx. 353bp (most common) or 416 bp (less frequent) depending on the strain that is amplified.

Shi BJ, Habili N and Symons RH. 2003. Nucleotide sequence variation in a small region of the *Grapevine fleck virus* replicase provides evidence for two sequence variants of the virus *Annals of Applied Biology.* 142, 349-355

Molecular detection of *Grapevine virus A* p10 gene using one step RT-PCR

Introduction
RT-PCR was used by MacKenzie 1997 to detect *Grapevine virus A* (GVA) in grapevine. The RT-PCR amplifies part of the p10 gene of GVA.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GVA infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (MacKenzie 1997)

H7038 AGGTCCACGTTTGCTAAG
C7273 CATCGTCTGAGGTTTCTACTA

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer H7038	0.4µl
10 µM primer C7273	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer H7038	0.4µl
10 µM primer C7273	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	2o µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
52ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products The expected product size is approx. 235bp.

MacKenzie DJ. 1997. A standard protocol for the detection of viruses and viroids using a reverse transcription-polymerase chain reaction technique. Document CPHBT-RTPCR1.00. Canadian Food Inspection Agency.

(Also see Stewart S and Nassuth A. 2001. RT-PCR based detection of *Rupestris stem pitting associated virus* within field-grown grapevines throughout the year*. Plant Disease* 85,617-620.)

Molecular detection of *Grapevine virus A* coat proein gene using one step RT-PCR

Introduction
RT-PCR was used by Minafra et al 1997 to detect *Grapevine virus A* (GVA) in grapevine. The RT-PCR amplifies part of the coat protein gene of GVA.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GVA infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Minafra et al 1997)

H587 GACAAATGGCACACTACG
C995 AAGCCTGACCTAGTCATCTTGG

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer H587	0.4µl
10 µM primer C995	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer H587	0.4µl
10 µM primer C995	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	2o µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
55ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 430bp.

Minafra A, Saldarelli P. and Martelli GP. 1997. *Grapevine virus A*: nucleotide sequence, genome organization, and relationship in the *Trichovirus* genus. *Archives of Virology* 142, 417-423.

Molecular detection of *Grapevine virus B* using one step RT-PCR

Introduction
RT-PCR was used by Minafra and Hadidi (1994) to detect *Grapevine virus B* (GVB) in grapevine. The RT-PCR amplifies part of the coat protein gene and putative RNA binding protein of GVB.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GVB infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Minafra and Hadidi 1994)

H6980 (GVB H28) GTGCTAAGAACGTCTTCACAGC
C7439 (GVB410) ATCAGCAAACACGCTTGAACCG

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer H6980	0.4µl
10 µM primer C7439	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer H6980	0.4µl
10 µM primer C7439	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	2o µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
55ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 469bp.

Minafra A and Hadidi A. 1994 Sensitive detection of grapevine virus A, B, or leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. *Journal of Virological Methods* 47,175-188. 1994.

Molecular detection of *Grapevine virus D* using one step RT-PCR

Introduction
RT-PCR was used by Terlizzi and Credi (2006) to detect *Grapevine virus D* (GVD) in grapevine. The RT-PCR amplifies part of the Movement protein gene of GVD

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GVD infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Terlizzi and Credi 2006)

GVDORF2F1 TAA TAG GGC CTA AGT C

GVDORF2R1 GGG CGT TGA ATA CAC CTT TAG C

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer GVDORF2F1	0.4µl
10 µM primer GVDORF2R1	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer GVDORF2F1	0.4µl
10 µM primer GVDORF2R1	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

PCR cycling conditions
1 cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
50ºC for 35 min
72ºC for 35 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 371bp .

Terlizzi F and Credi R. 2006. Mechanical transmission and identification of three Vitiviruses associated with grapevine rugose wood. *Extended Abstracts 15th Meeting of ICVG,* Stellenbosch, South Africa, 184-185

Molecular detection of *Grapevine red globe virus* using one step RT-PCR

Introduction
RT-PCR was used by Sabanadzovic et al (2000) to detect *Grapevine red globe virus* (GRGV) in grapevine. The RT-PCR amplifies part of the RNA dependent RNA polymerase ORF1 of GRGV

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from GRGV infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Sabanadzovic et al 2000)

DNA hexanucleotides	Commercially available
RD1	CYCARCAYAARGTVAACGA
RGAP	GGCCAAACTGGGTGCTCACG

Two-Step RT-PCR

20 µl Reverse transcription reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
RNA template or control	5µl
10 µM DNA hexanucleotides	2µl
Sterile (RNase, Dnase free) water	5µl
Heat to 95°C for five minutes and then chilled on ice.	
5× first strand buffer	4µl
0.1 M DTT	2µl
10 mM dNTP mix	1µl
RNase H⁻ Reverse Transcriptase (200 units/µl)	1µl
Total reaction volume	20 µl

Reverse transcription conditions:
1 cycle of:
42°C for 50 min
95°C for 2 minutes

PCR
Invitrogen Platinum® *Taq* DNA Polymerase kit was used.

25 μl RT-PCR reaction master mix

Reagent	Volume – 1 reaction
Sterile (RNase, Dnase free) water	17.15μl
10 × reaction buffer	2.5μl
50 mM $MgCl_2$	0.75μl
dNTP mixture 10 mM	0.5μl
10 μM primer RD1	1μl
10 μM primer RGAP	1μl
Platinum® Taq (5 units/μl)	0.1μl
DNA template of RT-PCR product	2μl
Total reaction volume	25 μl

PCR cycling conditions

35 cycles of:
94ºC for 30 min
58ºC for 35 min
72ºC for 35 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 386bp .

Sabanadzovic S, Abou-Ghanem N, Castellano MA, Digiaro M and Martelli GP. 2000. Grapevine fleck virus-like viruses in *Vitis* Archives of Virology 145, 553–565

Molecular detection of *Ilarvirus* species using nested PCR

Introduction
Nested PCR was used by Maliogka et al (2007) to detect *Ilarvirus* species in grapevine and other plant hosts. The assay amplifies part of the polymerase gene on RNA2 of *Ilarvirus* species including *Grapevine angular mosaic virus.*

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from Ilarvirus infected host (e.g. *Apple mosaic virus* in pome fruit)
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
First round primers: Published Primers (Maliogka et al 2007)

Ilapol up2 YTCIAMRTTYGAYAARTC
Ilapol do4 GGYTGRTTRTGIGGRAA

Nested PCR Primers: Published Primers (Maliogka et al 2008)

Ilapolnes up3 TCGAMRTTYGAYAARTCICA
Ilapolnest d5 TGGGGRAAYTTIGYYTCRA

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	1.6µl
10 µM primer Ilapol up2	2µl
10 µM primer Ilapol do4	2 µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 μl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	2.6μl
10 μM primer Ilapol up2	2μl
10 μM primer Ilapol do4	2 μl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10μl
SuperScript™ III RT/Platinum® Taq mix	0.4μl
RNA template or control	1μl
Total reaction volume	20 μl

RT-PCR cycling conditions

One cycle of:
42ºC for 50 min
94ºC for 4 min

5 cycles of:
94ºC for 30 s
38ºC for 30s
72ºC for 20 s

35 cycles of:
94ºC for 30 s
42ºC for 20 s
38ºC for 10 s
72ºC for 20 s

One cycle of:
72ºC for 2 min
24ºC hold

Nested PCR
Invitrogen Platinum® *Taq* DNA Polymerase kit was used.

25 μl RT-PCR reaction master mix

Reagent	Volume – 1 reaction
Sterile (RNase, Dnase free) water	16.15μl
10 × reaction buffer	2.5μl
50 mM $MgCl_2$	0.75μl
dNTP mixture 10 mM	0.5μl
10 μM primer Ilapolnes up3	2μl
10 μM primer Ilapolnes d5	2μl
Platinum® Taq (5 units/μl)	0.1μl
DNA template of RT-PCR product	1μl
Total reaction volume	25 μl

PCR cycling conditions

1 cycle of:
94ºC for 2 min

5 cycles of:
94ºC for 30 s
43ºC for 30 s
72ºC for 20 s

35 cycles of:
94ºC for 30 s
47ºC for 20 s
43ºC for 10 s
72ºC for 20 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size of the one-step RT-PCR product is 381bp and the expected size of the nested PCR product is 371bp.

Maliogka VI, Dovas CI and Katis NI. 2007. Demarcation of ilarviruses based on the phylogeny of RNA2-encoded RdRp and a generic ramped annealing RT-PCR. *Archives of Virology* 152, 1687–1698

Molecular detection of *Peach rosette mosaic virus* using one step RT-PCR

Introduction
RT-PCR was used by Kheder et al (2004) to detect *Peach rosette mosaic virus* (PRMV) in peach and grapevine. The diagnostic test amplifies a partial fragment of the coat protein in RNA-2 of PRMV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: PRMV in total RNA.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Kheder et al 2004)

PRMVV1 ÓGTGATGGAATATTTGGAC
PRMVC1 ÓCCATTGGCAACCGCCAGAGCTAC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer PRMVV1	0.4µl
10 µM primer PRMVC1	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer PRMVV1	0.4µl
10 µM primer PRMVC1	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
60ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 200 bp.

Kheder AA, Ibrahim IAM and Mazyad HM. 2004. Isolation and characterization of *Peach Rosette Mosaic Virus Egyptian Journal of Virology*. 1, 259-272

Molecular detection of *Raspberry ringspot virus* using one step RT-PCR

Introduction
RT-PCR was used by Ochoa-Corona et al (2006) to detect *Raspberry ringspot virus* (RsRSV) in raspberries. The diagnostic test amplifies a partial fragment of the coat protein in RNA-2 of RsRSV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: Partial clone of cDNA of the RsRSV coat protein ligated intopCR-4TOPO vector.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Ochoa-Corona et al 2006)

RpRSVF1 TGTGTCTGGTTTTGATGCT
RpRSVR1 GAGTGCGATAGGGGCTGTT

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer RpRSVF1	0.4µl
10 µM primer RpRSVR1	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer RpRSVF1	0.4µl
10 µM primer RpRSVR1	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
61ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 385 bp.

Ochoa-Corona, FM, Lebas, BS., Tang, JZ, Bootten, TJ, Stewart, FJ, Harris, R, Elliott, DR and Alexander, BJR. 2006. RT-PCR detection and strain typing of Raspberry ringspot virus. Proceedings of the XXth International Symposium on Virus and Virus-like Diseases of Temperate Fruit Crops & XIth nternational Symposium on Small Fruit Virus Diseases, Antalya, Turkey, May 22–26, 2006.

(Also see: Wei T, Lu G and Clover G. 2008. Novel approaches to mitigate primer interaction and eliminate inhibitors in multiplex PCR, demonstrated using an assay for detection of three strawberry viruses. *Journal of Virological Methods* 151: 132–139)

Molecular detection of *Rupestris stem pitting associated virus* replicase gene using one step RT-PCR

Introduction
RT-PCR was used by Habili et al (2006) to detect *Rupestris stem pitting associated virus* (RSPaV) associated with Shiraz decline in grapevine. The RT-PCR amplifies part of the replicase gene of RSPaV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from RSPaV infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Habili et al 2006)

Sy9F AGGATTCCAAACTGTAGAGCAA
Sy8R TTGGTCGTCATCTTCCAGTT

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer Sy9F	0.4µl
10 µM primer Sy8R	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer Sy9F	0.4µl
10 µM primer Sy8R	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
55ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 628bp.

Habili N, Farrokhi N, Lima F, Nicholas P and JW Randles JW. 2006. Distribution of Rupestris stem-pitting-associated virus variants in two Australian vineyards showing different symptoms. *Annals of Applied Biology* 148, 91–96

Molecular detection of *Rupestris stem pitting associated virus* coat protein gene using one step RT-PCR (RSP48/RSP49)

Introduction
RT-PCR was used by Nolasco et al (2000) to detect *Rupestris stem pitting associated virus* (RSPaV) in grapevine. The RT-PCR amplifies part of the coat protein gene of RSPaV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: RNA extracted from RSPaV infected grapevine
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Nolasco et al 2000)

RSP48 AGCTGGGATTATAAGGGAGGT
RSP49 CCAGCCGTTCCACCACTAAT

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer RSP48	0.4µl
10 µM primer RSP49	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer RSP48	0.4µl
10 µM primer RSP49	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	2o µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 min
52ºC for 30 min
72ºC for 30 min

One cycle of:
72ºC for 5 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 512bp.

Nolasco G, Mansinho A, Santos MT, Soares C, Sequiera Z, Sequeira C, Correia K and Sequeira OA. 2000. Large scale evaluation of primers for diagnosis of *Rupestris stem pitting associated virus*-1. *European Journal of Plant Pathology,* 106, 311–318.

Molecular detection of *Strawberry latent ringspot virus* using one step RT-PCR

Introduction
RT-PCR was used by Faggioli et al (2002) to detect *Strawberry latent ringspot virus* (SLRSV) in olives. The diagnostic test amplifies a partial fragment of the coat protein in RNA-2 of SLRSV.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: SLRSV in total RNA extracted from *Narcissus*.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Faggioli et al 2002)

SLRSV-5D CCCTTGGTTACTTTTACCTCCTCATTGTCC
SLRSV-3D AGGCTCAAGAAAACACAC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer SLRSV-5D	0.4µl
10 µM primer SLRSV-3D	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer SLRSV-5D	0.4µl
10 µM primer SLRSV-3D	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
55ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 291 bp.

Faggioli F, Ferretti L, Pasquini G and Barba M. 2002. Detection of *Strawberry latent ring spot virus* in leaves of olive trees in Italy using a one-step RT–PCR. *Journal of Phytopathology* 150, 636–639.

Molecular detection of *Tomato ringspot virus* using one step RT-PCR

Introduction
RT-PCR was used by Griesbach (1995) to detect *Tomato ringspot virus* (TomRSV) in tomatoes. The diagnostic test amplifies a partial fragment of the polyprotein region of TomRSV RNA1.

RNA extraction
RNA extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or the RNeasy® Plant Mini Kit (Qiagen).

Controls
Positive control: TomRSV cDNA, generated by U1 and D1 primer pair and ligated into pGEM®-Teasy vector (Promega). Use a 1 in 10000 dilution of the original stock.
Negative control: RNA from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Griesbach, 1995)
U1 5'-GAC GAA GTT ATC AAT GGC AGC-3'
D1 5'-TCC GTC CAA TCA CGC GAA TA-3'

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer D1	0.4µl
10 µM primer U1	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20 µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer D1	0.4µl
10 µM primer U1	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	2o µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 30 s
55ºC for 30 s
72ºC for 30 s

One cycle of:
72ºC for 2 min
24ºC hold

PCR products (10μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised under an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected product size is approx. 450 bp.

Griesbach JA.1995 Detection of *Tomato ringspot virus* by polymerase chain reaction. *Plant Disease*, 79, 1054-1056

Molecular detection of *Tombusvirus* species read through protein gene using one-step RT-PCR

Introduction
Reverse transcriptase polymerase chain reaction (RT-PCR) was used by Harris *et al* 2006 to detect *Tombusvirus* species. The primers amplify part of the polymerase read through protein gene of Tombusvirus species.

RNA extraction
RNA extraction was done according to the RNA extraction protocol using the MacKenzie buffer and the RNeasy® Plant Mini Kit (Qaigen).

Controls
Positive control: PeLV CP cDNA, generated by TomCPF and TomCPR primer pair, and ligated into pGEM®-Teasy vector (Promega). Use a 1 in 10000 dilution of the purified plasmid
Negative control: RNA extracted from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Harris *et al* 2006)

TBSVGralF1 AAGGGTAAGGATGGTGAGGA
TBSVGralR1 TTTGGTAGGTTGTGGAGTGC

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer TBSVGralF1	0.4µl
10 µM primer TBSVGralR1	0.4µl
2 × reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer TomCPF	0.4µl
10 µM primer TomCPR	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 35 s
61ºC for 35 s
72ºC for 45 min

One cycle of:
72ºC for 5 min
15ºC hold

Electrophorese PCR products (10μl) on a 1.5% agarose gel containing ethidium bromide and visualise using an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the productsThe expected size of the PCR product is approx. 590 bp.

Harris, R., Ochoa-Corona, R., Lebas, B., Timudo, O., Stewart, F., Alexander, B., 2006. Broad detection and diagnosis of viruses of the genus Tombusvirus by RT-PCR coupled to single strand conformation polymorphism analysis. In: Proceedings of the 7th Australasian Plant Virology Workshop, Rottnest Island, Perth, Australia, November 8–11, 2006, p. 48.

Molecular detection of *Tombusvirus species* coat protein gene using one-step RT-PCR

Introduction
Reverse transcriptase polymerase chain reaction (RT-PCR) was used by Russo *et al* 2002 to characterise *Pear latent virus* (PeLV). Two oligonucleotides were designed from highly conserved regions flanking the coat protein (CP) gene of several tombusviruses and were used to amplify the putative CP gene of PeLV and other Tombusvirus species.

RNA extraction
RNA extraction was done according to the RNA extraction protocol using the MacKenzie buffer and the RNeasy® Plant Mini Kit (Qaigen).

Controls
Positive control: PeLV CP cDNA, generated by TomCPF and TomCPR primer pair, and ligated into pGEM®-Teasy vector (Promega). Use a 1 in 10000 dilution of the purified plasmid
Negative control: RNA extracted from uninfected grapevine
No template control: Sterile distilled water

RT-PCR
Primers: Published Primers (Russo *et al* 2002)
TomCPF 5'- CCG CCG TAG CAT GAC CAA GTA-3'
TomCPR 5'- CCA TGA ACT GGT CTT GTT CAA-3'

One Step RT-PCR
Invitrogen SuperScript™ III One-Step RT-PCR system with Platinum® *Taq* DNA Polymerase kit was used.

20 µl RT-PCR reaction master mix for nucleic acid prepared using the QIAxtractor

Reagent	Volume
Sterile (RNase, Dnase free) water	6.8µl
10 µM primer TomCPF	0.4µl
10 µM primer TomCPR	0.4µl
2 × reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	2µl
Total reaction volume	20µl

20 µl RT-PCR reaction master mix for nucleic acid prepared using the RNeasy kit

Reagent	Volume
Sterile (RNase, Dnase free) water	7.8µl
10 µM primer TomCPF	0.4µl
10 µM primer TomCPR	0.4µl
2X reaction mix (Contains buffer, dNTPs and $MgCl_2$)	10µl
SuperScript™ III RT/Platinum® Taq mix	0.4µl
RNA template or control	1µl
Total reaction volume	20 µl

RT-PCR cycling conditions

One cycle of:
48ºC for 20 min
94ºC for 2 min

35 cycles of:
94ºC for 45 s
55ºC for 45 s
72ºC for 1.5 min

One cycle of:
72ºC for 5 min
15ºC hold

Electrophorese PCR products (10μl) on a 1% agarose gel containing ethidium bromide and visualise using an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected size of the PCR product is approx. 1240 bp.

Russo M, Vovlas C, Rubino L, Grieco F and Martelli, GP. 2002. Molecular characterization of a tombusvirus isolated from diseased pear trees in southern Italy. *Journal of Plant Pathology* 84, 161-166.

Molecular detection of *Xylophilus ampelinus* DNA using PCR

Introduction
Polymerase chain reaction (PCR) was used by Botha *et al* 1991 to detect *Xylophilus ampelinus*. This PCR test detects the ITS region of the ribosomal RNA operon.

DNA extraction
Total nucleic acid extraction is done according to the protocol using the MacKenzie buffer and the QIAxtractor (Qiagen) automated liquid handler or DNA extraction was done according to the DNA extraction protocol using the CTAB buffer and the Dneasy® Plant Mini Kit (Qiagen).

Controls

Positive control:	DNA of *Xylophilus ampelinus*
Negative control:	Nucleic acid from uninfected grapevine
No template control:	Sterile distilled water

PCR
Primers: Published Primers (Botha *et al* 2001)

S3 GGTGTTAGGCCGAGTAGTGAG
S4 GGTCTTTCACCTGACGCGTTA

PCR
Invitrogen Platinum® *Taq* DNA Polymerase was used.

One 25 µl RT-PCR reaction master mix – using a normal PCR mix

Reagent	Volume – 1 reaction
Sterile (RNase, Dnase free) water	18.15µl
10 × reaction buffer	2.5µl
50 mM $MgCl_2$	0.75µl
dNTP mixture 10 mM	0.5µl
10 µM primer S3	1µl
10 µM primer S4	1µl
Platinum® Taq (5 units/µl)	0.1µl
DNA template or control	1µl
Total reaction volume	25 µl

Pipette 24µl of reaction mix into each tube then add 1µl of DNA template

RT-PCR cycling conditions

One cycle of:
95ºC for 1 min

40 cycles of:
95ºC for 30 s
60ºC for 45 s
72ºC for 45 s

One cycle of:
72ºC for 2 min
25ºC hold

Electrophorese PCR products (10μl) on a 2% agarose gel containing ethidium bromide and visualise using an UV transilluminator. DNA molecular weight marker X (Roche Applied Science) was used to determine the size of the products. The expected PCR product size is approx. 277 bp.

Botha WJ, Serfontein S, Greyling MM & Berger DK (2001) Detection of *Xylophilus ampelinus* in grapevine cuttings using a nested polymerase chain reaction. *Plant Pathology* 50, 515–526.

Appendix 5 – A guide to symptoms expressed by grapevine woody indicators inoculated in the Australian environment.

This guide will supplement two existing books about graft transmissible diseases in grapevines (Martelli 1993; Krake et al 1999). The information presented here extends the knowledge of symptoms associated with specific virus species by providing images of symptoms in biological indicators inoculated with known sources of virus.

Introduction

Biological indexing takes advantage of a sensitive plant response to the presence of pathogens. Indicator plants are inoculated with material of another source and are observed for characteristic symptoms. Woody indictors are currently used for diagnosis of virus associated diseases in Australian PEQ and certification schemes. Chip buds of a candidate plant are grafted onto sensitive indicator grapevines (Figure 1). The indicators used in Australian certification programs and the expected symptoms for leafroll, rugose wood and fleck diseases are listed in table 1.3. This method is time consuming and symptoms may take several years to appear. Indicators are usually planted in the field and symptom development is observed during three growing seasons. Rupestris St George is observed in spring for symptoms associated with fleck disease and Cabernet Franc is observed from late summer through autumn for symptoms of leafroll disease. During winter of the third season the bark is stripped from the trunks of Rupestris St George, Kober 5BB and LN33 to observed rugose wood symptoms. Australian PEQ only uses LN33 to test for corky bark disease.

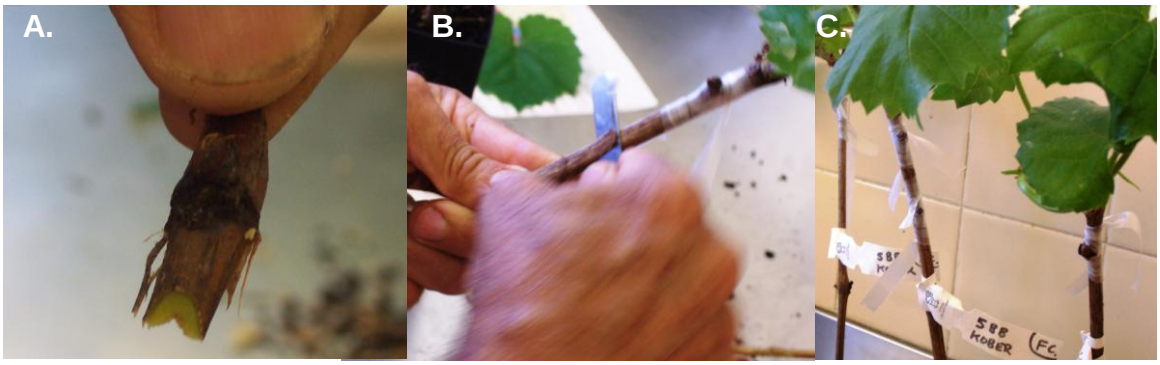


Figure 1.1 Chip bud inoculation of *V. berlandieri* × *V. riparia* cv. Kober 5BB for detection of GVA. A. A chip bud of a candidate grapevine used for inoculation. B. Creating a notch in the indicator vine for insertion of the chip bud. C. Indicator vines with chip buds held in place using budding tape.

Table 1.3 The indicators used to detect viruses associated diseases of grapevines and the symptoms that are observed.

Indicator	Disease	Symptoms	Associated virus
Cabernet Franc	Grapevine leafroll	Leaf reddening, often with green veins but sometimes with red veins, and leaf rolling	All GLRaVs, although symptom expression, particularly severity, can vary depending on the virus species.
Rupestris St George	Rugose wood complex - stem pitting	Pitting of the woody cylinder	RSPaV
Rupestris St George	Fleck disease	Vein clearing symptoms along veinlets. Severely affected leaves maybe twisted and curled upwards	GFkV
Kober 5BB	Rugose wood complex - stem grooving	grooving of the woody cylinder	GVA
LN33	Rugose wood complex – corky bark	Grooving and pitting of the stem, internodal swelling with a cracked surface, uneven or no lignification of canes	GVB

Woody indicators should be free of viruses and phytoplasmas, as these can affect symptom expression and they should be isolated from other grapevines to ensure that field infection does not occur and lead to false positive results. Biological indexing onto woody indicators is dependent of the successful inoculation of associated viruses, which are affected by graft take and presence of viruses in the grafted chip buds. Disease expression in the indicators is also affected by strain variation amongst the associated virus species, and some may not induce obvious symptoms on the selected biological indictors. In Australia biological indexing is mainly done in the field and it is also possible that environmental factors, such as temperature, light and nutrition, will affect symptom expression. Some indicators develop symptoms that cannot be readily attributed to a known causal agent, or combination of causal agents.

Rupestris stem pitting disease:

Rupestris stem pitting is a disease of the rugose wood complex that is diagnosed and detected by chip bud inoculation onto *Vitis rupestris* cv St George. It is associated with *Rupestris stem pitting-associated virus*.

Indicators can be chip bud inoculated in spring or early summer using two buds per grapevine

In *V. rupestris* St George, symptoms include pitting of the xylem tissue of the wood cylinder underneath the bark (Figure 1). Symptoms develop below the chip bud and extend downwards towards the roots. Symptoms may not be evident until the bark is completely removed to expose the woody cylinder, however at times severe symptoms might be observed on the outer surface of the bark. The disease is obvious on *V. rupestris* only and LN33 and Kober 5BB indicators are symptomless.

On biological indicators, bark may be peeled back in the field to expose the woody cylinder and reveal symptoms. Alternatively the trunks of woody indicators can be removed from the field, boiled for 60 minutes or autoclaved at 121°C for 30 minutes. As soon as they are cool enough to handle the outer bark, periderm and the phloem can be removed to observe symptoms affecting the xylem tissue.

Other grapevine varieties may harbour the causal agent but are symptomless. Some sensitive grapevine varieties may exhibit graft incompatibilities associated with the disease. Other symptoms can include swelling of the graft union and pitting of the rootstock when sensitive varieties grafted onto diseased rootstock.

References:

Golino. DA. 1993. Potential Interactions Between Rootstocks and Grapevine Latent Viruses American Journal of Enology and Viticulture 44:148-152

Krake, LR, Steel-Scott, N, Rezaian, MA and Taylor, RH. 1999. Graft Transmitted Diseases of Grapevines. CSIRO Publishing, Collingwood, Victoria.

Martelli G.P. (1993) Rugose wood complex. In Graft-transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis, pp. 45–54. Ed G.P. Martelli. Rome, Italy: FAO.

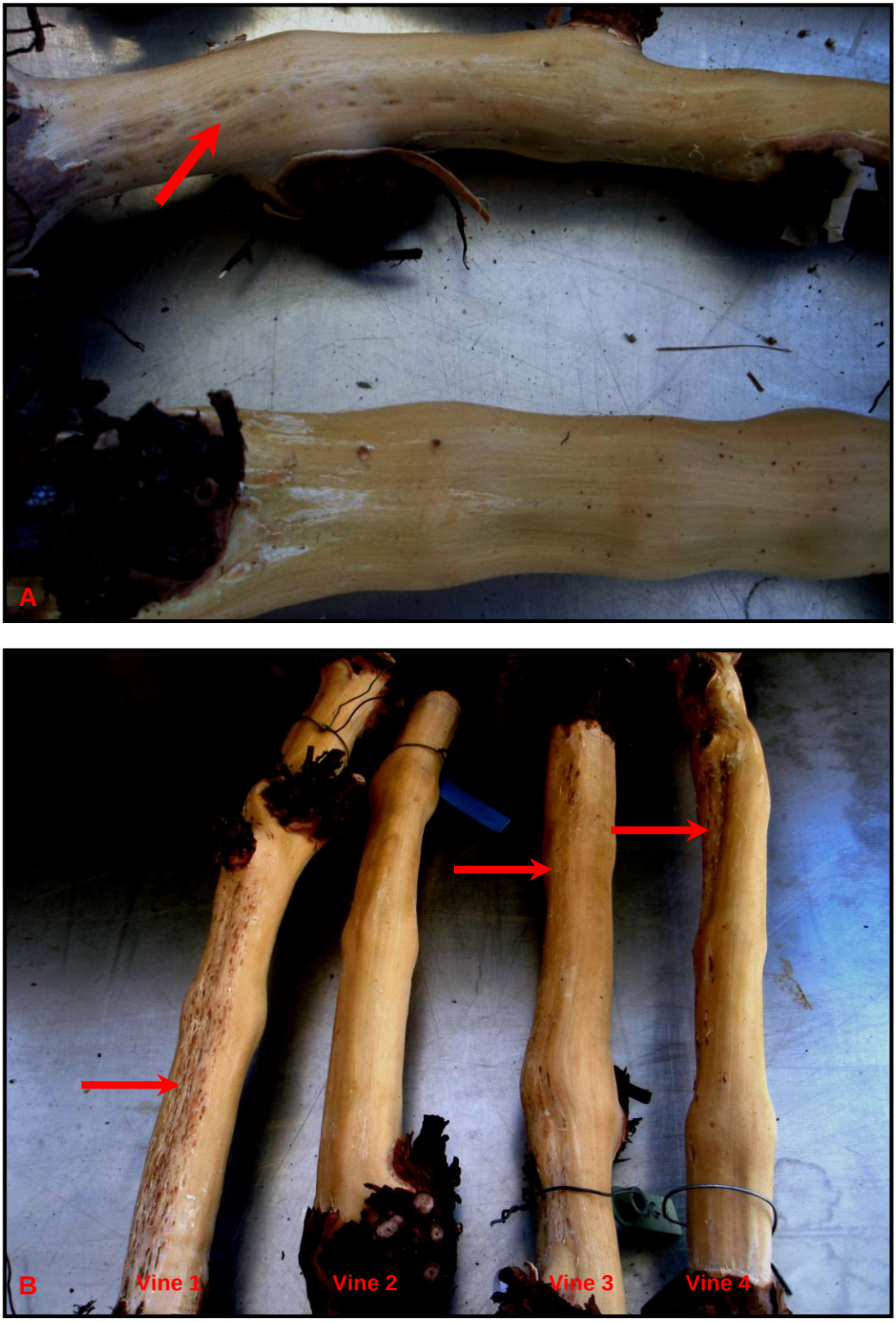


Figure 1. A. Stem pitting symptoms (top, arrow) on *Vitis rupestris* (Rupestris St George) inoculated with a strain of *Rupestris stem pitting associated virus* (RSPaV) from Cabernet Franc C24-1 compared to un-inoculated *V. rupestris* (Rupestris St George, bottom).
B. Stem pitting symptoms (Vines 1, 3 and 4, arrows) on *Vitis rupestris* (Rupestris St George) inoculated with a strain of RSPaV from Cabernet Franc C24-1 compared to un-inoculated *V.rupestris* (Rupestris St George, Vine 2).

Corky bark and LN33 stem grooving diseases:

Corky bark disease and LN33 stem grooving are diseases of the rugose wood complex that are diagnosed and detected by chip bud inoculation onto LN33 (Couderc 1613 x *Vitis vinifera* cv Thompson Seedless*)*. Corky bark is associated with *Grapevine virus B*. The association between LN33 stem grooving and a virus is not certain

Grapevine corky bark (GCB) symptoms include pits and grooves on the woody cylinder and swelling, cracking and corky appearance of the bark tissue in the internodes of canes and shoots of LN-33 caused by the proliferation of secondary phloem tissue (Figure 2). Canes have uneven lignification and may not lignify at all. Other symptoms that have been observed include growth stunting, reddening and drooping of leaves. Symptoms may also be seen on *V. rupestris.* The appearance of disease on LN33 and *V. rupestris* differentiates this disease from other rugose wood diseases that do not affect LN33.

Symptoms of LN33 stem grooving of the stem of LN 33 in a similar manner to corky bark disease (Figure 3). However it differs to corky bark disease as there is no phloem proliferation, internodal swellings or foliar discolorations.

Symptoms on LN33 are evident on the green shoots and canes of inoculated on biological indicators, bark may be peeled back in the field to expose the woody cylinder and reveal symptoms. Alternatively the trunks of woody indicators can be removed from the field, boiled for 60 minutes or autoclaved at 121°C for 30 minutes. As soon as they are cool enough to handle the outer bark, periderm and the phloem can be removed to observe symptoms affecting the xylem tissue.

Other grapevine varieties may harbour the causal agent but are symptomless. The severity of symptoms in other cultivars differs in severity. Some sensitive grapevine varieties may exhibit graft incompatibilities associated with the disease. Other symptoms can include pitting and grooving of the rootstock and sensitive scion varieties may exhibit swelling, cracking and corky appearance of the bark tissue in the internodes. Yield and quality of fruit on sensitive varieties is significantly affected.

References:

Golino. DA. 1993. Potential Interactions Between Rootstocks and Grapevine Latent Viruses American Journal of Enology and Viticulture 44:148-152

Krake, LR, Steel-Scott, N, Rezaian, MA and Taylor, RH. 1999. Graft Transmitted Diseases of Grapevines. CSIRO Publishing, Collingwood, Victoria.

Martelli G.P. (1993) Rugose wood complex. In Grafttransmissible Diseases of Grapevines, Handbook for Detection and Diagnosis, pp. 45–54. Ed G.P. Martelli. Rome, Italy: FAO.

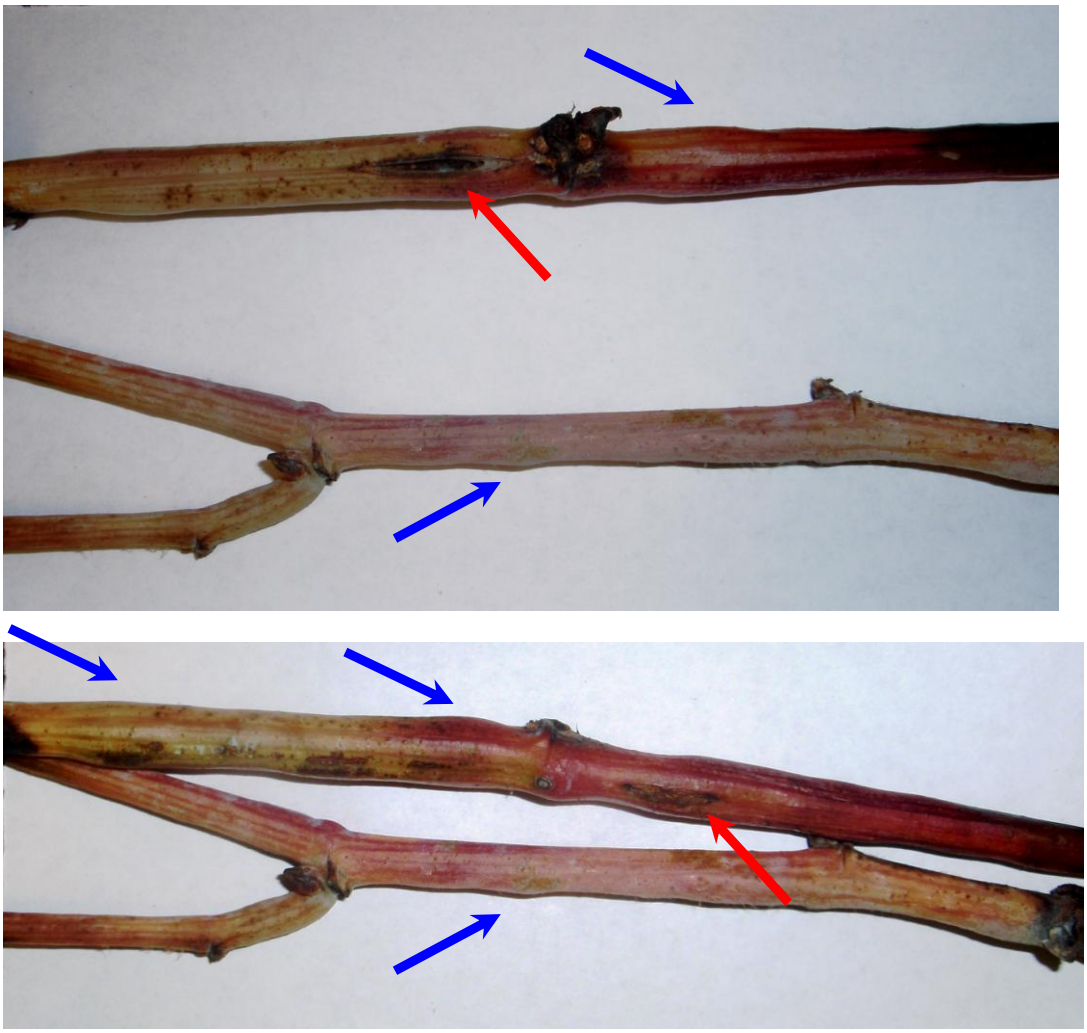


Figure 2. Corky bark symptoms on LN33 inoculated with a strain of *Grapevine virus B* that was observed during post entry quarantine. Note the internodal swellings (red arrows) and cracks (blue arrows) of unlignified shoots.



Figure 3. Possible mild rugose wood symptoms (top, arrow) on LN33 inoculated with *Grapevine virus B* (GVB) compared to un-inoculated LN33 (bottom) The GVB source was from Rupestris St George A5V11 which is also infected with *Grapevine leafroll associated virus-2, Grapevine fleck virus* and *Rupestris stem pitting associated virus*.

Kober stem grooving:

Kober stem grooving is a disease of the rugose wood complex that is diagnosed and detected by chip bud inoculation onto the hybrid Kober 5BB (*Vitis. berlandieri X V. riparia*). It is associated with *Grapevine virus A*.

Indicators can be chip bud inoculated in spring or early summer using two buds per grapevine

In Kober5BB, symptoms include grooving of the xylem tissue of the wood cylinder underneath the bark. Symptoms may not be evident until the bark is completely removed to expose the woody cylinder, The disease is obvious only on Kober 5BB and *V. rupestris* only and LN33 indicators are symptomless.

On biological indicators, bark may be peeled back in the field to expose the woody cylinder and reveal symptoms. Alternatively the trunks of woody indicators can be removed from the field, boiled for 60 minutes or autoclaved at 121°C for 30 minutes. As soon as they are cool enough to handle the outer bark, periderm and the phloem can be removed to observe symptoms affecting the xylem tissue.

Other grapevine varieties may harbour the causal agent but are symptomless. Some sensitive grapevine varieties may exhibit graft incompatibilities associated with the disease. Other symptoms can include swelling of the graft union and pitting of the rootstock when sensitive varieties grafted onto diseased rootstock.

References:

Golino. DA. 1993. Potential Interactions Between Rootstocks and Grapevine Latent Viruses American Journal of Enology and Viticulture 44:148-152

Krake, LR, Steel-Scott, N, Rezaian, MA and Taylor, RH. 1999. Graft Transmitted Diseases of Grapevines. CSIRO Publishing, Collingwood, Victoria.

Martelli G.P. (1993) Rugose wood complex. In Graft-transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis, pp. 45–54. Ed G.P. Martelli. Rome, Italy: FAO.

Fleck Disease:

Grapevine fleck disease is diagnosed and detected by chip bud inoculation onto *Vitis rupestris* and is associated with *Grapevine fleck virus*.

Fleck symptoms include vein clearing of the lower leaves and is most easily observed by holding the leaves against the light (Figure 4). The clearing symptoms diffuse to form spots or a mosaic pattern. Severely affected leaves can be distorted and may curl upward. Symptoms on *V. rupestris* are evident on the lower leaves of inoculated biological indicators, in spring and disappear with hot temperatures.

Most varieties and rootstocks are symptomless

References:

Krake, LR, Steel-Scott, N, Rezaian, MA and Taylor, RH. 1999. Graft Transmitted Diseases of Grapevines. CSIRO Publishing, Collingwood, Victoria.

Martelli G.P. (1993) Rugose wood complex. In Graft-transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis, pp. 45–54. Ed G.P. Martelli. Rome, Italy: FAO

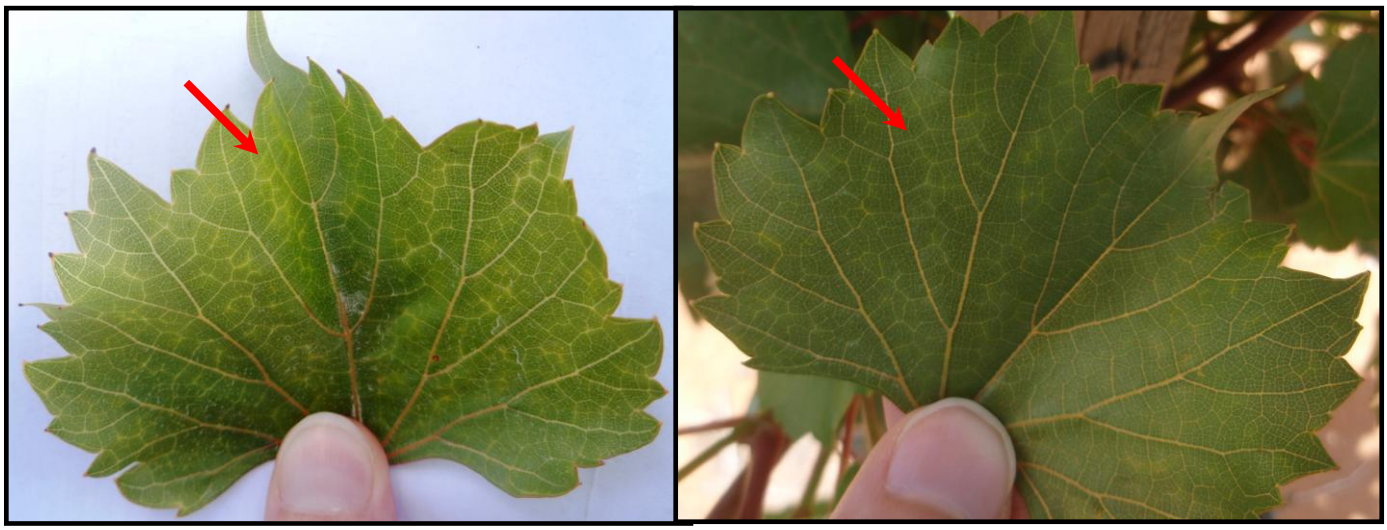


Figure 3. Fleck symptoms on *Vitis rupestris* (Rupestris St George). Note the vein clearing symptoms on minor veins (arrow). This indicator was inoculated with *Grapevine fleck virus* from Chardonnay 84, which is also infected *Rupestris stem pitting associated virus*.

Grapevine Leafroll disease:

Grapevine leafroll disease is diagnosed and detected by chip bud inoculation onto sensitive varieties such as Cabernet franc and Pinot Noir. Cabernet Franc is most commonly used in Australia. In Australia grapevine leafroll disease is associated with *Grapevine leafroll associated virus* (GLRaV) 1 (Figures 4, 5 and 6), GLRaV-2 (Figure 7, 8 and 9), GLRaV-3 (Figure 10), GLRaV-4, GLRaV-5 and GLRaV-9 (Figure 7).

Grapevine leafroll symptoms include downward rolling of the leaves which exhibit interveinal reddening in red varieties and yellowing in white varieties. Often the veins remain green, but in some cases they may redden or yellow as the disease progresses during the season..

Symptoms on LN33 are evident on the leaves during late summer to aurumn

Other grapevine varieties may harbour the causal agent of leafroll disease but are symptomless. The severity of symptoms in other cultivars differs in severity. Some strains of GLRaV0-2 are associated with graft incompatibilities in some varieties. Yield and quality of fruit on sensitive varieties is significantly affected.

References:

Krake, LR, Steel-Scott, N, Rezaian, MA and Taylor, RH. 1999. Graft Transmitted Diseases of Grapevines. CSIRO Publishing, Collingwood, Victoria.

Martelli G.P. (1993) Rugose wood complex. In Graft-transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis, pp. 45–54. Ed G.P. Martelli. Rome, Italy: FAO

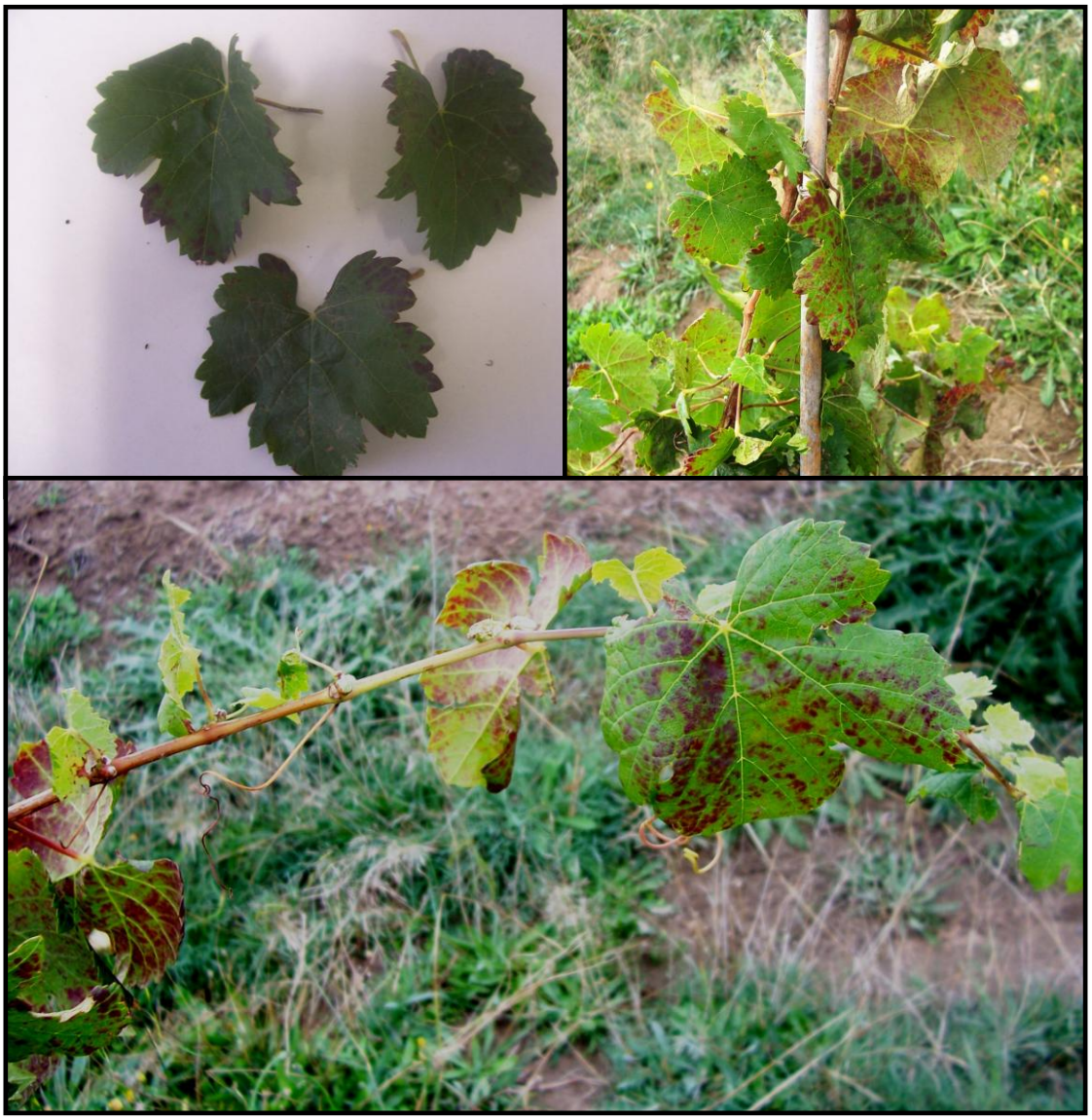


Figure 4. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus 1* from the variety Shiraz RVC14 which is also infected with *Rupestris stem pitting associated virus* Symptoms were observed in autumn 2007 at the Yarra Valley.



Figure 5. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus 1* from the variety Shiraz RVC14 which is also infected with *Rupestris stem pitting associated virus*. Symptoms were observed in autumn 2008 at Loxton.

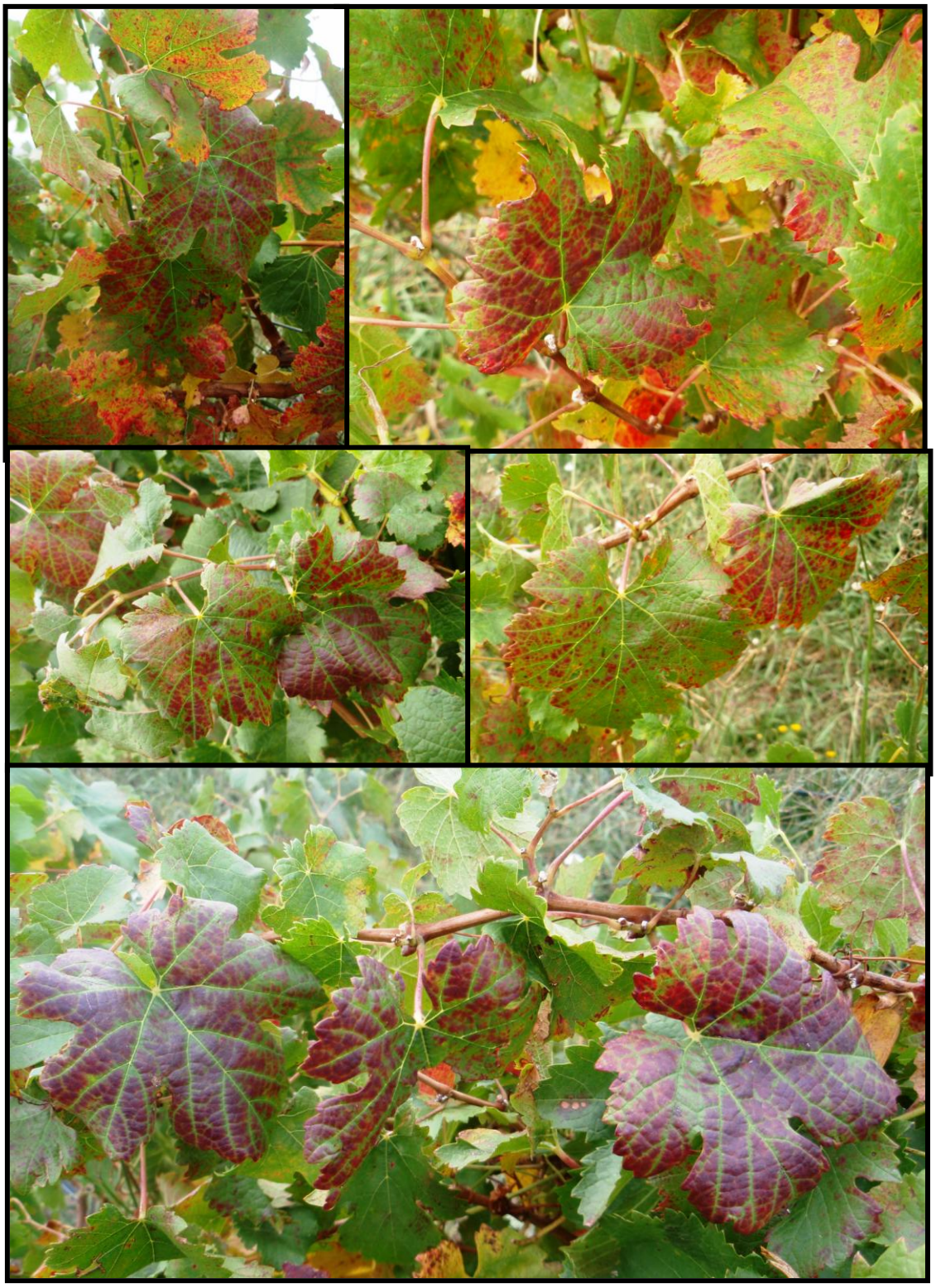


Figure 6. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus 1* from the variety Shiraz RVC14 which is also infected with *Rupestris stem pitting associated virus*. Symptoms were observed in autumn 2008 at the Yarra Valley.



Figure 7. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus 2* from the variety Chardonnay Pen58 which is also infected with *Rupestris stem pitting associated virus* and *Grapevine fleck virus*. Symptoms were observed in autumn 2007 at the Yarra Valley.



Figure 8. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus* 2 from the variety Chardonnay Pen 58 which is also infected with *Rupestris stem pitting associated virus* and *Grapevine fleck virus*. Symptoms were observed in autumn 2008 at the Yarra Valley. Note the variation in symptom severity between leaves and vines.



Figure 9. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus 2* from the variety Chardonnay Pen 58 which is also infected with *Rupestris stem pitting associated virus* and *Grapevine fleck virus*.. Symptoms were observed in autumn 2008 at Loxton. Note the variation in symptom severity between leaves and vines.



Figure 10. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus 3* from the variety Graciano WA6V6 which is also infected with *Rupestris stem pitting associated virus*. Symptoms were observed in autumn 2007 at the Yarra Valley.



Figure 11. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus 3* from the variety Graciano WA6V6 which is also infected with *Rupestris stem pitting associated virus*. Symptoms were observed in autumn 2008 at the Yarra Valley.



A. **B.**

Figure 12. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus 3* from the variety Graciano WA6V6 which is also infected with *Rupestris stem pitting associated virus*. A. Symptoms were observed in autumn 2008 at Loxton. B. Symptoms were also observed in autumn 2009 at Loxton



Figure 13. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus* -9 from the variety Shiraz BVRC 12 which is also infected with *Rupestris stem pitting associated virus*. Symptoms were observed in autumn 2008 at Loxton.



Figure 14. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus* -9 from the variety Shiraz BVRC 12 which is also infected with *Rupestris stem pitting associated virus*. Symptoms were observed in autumn 2007 at the Yarra Valley.



Figure 15. Reddening and subtle rolling of leaves symptoms on the sensitive indicator Cabernet Franc inoculated with *Grapevine leafroll associated virus* -9 from the variety Shiraz BVRC 12 which is also infected with *Rupestris stem pitting associated virus* Symptoms were observed in autumn 2008 at the Yarra Valley.

Other information:

The following websites provide detailed information and images of grapevine virus associated diseases:

http://groups.ucanr.org/iv/index.cfm
http://www.fao.org/docrep/t0675e/T0675E00.htm

Sample No		sample description	State	GVA H587/C995	GVA 7038/7273	GVB	GFkV	RSPaV 48/49	RSPaV Sy9f/Sy9r	Maliogka LR4,	Maliogka LR5	Maliogka LR6	Maliogka Ampelovirus subgroupI	Maliogka Costeroviridae	LR1	LR2	LR3	LR4	LR5	LR9
GS	41	Red Frontignac own roots m100 20 years	NSW	1			1	1						1	1					?
GS	40	Cabernet Sauvignon 15 years own roots MO21	NSW				?	1	1											
GS	42	Semillon DA16162 own roots 14 years old Mo84	NSW					1	1					?		?				
GS	50	Semillon own roots 34 years old	NSW						1											
GS	52	Shiraz 1654 mo53 own roots 12 years old	NSW					1	1											
GS	53	Riesling mo52 15 years old own roots	NSW					1	1											
GS	54	Semillon own roots 18 years old	NSW					1	1											
GS	55	Cabernet sSauvignon PVG9V3 own roots mo 22 15 years old	NSW					1	1											
GS	58	Shiraz unknown clone own roots	NSW	?				1	1											
GS	93	Semillon 1982 Rootstock	NSW					1	1											
GS	94	Semillon 1908 Rootstock	NSW					1	1											
GS	95	Semillon 1960's Rootstock	NSW					1												
GS	96	Shiraz 1975 Rootstock	NSW					1	1											
GS	97	Shiraz 1975 Rootstock	NSW					1	1											
GS	98	Shiraz 1975 Rootstock	NSW					1	1											
GS	99	Shiraz 1975 Rootstock	NSW					1	1											
GS	100	Shiraz 1975 Rootstock	NSW					1	1											
GS	101	Shiraz 1975 Rootstock	NSW					1	1											
GS	102	Shiraz 1975 Rootstock	NSW					1	1											
GS	103	Shiraz 1975 Rootstock	NSW					1												
GS	43	Pinot Noir Mariafield Mo 40 own roots 25 years old	NSW				1	1	1											
GS	44	Merlot FVD3V14 mo32 own roots 23 years old	NSW				1	1	1											
GS	45	Durif FVH7V13 mo34 own roots 23 years old	NSW					1	1							1				
GS	46	Shiraz ESA Mo30 own roots	NSW	1	1			1	1											
GS	48	Traminer own roots 10 years old	NSW					1	1				1	1			1			
GS	47	Cabernet Sauvignon own roots mo39 35 years old	NSW	1	1		?	1	1				1	1						1
GS	38	White Frontignac Mo98 own roots 23 yearsold	NSW	1	1		?		1											1
GS	39	Marsanne own roots 41 years old mo93	NSW	1	1		?	1	1				1	1	?	1	1			1

Sample No		sample description	State	GVA H587/C995	GVA 7038/7273	GVB	GFkV	RSPaV 48/49	RSPaV Sy9f/Sy9r	Maliogka LR4,	Maliogka LR5	Maliogka LR6	Maliogka Ampelovirus subgroupI	Maliogka Costeroviridae	LR1	LR2	LR3	LR4	LR5	LR9
GS	49	Pinmot Noir MV6 mo 33 own roots 22 years old - some leafroll like reddening	NSW				1	1	1				1	1			1			
GS	51	Cabernet Sauvignon 125 mo51 ow roots 15 years old	NSW	1	1			1	1				1	1						1
GS	57	Sauvignon Blanc own roots mo44 23 yaesr old	NSW	1				1	1											1
GS	90	Semillon 1923 Rootstock- Nil	NSW	1	1			1	1				1	1						
GS	91	Semillon 1969 Rootstock- Nil	NSW	1	1			1	1				1	1	1				1	
GS	92	Semillon 1946 Rootstock- Nil	NSW	1				1	1				1	1					1	
GS	56	Sauvignon Blanc own roots 26 yesr old	NSW	1	1			1	1						1					
GS	118	Semillon LRC82 (1-4)	QLD					1	1											
GS	119	Chardonnay 110V5 IC69.8129 (41-44)	QLD					1	1											
GS	120	Cabernet sauv. CW74-44 (45-48)	QLD				1	1	1											
GS	121	Merlot D3V7 (29-32)	QLD					1	1											
GS	122	Shiraz PT23 AN61.0020 (13-15)	QLD					1	1			1		1			1			
GS	173	Chambouroin 9 years old	QLD					1	1											
GS	174	Merlot clone D3V1 D3V14 10 years old	QLD				1	1	1				?							
GS	175	Chardonnay	QLD				1	1	1											
GS	176	Viognier	QLD				1	1	1				?	1	1	1				
GS	177	Chambouroin 10 years old	QLD					1	1						?					
GS	178	Shiraz 11 years old	QLD					1	1											
GS	179	Chardonnay 3 years old	QLD	1				1	1						1					
GS	180	Merlot on Ramsey 10 years old	QLD					1	1				?							
GS	181	Shiraz clone PT23 PT23N 10 years old	QLD					1	1											
GS	182	Chardonnay 8 years old	QLD					1												
GS	142	Red Globe clone olmo	SA				1							1		1				
GS	146	sauvignon blanc clone 132	SA	1	1								1	1	1					1
GS	148	Riesling clone 140	SA	1	1					1?										
GS	188	heinze grenach	SA	1	1		1							1	1	1				
GS	68	Shiraz own roots planted 1959	SA					1	1											
GS	69	Shiraz own roots planted 1967	SA					1	1				?			?				
GS	70	Semillon own roots planted 1957 Harry Tulloch clone?	SA					1	1											

Sample No		sample description	State	GVA H587/C995	GVA 7038/7273	GVB	GFkV	RSPaV 48/49	RSPaV Sy9f/Sy9r	Maliogka LR4,	Maliogka LR5	Maliogka LR6	Maliogka Ampelovirus subgroupI	Maliogka Costeroviridae	LR1	LR2	LR3	LR4	LR5	LR9
GS	71	Semillon own roots planted 1959	SA					1	1											
GS	81	Pinot Gris D1V7	SA					1	1											
GS	82	Pinot Gris D1V7	SA					1	1											
GS	83	Pinot Gris D1V7	SA					1	1											
GS	84	Pinot Gris D1V7	SA					1	1											
GS	85	Cabernet sauvignon on 5C Teliki 8-10 yearts old	SA					1	1											
GS	86	Cabernet sauvignon on 5BB kober 8-10 yearts old	SA					1	1											
GS	155	101-14 clone 123H	SA					1	1											
GS	190	shirz 1654	SA					1	1											
GS	198	waechters mass Shiraz	SA					1	1											
GS	78	Viogner HTK	SA					1	1											
GS	204	Ramsey	SA					1	1											
GS	72	Riesling own roots planted 1935	SA	1	1			1	1											
GS	73	Riesling own roots planted 1970	SA	1	1			1	1											
GS	74	Riesling own roots planted 1969	SA	1	1			1	1											
GS	79	Viogner HTK	SA	1	1			1	1											
GS	195	Paulsen 1103	SA	1				1												
GS	201	Ruggeri 140	SA	1				1	1											
GS	77	Viogner HTK	SA	1				1	1											
GS	104	Pinot noir MV6	SA				1	1												
GS	105	Pinot noir MV6	SA				1	1												
GS	106	Pinot noir MV6	SA				1	1												
GS	107	Pinot noir MV6	SA				1	1												
GS	108	Pinot noir MV6	SA				1	1												
GS	141	Chardonnay clone Q390-09	SA				1	1	1	1?										
GS	147	shiraz clone 1125	SA				1	1	1											
GS	150	Chardonnay clone 84	SA				1	1	1	1?										
GS	153	Ramsey clone A11V12	SA				1	1	1											
GS	156	5C Teleki clone 10-48-49 Gm	SA				1		1											

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GS	168	Pinot Noir clone 115	SA				1	1	1											
GS	197	Ruggeri 140	SA				1	1												
GS	203	Paulsen 1103	SA					1												
GS	76	Viogner HTK	SA					1	1				1	1			1			
GS	157	Malbec cw14	SA					1	1					1			1			
GS	164	Cabernet Franc clone Francese	SA					1						1			1			
GS	75	Riesling own roots planted 1980	SA	1	1		1	1	1											
GS	144	Riesling clone 114	SA	1	1		1	1	1											
GS	165	Grenache clone 139HT	SA	1	1		1	1	1											
GS	80	Viogner HTK	SA	1				1	1				?			1				
GS	136	Graciano Clone WA6V6	SA	1	1				1					1			1			
GS	138	temperanillo clone D8V12	SA	1				1	1				1	1			1			
GS	139	Moss sultana	SA	1	1			1	1	?H			1	1						
GS	143	Cabernet sauvignon clone 125	SA	1	1			1	1				1	1						1
GS	152	sultana clone H5	SA	1				1	1	1	1		1	1				1		
GS	154	Marsanne clone NE vic	SA	1	1			1	1		1		1	1						1
GS	161	Brown frontignac LC8	SA	1	1			1	1				1	1			1		1	1
GS	191	Grope shiraz mas selection	SA	1				1	1				1							
GS	199	waechters mass mataro	SA	1				1	1										1	
GS	200	Shiraz	SA	1				1	1				1	1					1	
GS	205	waechters mass Shiraz	SA	1	1			1	1						1					
GS	151	Chenin blanc clone Steen	SA				1	1	1			1		1			1			
GS	158	Brown frontignac clone BVRC12	SA				1	1	1			1		1	1					
GS	159	Brown frontignac clone BVRC16	SA				1	1	1					1	1					
GS	163	Chardonnay Pen 58 (1959)	SA				1	1	1					1		1				
GS	167	Grenache clone LCE11-2	SA				1	1	1				1	1		1				1
GS	187	mendoza chardonnay	SA				1	1	1					1	1					
GS	193	101-14	SA				1	1	1			1							1	
GS	149	Riesling clone flv544-6	SA	1	1	1		1	1			1		1		1				

Sample No		sample description	State	GVA H587/C995	GVA 7038/7273	GVB	GFkV	RSPaV 48/49	RSPaV Sy9f/Sy9r	Maliogka LR4,	Maliogka LR5	Maliogka LR6	Maliogka Ampelovirus subgroupI	Maliogka Costeroviridae	LR1	LR2	LR3	LR4	LR5	LR9
GS	137	Pinot Noir clone MV6	SA	1	1		1	1						1			1			
GS	140	Chardonnay clone 277	SA	1	1		1	1	1					1			1			
GS	145	Riesling clone 138	SA	1	1		1	1	1				1	1						
GS	160	Brown frontignac LC7	SA	1	1		1	1	1		1		1	1					1	
GS	162	Emperor clone 3A	SA	1	1		1	1	1		1	1	1	1			1		1	
GS	166	Grenache clone LCE11-1	SA	1	1		1	1	1				1	1	1					1
GS	189	rohrlach grenach e	SA	1	1		1	1	1						1					
GS	192	Chardonnay	SA	1	1		1	1	1						1					
GS	194	Teleki 5C	SA	1			1	1									1			
GS	196	Ramsey 60	SA	1			1	1	1											
GS	202	101-14	SA	1			1	1	1			1								
GS	206	Merlot	SA	1	1		1	1	1					1	1	1				
GS	88	richter 110	Vic																	
GS	186	Chardonnay 13 years old	Vic																	
GS	11	Shiraz SP1, OR(own roots?) 48 yo	Vic					1	1											
GS	13	Shiraz, own roots planted 1868! 141 years old!	Vic					1	1											
GS	15	Shiraz 40 years old	Vic					1	1											
GS	17	Cabernet Sauvignon, own roots, 18 years old	Vic					1	1											
GS	59	Shiraz own roots 32 years old	Vic					1	1											
GS	60	Shiraz own roots 10 years old	Vic					1	1											
GS	61	Mataro Heritage on 110 Richter 4.5 yearts	Vic					1	1											
GS	110	Semebat. IC79.8302	Vic					1	1											
GS	183	Chardonnay Pen 58 30 years old	Vic					1												
GS	184	Cabernet Sauvignobn on R99 rootstock 20 years old	Vic					1												
GS	217	Flat stem	Vic	1	1			1	1											
GS	218		Vic	1				1	1											
GS	185	Cabernet Sauvignon on Schwartzmann 8 years old	Vic	1	1			1	1											
GS	113	Marsanne FVE8V1 IC68.8036	Vic					1	1		1	1?	1	1	1				1	
GS	117	Alicante Bouschet FVC1V3 IV64.2089	Vic					1	1					1			1			

Sample No		sample description	State	GVA H587/C995	GVA 7038/7273	GVB	GFkV	RSPaV 48/49	RSPaV Sy9f/Sy9r	Maliogka LR4,	Maliogka LR5	Maliogka LR6	Maliogka Ampelovirus subgroupI	Maliogka Coste roviridae	LR1	LR2	LR3	LR4	LR5	LR9
GS	67	Chardonnay orwn roots 24years old	Vic					1	1				?	1						
GS	14	Chardoonay (RMI) own roots 29 years old	Vic					1	1				1			1				
GS	36	Shiraz - Mt Langhi Ghiran. origions in great western	Vic					1	1				1	1						
GS	112	Rousanne 1974/CX/Vassas IC74.8289	Vic				1	1	1											
GS	109	Pinot noir- more details to be provided	Vic				1	1	1											
GS	65	Shiraz own roots 40 years old	Vic				1	1	1											
GS	12	Cabernet Sauvignon CR1, on Chenin, 29 yo,	Vic	1	1			1	1				1	1						1
GS	28	See CHS for details FR/4561/1- reddening, leafroll symptoms	Vic	1	1			1	1		1		1	1	1					
GS	37	Cabernet Sauvingnon vine decline	Vic	1	1		1	1	1				1	1						1
GS	64	Pinot noir orwn roots 36 years old	Vic	1	1			1	1				1	1	1					1
GS	66	Pinot noir orwn roots 27years old	Vic	1	1			1	1				?	1	1					
GS	87	marzemino r1	Vic	1	1			1	1					1	1					
GS	89	pinot gris A1	Vic	1	1			1	1				1	1	1					
GS	207	Varieties largely unknown!!	Vic	1				1	1				1		1				1	
GS	208	Varieties largely unknown!!	Vic	1	1			1	1				1	1						1
GS	212	Varieties largely unknown!!	Vic	1	1			1						1	1	1	1			
GS	16	Shriaz (IMI) own roots 40 years old	Vic				1	1	1				1	1			1			
GS	114	Kadarka IC74.8461 ex Budapest via Colmar	Vic				1	1	1			1?		1			1			
GS	115	Fernao Pires 1961/VX/UCD IV61.2037	Vic				1	1	1					1						
GS	116	Bonvedro ex Vassal, France	Vic				1	1	1					1			1			
GS	111	Rubired FVC5V14 IV63.2068	Vic				1	1	1		1		1							
GS	169	Cabernet Sauvignon	Vic				1	1											1	
GS	170	Cabernet Sauvignon	Vic				1	1			1		1	1					1	
GS	171	Cabernet Sauvignon	Vic				1	1	1		1		1						1	
GS	210	Varieties largely unknown!!	Vic				1	1	1				1	1		1				
GS	213	Varieties largely unknown!!	Vic				1	1	1					1			1			
GS	62	Grenache Heritage on 140 Ruggeri 4.5 years old	Vic	1	1		1	1	1					1	1					
GS	63	Pinot noir orwn roots 36 years old	Vic	1	1		1	1	1					1	1					
GS	209	Varieties largely unknown!!	Vic	1	1		1	1	1				1	1	?					1

Sample No		sample description	State	GVA H587/C995	GVA 7038/7273	GVB	GFkV	RSPaV 48/49	RSPaV Sy9f/Sy9r	Maliogka LR4,	Maliogka LR5	Maliogka LR6	Maliogka Ampelovirus subgroupI	Maliogka Costeroviridae	LR1	LR2	LR3	LR4	LR5	LR9
GS	211	Varieties largely unknown!!	Vic	1	1		1	1	1	1			1	1		1	1			
GS	214	Varieties largely unknown!!	Vic	1	1		1	1		1			1	1	1		1		1	1
GS	215	Varieties largely unknown!!	Vic	1	1		1	1	1	1			1	1	1		1	1	1	
GS	216	Varieties largely unknown!!	Vic	1	1		1	1	1	1			1	1	1		1	1	1	
GS	172	Cabernet Sauvignon	Vic	1	1	1	1	1	1				1	1					1	
GS	27	D Pinot own roots 12 years	WA											?	?					
GS	34	cabernet sauvignon own roots 10 years old	WA																	
GS	132	Shiraz 11 yrs old	WA					1	1											
GS	133	Cabernet sauvignon SA126 clone, 31 yrs old	WA					1	1											
GS	134	Semillon FVD10V12 clone, 5 yrs old	WA					1	1											
GS	1	Shiraz, own roots, 10 yo, planted 1998	WA					1	1											
GS	2	Chardonnay clone 1 own roots 5 yo planted 2003 - herbicide damage	WA					1	1											
GS	3	Shiraz, own roots, 10 yo, planted 1998	WA					1	1											
GS	4	Cabernet Sauvignon, own roots, 10 yo, planted 1998	WA					1												
GS	5	Semillon, own roots, 10 yo, planted 1998	WA					1	1											
GS	6	Chardonnay C 1 own roots 11 yo planted 1977	WA					1	1											
GS	7	Chardonnay C 7 own roots 11 yo planted 1977	WA					1												
GS	8	Sauvignon Blanc, own roots, 10 yo, planted 1998	WA					1	1											
GS	9	Merlot, own roots, 10 yo, planted 1998	WA						1											
GS	18	Semillon, own roots, 12 yo	WA					1												
GS	19	B Sauvignon Blanc own roots 6 years	WA					1	1											
GS	21	E Sauvignon Blanc own roots 12 years	WA					1	1							?				
GS	29	Semillon own roots 10 years old	WA					1	1											
GS	33	Chardonnay Gin Gin clone own roots 10 years old	WA					1	1											
GS	35	sauvignon blanc own roots 10 years old	WA					1	1											
GS	26	D Chardonny Gin Gin own roots 12 years	WA											1	1					
GS	10	Riesling, own roots, 10 yo, planted 1998	WA	1	1			1	1											
GS	135	Chardonnay (Gin Gin), 6 yrs old	WA					1	1						1					

Sample No		sample description	State	GVA H587/C995	GVA 7038/7273	GVB	GFkV	RSPaV 48/49	RSPaV Sy9f/Sy9r	Maliogka LR4,	Maliogka LR5	Maliogka LR6	Maliogka Ampelovirus subgroupI	Maliogka Costeroviridae	LR1	LR2	LR3	LR4	LR5	LR9
GS	22	E Chardonny clone 1 own roots 12 years	WA					1	1					1			1			
GS	30	Shiraz own roots 10 years old	WA					1	1		1		1	1			1		1	
GS	31	cabernet sauvignon own roots 10 years old	WA				1	1	1											
GS	23	A Shiraz schwazman rootstock 6 years reddening	WA	1	1				1					1			1			
GS	25	A Rielsling on schwazman rootstock 6 years	WA	1	1			1	1					1			1			
GS	20	A Verdhello own roots 12 years	WA	1				1	1				1	1			1		1	1
GS	123	Chardonnay P58 clone, 8yrs old	WA	1	1			1	1				1	1					1	1
GS	125	Chardonnay P58 clone, 8yrs old	WA	1	1			1	1				1	1						1
GS	126	Chardonnay P58 clone, 8yrs old	WA	1	1			1	1				1	1						1
GS	128	Chardonnay P58 clone, 8yrs old	WA	1	1			1	1				1	1						1
GS	129	Chardonnay P58 clone, 8yrs old	WA	1	1			1	1				1	1						1
GS	130	Chardonnay P58 clone, 8yrs old	WA	1	1			1	1			1	1	1						1
GS	131	Chardonnay P58 clone, 8yrs old	WA	1	1			1	1				1	1						1
GS	32	Chardonnay own roots 10 years old	WA				1	1	1					1			1			
GS	24	D Merlot on Schwazmann rootsock 6 years old some reddening of leaves	WA				1	1	1					1	1		1			
GS	124	Chardonnay P58 clone, 8yrs old	WA				1	1	1			1		1		1				
GS	127	Chardonnay P58 clone, 8yrs old	WA				1	1	1					1		1				

Australian Government
Grape and Wine Research and Development Corporation

END OF PROJECT FINANCIAL STATEMENT

Statement of Receipts and Expenditure

At the conclusion of each Project, the GWRDC requires a Statement of Receipts and Expenditure of GWRDC funds received in relation to the Project.

Project Title:	Development and validation of diagnostic protocols for the detection of endemic and exotic pathogens of grapevines

GWRDC Project Number	Project Start Date	Project End Date
05/04 (03293)	**1 January 2006**	**31 December 2009**

Financial Year (e.g. 2006/07)	**(a)** ***Funds brought forward from previous year***	**(b)** **#Approved GWRDC Budget (full year)**	**(c)** **Total budget available** *(a)+(b)*	**(d)** **Actual expenditure ($)**	**(e)** **Difference ($)** *(c)-(d)*
Year 2: 2005/2006	**0**	72,669	72,669	5,922	66,747
Year 2: 2006/2007	66,747	103,706	170,453	124,101	46,352
Year 3: 2007/2008	46,352	107,880	154,232	142,680	11,552
Year 4: 2008/2009	11,552	162,979	174,531	91,799	82,732
Year 5: 2009/2010	82,732	0	82,732	84,741	(2,009)
TOTAL		447,234		449,243	

* "Funds brought forward" refers to any unspent GWRDC funds from the previous year and approved by the GWRDC to be spent the following year (i.e. column *(e)* from previous year).
# "Approved GWRDC Budget" is the funding amount specified in the Project Agreement, including any approved Variations after Project commencement.

Was funding expended as approved in the Project Agreement? Yes ☒ No ☐

*If **NO**, please provide reasons:*

Has the cash and/or in-kind funding from Contributing Agencies been received and applied to the Project as approved in the Project Agreement? Yes ☒ No ☐

*If **NO,** please provide reasons:*

I hereby certify that this statement is true and accurate.

Signature of duly authorised representative ……

…….. ……..
Name: Pauline Jubb *Title: Administration Contact* *Date:*