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

Fiona E. Constable[1], Phil Nicholas[2], Julian Connellan[3], Tony Bass[2], Nuredin Habili, and Brendan C. Rodoni[1]

[1]Department of Primary Industries, Knoxfield, Private Bag 15, Ferntree Gully Delivery Centre, Victoria 3156, Australia. [2]South Australian Research and Development Institute, Loxton Research Centre Loxton South Australia 5333, Australia. [3]NSW Department of Primary Industries, Griffith node National Wine and Grape Industry Centre, PO Box 1087, Griffith, New South Wales 2680 Australia [4]Waite Diagnostics School of Agriculture & Wine, Waite Campus, The University of Adelaide, PMB1, Glen Osmond, South Australia 5064 Australia

The improved productivity and sustainability of the Australian viticulture industry is dependent on the provision of high health planting material. 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 vine material to nurseries, via regional vine improvement groups. Certification is supported by indexing, ELISA and PCR for pathogen detection.

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 other in Australia. Currently, we have a four year research project that aims to contribute to the development of grapevine certification protocols for Australia and will:

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

To determine the specificity and reliability of primers for PCR testing, bioinformatics analyses were done to select several published primer pairs for each endemic and exotic virus. Several tests were established in the laboratory and tested against a range of isolates. Variation in detection is being observed for GLRaV-1,-3, -5, GVA, and GVB.

For detection of GLRaV-3, five different primer pairs were selected based on bioinformatics analysis. When these primer pairs were trialled against several isolates of GLRaV-3, the most reliable primer pair was P3U/P3D (Turturo *et. al.* 2005). The primer pair C547/H229 (Minafra and Hadidi 1994) occasionally generated products of larger than the expected size for some isolates of GLRaV-3. Sequence analysis indicated that the larger products are significantly different (79% sequence similarity) from other GLRaV-3 isolates but closely related (98%) to a divergent GLRaV-3 strain from Italy (GenBank accession No. DQ314610).

For detection of GVA, three primer pairs were selected and trialled against several isolates of GVA. None of the primer pairs detected all the GVA isolates that were used. The primer pair C995/H587 (Minafra *et. al.* 1997) detected most GVA isolates however some GVA strains were only detected by the 7038/7273 primer pair (MacKenzie 1997).

One isolate of GVA that was only detected using the C995/H587 primer pair had not been detected previously by woody indexing, ELISA or other PCR assays. A larger product (868 bp), which generated from this isolate using the H587 and 7273 primer and included the ORF4 (coat protein gene) and most of ORF5 (RNA binding protein gene), was cloned for sequencing. Sequence analysis of this amplicon indicated that this GVA isolate had less than 90% sequence similarity with other GVA isolates. This GVA isolate was detected in vines that had undergone heat-treatment and meristem culture for virus eradication and had tested negative when indexed for virus using woody indicators, ELISA and PCR. GVA was also detected in the original untreated material and there was 99% sequence similarity between

the GVA isolates from the treated and untreated vines. This result suggests that GVA had not been eradicated through heat treatment and meristem culture.
To investigate the sampling and sensitivity of ELISA and PCR under Australian conditions, two replicate trials were established in a hot climate region (Sunraysia, Victoria) and a cool climate region (Yarra Valley, Victoria). Each trial contains two varieties (Shiraz and Chardonnay) and for each variety there are five treatments in which five replicate vines were either un-inoculated or inoculated with GLRaV-2, GLRaV-3, GVA or GFkV. These vines were chip bud inoculated in October 2006 (Sunraysia) and November 2006 (Yarra Valley) and have been tested monthly by ELISA and PCR since December and January respectively. At both sites, six to seven weeks post-inoculation GLRaV-3 was detected by ELISA and PCR and GLRaV-2 and GFkV were detected by PCR only. GVA has only been detected in April 2007 in one vine at Sunraysia. Between December 2006 and April 2007 more positive results for each virus were obtained using PCR compared to ELISA indicating that PCR is more sensitive than the serological assay.

In an additional trial, to observe the movement of virus within a vine with time, the variety Chardonnay was inoculated with the same isolates of GLRaV-2, GLRaV-3, GVA or GFkV. Three replicate vines are destructively sampled in 20 cm sections over the whole vine in the spring and autumn of each year for three years. The first sampling was conducted in Sunraysia in December 2006, eight weeks post-inoculation. GLRaV-2, GLRaV-3 and GFkV were detected in the roots and the trunks of inoculated vines. GFkV was the only virus detected in the shoots. GVA was not detected in any of the plant sections.

Symptom development on woody indexing is being documented in a trial that has been replicated in three different environments: a hot climate (Riverland region, South Australia); cool climate (Yarra Valley, Victoria); and in potted vines, maintained in a screenhouse (Knoxfield, Victoria). The sensitive indicator varieties Cabernet Franc, LN33, Kober 5BB and Rupestris St George have been inoculated with GLRaV-1,-2,-3,-9, GVA, GVB, GFkV or RSPaV. At the Riverland region and in the Yarra Valley, the indicators were chip bud inoculated in October 2006 and the screenhouse trial was inoculated in February 2007. Leafroll symptoms on vines inoculated with GLRaV-1,-2,-3 and -9 were observed in April 2007 in the Yarra Valley only. Symptoms were not observed at the Riverland or Knoxfield. These results indicate that climatic/environmental factors may be important in the development of symptoms on indicators. Testing is underway to determine if virus was transmitted to each of the indicators.

References

1. MacKenzie, D.J. 1997. A standard protocol for the detection of viruses and viroids using a reverse transcription- polymerase chain reaction technique. Document CPHBT-RTPCR1.00 The Canadian Food Inspection Agency.
2. Minafra, A., Saldarelli, P. and Martelli, G.P. 1997. Grapevine virus A: nucleotide sequence, genome organization, and relationship in the Trichovirus genus. Archives of Virology 142, 417-423.
3. Minafra, A. and Hadidi, A. 1994. Sensitive detection of grapevine virus A, B, or leafroll-associated virus III from viruliferous mealybugs and infected tissue. Journal of Virological Methods 47,175-188.
4. Turturo, C., Saldarelli, P., Yafeng, D., Digiaro, M., Minafra, A., Savino, V. and Martelli, G. P. 2005. Genetic variability and population structure of *Grapevine leafroll-associated virus 3* isolates. Journal of General Virology 86, 217–224.

Acknowledgements

We thank the Australian Vine Improvement Association and the Victorian and Murray Valley Vine Improvement Association for their support and supplying material for this project. This project is funded by The Grape and Wine Research and Development Corporation.