National Program for the management phytoplasmas in Australian grapevines

FINAL REPORT to
Grape and Wine Research and Development Corporation

Project Number: **CRV 95/2**

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Research Organisation: **Cooperative Research Centre for Viticulture**
Date**: October 2000**

National Program for the management of phytoplasmas in grapevines: Final Report

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Grape and Wine Research and Development Corporation

Interim Report

Project Number: CRCV 95/2

National Program for the management of phytoplasmas in Australian grapevines

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Acknowledgments

We would like to acknowledge the technical assistance of Tim Griffin, Jingye Zhang and Rebecca Miles. We also appreciate the extensive contributions in time as well as access to vineyards from Carmel and Grant Carey, Noel and Midge Cameron, Frank Sos, Keith Nichols, Vince Barilla, Lindsay Herath, Gary Thomas, Barry Avery and Len Schleifert. This work has been supported by Grape and Wine Research and Development Corporation (GWRDC), the Cooperative Research Centre for Viticulture (CRCV), the Dried Fruit Research and Development Council (DFRDC), Agriculture Victoria (DNRE), Primary Industries Research of South Australia (PIRSA), University of Adelaide, Northern Territory University.

Thanks to other technical staff at Sunraysia Horticultural Centre (SHC) Sonja Needs, Leonie Pitt and Fred Hancock, and at Southcorp, Kerrilyn Quick. In kind assistance was received from Southcorp and Mildara Blass. Thanks also to Bernd Schneider. Thanks to Donna Aitken (IHD) for assisting in compiling the Interim report.

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Project Summary

National Program for the Management of Phytoplasmas in Australian Grapevines

Introduction

Australian Grapevine Yellows (AGY) was first reported in 1975 (Magareyand Wachtel 1983). Since then, there has been considerable concern and uncertainty in the viticulture industry about its impact on winegrape production. Early surveys of AGY symptoms showed widespread occurrence in the warmer inland districts of Sunraysia, MIA and the Riverland (Magarey and Wachtel 1986). In some vineyards up to 50% of vines had one or more shoots showing AGY symptoms. Chardonnay and Riesling were most often affected, but symptoms were also found in other varieties and in other regions.

In 1995/96 the GWRDC and CRCV funded a project, "National program for the management of phytoplasmas in Australian grapevines" to investigate the real. Economic impact of the AGY disease and associated phytoplasmas and to devise and implement control strategies. The program included collaborative researchers from Agriculture Victoria, Adelaide University, Northern Territory University and Primary Industries South Australia. A steering committee with strong industry involvement was established to oversee the project.

Objectives

This national program identified 8 key points to investigate after discussions with industry and researchers, including:

1) The extent of AGY disease distribution in Australian vineyards and spread compared to previous surveys.
2) The full range of symptoms associated with phytoplasma in grapevines.
3) The identification of the vector(s) and alternative hosts of AGY phytoplasma (including symptom-less infections in other grape varieties).
4) The potential for the spread of AGY disease by vegetative propagation, and its control.
5) Control and management options for AGY disease in regions where it is already present.
6) The need for phytosanitary protocols to restrict the introduction and spread of phytoplasma, particularly flavescence doree phytoplasma.
7) Characterisation of phytoplasmas in Australian grapevines.
8) The origins of Australian phytoplasmas.

Research within the project centred on developing tools for detecting phytoplasmas, determining the association between phytoplasmas and symptoms, mapping the incidence and distribution of AGY symptoms, testing potential vectors and alternative hosts for the AGY phytoplasma and investigating hot water treatment for propagation materials.

Methods for detecting phytoplasma

Universal polymerase chain reaction (PCR) molecular methods were developed to detect any phytoplasma that might infect grapevines and provided a reliable test for detecting the organism.

PCR methods are used to exponentially multiply specific segments of the DNA of an organism to a concentration that can be visualised. The PCR methods can also be used to detect phytoplasmas in insects and other plant species when screening for vectors and alternative hosts for the AGY phytoplasma. A specific PCR method was also developed to detect and confirm infection by the AGY phytoplasma. The universal PCR methods have been adopted for commercial diagnosis of phytoplasma infections in grapevines and other crops by Crop Health Services at IHD Knoxfield, Victoria and Waite Diagnostics. The original PCR protocols for detecting phytoplasma and the techniques used to process insect and plant samples, before PCR analysis were modified in 1998. The altered PCR protocols have used at IHD Knoxfield to improve detection and shorten time needed for sample processing.

Phytoplasmas have been consistently detected by universal PCR techniques in over 80% of symptomatic shoots if tested in January or early February when AGY symptoms are most apparent. A small number (3%) of asymptomatic vines have also tested positive for phytoplasma (Gibb et al. 1999). A survey conducted in 1995/96 showed that phytoplasmas are found in most viticulture regions in Australia in a broad range of both red and white varieties.

Three different phytoplasmas have been identified in Australian grapevines, AGY phytoplasma, a Tomato Big Bud-type phytoplasma and a variant of the AGY phytoplasma that was only found in the Ovens Valley. DNA typing work from Northern Territory University suggests that the AGY phytoplasma is unique to the Australasian region (Padovan et al.1996). None of the phytoplasmas associated with grapevine yellows in Europe and the U.S.A. have been detected in Australia.

The development of an antibody-based test, Enzyme Linked ImmunoSorbent Assay (ELISA) was investigated as the test was considered to be more robust and cost effective than PCR methods. However the sensitivity of ELISA was found to be inadequate to detect phytoplasmas in grapevines and the work was terminated.

Mapping AGY incidence

The incidence of grapevines affected with AGY symptoms was mapped over five seasons in Sunraysia, at Gol Gol, Karadoc and Wemen (Table 1). These results indicate that AGY symptom expression within vineyards and between vineyards is variable from season to season. Vineyard mapping data associated with AGY symptoms from 1976-1984 (Magarey et al., 1986, this report) also showed variability in incidence of vines with AGY symptoms from season to season and vineyard to vineyard. These data suggest that conditions that differ between seasons and sites have an effect on the expression of AGY symptoms in grapevine.

Table 1. Incidence (%) of grapevines affected with AGY symptoms in the 1995/96, 1996/97, 1997/98, 1998/99, 1999/2000 seasons at three study sites in Sunraysia.

Location	Season				
	1995/96	1996/97	1997/98	1998/99	1999/00
Gol Gol	20%	54%	19%	45%	46%
Karadoc	2%	4%	6%	21%	41%
Wemen	0.7%	24%	21%	17%	12%

The mapping data collected over three seasons from spring 1995 through autumn 1998 was analysed by a spatial analysis package, Patchy® (1993), developed by Michael Maixner to study distribution and spread of Vergilbungskrankheit phytoplasma in Germany (Constable et al. 1997 & 1998). The analysis showed a clustered distribution of vines with AGY symptoms for three seasons at Gol Gol and for two seasons, 1995/96 and 1996/97 at Karadoc. The distribution of vines with AGY symptoms at Karadoc in 1997/98 was random. The clustered distribution observed in most years suggests that an insect vector may be transmitting the phytoplasma within the vineyard either from vine to vine or from weed to vine. Alternatively, a single inoculative leafhopper may infect a cluster of vines in a single visit to the vineyard.

Phytoplasma vectors

Planthoppers, leafhoppers and psyllids transmit most phytoplasmas. In France, flavescence doree (FD) phytoplasma is transmitted from vine to vine via an introduced insect vector, which lives solely on grapevines. Very few planthoppers or leafhoppers were found on grapevines at the French study sites. Closely related phytoplasmas, causing Vergilbungskrankheit (VK) from Germany and Phormium yellow leaf from New Zealand, are transmitted by members of the Cixiidae family of planthoppers.

More than 15 different leafhopper and planthopper species have been collected from vineyards with a high incidence of AGY symptomatic vines in the Sunraysia district, Gol Gol and Wemen. The three dominant species have been *Orosius argentatus,* a known vector of the Tomato Big Bud (TBB) phytoplasma, *Austroasca viridigrisea* and *Zygina zealandica.* Surveys of candidate vector species were also made in South Australia using sticky trap sampling. *O*. *Argentatus* was the most abundant leafhopper captured followed by *Austrogallia torrida, A. viridigrisea, Batracomorphus angustanus* and Z. *zealandica.* Few Cixiidae species have been found in the study sites. Over 5000 leafhoppers and planthoppers have been screened by PCR for phytoplasma and 4 leafhopper samples of up to 10 *O*. *argentatus* have tested positive for the AGY phytoplasma.

A labelling system to detect phytoplasma in insect vectors was developed in collaboration with researchers at the University in Udine (Italy).

The position of phytoplasma within a vector was correlated with acquisition time and latency period (14-18 days), after which the insect is able to transmit phytoplasma.

In laboratory trials, PCR tests were used to show that the TBB phytoplasma could be detected in *O. argentatus* within two days of feeding on TBB positive plants. This suggests that the field collected *O. argentatus* which tested PCR positive for the AGY phytoplasma may have tested positive after feeding on AGY positive plants. This does not indicate that *O. argentatus* is a vector of the AGY phytoplasma.

Transmission trials using leafhopper species commonly found in and around vineyards have not yet been successful in establishing acquisition or transmission of AGY phytoplasma by test leafhoppers. Special attention has been given to *O. argentatus* because of its abundance, the positive PCR results for AGY and its vector status for TBB phytoplasma. Symptomatic grapevines were used as acquisition hosts and several plant species have been used as inoculation test plants. If leafhoppers are unable to acquire the phytoplasma from grapevines, acquisition of phytoplasma by test insects may not be possible until another AGY phytoplasma host is established.

The possible timing of infection was also investigated by placing likely alternative AGY plant hosts in an infected vineyard at two-week intervals. The 'bait' plants were then placed in an insect-proof screen house and observed for symptoms of phytoplasma infection. Several plants have exhibited symptoms and have been tested by PCR analysis. To date none have tested positive for AGY phytoplasma, although TBB phytoplasma has been detected. Many of the test plants may not become symptomatic for several months.

Alternative plant hosts for phytoplasmas

In Germany, transmission of Vergilbungskrankheit phytoplasma is from weed to vine by a vector living the majority of its life on the alternative weed host. In 1997/98 a thorough survey of weeds in two vineyards with a high incidence of vines with AGY symptoms was conducted. Every six weeks weeds were collected and identified and then screened for phytoplasma using PCR. More than 30 plant species have been identified and more than 80% of these are broadleaf. None of the samples tested by nested PCR were positive for AGY phytoplasma, although TBB phytoplasma was detected in some. At another site, two samples of lucerne tested positive for the AGY phytoplasma (pers. comm. Rod Bonfiglioli). In 1998/99 and 99/2000 only weeds with symptoms associated with phytoplasma infections were collected and tested by PCR. None of the samples tested positive for the AGY phytoplasma.

Effects of AGY on yield

When AGY was first investigated in 1976 there was concern that the effects of the disease would be as severe as those caused by particular European grapevine yellows diseases. For example, flavescence doree can cause up to 100% yield loss. Early research in the Riverland suggested that yield losses up to 13% could occur in vineyards severely affected by AGY (Magarey and Wachtel 1986). However, analysis of yield data from 1996-1998 showed that there was no significant difference in yield between asymptomatic and symptomatic vines expressing AGY shoot symptoms at two study sites, in Sunraysia at Gol Gol and Wemen.

However, at a third study site, at Karadoc, there was a moderately significant reduction in yield in 1996 and 1997.

A small trial was also conducted at Wemen in 1996/97 to compare the yield of vines that displayed unlignified shoots at the beginning of the season with vines that did not. There was a significantly lower yield in affected vines. All the vines in this study that had unlignified shoots displayed AGY symptoms later in the season and all vines but one displayed restricted growth symptoms.

Linking phytoplasmas and vine symptoms

While there is strong evidence for the association of phytoplasma with typical AGY symptoms, the link between the AGY phytoplasma and restricted growth and late season leaf curl symptoms is still unfolding. Some data suggested that vines are more prone to restricted growth symptoms if they are positive for AGY phytoplasma or display AGY shoot symptoms. Other data shows no significant correlation. There are many vines that display restricted growth and no AGY symptoms, and vines displaying AGY symptoms that have never shown restricted growth symptoms.

Phytoplasmas are complex organisms to study because they cannot be cultured outside their host plant or insect. It is therefore difficult to infect a "healthy" grapevine under controlled conditions to observe symptom development and verify the cause of a disorder.

Association of AGY, restricted growth and late season leaf curl

Using PCR methods

To determine associations between vine symptoms and phytoplasmas, a study was conducted during the 1995/96 to 1997/98 seasons in three Chardonnay vineyards in Sunraysia. Detailed symptoms were recorded from individual vines, particularly related to AGY, restricted growth and late season leaf curl. Samples from each type of symptom from each vine were taken for PCR diagnosis. Some vine samples from other regions were collected and tested as well. The PCR tests showed a strong association between the AGY phytoplasma and AGY symptoms, but not between phytoplasma and restricted growth symptoms or between phytoplasma and late season leaf curl symptoms (Gibb et al. 1999).

Bonfiglioli et al. (1995) PCR studies also found no association between phytoplasma and restricted growth, but did find a correlation between the presence of phytoplasma and late season leaf curl symptoms. However, the associated phytoplasma was not identified.

To further investigate the association of restricted growth symptoms with AGY, a trial was set up in 4 vineyards in Sunraysia. Twelve vines that displayed restricted growth in early December 1997 were paired with 12 unaffected vines. In January 1998, 10 of the 12 vines with restricted growth either displayed AGY shoot symptoms or were PCR positive for AGY. Only one of the 12 unaffected vines was PCR positive for AGY with no visible symptoms. No other pathogens were consistently isolated from the vines.

Mapping symptoms

A preliminary analysis of mapping information from one study site showed that AGY affected grapevines often display restricted growth symptoms in the following season (Bonfiglioli et al. 1997). A further three-year study included mapping of vines within three Sunraysia vineyards. Vines were observed regularly during the three years and a statistical analysis conducted to determine whether AGY, restricted growth (mild and severe) and late season leaf curl (mild and severe) persisted year to year and if there was any significant association between them. The statistical analysis showed that:

- AGY, restricted growth or late season leaf curl symptoms may affect the same grapevine in several subsequent years.
- AGY symptoms were significantly associated with mild restricted growth symptoms in the following season.
- AGY symptoms were significantly associated with mild late season leaf curl symptoms.
- AGY symptoms were not significantly associated with severe restricted growth symptoms.

Restricted Spring Growth National Task Force

Concern that phytoplasma infection may also cause the restricted spring growth (RSG) syndrome led to the incorporation of the CRCV project "Understanding and Management of Restricted Spring Growth" into the phytoplasma program.

The RSG project was initiated in 1994 after widespread reports from growers of RSG symptoms and yield reductions in vineyards. A National Task Force with representatives from AgVic, PIRSA, NSWAg, CSIRO Horticulture, wine companies, GWRDC and CRCV, was established in October 1994 to investigate and coordinate RSG syndrome related activities. Initially the project was funded solely by CRCV with the following objectives:

- Coordinate investigations into the RSG syndrome through a national liaison group with key activities including publication of a literature review.
- Transfer information to growers on the causes of the RSG syndrome.

A literature review was completed and published in October 1995. This review concluded that the unusual grapevine growth during the 1994/95 season was not the result of a single cause, but rather a product of a number of factors, including climatic effects and cultural practices. The main recommendation was to monitor conditions and apply light winter irrigation and pre-budburst irrigation in unusually dry conditions.

In 1994/95 three vineyards that were badly affected with RSG symptoms were selected to assess whether vines can recover from this disorder. In 1995/96 yields increased at all sites with the worst affected site (Karadoc) increasing from approximately 2.0t/ha to 22.0t/ha. However, yields were reduced again in 1997, but not to the same level as in 1994/95, and were followed by a significant increase again in 1998. The link between low rainfall and RSG symptoms was tested in a pot trial. Potted vines were subjected to water stress during dormancy and results showed 20% lower starch content in stressed vines compared to non-stressed but there was no evidence of RSG symptoms.

In 1996 a trial was established to look at the effect of cropping levels on young Chardonnay on Ramsey vines. Four treatments were applied: control (no bunch removal), and bunch removal of 25%, 50% and 75% in November 1996. There was no bunch removal in 1997/98. The harvest results from this trial in 1997 and 1998 suggest that over-cropping can reduce yields in the following year.

A communication strategy to convey information about the RSG syndrome was developed in 1995 that utilised local television, regional radio, journals and a series of seminars to inform growers of the issues associated with the RSG syndrome. This information has been released at times during the season when management recommendations can be applied. Since the incorporation of the RSG project into the AGY program much of the extension information for 1996/97 and 1997/98 has been delivered in association with the project team working on AGY.

Current Management Options

Preliminary management options have been developed and continue to be investigated. These include heat treatment for cuttings, control of broad leaf weeds, use of quality planting material and the maintenance of good vine health.

The use of quality planting material has been encouraged to reduce the risk of spread via cuttings. Most vine improvement associations provide material to nurseries from source blocks that are inspected for AGY symptoms. If symptoms are observed then the affected vines are not used for propagation material. If the level of infection is too high within a source block then that vineyard is no longer used.

Local and overseas research has identified a thermal death point of phytoplasma in plant tissue at 50°C when material is maintained at this temperature for more than 20 minutes and this has been adopted for managing the spread of AGY phytoplasma in cuttings and planting material. A hot water treatment of 50°C for 30 minutes is the standard protocol used by V AMMVIA for all grape varieties and has been incorporated into A VIA vine accreditation practices.

Communication strategy

A communication strategy for distribution of information about the effects of phytoplasma in Australian grapevines was developed at the beginning of the project. Information about phytoplasma and the associated disorders has been disseminated through seminars, field days, workshops and industry articles. An industry led Steering Committee has directed the communication activities of this project.

Project team members have participated in several seminars, professional meetings, field days and workshops. A module about AGY has been incorporated into the IPM Viticulture: *Research to Practice* manual. Local print, television and radio media have also been used to disseminate results of this project to growers.

In spring 1997, a colour brochure describing the symptoms of AGY and the related disorders RSG and late season leaf curl was released. The brochure describes other disorders that might be misdiagnosed as AGY and basic management options for the control of phytoplasma in grapevines. The Australian Grapevine Yellows colour brochure has been disseminated widely by the Phylloxera and Grape Industry Board of South Australia, various agriculture or primary industry departments, CRCV, GWRDC and via the IPM Viticulture: Research to Practice workshops.

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Chapter 1 The full range of symptoms associated with phytoplasmas in grapevines



1.1 The association between diseases and phytoplasma using PCR *Karen Gibb, Fiona Constable and Jane Moran*

1.2 Australian grapevine yellows, restricted growth and late season leaf curl symptoms are associated
Fiona Constable, Antony Ugoni, Jane Moran and Yasmin Wilson.

1.3 A description of the progression of symptoms in vines affected with Australian Grapevine Yellows
Rod Bonfiglioli

1.4 Understanding and management of restricted spring growth (RSG) *Yasmin Wilson and Robert Hayes*

1.5 The association between restricted growth and various pathogens: Pilot study on factors inducing growth problems in Chardonnay in the Sunraysia, Riverland and Murrumbidgee Irrigation Areas (MIA)
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1.6 Yield trials from AGY affected grapevines
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1.7 Characterisation of phytop1asma in Australian Grapevines *Fiona Constable, Karen Gibb and Jane Moran*

Chapter 1. The full range of symptoms associated with phytoplasmas in grapevines.

1.1 The association between Australian grapevine yellows, restricted growth and late season leaf curl symptoms and phytoplasmas using the polymerase chain reaction method.

Karen Gibb, Fiona Constable and Jane Moran

INTRODUCTION

Like the grapevine yellows diseases found overseas, AGY has been associated with a phytoplasma. Magarey and Wachtel (1988), who observed phytoplasmas in AGY affected vines using electron microscopy, provided evidence for an association. Also, experiments injecting oxy-tetracycline hydrochloride demonstrated that AGY affected vines recovered after antibiotic treatment (Magarey and Wachtel, 1986).

Symptoms of AGY typically include:

- Irregular yellowing and backward curling of the leaves, which drop from the vine prematurely.
- The yellow leaf tissue may become necrotic.
- Overlapping, "shingling", of leaves on an affected shoot.
- Death of affected shoots tips.
- Affected shoots fail to harden off and remain rubbery.
- Abortion of flowers on affected shoots or shrivelling of berries

Other symptoms often observed in Australian vineyards and possibly associated with phytoplasmas are restricted growth and late season leaf curl. Restricted growth is characterised by uneven and failed budburst. Shoot growth is stunted with shortened internodes and small leaves. Shoots and cordons on affected vines may die back. Affected vines usually exhibit poor growth throughout the season. Leaves on late season leaf curl affected shoots remain green, curl backwards and remain on the vine after other leaves fall. The symptoms generally appear from early autumn onwards.

Phytoplasmas are difficult organisms to study particularly as they are unable to be cultured and are dependent on their insect or plant hosts for survival and multiplication. In woody plant hosts, such as grapevines, they are often present in low titre and may be unevenly distributed and thus can be difficult to detect. In the past, the only means by which an association between disease and phytoplasma could be established was by use of electron- or fluorescent- microscopy techniques or by transmission to indicator plants.

In recent years, the polymerase chain reaction, PCR, has been developed for the detection of phytoplasma in infected plants (Deng and Hiruki, 1991, Ahrens and Seemuller, 1992, Lee et al. 1993, Smart et al. 1996). This method is far more sensitive and rapid and has the ability to detect all potential phytoplasmas or can be developed to specifically detect one "species" of phytoplasma, such as the AGY phytoplasma (Gibb et al. this report). PCR analysis facilitates the screening of large numbers of samples in epidemiology studies and therefore can be used to show more accurately associations between phytoplasma and disease.

We used PCR analysis to survey more than 1000 samples from grapevines showing one or a combination of AGY, restricted growth and/or late season leaf curl or that were a symptomatic to determine the association between disease and phytoplasma.

MATERIALS AND METHODS

Sampling

Grapevine tissue samples were collected from three vineyards in Sunraysia. The variety Chardonnay was grown at each site. The vineyard was established at Gol Gol, in 1992 and 1993, at Karadoc in 1992 and at Wemen in 1993. At Gol Gol, vines were grown on a variety of rootstocks including Ramsay, KY325l, Schwarzrnann, and Teliki. At Karadoc all vines were on own roots except for replanted vines, which were on Ramsay. At Wemen all vines were on Ramsay rootstock. In addition samples were collected from other vineyards in Sunraysia and from other grape growing regions. A small number of varieties other than Chardonnay were also tested.

Shoots with symptoms typical of AGY were used as source material for phytoplasmas. Grapevines with late season leaf curl symptoms and restricted growths were also used in phytoplasma experiments. A symptomatic grapevine samples were also collected and tested.

Samples were labelled as affected by AGY if shoots on a vine displayed the following symptoms:

- Irregular yellowing and backward curling of the leaves, which drop from the vine prematurely.
- Overlapping of leaves on an affected shoot

Other symptoms of AGY may also include:

- The yellow leaf tissue may become necrotic. Tips of affected shoots may die.
- Affected shoots fail to harden off and remain rubbery.
- Flowers on affected shoots may abort or berries may shrivel.

These symptoms were only considered if found in association with the yellowing, backward curling and overlapping of leaves on affected shoots.

Samples were labelled as affected by RG if vines displayed the following symptoms

- Uneven and failed bud burst resulting in canes and cordons that are partially or entirely bare of leaves.
- Shoot growth is stunted with shortened internodes and small leaves.
- Affected vines usually exhibit poor growth throughout the season.
- Vines lacked vigour when compared to unaffected vines
- The canopy appears thin compared to unaffected vines

Other symptoms of restricted growth may also include:

- Dead shoots from the previous year -giving a twiggy appearance.
- Little or no bunch development

Samples were labelled as affected by LSLC if vines displayed the following symptoms:

- Leaves on affected shoots remain green.
- Leaves on affected shoots curl backwards.
- Leaves on affected shoots remain on the vine after other leaves fall.
- The symptoms appear from March onwards.

PCR testing

DNA extraction and nested PCR was carried out according to the methods described previously in this report (Gibb et al.).

RESULTS

Table 1. Number of samples, tested for phytoplasma using PCR, showing different symptoms and the number, which were positive (1995/96-1997/98)

Disease	Number tested	Number positive (%)	
AGY	223	131	(59%)
LSLC	126	12	(10%)
RG	203	11	(5%)
AGY/RG	66	40	(61%)
AGY/LSLC	35	9	(26%)
AGY/RG/LSLC	11	5	(45%)
RG/LSLC	28	1	(4%)
Asymptomatic	381	10	(3%)

Using nested PCR, grapevines with a range of diseases were tested between 1995 and 1998. Diseases were assessed as Australian grapevine yellows (AGY), late season leaf curl (LSLC), restricted growth (RG) or a combination of diseases including AGY/RG, AGY/LSLC, AGY/RG/LSLC, RG/LSLC and a symptomatic (Table 1). When AGY symptoms were observed either alone or with RG, approximately 60% of vines were phytoplasma positive. Of the 35 grapevines with AGY and LSLC, only 26% were phytoplasma positive, but this is significantly greater than for a symptomatic vines (3%). Only 11 vines with all three disorders were tested, but of these 46% were phytoplasma positive. For vines with LSLC, 10% were phytoplasma positive. Only 5% of vines with RG and 4% of vines with RG and LSLC were phytoplasma positive.

DISCUSSION

Results showed a strong association between AGY and presence of phytoplasma in vines where both AGY and mixed symptoms were recorded. Like grapevine yellows diseases observed overseas, the cause of AGY is most likely to be phytoplasma infection. These results confirm the findings of Magarey and Wachtel (1988), who noted the presence of phytoplasma, by electron microscopy, in vines displaying AGY symptoms. The results for AGY, are confirmed by other similar studies (Bonfiglioli et al. 1995, Padovan et al. 1995).

The result for restricted growth support the findings of Bonflglioli et aI. (1995) and Padovan et al. (1995), which also show a low association between phytoplasma and restricted growth. Restricted growth symptoms may have many causes including drought through winter and winter chilling (found in association with restricted spring growth in 1994 (Wilson, 1995)), bud mite or fungaI pathogens such as *Phaeoacremonium sp.* (Ferreira et aI. 1994; Crous et aI. 1996.).

This study showed no strong association between late season leaf curl and phytoplasma. However, the possibility of late season leaf curl as a phytoplasma induced disorder cannot be discounted because of the results of Bonflglioli et al. (1995), who noted a high association between the presence of phytoplasma and what was referred ti as kate seasib AGY symptoms.

It may be that there is a difference in the interpretation of late season symptoms between the two research groups. It has been suggested by Bonfiglioli et al. (1997) that grapevine leafroll viruses may also cause symptoms similar to late season leaf curl at the same time of year. Nutritional factors and cultural practices may also contribute to late season leaf curl symptoms.

The low correlation between restricted growth symptoms and phytoplasma and late season leaf curl and phytoplasma suggests that phytoplasmas may only be one of the causes for these disorders. This conclusion is supported by further analysis of mapping data for restricted growth, AGY and late season leaf curl symptoms as provided in this report. The analysis indicated a significant association between mild restricted growth symptoms and mild late season leaf curl symptoms (Constable et al. this report).

Although, phytoplasmas are the most likely cause of AGY symptoms, we have observed similar symptoms on shoots with restricted phloem caused by broken shoots and tendril restrictions. Phytoplasma was often not detected in these shoots. These observations highlights the need for diagnostic testing of symptomatic material at the correct time of year (Gibb et al., this report) to determine whether material is actually phytoplasma positive and not displaying symptoms caused by other factors.

Our results show that the aetiology of restricted growth and late season leaf curl is complex but that phytoplasmas may be one of the contributing factors. Transmission studies will be useful in determining association between phytoplasma and these syndromes.

The results of this study have been published in Vitis (Gibb etal. 1999).

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1.2 Australian grapevine Yellows, restricted growth and late season leaf curl symptoms are associated

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ABSTRACT

Three vineyards in the Sunraysia region of Victoria and New South Wales, Australia, were assessed once in spring, twice in summer and once in autumn, during three years, for grapevines with symptoms of Australian grapevine yellows (AGY), restricted growth (RG) and late season leaf curl (LSLC). The statistical analysis for relative risk (RR) indicated that symptoms of AGY, mild RG and mild LSLC were statistically more likely to affect grapevines from year to year. The results for RR in this study also show an association between symptoms of AGY and mild RG or mild LSLC symptoms, implicating phytoplasma as one possible cause of all three diseases, as the AGY phytoplasma has been consistently associated with AGY symptoms.

INTRODUCTION

Australian Grapevine Yellows (AGY), a disease affecting grapevines *(Vilis vinifera* L.), was first described in 1976 (11). The disease is observed throughout many Australian grape-growing regions with a greater prevalence in the warmer irrigated districts of Sunraysia (Vic), the Riverland (SA) and the Murrumbidgee Irrigation Area (NSW) (12). Restricted growth (RG) and late season leaf curl (LSLC) symptoms are also commonly observed affecting grapevines in the warmer inland districts of Australia. RG symptoms are often observed affecting grapevines in spring and symptomatic plants may remain conspicuous throughout the season (2). Symptoms include delayed and uneven bud- break, shortened internodes and smaller leaves. A more severe form of RG is observed where grapevines display little or no bud-break, severely stunted shoots with very small leaves and much of the previous year's wood is dead. Grapevines with RG symptoms may produce little or no crop. LSLC symptoms appear from late summer and include leaves that roll tightly downward and overlie one another in a shingled appearance (late AGY, 2).

In the warmer inland districts RG and LSLC symptoms are often observed affecting grapevines that have displayed AGY symptoms. A phytoplasma, most commonly the AGY phytoplasma, has been consistently associated with symptoms of AGY using polymerase chain reaction (PCR) methods for phytoplasma detection (2, 7, 14). No strong association has been determined for RG symptoms and phytoplasma using PCR methods (2, 7, 14). A strong association was shown using PCR methods between symptoms of LSLC and the presence of an unidentified phytoplasma (2). However, the results of others, using similar PCR detection methods for phytoplasma, did not indicate a strong association between LSLC symptoms and phytoplasma (7).

A vineyard in the Sunraysia district, Victoria, Australia was assessed for the presence of symptoms and, from the analysis of the data, it was proposed that AGY, RG and LSLC symptoms were all related and likely to be caused by phytoplasma infection (1).

It has also been observed that spurs expressing AGY symptoms in previous seasons may also express symptoms of delayed bud-break in subsequent seasons and in some instances these spurs were reduced in size but did not display AGY symptoms again (12). However, entire grapevines becoming affected with RG symptoms in the following season were not reported.

Symptom remission of AGY -affected shoots and of whole grapevines in subsequent seasons has also been reported (12). Remission or recovery has also been commonly observed for grapevines affected with similar phytoplasma-associated diseases in Europe, including the grapevine yellows disease in Italy (14) and the flavescence doree disease in France (3, 4).

In this study we used the statistical analysis of relative risk (RR, 16) to determine if a significant association exists between AGY and LSLC symptoms or AGY and RG symptoms, thus determining whether phytoplasma might be a potential cause for each symptom. We also calculated RR to determine whether RG, AGY or LSLC symptoms are more likely to persistently affect grapevines over more than one season.

METHODS AND MATERIALS

Study locations. Three vineyards were chosen for this study: Gol Gol, NSW, Karadoc, Vic and Wemen, Vic. At both Gol Gol and Karadoc an area of 18 rows each with 50 grapevines was selected. At Wemen, three areas each of ten rows with ten grapevines were selected. The variety Chardonnay was grown at each location. The vineyard was established at Gol Gol in 1992 and 1993, at Karadoc in 1992 and at Wemen in 1993. At Gol Gol, grapevines were grown on a variety of rootstocks, which were Ramsey (rows 1- 7), K51-32 (rows 8-10), Schwarzmann (rows 11-12), Ramsey (rows 13-14) and Teleki- 5C (rows 15 -18). At Karadoc all grapevines were grown on their own roots except for replanted grapevines, which were on Ramsey rootstock. At Wemen all grapevines were grown on Ramsey rootstock. The soil type at Gol Gol was a sandy clay loam except around rows eight to ten where the soil was a light to medium clay. At Karadoc, the soil type was heavy clay. At Wemen the soil type was deep red sand.

Vineyard surveys. Incidence of disease was assessed in each vineyard on a monthly basis from January through April in 1995/96 and in October through May in 1996/97. In 1997/98 incidence of disease was assessed in each vineyard in October, December, late January and April. Each grapevine was carefully inspected for symptoms of AGY, RG and LSLC symptoms. A grapevine was recorded with AGY symptoms if one or more shoots displayed irregular veinal and interveinal yellowing and downward rolling of leaves that overlaid one another in a shingled appearance. Two levels of severity were recorded for RG symptoms, mild and severe. A grapevine was labelled with symptoms of mild RG if normal growth was retarded resulting in shortened shoots, smaller leaves and if the grapevine had an overall appearance of stunting or slow growth compared to unaffected grapevines. Symptoms of severe RG were recorded if the grapevines displayed little or no bud-break, severely stunted shoots with very small leaves and if much of the previous year's wood was dead. LSLC was defined by the appearance of symptoms from late summer where leaves on affected shoots rolled downward tightly, were darker green and overlaid one another in a shingled appearance.

Mild LSLC symptoms were recorded where less than half the shoots on a grapevine were affected and severe LSLC symptoms were recorded where more than half the shoots *of* the grapevine were affected. Affected grapevines were recorded with either mild LSLC or severe LSLC but could not be recorded with both diseases. Similarly, affected grapevines were recorded with either symptoms *of* mild RG or severe RG but could not be recorded with both diseases.

Analysis of survey data. The data collected was dichotomous; that is, grapevines were recorded as affected or not affected for each of AGY, mild RG, severe RG, mild LSLC and severe LSLC symptoms. Due to the dichotomy *of* the data a statistical analysis of proportions (risks) was performed. The analysis was done with a generalized linear model (10) using a log link and a binomial family of distributions. The choice of this model enabled comparisons *of* risk for each disease to be made while adjusting for the possibility *of* confounding factors such as differences between locations, previous disease and proportion of affected neighbours. In this study, a neighbour refers to a grapevine that is adjacent to the grapevine being analysed. There can be up to eight neighbours for each grapevine except on the edge of the study location or where vines are missing. The log link allowed the results to be interpreted as a relative risk (RR, 5). Increases (or decreases) *of* risk are indicated by the magnitude *of* the RR and its deviation from the value of 1 (one). For the analysis *of* RR for each disease, a value *of* one refers to the reference group to which other variables were compared. Statistically significant results are identified by the exclusion of 1 from the corresponding 95% confidence intervals.

In this study, RR indicated the increase (or decrease) in risk of a grapevine being affected by a disease in 1997/98 when compared to some reference group. The relative risk was determined by comparing the proportion of grapevines affected with a disease in 1997/98 that had a particular risk factor and to the proportion grapevines also affected with the same disease in 1997/98 but without the risk factor. The risk factors considered that may contribute to the risk of a disease affecting grapevines in 1997/98 included the same disease or another disease affecting the same grapevines in 1995/96 and/or 1996/97. The reference group was the group of grapevines that did not have the risk factor in 1995/96 or 1996/97. The proportions *of* neighbours affected by the same disease or another disease in 1995/96 or 1996/97 were also considered as risk factors in our analysis. The risk of a disease affecting grapevines at both Karadoc and Wemen compared to Gol Gol, which was used as the reference group, was also determined. The crude RR (i.e. unadjusted) is calculated for the disease and a risk factor, without considering the influence *of* other variables that may contribute to the disease. The adjusted RR takes into consideration other variables that may confound the result such as diseases that affected the same grapevine in previous seasons, proportion of neighbours affected with a disease, and location differences. A difference in value between the crude RR and the adjusted RR indicates that there is a confounding effect of other variables on the statistical result because they may contribute to the occurrence of a disease or they may conceal the association between a risk factor and a disease.

The RR estimated for "proportion *of* neighbours" denotes the increased risk of a disease for a grapevine that has 100% *of* neighbours affected (with the indicated disease) compared to a grapevine with no neighbours affected.

To compare grapevines, in which proportions of affected neighbours differ by less than 100%, the estimated RR is raised to the power of the difference in the proportions of affected neighbours. For example, comparing a grapevine with 30% affected neighbours to a grapevine with 10% affected neighbours when the estimated RR is 2, would yield a RR of $2^{(0.30-0.10)} = 1.15$.

Some grapevines were removed, had died or were replants after the survey was begun and because the data set for these grapevines was not complete for the three seasons they were not included in the analysis. The analysis of RR for grapevines with symptoms of severe LSLC was not conducted due to low numbers of affected grapevines and because severe LSLC symptoms were not recorded at Gol Gol in 1995/96.

RESULTS

The incidence of grapevines affected with each disease at the three study locations over the three seasons is given in Table 1.

Analysis of the relative risks for grapevines with AGY. The crude and adjusted RR of AGY symptoms affecting grapevines in 1997/98 for is given in Table 2. The crude RR indicated a significantly greater risk of AGY symptoms affecting grapevines in 1997/98 if the same grapevines were also affected with AGY symptoms in one or both of the two previous seasons. The adjusted RR indicated a significantly greater risk of AGY symptoms affecting grapevines in 1997/98 if AGY symptoms also affected the same grapevines in 1995/96 only or in both 1995/96 and 1996/97. The crude RR indicated a significantly greater risk of AGY symptoms affecting grapevines in 1997/98 if mild LSLC symptoms affected the same grapevines in 1996/97 only or in both 1995/96 and 1996/97. The adjusted RR also indicated a significantly greater risk of AGY symptoms affecting grapevines in 1997/98 if the same grapevines were affected with mild LSLC symptoms in 1995/96 and/or 1996/97. Both the crude and adjusted RR indicated a significantly greater risk of AGY symptoms affecting grapevines in 1997/98 if there was an increase in the proportion of AGY affected neighbours in 1995/96 or 1996/97. The crude RR indicated that grapevines were less likely to be affected with AGY symptoms in 1997/98 if there was an increase in the proportion of mild LSLC affected neighbours in 1995/96 and this result was significant. The adjusted RR is greater than the crude RR but the result is moderately significant (p=0.066) and still indicated that grapevines are less likely to display AGY symptoms in 1997/98 if there is an increase in the proportion of mild LSLC affected neighbours in 1995/96.

Analysis of the relative risks for grapevines with mild RG symptoms. The crude and adjusted RR for grapevines affected with mild RG symptoms in 1997/98 is given in table 3. The crude RR and adjusted RR both indicate a significantly greater risk of mild RG symptoms affecting grapevines in 1997/98 if mild RG symptoms affected the same grapevines in 1995/96 and/or 1996/97. The crude RR also indicated a significantly greater risk of mild RG symptoms affecting grapevines in 1997/98 if AGY symptoms affected those grapevines in 1996/97 only or in both 1995/96 and 1996/97. However, the adjusted RR indicated a significantly greater risk of mild RG symptoms affecting grapevines in 1997/98 if AGY symptoms affected the same grapevines in 1996/97 only.

The crude RR indicated a significantly greater risk of mild RG symptoms affecting grapevines in 1997/98 with an increase in the proportion of AGY affected neighbours in 1995/96 and 1996/97. However, the adjusted RR is less than the crude RR for proportion of AGY affected neighbours in 1995/96 and is not statistically significant. The adjusted RR for proportion of AGY affected neighbours in 1996/97 could not be calculated due to collinearity.

Analysis of the relative risks for grapevines with severe RG symptoms. The crude and adjusted RR for grapevines affected with severe RG symptoms in 1997/98 is given in table 4. The crude RR indicated that there is a moderately significant greater risk of severe RG symptoms affecting grapevines in 1997/98 only if severe RG symptoms affected the same grapevines in 1996/97 ($p=0.055$). The adjusted RR is less than the crude RR and indicated that there is no significant risk of severe RG symptoms affecting grapevines in 1997/98 if they were affected with severe RG symptoms in one or both of the previous seasons. The crude RR calculated for those grapevines previously affected with AGY symptoms in both seasons indicated that they are significantly less likely to be affected with severe RG symptoms in 1997/98 ($p=0.05$). There is little change in the adjusted RR for grapevines affected with AGY symptoms in both seasons and this risk remains moderately significant ($p=0.068$). The crude RR indicated a significantly greater risk of severe RG symptoms affecting grapevines in 1997/98 if there was an increase in the proportion of severe RG affected neighbours in 1996/97. The crude RR also indicated a greater risk that is moderately significant associated with an increase in the proportion of severe RG affected neighbours in 1995/96 ($p=0.062$). The adjusted RR indicated a significant risk of severe RG affecting grapevines in 1997/98 if there is an increase in proportion of severe RG affected neighbours in 1996/97 only.

Analysis of the relative risks for grapevines with mild LSLC symptoms. The crude and adjusted RR for vines with mild LSLC symptoms in 1997/98 is given in table 5. In this analysis AGY symptoms affecting the same grapevine in 1997/98 and the proportion of neighbours affected by AGY or mild LSLC symptoms in 1997/98 were also considered when determining the crude and adjusted RR. The crude RR indicated a significantly greater risk of mild LSLC symptoms affecting grapevines in 1997/98 if the same grapevines were affected with mild LSLC symptoms in 1995/96 or 1996/97. The adjusted RR only indicated a significantly greater risk of mild LSLC symptoms affecting grapevines in 1997/98 when mild LSLC symptoms affected the same grapevines in 1996/97. The crude and adjusted RR indicated a significantly greater risk of mild LSLC symptoms affecting grapevines in 1997/98 if the same grapevines were affected with AGY symptoms in all three seasons, in both 1996/97 and 1997/98, in 1997/98 only, in both 1995/96 and 1996/97 and in 1996/97 only. The crude RR indicated a significantly increased risk of mild LSLC symptoms affecting grapevines in 1997/98 with an increase in the proportion of mild LSLC affected neighbours in each of the three seasons. However, the adjusted RR is significant only for an increase in the proportion of mild LSLC affected neighbours in 1995/96 or 1997/98. The crude RR indicated a significantly greater risk of mild LSLC symptoms affecting grapevines with an increase in the proportion of AGY affected neighbours in 1995/96 and 1996/97. However, the adjusted RR indicated that there is no significant risk associated with an increase in the proportion of AGY affected neighbours in any season.

DISCUSSION

The RR is a useful statistical measure that compares the proportion of individuals in a group with disease with a particular risk factor to the proportion of individuals with disease without the same risk factor. The use of the RR allows both calculation of the statistical significance and magnitude of any association between a disease and risk factor. For example, AGY symptoms affecting a grapevine in both 1995/96 and 1996/97 are associated with AGY symptoms affecting a grapevine in 1997/98. In our data, grapevines affected with AGY in both 1995/96 and 1996/97 are 1.63 (95% CI 1.36-1.96 $p<0.05$, table 3) times more likely to be affected with AGY symptoms m 1997/98, compared to grapevines not affected with AGY in both 1995/96 and 1996/97. Most research into the symptomatology associated with phytoplasmas in Australian grapevines is limited to field populations, where other factors may contribute to the occurrence of symptoms. When determining the association between a disease and risk factor, other risk factors may also contribute to the presence (or absence) of that disease and confound the initial result. The linear models used in this paper can adjust for the possibility of such confounding. This enables an adjusted estimate of the association between disease and risk factor (adjusted RR) to be made. In the analysis presented, we were able to take such confounding factors such as study locations and influence of diseased neighbours into consideration. Where the crude RR differs to the adjusted RR it is likely that these other factors may also contribute to the risk.

The statistical analysis of RR presented in this study indicated that AGY symptoms affecting a grapevine in one season are significantly associated with AGY symptoms affecting the same grapevine in previous seasons, indicating a significantly greater risk that the symptoms will persistently affect grapevines from season to season. Since the AGY phytoplasma has been associated with AGY symptoms (7, 14), then it may also persist from year to year within a grapevine. However, we have also observed remission of symptoms in affected grapevines in the field, there some grapevines do not display symptoms each season. It may be that the location and or number of phytoplasmas within the grapevine influences AGY symptom expression. Another explanation for this result is that the AGY phytoplasma does not persist in grapevines and the reoccurrence of AGY symptoms in subsequent seasons is due to a new infection event. However it is likely that both persistence of phytoplasmas within a grapevine and new infections play a role in persistence of AGY symptoms in affected grapevines over several seasons.

Our results showed that there was significantly greater risk of a grapevine being affected with AGY symptoms if neighbouring plants exhibited AGY symptoms in previous seasons. Additionally, no alternative host has been identified for the AGY phytoplasma from infected vineyards. It may be that phytoplasma is spread from infected grapevines to neighbouring grapevines within a vineyard. Most phytoplasmas are spread to plants via insect vectors, mainly leafhoppers and planthoppers (9). Another explanation for aggregation of AGY affected grapevines may be that grapevines were inoculated in clusters by an insect vector entering the vineyard from outside. However, the vector for the AGY phytoplasma in Australian grapevines remains unknown.

Each season there was a substantial variation between locations in the incidence of grapevines affected with AGY symptoms. This indicated that differences in location may influence the expression of symptoms in grapevines within the vineyard and may influence spread of the phytoplasma. Such differences may include differences in vector populations within and passing through different vineyards, differences in vineyard management or even location and soil type.

Mild RG symptoms affecting grapevines were also significantly associated with AGY symptoms affecting the same grapevines in the previous season. Since AGY symptoms have been associated with the AGY phytoplasma, mild RG symptoms might also be associated with the AGY phytoplasma. However, there was no significant increase in risk of mild RG symptoms affecting grapevines if there was an increase in the proportion of neighbours affected with mild RG or AGY symptoms in previous seasons. This result suggests that if mild RG symptoms are associated with the AGY phytoplasma then they are part of a progression of symptoms associated with infection in the previous year rather than an immediate response to new infection. The progression of AGY symptoms in one season to RG symptoms in the next has also been suggested by another analysis (1).

Severe RG symptoms affecting grapevines were not significantly associated with AGY symptoms affecting the same grapevines in previous seasons and may not be associated with the AGY phytoplasma. Also, severe RG symptoms were not associated with the presence of severe RG symptoms in previous seasons. However incidence of this disorder increased at all three locations over the three seasons and there was a significant association between the increase in risk of a grapevine becoming affected with severe RG with an increase in the proportion of neighbours affected with severe RG in previous seasons. It is possible that the spread of a causal agent of severe RG symptoms is from affected grapevine to grapevine and that remission of symptoms occurs in affected grapevines in the next season. However the number of grapevines with severe RG symptoms was low, and the statistical results reported here may not apply when the incidence of grapevines with severe RG symptoms is higher.

Like AGY symptoms, there was a significantly greater risk of mild LSLC symptoms affecting the same grapevines from season to season. Our also results show a significant association between symptoms of AGY and mild LSLC affecting the same grapevines and therefore it is possible that LSLC symptoms are associated with phytoplasmas. However, unlike the results of others (1), our results do not suggest that LSLC symptoms are the first expression of a phytoplasma infection in Australian grapevines. Both LSLC and AGY symptoms were associated with each other in previous seasons. It may be that LSLC symptoms are simply a late expression of phytoplasma infection. Additionally, mild LSLC affected grapevines were not significantly associated with an increase in the proportion of AGY affected neighbours in any season, and AGY symptoms were not associated with an increase in the proportion of neighbours previously affected with mild LSLC symptoms. This also suggests that mild LSLC symptoms may not be a first expression of symptoms due to phytoplasma infection. Our results showed. a significantly greater risk of mild LSLC symptoms affecting grapevines if there was increase in the proportion of mild LSLC affected neighbours in the same season or in the 1995/96 season.

These results indicate aggregation of the disease, which is possibly due to the spread of the causal agent from infected grapevine to grapevine. However, aggregation of mild LSLC affected grapevines may also be due to vectors inoculating plants in clusters.

RG and LSLC are generic terms used to describe symptoms that can affect Australian grapevines. The results in this study show an association between symptoms of AGY and mild RG and mild LSLC, implicating phytoplasma as a possible cause of all three diseases. Further analysis of relative risk over more than three years of data will be useful in confirming relationships between the diseases particularly when we do not know the history of each vineyard prior to the collection of data from 1995/96. The aetiology of RG and LSLC is complex and the AGY phytoplasma may not be the sole cause. The tomato big bud phytoplasma has also been detected in Australian grapevines and in vines -displaying LSLC symptoms (7) and it may be that this phytoplasma is a potential cause of LSLC symptoms rather than the AGY phytoplasma. Other pathogens such as viruses and fungi may also cause similar symptoms to RG and LSLC. Physiological problems such as winter chilling (13, 17) causing RG symptoms or high chloride levels stimulating leaf curl symptoms (8) may also contribute to the occurrence of symptoms in affected grapevines. We have also shown that symptoms of mild RG, mild LSLC and AGY can persist in a grapevine from year to year and it may be that the cause of these diseases also persists. However, we do not know if the reoccurrence of any disease is due to one or a combination of persistence of phytoplasma and new infection events.

ACKNOWLEDGMENTS

We thank Tim Griffin from the Institute for Horticultural Development for technical assistance in the field. Many thanks also to Noel and Midge Cameron, Carmel and Grant Carey and Frank Sos for allowing the use of their vineyards as study sites. This research was supported by the Co-operative Research Centre for Viticulture, Grape and Wine Research and Development Corporation, Dried Fruits Research and Development Council, the Murray Valley Wine Industry Development Committee and Agriculture Victoria.

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Table 1. Incidence (%) of grapevines affected with AGY, mild RG, severe RG, mild LSLC and severe LSLC symptoms in the 1995/96, 1996-97 and 1997/98 seasons at three study locations in Sunraysia. The total number of grapevines observed in 1995/96 at Gol Gol = 826, at Karadoc = 884 and at Wemen = 300. The total number of grapevines observed in 1997/98 at Gol Gol = 890, at Karadoc = 876 and at Wemen = 229. The total number of grapevines observed in 1997/98 at Gol Gol = 890, at kardoc = 876 and at Wemen = 229.

Disease	Location	Season		
		1995/96	1996/97	1997/98
AGY	Gol Gol	20%	54%	19%
	Karadoc	2%	4%	6%
	Wemen	0.7%	24%	21%
Mild RG	Gol Gol	5%	38%	40%
	Karadoc	30%	40%	31%
	Wemen	6%	37%	31%
Severe RG	Gol Gol	0.5%	2%	6%
	Karadoc	3%	4%	6%
	Wemen	0.3%	2%	5%
Mild LSLC	Gol Gol	25%	26%	18%
	Karadoc	20%	17%	11%
	Wemen	2%	15%	11%
Severe LSLC	Gol Gol		5%	2%
	Karadoc	0.9%	10%	4%
	Wemen	0%	3%	0%

Table 2. The crude and adjusted relative risks of associations between AGY symptoms affecting the grapevines in 1997/98 and several variables that may influence the occurrence of those symptoms. The variables considered are AGY or mild LSLC symptoms affecting the same grapevines in 1995/96 and/or 1996/97, the proportion of neighbours affected with AGY or mild LSLC in 1995/96 or 1996/97 and location. Significant risk is indicated by the exclusion of 1 from the 95% confidence interval (CI). The total number of grapevines used in this analysis = 1944.

Variable			Relative Risk (crude)	95% CI	Relative Risk (adjusted)	95% CI
AGY	1995/96	1996/97				
	N[a]	N	1		1	
	Y[b]	N	2.76	1.88-4.06	1.64	1.07-2.50
	N	Y	1.93	1.52-2.44	1.16	0.90-1.51
	Y	Y	3.20	2.40-4.26	1.63	1.15-2.33
Mild LSLC	1995/96	1996/97				
	N	N	1		1	
	Y	N	1.18	0.87-1.58	1.48	1.10-2.00
	N	Y	1.53	1.17-2.00	1.55	1.20-2.00
	Y	Y	1.62	1.10-2.38	1.72	1.20-2.46
Proportion neighbours						
	AGY 1995/96		5.00	3.55-7.06	2.05	1.22-3.46
	AGY 1996/97		4.62	3.41-6.26	2.18	1.33-3.60
	Mild LSLC 1995/96		0.50	0.29-0.87	0.57	0.32-1.04
	Mild LSLC 1996/97		1.04	0.56-1.93	1.14	0.62-2.11
Location						
	1		1		1	
	2		0.3	0.22-0.39	0.68	0.44-1.00
	3		1.23	0.98-1.55	2.39	1.71-3.33

[a]N indicated absence of the variable in that season.
[b]Y indicated presence of the variable in that season

Table 3. The crude and adjusted relative risks of associations between mild RG symptoms affecting the grapevines in 1997/98 and several variables that may influence the occurrence of those symptoms. The variables considered are mild RG or AGY symptoms affecting the same grapevines in 1995/96 and/or 1996/97, the proportion of neighbours affected with mild RG or AGY symptoms in 1995/96 or 1996/97 and location. Significant risk is indicated by the exclusion of 1 from the 95% confidence interval (CI). The total number of grapevines used in this analysis = 1947.

Variable			Relative Risk (crude)	95% CI	Relative Risk (adjusted)	95% CI
Mild RG	1995/96	1996/97				
	N	N	1		1	
	Y	N	1.52	1.23-1.87	1.48	1.19-1.84
	N	Y	1.59	1.40-1.80	1.53	1.35-1.73
	Y	Y	1.42	1.18-1.72	1.50	1.24-1.83
AGY	1995/96	1996/97				
	N	N	1		1	
	Y	N	0.99	0.70-1.40	0.82	0.58-1.17
	N	Y	1.39	1.22-1.58	1.21	1.03-1.40
	Y	Y	1.63	1.36-1.96	1.21	0.96-1.52
Proportion neighbours						
	Mild RG 1995/96*		0.95	0.71-1.27		
	Mild RG 1996/97		1.08	0.84-1.39	0.94	0.73-1.21
	AGY 1995/96		1.85	1.45-2.34	1.24	0.91-1.69
	AGY 1996/97*		1.63	1.36-1.95		
Location						
	1		1		1	
	2		0.79	0.70-0.89	0.92	0.78-1.08
	3		0.58	0.47-0.71	0.64	0.51-0.80

*Due to collinearity the adjusted RR for mild RG affected neighbours 96 and AGY affected neighbours 1996/97 could not be calculated.

Table 4. The crude and adjusted relative risks of associations between severe RG symptoms affecting the grapevines in 1997/98 and several variables that may influence the occurrence of those symptoms. The variables considered are severe RG or AGY symptoms affecting the same grapevines in 1995/96 and/or 1996/97, the proportion of neighbours affected with severe RG or AGY symptoms in 1995/96 or 1996/97 and location. Significant risk is indicated by the exclusion of 1 from the 95% confidence interval (CI). The total number of grapevines used in this analysis = 1947.

Variable			Relative Risk (crude)	95% CI	Relative Risk (adjusted)	95% CI
Severe RG	1995/96	1996/97				
	N	N	1		1	
	Y	N	1.38	0.36-5.27	1.28	0.34-4.83
	N	Y	2.27	0.98-5.23	1.40	0.57-3.41
	Y	Y	2.87	0.48-17.34	1.56	0.26-9.31
AGY	1995/96	1996/97				
	N	N	1		1	
	Y	N	1.38	0.62-3.04	1.52	0.66-3.53
	N	Y	0.95	0.63-1.46	0.88	0.54-1.44
	Y	Y	0.14	0.02-1.00	0.15	0.02-1.15
Proportion neighbours						
	Severe RG 1995/96		11.51	0.88-150.20	2.05	0.01-44.10
	Severe RG 1996/97		27.42	6.36-118.37	18.91	3.77-94.95
	AGY 1995/96		0.47	0.16-1.53	0.41	0.11-1.57
	AGY 1996/97		0.82	0.45-1.53	0.76	0.27-2.17
Location						
	1		1		1	
	2		0.97	0.67-1.41	0.58	0.32-1.04
	3		0.57	0.30-1.07	0.44	0.22-0.90

Table 5. The crude and adjusted relative risks of associations between mild LSLC symptoms affecting the grapevines in 1997/98 and several variables that may influence the occurrence of those symptoms. The variables considered are mild LSLC in 1995/96 and/or 1996/97, AGY symptoms affecting the same grapevines in 1995/96 1996/97 and/or 1997/98, the proportion of neighbours affected with mild LSLC or AGY symptoms in 1995/96, 1996/97 or 1997/98 and location. Significant risk is indicated by the exclusion of 1 from the 95% confidence interval (CI). The total number of grapevines used in this analysis = 1939.

Variable				Relative Risk (crude)	95% CI	Relative Risk (adjusted)	95% CI
mild LSLC	1995 /96	1996 /97	1997 /98				
	N	N		1		1	
	Y	N		1.56	1.18-2.05	1.22	0.92-1.62
	N	Y		1.72	1.32-2.25	1.34	1.02-1.75
	Y	Y		1.08	0.65-1.79	0.66	0.39-1.10
AGY							
	N	N	N	1		1	
	Y	N	N	0.88	0.34-2.29	0.88	0.33-2.30
	N	Y	N	2.37	1.83-3.07	1.99	1.47-2.70
	Y	Y	N	1.93	1.17-3.19	1.92	1.09-3.37
	N	N	Y	2.67	1.95-3.64	2.55	1.84-3.54
	Y	N	Y	1.90	0.77-4.65	2.03	0.81-5.07
	N	Y	Y	2.10	1.36-3.24	1.92	1.21-3.04
	Y	Y	Y	3.88	2.55-5.90	3.38	2.03-5.61
Proportion neighbours							
	Mild LSLC 1995/96			2.35	1.50-3.69	1.91	1.13-3.20
	Mild LSLC 1996/97			2.54	1.43-4.53	1.55	0.85-2.83
	Mild LSLC 1997/98			5.12	2.71-9.69	2.41	1.23-4.74
	AGY 1995/96			1.81	1.13-2.91	0.77	0.39-1.49
	AGY 1996/97			2.57	1.86-3.55	1.43	0.82-2.52
	AGY 1997/98			1.48	0.84-2.63	0.57	0.28-1.17
Location							
	1			1		1	
	2			0.55	0.44-0.70	1.03	0.71-1.49
	3			0.72	0.53-0.98	1.19	0.78-1.80

1.3 A description of the progression of symptoms in vines affected with Australian Grapevine Yellows

Rod Bonfiglioli

The association of phytoplasma with AGY symptoms in affected vines has been adequately verified by the use of the polymerase chain reaction (PCR). We have shown that there is a close association between these typical symptoms and other non- specific symptom groups observed in mapping surveys. We find disordered budburst (usually early) in the spring followed by the appearance of the typical symptoms (P. Magarey and M. Wachtel, pers. com.) in the summer, out-of-season growth in the winter and poor growth in the following spring. We also observed that vines that had typical symptoms in one year often carried little or no fruit the following year.

We do not fully understand the nature of the association of various symptoms in grapevine. The closest associated groups of non-specific symptoms include disordered budburst and out-of-season growth. These symptoms are well recorded in the literature for other woody host plants, specifically *Prunus* species and other fruit trees. We have also observed a phloem necrosis on vines that displayed AGY symptoms in one year followed by a winter or spring with hard frosts. It is our observation that symptomatic vines are more sensitive to damage by frost. This observation is consistent with the observations of woody host plants in European studies. The most logical explanation is that the phloem is damaged by the infection with phytoplasmas and does not mature properly before the end of the season and is therefore more sensitive to frost.

1.4 Understanding And Management Of Restricted Spring Growth (RSG)

Yasmin Wilson and Robert Hayes

BACKGROUND

During the 1994-1995 season, wine, dried and table-grape growers reported substantial growth and yield reductions in vineyards, with large numbers of vines displaying restricted growth. Increased concern over the severity and widespread nature of the Restricted Spring Growth (RSG) problem led to the formation of a National Task Force in October 1994 to investigate and coordinate activities into RSG. Membership consisted of representatives from Victorian, South Australian and New South Wales Departments of Agriculture, CSIRO Horticulture, wine industry, GWRDC and CRCV (Appendix 1).

The first role of the RSG Task Force was to coordinate an extensive survey of growers in Sunraysia (Vic.), Riverland (SA) and the Murrumbidge Irrigation Area (NSW).

The aims of this survey were to (1) define the extent of the RSG problem in viticultural areas and (2) use the results to provide directions for further investigative work into RSG. Results from the survey suggested that the cause of RSG could be attributed to numerous factors including grapevine age, variety, rootstock, soil type, pruning method, fertiliser treatment and soil moisture (Mattschoss, 1995).

To continue further investigation into RSG, a CRCV funded project, with collaboration from GWRDC, commenced in July 1995. This project continued until July 1996, when it amalgamated with the national CRCV /GWRDC project "Control of phytoplasmas in Australian grapevines". The objectives of the RSG work were modified after amalgamation with the phytoplasma project.

INTRODUCTION

Since the unfavourable 1994/95 grape growing season, the potential for Restricted Spring Growth (RSG) to occur again has been of concern for the wine, dried fruit and to a lesser extent the table grape industries. Initial investigations by the RSG Task Force suggested that the growth problems were related to unusual environmental and climatic conditions that caused damage to the vascular conducting tissue, and subsequent reduction in carbohydrate reserves of the grapevine.

Further findings supported the theory that the restricted growth was not the result of a single or specific cause, but rather a combination of factors, such as climatic, environmental, cultural and pathological disruptions (Wilson, 1995).

The characteristic symptoms of RSG are poor and uneven budburst, often with a large proportion of buds failing to burst (Mattschoss, 1994). Subsequent shoot growth is retarded or stunted with smaller than normal leaves and short internodes. Despite a lack or delay of budburst along the cordons there is also a tendency towards renewed shoot growth occurring from the trunk or within the crown (Hayes et al. et al. 1995). On mature vines only the canes and cordons tend to be affected, whereas younger vines can be killed entirely. Depending on the severity of the RSG, some vines may even display varying degrees of trunk damage.

This appears as brown discolouration of the phloem tissue on the south-west side of the trunk, possibly as a result of cold injury. Sometimes trunk splitting, which extends into the xylem, may be observed. Furthermore, RSG is characterised by its random distribution throughout the vineyard.

Each year grapevine growth is dependent on an optimum balance between vegetative growth, required for sustaining carbohydrate reserves for canopy development and fruit ripening. Over cropping of a grapevine crop, combined with external stresses such as water deficit from drought or lack of irrigation, over- fertilising or pest and disease problems can limit grapevine productivity. Alternatively, insufficient food reserves (sugars and starches) in the root, trunk, arms and canes, can make a vine susceptible to cold temperatures during the dormant period. Investigations into freeze injury have noted that the degree of tissue damage is dependent on the concentration of natural antifreeze compounds (carbohydrates) contained in the tissues (Meiering et al. et at. 1980)

Similar occurrences of restricted growth have been observed in the past, generally in conjunction with unusual weather conditions (Walters & Ludbrook, 1940; MacIntosh, 1983). Likewise, overseas experience has revealed that similar growth restrictions due to environmental conditions occur in Washington State, South Africa and California and are known as winter chilling injury (Wample pers. comm.), growth arrestment syndrome (Orth pers. comm.) and delayed growth (Christensen pers. comm) respectively.

More recently a hypothesis has been suggested that RSG is associated with the phytoplasma disease called Australian Grapevine Yellows. The relationship between RSG and the presence of phytoplasma is not well understood and has been under study (See Gibb et al. this report).

In this project we investigated the relationship between RSG and environmental conditions, and provided growers and industry with information on, and management strategies to mitigate the effects of RSG.

OBJECTIVES

1. Project Coordination

To coordinate investigations into RSG through a national liaison group with key activities including publication of a literature review.

2. Industry/Grower Communication

To advise wine, dried and tablegrape growers on the possible causes of RSG and management strategies for reducing the effects of RSG.

3. RSG Trials

Monitor performance of vineyards with a history of RSG.

4. Carbohydrate reserves/Environmental data

Establish the relationship between RSG and soil moisture, temperature and carbohydrate reserves.

MATERIALS AND METHODS

1. Project Coordination (internal)

The RSG task force was established in October 1994 and continued until July 1996, when the RSG project amalgamated with the AGY project. Direction for the RSG work was then coordinated from the larger national group.

2. Industry/Grower Communication (external)

A communication strategy was developed in 1995 to inform growers of the issues associated with RSG. This information has been continually delivered to growers and industry, targeting certain times during the season, particularly during post-harvest and winter.

3. RSG Trials

3.1 Grower Trials

In 1994/1995 a grower survey was distributed to determine the level of poor vine growth across the district (Mattschoss, 1995). From this survey, three vineyards badly affected with RSG were selected for further study. Our goals were to (1) monitor growth and yield in subsequent years and (2) observe effects and recovery of different varieties (Table 1) on these three vineyards.

Table 1. Details of each RSG trial site established in Sunraysia, Vic.

Site	Location	Variety/Rootstock	Irrigation System	Pruning method	Year Planted (approx.)
1	Karadoc	Chardonnay/Own roots	Overhead	Minimal	1992
2	Dareton	Sultana/Ramsey	Drip	Cane	1990
3a	Yelta	Colombard/Own Roots	Drip	Minimal	1986
3b	Yelta	Colombard/Own Roots	Overhead	Minimal	1986
*4	Robinvale	Chardonnay/Ramsey	Overhead	Minimal	1986

* New Robinvale site selected in 1996, after Dareton site discontinued.

The trial was originally designed so that subsequent treatments could be imposed on the sites if needed (ie. fertiliser/irrigation), however this was never performed. Sites 1, 2 and 4 contained 240 test vines over a 10-row plot. Site 3 included two different irrigation systems. Originally this site was designed to monitor growth in subsequent years, however as the trial progressed it became advantageous to also compare crop yields from vines in the two irrigation systems. The drip section (Site 3a) comprised 84 test vines over four rows, and the overhead section (Site 3b) comprised 96 test vines over four rows. Yield (Kg/vine) measurements were collected from 1995-1998 on each site.

In 1996 a new Chardonnay on Ramsey site was established at Robinvale following the top working of the Sultana site at Dareton. The Robinvale site had suffered a range of growth related problems, such as retarded growth and yield reductions, following the 1994 season.

To assist with irrigation scheduling on the Robinvale vineyard, the grower installed an EnviroScan irrigation monitoring system in 1996.

3.2 Chardonnay cropping trial

In 1996 a trial was established at the Sunraysia Horticultural Centre (Irymple) to determine the effect of cropping levels on young Chardonnay vines, which were "pushed" to produce maximum yields in the first cropping year. The Chardonnay vines were 18 months old and grafted to Ramsey rootstock. Four treatments were established, consisting of a control (no bunch removal), and bunch removal of 25%, 50% and 75% on 19/11/96 using a randomised block design with 6 replicates, each comprising four vines. Each replicate was separated by three non-treated rows. To ensure bunches could be easily identified and removed at harvest, the buffer vines were cut back if they overlapped the treated vines. All trial vines were hand-harvested m February 1997. Bunch number and vine yield measurements were assessed in the field. The bunch removal treatments were not performed in 1997, however yield data was collected again in 1998. The vines were spur-pruned in winter 1997.

4. Carbohydrate reserves Environmental data

4.1 Potted Sultana trial (carbohydrate reserves)

To assess whether a dry autumn and winter were contributing to spring growth problems, a trial was established in 1996 to measure the total starch of own rooted Sultanas after severely water stressing the vines during autumn and winter 1996. Sultana was chosen for this trial on the basis that it was severely affected by RSG in 1994, and also a variety not reported as being affected by AGY (Padovan et aI 1995).

The Sultanas were established 20 years ago in 70cm diameter x 70cm deep cement pots. All forty vines were drip irrigated. Twenty vines were irrigated to provide a soil moisture level where the drains were just running. Another twenty vines had the soil surface covered by a waterproof material to prevent rainfall from reaching the soil around the test vines. Test vines received limited irrigations from 4 April 1997 to 26 May 1997, and were not irrigated again until 28 July 1997. Dormant canes were collected from both the irrigated and stressed vines on 1 July 1997 and the starch content determined as follows:

Sub-samples of vine shoots were taken from between the first two basal nodes, placed in paper bags and oven dried at 85.0°C overnight. The samples were then ground through a 1.0mm sieve mesh and stored in the oven until starch digestion was complete. Starch levels were assayed enzymatically using the method described by Krstic (1997). The procedure Involved adding 100mg of dry sample to a 20.0ml centrifuge tube. To ensure complete removal of residual sugars, 5.0ml 80% aqueous ethanol (v/v) was added and incubated at 80-85°C for 5 minutes. Contents were vortexed and then centrifuged at 3000rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 5.0ml 80% aqueous ethanol (v/v), vortexed, incubated at 80-85°C for 5 minutes and centrifuged at 3000rpm for 10 minutes. The supernatant was discarded and 3.0ml of thermostable α-amylase in MOPS buffer was added, the tube was vortexed and then incubated at 100°C for 6 minutes, vortexing every 2 minutes. The tube was transferred to a 50.0°C water bath after which 4.0ml of sodium acetate buffer and 1.0ml amyloglucosidase were added.

The tube was vortexed and incubated at 50.0°C for 30 minutes. After incubation the sample volume was adjusted by adding distilled water to the 10.0ml volumetric mark. The tube was then vortexed and centrifuged at 3000rpm for 10 minutes. Aliquots of tube contents (0.lml) were transferred to glass cuvettes. Added to the tubes were 3.0ml of GOPOD reagent, 0.1ml glucose standard and 0.1ml reagent blanks (distilled water). The tubes were incubated at 50.0°C for 20 minutes before reading the light absorbance of each sample against the reagent blank at 510nm on a spectrophotometer.

RESULTS

2. Industry/Grower Communication (external)

A communication strategy was developed to assist the RSG Task Force in disseminating information to growers and industry (Table 2).

Table 2. Communication Strategy for Restricted Spring Growth.

Target	Message/ Response	When	How	Who
RSG Task Force	Update on progress of project	Bi monthly	Project meetings and copies of information	Robert Hayes Rod Mattschoss Yasmin Wilson
Industry	Logical explanation to problem	Immediate	Literature review, press releases, letters to associations	RSG Task Force, Yasmin Wilson
	Problem was being addressed through R&D	Ongoing	Progress reports, seminar presentations	Robert Hayes, Yasmin Wilson
Growers	Understanding of problem and strategies to mitigate effects	Bi monthly	Press releases, industry newsletters, seminars, regional TV, radio	Robert Hayes, Yasmin Wilson
	Water in autumn/winter if needed	Autumn/ winter	Harvest hints, industry newsletters, radio, newspapers	Yasmin Wilson
CRCV/ GWRDC	Status of project	As needed	Progress reports	Robert Hayes
AV	Awareness of problem and project	Quarterly	Briefings to senior management	Robert Hayes

Information on probable causes and management strategies for RSG were delivered via local television, regional radio, journals, newsletters and seminars (Appendix 2.).

3. RSG Trials

3.1 Grower trials

Yields for 1995 increased for all sites, with the worst affected site (Karadoc) increasing from approximately 2.0t/ha to 22.0t/ha (Table 3). However, yields reduced again in 1997 followed by a significant increase again in 1998.

Table 3. Yield data for RSG grower trial sites.

Table 3. Yield data for RSG grower trial sites.

SITE	VARIETY	AVERAGE YIELD (T/ha)			
		1995	1996	1997	1998
Karadoc	Chardonnay	**2.6** (±6.5)	**22.8** (±19.0)	**9.1** (±11.1)	**21.1** (±6.7)
Yelta (drip)	Colombard	**29.6** (±8.8)	**36.2** (±4.2)	**19.2** (±4.2)	**39.1** (±4.7)
Yelta (o/h)	Colombard	**39.5** (±8.2)	**40.1** (±3.5)	**28.5** (±3.9)	**33.6** (±4.2)
Dareton	Sultana	**25.5** (±16.5)	**61.0** (±14.6)	-	-
Robinvale	Chardonnay	-	-	**22.8** (±22.0)	**27.1** (±5.2)

Values are follow by standard deviations in parentheses.

At the Robinvale site, Chardonnay water use during 1997 was determined from the EnviroScan readings provided by Barry Avery (Figure 1.).

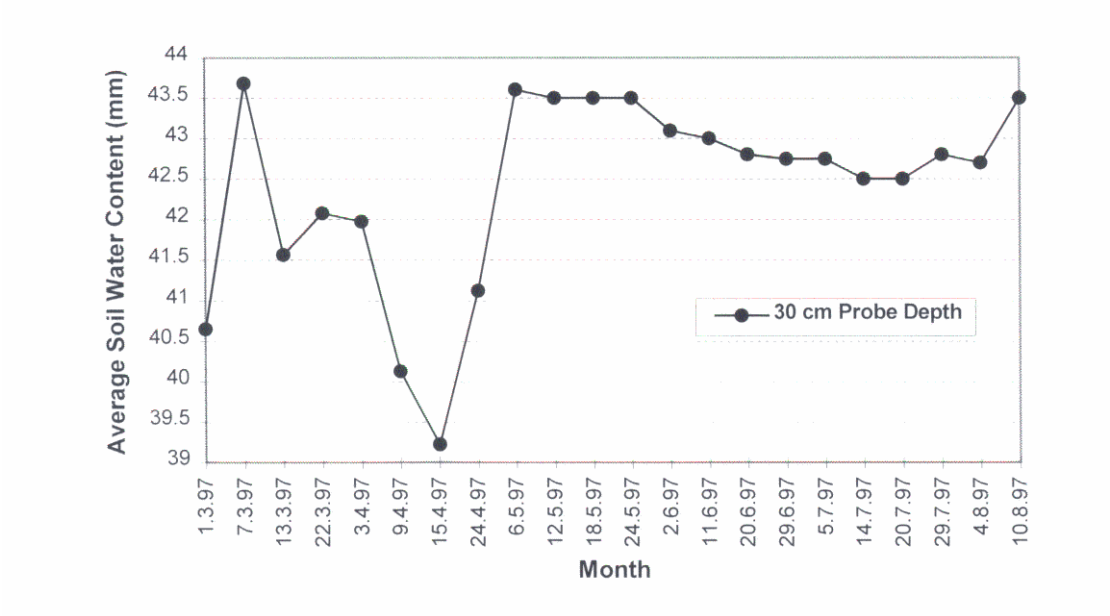


Figure 1. Average soil water content at 30cm in Chardonnay vineyard, Robinvale.

3.2 Chardonnay cropping trial

The summary of yield data for this trial is given in Table 4.

Many vines had bunch rot and berry shrivel, but based on visual observations, there did not appear to be any differences in the levels of these symptoms between treatments. Two trial vines collapsed toward the end of the season, resulting in complete berry shrivel and could therefore, not be harvested in the trial.

Bunch removal treatments had been applied to these two vines. Hot conditions prevailed, during February, with the average maximum temperature just exceeding 35° C.

Table 4. Summary of yield data for Chardonnay.

Treatments	Average yield (T/ha) 1997	Average Yield (T/ha) 1998	Average Bunch number	Average berry weight (g)
1.Control – no bunch removal	23.8 a	23.3a	196a	0.97a
2. 25% of bunches removed	25.0a	30.1a	152 ac	1.10b
3. 50% of bunches removed	13.2b	29.6a	106bc	1.19b
4. 75% of bunches removed	6.70b	28.0a	64b	1.14b

* Means not followed by the same letter differ significantly within a year *(α<0.05)*

4. Carbohydrate reserves/Environmental data

4.1 Potted Sultana trial (carbohydrate reserves)

Studies on potted vines in 1997 showed that reductions in starch levels were induced (Table 5). No differences in spring shoot growth were observed.

Table 5. Starch content for potted Sultana vines Treatment

Treatment	**Average % Starch**
Uncovered	5.65
Covered	4.55

4.2 Meteorological Data

Data obtained from the Mildura weather station shows that 1994 and 1997 were substantially drier during autumn and winter and that 1994 also had more frost days than 1995, which was close to the 30-year average for frost days and rainfall (Table 6).

Table 6: Mildura weather data

Year	**Annual rainfall (mm)**	**Rainfall from March to August (mm)**	**Annual frost days***
1997	202.1	63.5	27
1982	121.6	51.4	35
1989	359.0	274	44
1994	161.8	46.4	48
1995	302.8	114.0	24
1997	293.0	74.2	35
30 Year Average	296.8	144.9	27

* Frost day terrestrial minimum < -0.9°C.

The average monthly terrestrial minimum temperatures for April to September 1982, 1994 and 1997 were substantially less than for the same period in 1995 which was similar to the 30 year average (Figure 2.).

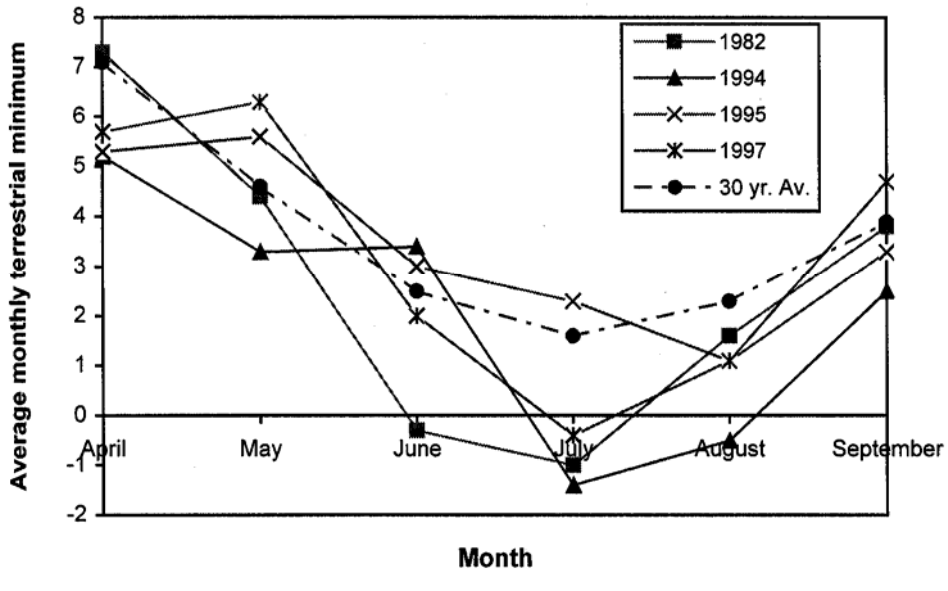


Figure 2. Average Mildura terrestrial minimums April to September 1982, 1994, 1995, 1997 and 30-year average.

DISCUSSION

1. Project Coordination (internal)

An integral component of the National RSG task force was to draw together resources from Riverlink agencies (Vic., SA, NSW) to provide direction and coordinate the delivery of information and monitoring of the CRCV project. This group generally worked well, however lack of a formal structure within the task force led to problems relating to accountability and roles.

Furthermore, once the RSG project amalgamated with the AGY project the Task Force was disbanded, although this was not clearly defined or stated. Some task force members maintained roles within the AGY project team or steering committee

2. Industry/Grower Communication (external)

The literature review, which was developed as part of Objective 1, was used extensively throughout the communication strategy. This review, which was completed and published in October 1995, concluded that the unusual grapevine growth during the 1994/95 season was not the result of a single cause, but rather a multitude of factors, including climatic effects and cultural practices. An integral part of this review was to provide recommendations on management strategies that could be adopted to mitigate future losses.

The main conclusion was the importance of monitoring weather conditions and applying light winter irrigations and pre-budburst irrigations during unusually dry seasons. A visit by Yasmin Wilson to California and Washington State in September 1996, assisted in clarifying that the RSG hypotheses have some similarities with other growth phenomena, such as Winter Chilling Injury, caused by unseasonable climatic conditions. Also a visit by Robert Hayes to South Africa in November .1997 revealed that similar environment related growth problems had been observed m both South Africa and California, where they are known as growth arrestment syndrome (Orth, pers. comm.) and delayed growth (Christensen, pers. comm.), respectively.

Industry members have been prepared to assist with and provide funding for delivery of information on management strategies for RSG i.e. the Murray Valley Winegrape Industry Development Committee, have funded harvest hints addressing the need for post-harvest irrigation. The techniques used to extend information on RSG have been effective. Messages on management strategies have been transferred to industry and growers in many regions resulting in reports from the MIA of irrigators lobbying for winter irrigation to be available, especially in dry years. In the Riverland information on avoiding RSG has been regionally investigated and published for local growers (Anonymous, 1998). Similarly reports by regional winegrape grower organisations on avoiding RSG have been published in the local Murray Valley Winegrape Grower newsletter (June Vol 2 Issue 5 1996, April Vol 3 Issue 2, 1997).

Because of the suggestion that RSG might be associated with the presence of a phytoplasma, there has been much confusion regarding the terms and symptoms used to describe poor vine growth.

The growth restrictions associated with RSG result from a particular event, one of which occurred in 1994 and caused widespread growth restrictions and subsequent yield reductions across a large number of varieties. The characteristic symptoms of RSG are poor and uneven budburst, often with a large number of buds failing to burst. Additionally there can be a tendency towards new shoot growth occurring from the trunk or within the crown. Generally canes and cordons are affected on older vines, whereas younger vines can be killed by RSG. Affected vines generally "grow out" during the season to produce normal cropping levels in subsequent years. This event also appears to be associated with drier and colder years, hence the recommendation for soil moisture levels to be maintained during autumn and winter.

However many vines, individually or in small numbers, have been observed with similar symptoms to RSG in all years, not just the "RSG years". These symptoms have been predominantly observed in Chardonnay and may be due to a number of factors ie. wood and/or soil pathogens, phytoplasma, poor graft unions, tendril restrictions, nutritional imbalances, toxicities (salt, boron), mechanical injury or poor vineyard management. The vines do not grow out of the restricted growth during the season and yields continue to be below average. These observations do not support the hypothesis proposed for RSG, and it is recommended that these symptoms and other restricted growth symptoms which do not have a definitive cause be given the generic name of restricted growth (RG), as opposed to environment related symptoms of RSG.

There is no evidence to suggest that RSG, RG and AGY and other growth restricting pests or diseases are mutually exclusive, and it is conceivable that any or all could be present concurrently within an individual vine.

3. RSG Trials

3.1 Grower trials

Following the RSG event in 1994, the Chardonnay (Karadoc) and Sultana (Dareton) sites had substantially lower yields in 1995 compared to the following year (1996) when the RSG symptoms were reduced and absent after budburst at each site respectively (Table 3).

At the Karadoc site yields did increase for 1996 and 1998, but not uniformly across the trial area as evidenced by the larger standard deviation (Table 3.). This may be due to some vines being severely affected by RSG in 1994 and not recovering by the 1995/96 season. Considering the vineyard was young (2 years old) when the RSG event occurred, it is possible many of the vines were severely affected by the unfavourable weather conditions of 1994. While yield data was not collected for the Karadoc site in 1994 (first cropping), indications are that it was substantially higher than 1995 (Sos pers. comm.). Demand from such a crop on young vines, along with unseasonable conditions, could have contributed to the reduction in yield for the 1995 harvest. Consequently in 1996 the healthier vines did produce much larger yields per vine so as to "mask" the severely restricted vines. A number of vines have been replanted since 1995. Variation in canopy size between vines of different age may I have also influenced the yield results.

At the Dareton site, the increase in yield was generally uniform from 1995 to 1996 with similar standard deviations for each year (Table 3). In 1995 there was probably a uniform distribution of RSG across the site, with subsequent recovery of the vines in the following season.

The Colombard (Yelta) site showed minimal yield reductions that could be attributable to RSG (Table 3). Overall in 1995 there appears to have been more variation in cropping levels from vine to vine with higher standard deviations than 1996-1998 (Table 3). No conclusions can be drawn from the effect of drip to overhead irrigation on yield, as the results do not follow a consistent pattern. The variation in yield from 1995-1998 could possibly be due to a combination of either seasonal temperature or water fluctuations, type of irrigation system (drip versus overheads) or irrigation scheduling of these systems. Overall the vines appear to have recovered from the RSG event enough to produce consistent yields.

The Chardonnay (Robinvale) site showed yields increasing from the 1997 to 1998 harvest with less vine to vine variation (lower standard deviation) for 1998 compared to 1997 (Table 3). Anecdotal evidence suggests that by improving irrigation scheduling, particularly during the post-harvest period, consistent yields can be achieved from vines growing in poor soil conditions (i.e. heavy clay), and which have previously been affected by RSG (Avery, pers. comm.) (Figure 1).

From March to April 1997 the Chardonnay vines were actively using water (Figure 1). This is apparent as the average soil moisture content gradually decreased from 43.5mm to 39.0mm, irrespective of irrigation or rainfall, before rising to a plateau in June. This is contrary to the belief that vines do not require much water during the post-harvest period. The climatic conditions for March to August 1997 were much drier and colder than the 30-year average (Table 6, Figure 2). Consequently if the grower had not meet the water requirements of the vines during this period, then they may have displayed RSG symptoms in spring 1997. However by closely monitoring the water use pattern and scheduling appropriate irrigations the vines were able to produce a healthy crop in 1998.

Apart from unfavourable weather conditions, there are also a number of other factors associated with the Karadoc site that may well have influenced the results. A visit by Phillip Myburg (Soil specialist from South Africa) revealed that apart from the heavy clay soil, the topsoil was very shallow in places, with some vines having a root depth of only 20cm. Myburg commented that the soil was not suitable for healthy vine growth and suggested improving soil structure by adding gypsum and hilling the soil around the vine trunks to increase the rooting zone. Whilst the vineyard is irrigated by overhead sprinklers, it is not drained causing water logging in certain areas. The combination of water logging and clay soils could have caused anaerobic soil conditions that in turn may have lead to root death. Once the root system is disrupted, then physiological responses begin in the vine that result in restricted growth symptoms in the following spring.

Additionally, many of the vines have damaged trunks that resulted from parts of the machine harvester hitting the trunks too hard. Another factor, which could be affecting the vines, is the variation in pruning technique, viz: 1994 (no pruning), 1995 (lightly skirted), 1996 (no pruning) and 1997 (hedge pruned).

The Karadoc site was mapped for the incidence of the AGY phytoplasma. The major symptoms in this vineyard were typical of RSG and RG, with approximately 0.5% of I vines displaying AGY symptoms (Constable et al. et al. 1996).

3.2 Chardonnay cropping trial

The yield increase in 1997 for vines with 25 percent of bunches removed prior to veraison (25 T/ha compared to 23.8 T/ha in control) may be attributed to the significantly higher average berry weight which compensated for reduced bunch numbers (Table 4). This is consistent with previous findings (Kliewer et al. 1983; van Schalkwyk et al. 1995), where early bunch removal did not result in an equal reduction in yield. While vine yield can be effectively reduced when bunch removal is applied during the period from before bloom until veraison, maximum crop reduction occurs when bunches are removed after veraison, as berry set is complete and cell division and growth has ended (Bravdo et al. 1984, van Schalkwyk et al. 1995).

The results from vines with 50 percent and 75 percent of bunches removed indicated significant yield reductions compared with the control, with yields of about 13.2 and 6.7 T/ha, respectively (Table 4). Unlike the results for the removal of 25 percent of bunches, there did not appear to be any compensation by an increasing bunch number, despite the significant increase in average berry weight (Table 4). While it has been found that cultivars with small bunches compensate to a lesser extent (van Schalkwyk let al. 1995), this does not explain the significant yield difference between 25 percent bunch removal and 50 percent removal. The harvest date was later than preferred, by the vineyard manager, and it is possible that the more advanced maturity of the more severely thinned vines (50 and 75 percent bunch removal) caused a decline in average bunch weights. Berries lose their resistance to water loss, which results in shrivelling, and tend to experience rapid increases in shatter as they exceed optimum maturity levels (Winkler et al. 1962).

All treatments showed an increase in yield from 1997 to 1998. While the lowest yield occurred for vines with no bunches removed in 1996 the difference was not significant (Table 4). Overall it appears that in these young Chardonnay vines it was possible to control cropping levels, without affecting yields in subsequent years. It is important to control cropping levels during the first few seasons of a vine's growth so as a strong canopy framework and root system can be developed. Once established the vine is less likely to be affected by unfavourable conditions (ie.climatic stress, pest and! or disease) and is more likely to produce consistent yields from year to year.

4. Carbohydrate reserves Environmental data

4.1 Potted Sultana trial (carbohydrate reserves)

The potted Sultana vines were subject to induced moisture stress during autumn and winter 1997 but not to induced cold. Compared to the unstressed vines, the starch content of the stressed vines (covered) was 20% lower (Table 5). Since there were no observable differences in growth in the spring, it is not possible to conclude whether the difference was due to the moisture stress, seasonal conditions, or the inherent variation in vines .

During early spring, grapevines are heavily dependent on carbohydrate reserves of the canes and other storage organs (Balasubrahmanyam et al. 1978). An indication of the previous season's health and vigour is indicated by the amount of carbohydrate stored in the grapevine. Thus if carbohydrate levels are inadequate then the new spring growth could be slower and less vigorous (Wilson, 1995).

Despite having a reduction in starch reserves, the stressed Sultana vines did not display typical RSG symptoms. This could be due to the sub-optimal trial conditions. Temperatures were not cold enough during the post-harvest and dormant period. Despite experiencing winter temperatures colder than the 30-year average, 1997 did not have the sudden temperature decrease from autumn to winter as occurred in 1994 (Figure 2). Wample and Bary (1992) have reported that seasonal variations in soluble and insoluble carbohydrates appear to be related to changes in air temperature and the dormancy status of the tissue. However, subsequent observations showed no major differences in budburst or early shoot growth.

Alternatively the physiological differences between winegrape varietals and Sultana may influence their relative susceptibility to RSG. No RSG was seen in Sultanas in 1998, where as many Chardonnay and other winegrape varietals were observed with RSG.

4.2 Meteorological Data

Key climate data was examined for selected years (Table 6) to determine if autumn and winter environmental conditions may have contributed to the observed variation in yields. The 1982/83, 1994/95 and 1997/98 seasons had all documented similarities in restricted growth problems (MacIntosh, 1983; Wilson, 1995; Hayes, 1997) and climatic data ie. particularly dry conditions during the post harvest and dormant period (autumn and winter) (Table 6).

Although annual rainfall can be average or above, the critical period for rainfall is during autumn and winter. While some years had low rainfall in autumn/winter, or a high number of frost days, if they did not have both (ie 1977 65.3mm/27 frosts, 1989 274mm/44 frosts) then these years did not coincide with reported RSG events. These results suggest that a combination of low rainfall in autumn/winter and an above average number of frost days (Table 6) can trigger RSG.

Additionally, some relevant temperature data for the same years were also examined (Figure 2). In 1994, when reports of RSG were prevalent, the average minimum terrestrial temperature increased slightly in autumn followed by a sudden decrease into winter (Figure 2). The average winter temperature remained much colder than the 30-year average leading into spring. In contrast, 1982, which also experienced cold conditions in winter, did not have a particularly warm autumn. Rather, the average minimum temperature gradually decreased from May until reaching the coldest average minimum temperature in July, before warming up to temperatures similar to the 30 year average in September (Figure 2.).

Compared to 1982 and 1994, 1997 experienced fewer temperature fluctuations, but with the conditions during winter were much lower than the 30- year average. However, the average minimums were not as severe, nor as lengthy as those recorded in 1982 and 1994 (Figure 2).

Overall 1982, 1994 and 1997 experienced the coldest average minimum temperatures, as well as having longer cold periods than the 30-year average and these conditions appear to be regional. In 1994 there were widespread reports of retarded growth in vines across all viticultural industries, including Sultana, which was the largest planting at that time in Sunraysia. In contrast, the reports of RSG in 1997 were not as widespread and non-existent in Sultana, possibly as a result of the milder environmental conditions less likely to trigger RSG (Table 6, Figure 2).

Sudden temperature changes can affect the acclimatisation rate of grapevines, which in turn increases the risk of cellular damage to the bud and cane tissue (Strik et al. 1992). Additionally the risk of cold injury during winter depends on the concentration of natural antifreeze compounds (carbohydrates) contained in the grapevine tissues (Meiring et al. 1980). Before entering the dormant period, grapevines accumulate food reserves (starch and sugars).

Generally grapevines that have not matured sufficiently and are exposed to sudden temperature fluctuations will be damaged or killed as a result of extreme cold temperatures during winter. Thus, it is possible that the warm period during May - June 1994, may have caused the vines to produce new growth, thus preventing the accumulation of food reserves for the dormant period. Consequently the cold temperatures, which prevailed during the winter, may have then caused various degrees of injury on the canes, trunk or roots of the vines. When the temperatures warmed up for budburst, the injury could be observed as a retarded growth in the spring. Immature canes of old grapevines may be damaged by cold temperatures resulting in uneven budburst, whereas a young grapevine, regardless of wood maturity, may be killed entirely due to its smaller volume of woody tissues (Wilson, 1997).

CONCLUSION

The 1994/1995 growing season had unfavourable climatic conditions (ie. Mild, dry autumn and cold dry winter), which undoubtedly contributed to the RSG observed across the wine, dried tablegrape industries.

The establishment of the National RSG Task Force in October 1994 assisted with the coordination of investigations and the dissemination of information on RSG to growers and industry. Results and investigations by the Task Force suggest that a multitude of factors influence a grapevine's susceptibility to RSG.

Field studies on RSG affected vineyards in Sunraysia showed that it is possible for vines to recover and produce consistent yields in the following years. Fluctuating yields at the Karadoc site are probably not due to RSG alone, but a combination of the poor soil type and over cropping of young vines. In comparison the Robinvale vineyard, established in heavy soil, recovered from the RSG event to produce consistent crop yields. This is likely due to improved irrigation management and scheduling.

Investigations to determine whether cropping levels of young chardonnay vines could influence growth and yields in subsequent seasons indicated that removal of bunches prior to veraison could produce higher yields in the following year compared to vines with no bunch removal, however the differences were not significant.

Studies on potted vines subject to moisture stress during autumn and winter 1997, showed substantial induced reductions in starch levels, although no observable differences in spring shoot growth occurred.

Analysis of meteorological data confirms that annual rainfall, and soil surface temperatures during autumn and winter of 1994 and 1997 were lower, and the number of frost days was higher, than the long- term average. These conditions support the hypothesis that growth problems, such as RSG, may have been induced by the unfavourable weather conditions. Consequently a recommendation by the Task Force has been to monitor the season and apply light autumn and winter irrigations during unusually dry conditions.

Occasionally retarded growth symptoms, similar to RSG, have been seen, predominantly in Chardonnay. These symptoms can be caused by a range of factors and can occur in all years. Since RSG appears to be environmentally related, it is recommended that these other symptoms, which do not have a definitive cause, be given the generic term restricted growth (RG).

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Acknowledgments
The authors would like to thank members of the National RSG Task Force and AGY project team for their assistance with the project. Many thanks to Frank Sos, Lindsay Herath, Gary Thomas and Barry A very for allowing field trials to be set up on their vineyards. Funding assistance from the CRCV, GWRDC and MVWIDC. Thanks for the technical assistance from Sonja Needs, Leonie Pitt and Fred Hancock.

APPENDIX 1

Members of the National RSG Task Force

Robert Hayes -Agriculture Victoria
Rod Mattschoss -Agriculture Victoria
Peter Clingeleffer -CSIRO (Merbein)
Ross Turkington -NSW Agriculture (Griffith)
Harry Creecy -NSW Agriculture (Griffith)

Richard Hamilton – PISA
Graeme Wellman – Mildura Blass
Rob Gibson – Southcorp Wines
Jim Hardie – CRCV
Peter Hayes – GWRDC
Yasmin Wilson – Agriculture Victoria (joined March 1995)

Category	Details
Publications	• Hayes, R.J., Wilson, Y. (1997). Environmental Conditions Leading to Restricted Growth and Yield Losses in Sultanas. Presented at: International Table and Raisin Grape Symposium, Cape Town 1997. • Wilson Y. (1997). Winter Chilling Injury. Technical Issue, The Aust. Grapegrower and Winemaker. No. 402a. Pages 157-158. • Wilson Y and Hayes R. (1996). RSG and AGY - sorting fact from fiction. Technical Issue, The Aust. Grapegrower and Winemaker. No. 390a. Pages 139-146. • Wilson Y. M. (1995). Restricted Spring Growth of Grapevines in South Eastern Australia. Literature review. Sunraysia Horticultural Centre, Agriculture Victoria. pp 2, 5, 23 • Mattschoss R. and Wilson Y. (1995). Understanding and managing restricted spring growth. Poster abstract for 9th Australian Wine Industry Technical Conference, Adelaide, SA, 16-19 July, 1995. • Mattschoss R. (1994). Restricted spring growth- the hidden cause. The Aust. Grapegrower & Winemaker. December. (372). 65-66.
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1.5 The association between restricted growth and various pathogens

Fiona Constable, Brendan Rodoni, and Yasmin Wilson.

INTRODUCTION

In spring 1997 widespread reports of restricted growth symptoms were observed in the Sunraysia, Riverina and Riverland districts, in most grape varieties but particularly in Chardonnay. In November 1.997 a trial was established to investigate pathological factors which may Induce restricted growth symptoms In Chardonnay. Paired vines, affected and unaffected, from four sites, in the Sunraysia district, were analysed for the presence of pathogens.

METHODS

Sites

Four sites with Chardonnay vines in the Sunraysia region at Gol Gol, Irymple, Pomona, and Wemen were chosen for investigation. Growers were asked to reply to a questionnaire about the history of the site with respect to vineyard establishment, management and environmental conditions.

Vines

An area of 20 x 20 vines was mapped for incidence of restricted growth in spring and Australian grapevine yellows in January. A vine was recorded with AGY if one or more shoots displayed irregular veinal and interveinal yellowing and downward rolling of leaves, which overlie one another in a shingled pattern. Two levels of severity were mapped or restricted growth symptoms, mild and severe. A vine was labelled with mild restricted growth if normal growth was retarded resulting in shortened shoots, smaller leaves and the vine has an overall appearance of being stunted or slow compared to unaffected vines. Vines with severe restricted growth display little or no bud development, severely stunted shoots with very small leaves and dead wood from previous years.

Two affected and two unaffected vines each, were chosen for examination at Irymple, Pomona, and Wemen. At Gol Gol, six pairs of vines were chosen so that a more significant association between disease and physiological differences of vines, presence of pathogens and impact of environmental conditions and/or management practices could be made. Restricted growth affected vines displayed retarded growth resulting in shortened shoots, little or no bud development on at least part of the vine, dead wood from the previous season, smaller leaves and the vine has an overall appearance of being stunted or slow compared to unaffected vines. An unaffected vine, with no obvious signs of any disease, was chosen as close to the affected vine as possible, usually directly next to or opposite the vine to ensure that there was little difference in the type of soil, irrigation and nutrients available to each pair of vines. The 12 pairs of healthy and affected grapevines were removed from the vineyards in summer and were assessed for the presence of pathogens in the laboratory. Physiological characteristics of the 12 pairs of healthy and affected vines including the development of root systems, trunk and canopy were visually assessed in the field prior to removal and in the laboratory.

CHS -Pathogens other than phytoplasma

All vines were assessed for the presence of potential pathogens by Crop Health Services (CHS) at IHD. For fungal and bacterial culturing, isolations were taken from roots, graft union, trunk, cordons and shoots/leaves. Soil was also baited for the presence of Pythiaceous fungi. Soil and root samples were tested for nematodes. Leaves from each vine were inoculated onto herbaceous indicators primarily to test for the presence of nepoviruses.

Waite Diagnostics -Virus testing

Grapevine samples supplied as fresh shoot material for testing for viruses and phytoplasmas using reverse transcriptase (RT)-PCR.

Phytoplasma

Samples were taken from roots, trunks, cordons and shoots of each vine. Each cordon was sampled individually and random samples of shoots from each cordon were taken. For roots, trunks and cordons, the outer bark was peeled away from the wood and the vascular tissue was removed by scraping with a sterile scalpel blade and used for DNA extraction. DNA was extracted from each sample according to the method described in this report. Nested PCR, also described in this report, was used to detect phytoplasma. Restriction fragment length polymorphism (RFLP) analysis was used to determine which phytoplasma was present.

RESULTS/DISCUSSION

Vineyard information, compiled from the questionnaire, is given in Table 1.

Table 1, General Vineyard information for each study site.

Region	Sunraysia	Sunraysia	Sunraysia	Sunraysia
Vineyard location	Irymple	Darling River - North Wentworth	Gol Gol	Wemen
Owner/ company	SHC (Ag. Vic.)	Mildara Blass Ltd	Cottrell Nominees	GR and CT Carey
Year planted	September 1995	1994	1993	1993/94
Variety	Chardonnay	Chardonnay	Chardonnay	Chardonnay
Clone	-	I 10V1	I 10V1?	-
Rootstock	Ramsey	Own Rooted	Ramsey	Ramsey, 9 rows of Schwartzman
Graft technique	Bench	N/A	Bench	Field Chip Bud (3acres) Spring Banded grafts (2 acres) 1 year old grafted vines (5acres)
Trellis Pruning method	2 wire vertical spur or minimal	2 wire vertical cane and spur	2 wire vertical spur	2 wire vertical minimal side hedging
Soil type	sandy loam	sandy loam	sandy loam over limestone	deep red sand
Irrigation system	drip	Drip	overhead sprinklers	undervine sprinklers
Tonnes/Ha 1998	31.5	17.94	5.11 from trail section 2.11 overall	25
Tonnes/Ha 1997	25	10.14		12.5
Tonnes/Ha 1996				12.5
Tonnes/Ha 1995				0.5

Incidence of restricted growth and AGY at each site are given in table 2.

Table 2. Incidence (%) of restricted growth (mild and severe) and AGY at each study site in the 1997/98 season

Disease	Gol Gol	Pomona	SHC	Wemen
mild RG	19	1	7	23
AGY	11	0.8	5	11
mild RG and AGY	10	0.3	9	8
severe RG	1	0.3	0	3
severe RG and AGY	0.5	0	0	2

* RG = restricted growth

The incidence of each disease varied between sites. Gol Gol and Wemen had a

substantially higher incidence of both mild restricted growth and AGY symptoms compared to the other sites. All sites contained vines that displayed both disorders. No severe restricted growth symptoms were recorded at Pomona and incidence was low at the other sites. AGY and severe restricted growth symptoms only occurred together in vines at Gol Gol and Wemen. The symptoms recorded were as described except at SHC where the vines displaying AGY symptoms had many shoots that were affected and affected vines were paler green in colour as if nutrient deficient. Description of vines at sampling for pathology testing in the lab is given in table 3. The presence of AGY and restricted growth symptoms is given the summary of pathogen testing in table 4.

CHS -pathology testing.

A summary of pathogens detected in each vine is given in table 4. CHS staff were not aware of which plant from each of the paired vines was suffering from restricted growth although in many instances symptoms were obvious. All 24 vines received the same amount of attention and therefore the results were not biased.

There are some significant individual results that can be highlighted and some trends emerging from CHS work:

- Initial results from the fungal culturing indicate that *Botryosphaeriasp.* dominates and is found in both restricted growth affected and "healthy" vines.
- No *Phytophthora sp.* were detected in any of the soil samples.
- Only one vine was found to be infected with *Agrobacterium sp.* (most probably *A. vilis*).
- No nepoviruses were found in any of the vines.
- Six vines, four from one site (vines 1-4) and one vine from each of GoI Gol and Pomona, were found to have potential nematode problems with the numbers isolated significantly high and possibly contributing to any decline occurring on these plants.
- *Phaeoacremonium sp.* was detected in vine 8 along with *Alternaria alternata*, yet this vine did not exhibit restricted growth symptoms.
- *Alternaria altternata* was detected in a total of three vines, two of which did not exhibit restricted growth symptoms.
- *Eutypa lata* was detected in vine 12 and interestingly this vine was also positive for the AGY phytoplasma in most parts of the vine tested.

Waite Diagnostics -virus testing

The result of virus testing for each grapevine is given in table 5.

- Grapevine leafroll associated Virus (GLRaV)-1 and grapevine fanleaf virus were not detected in any grapevine sample
- GLRa V -4 was not detected in any sample that was tested
- GLRaV-3 was detected in vine 1
- Grapevine virus-A was detected in vine 21
- Grapevine fleck virus was detected in vines 11, 12, 13 and 15. Vines 11 and 13 were considered healthy and vines 13 and 15 were affected with restricted growth.
- Rupestris stem pitting associated virus was detected in 11 of the 12 samples from restricted growth affected grapevines and in 11 of the 12 samples from healthy grapevines.

Phytoplasma testing

Results of our phytoplasma detection are given in table 4 and the results for Waite Diagnostics phytoplasma detection are given in table 5. AGY shoot symptoms were observed in eight of 12 restricted growth affected vines at the time of sampling .The combined results from Waite diagnostics and our own phytoplasma testing identified ten of 12 restricted growth affected grapevines that were positive for phytoplasmas. We detected the AGY phytoplasma by PCR and RFLP analysis in shoots and cordons from seven of the eight vines with both restricted growth and AGY symptoms. In one of the seven vines that were PCR positive, the AGY phytoplasma was detected in the trunk, roots, cordons and shoots. The combined results from Waite Diagnostics and our own PCR testing identified three of the four vines with restricted growth only that were positive for phytoplasma. We detected the AGY phytoplasma in the trunk by PCR and RFLP analysis in one of these grapevines.

No AGY symptoms were observed in paired vines unaffected with restricted growth symptoms. We detected the AGY phytoplasma in cordons or from roots of two of the 12 unaffected vines. Waite Diagnostics identified a third unaffected grapevine that was also positive for phytoplasma. Waite Diagnostics did not determine the type of phytoplasma present in any of the samples.

Table 3. Description of each part of the sampled vines at time of pathogen testing

Vine	Roots	Graft union	Trunk	Cordon	shoots
1	Healthy	Longitudinal cuts revealed black dead tissue - the vine had tried to grow around this area	Healthy	some internal black spotting	Healthy
2	Healthy	Minimal discolouration	Flattened with some internal browning. Necrosis associated with some injury sites	In some areas there appeared to be some shrinkage inside the bark and the bark was flaky	In some areas there appeared to be some shrinkage inside the bark and the bark was flaky
3	Healthy	Healthy	Internal necrosis associated with a wound site, as well as an internal necrotic region along most of the trunk.	Internal black spotting	Yellowing of leaves, interveinal necrosis and puckering on young leaves
4	Healthy	Healthy	Healthy	Some wound induced discolouration	Some wound induced discolouration
5	Black spotting, wound injuries, generally the root tissue was easy to cut and appeared pitted	A lot of internal necrosis - It appeared a if a trichoderma plug had been introduced into this area	A sucker grew just above the graft union that had yellows symptoms. A black pot/smile was persistent along the entire length of the trunk.	Healthy	Healthy
6	Healthy with minimal black spotting, although the pith appeared blacker than other vines.	Some blackening (15%) of the union but this did not extend up or down the trunk. Otherwise healthy.	Some internal black spotting. Necrosis associated with wound areas.	One cordon was flattened, resulting in cane splitting which in turn was associated with internal necrosis	Healthy
7	Healthy	Healthy - although the area between the graft union and roots showed a wedge shaped necrosis - this did not extend into the roots or go into the union	Rough and knobbly on one side. Black spotting and a purplish discolouration extended for the entire length above the union.	Extensive discolouration, the bark was loose and stringy	The canopy lacked vigour
8	Healthy but looked as if it had been pot bound	healthy	healthy - some discolouration associated with wound sites towards the top	Healthy	Healthy

9	Healthy	Healthy	Healthy - necrosis associated with wound sites	Cordon 3 was flattened in some areas and had a major split	Shoots from cordon 2 looked virus like with shortened internodes
10	Healthy	60% associated with a necrotic wedge	A necrotic wedge extended from the lower trunk through to 10mm below the cordons - 20% of the lower trunk was affected, 60% of the graft union and 40% of the trunk above the graft union	Lacked vigour, internal necrosis in older parts, some tip death	Healthy
11	Some black spotting	Healthy	Below he graft union was healthy but above the union black spotting in creased as you moved towards the top. Many pruning wounds	healthy except for cordon 1 which was split	Healthy
12	Healthy but black spotting started at the lower trunk		A furrow begins at the graft union and continues two-thirds the way up the trunk. In internal necrosis is associated with the furrow. Black spotting increases towards the top of the trunk. There are a significant number of wound sites	Cordons two and 4 were dead. Cordons 1 and 3 had massive internal discolouration especially in he older portions.	Leaves showed some signs of nutritional stress and yellowing.
13	Healthy	Healthy	Lower trunk is healthy but black spotting in creases as you move up the trunk.	Healthy	Healthy

14	Some discolouration	healthy	Some black spotting above and below the graft union. The frequency of black spots increased as you move up the trunk, At the top of the trunk a distinct ring was apparent and most black spotting appeared in side this - it appeared as if the tissue inside the ring was dying off	Healthy	Healthy
15	Slight discolouration and black spotting on some roots	A small indentation down one side but otherwise healthy	Below the graft union the trunk was soft and easy to cut - it appeared pitted or corky. Just above the union the trunk was healthy but an internal discolouration increased as you move up the trunk. There were many wound sites towards the top of the trunk	Cordons 2 and 3 had a lot of discolouration. Cordon 3 was almost dead. Cordon 2 was flattened and twisted and internally had a necrotic wedge originating from the base of the cordon	
16	Healthy although the trunk section extending from the root ball was to the graft union was discoloured, A black ring that looked like a mass of black spots surrounded the cortex	Healthy	Healthy	Healthy	Healthy
17	Some main lateral roots had internal discolouration that extended into the root ball. The cortex root tissue from the root ball to the graft union also had black discolouration	Healthy	Some black spotting towards the top of the trunk that appeared to be associated with pruning wounds	There was much internal black discolouration	Some dieback of shoots but the foliage appeared normal

18	Healthy but there was a major region of discolouration between of the trunk between the root ball and the graft union	Knobbly/bumpy externally but internally appeared healthy	Black spots and wound sites increased as you moved towards he top of the trunk	Considerable discolouration in all cordons and the tips were showing dieback type symptoms. In one cordon dieback was associated with a wedge like region of necrosis	
19	Healthy	Healthy but with a slight brown discolouration	Some necrosis appeared internally towards the top.	Healthy	Healthy
20	Healthy although the trunk section below the graft union had brown discolouration with some black spotting	Healthy	Towards the top the brown discolouration and black spotting reappeared	Cordons 3 and 4 were dead. Cordons 1 and 2 had over 70% dieback from the tips. The lower parts of the cordons had extensive (>70%) wedge shaped necrosis	
21*	Healthy	Healthy	Healthy but with some discolouration below the graft and towards the top near some wounding sites	Healthy	Healthy
22*	Healthy but the was extensive discolouration of the trunk between the root ball and the graft union	There were 4 major patches of black discolouration in the graft union and overall it had a dull brown appearance	The external appearance was unhealthy. Internally it appeared dull brown and riddled with purple blotching and some black spotting, At the top a necrotic wedge had developed from a wound site	5 of the 6 cordons were dead	From the live cordon the appeared to be only one healthy shoot

23*	Unhealthy and lateral shoots had some discolouration. The root ball looked as if it had been pot bound. There was some discolouration leading up to the graft union	In the graft union there was a major area of brown discolouration. The wood appeared dry and off colour. There was no major dieback	Generally unhealthy, skinny, the wood is off-colour and the bark is loosely attached. Some marginal browning of cambium/phloem tissue occurs. Just below the graft union between the outer bark and stem was a white gummy exudate some black spotting is evident towards the top	Unthrifty	Unthrifty
24*	Healthy but appeared to have been pot bound at one point	Healthy	Black spotting appeared towards the top, apparently associated with pruning wounds	Healthy	Healthy

* These vines were recorded as being on own roots however when examined they appeared to be grafted

Table 4. Summary of the disease status for restricted growth and AGY symptoms and pathogen testing for each vine, excluding viruses

Vine	Site	RG	AGY	Phytoplasma				Nepo-virus	*Botryosphaeria*	Other Pathogen	Nematodes
				S	C	T	R				
1	Irymple	-	-	-	-	-	-	-	+	*Alternaria alternata*	*Paratylenchus sp.* (1494/200ml soil)
2	Irymple	Y	Y	+	-	-	-	-	+	*Alternaria alternata*	*Paratylenchus sp.* (726/200 ml soil)
3	Irymple	Y	Y	+	-	-	-	-	+		*Paratylenchus sp.* (1098/200ml soil)
4	Irymple	-	-	-	-	-	-	-	+		*Paratylenchus sp.* (240/200 ml soil)
5	Wemen	Y	-	-	-	-	-	-	+		
6	Wemen	-	-	-	-	-	-	-	+		
7	Wemen	Y	Y	-	+	-	+	-	+	*Pythium sp.* *Agrobacterium sp (A. vitis?)*	
8	Wemen	-	-	-	-	-	-	-	+	*Alternaria alternata* *Phaeoacremonium sp.*	

9	Gol Gol	Y	-	-	-	+	-	-	+		*Paratylenchus sp.* (36/200ml soil, 253/10 g roots) *Meloidogyne sp* (30/200 ml soil).
10	Gol Gol	Y	-	-	-	-	-	-	+		Paratylenchus sp. (28/10g roots)
11	Gol Gol	-	-	-	-	-	-	-	+		*Paratylenchus sp.* (6/200ml soil, 26/10 g roots)
12	Gol Gol	Y	Y	+	+	+	+	-	+	*Eutypa lata*	*Paratylenchus sp.* (18/200 ml soil)
13	Gol Gol	-	-	-	-	-	-	-	+		
14	Gol Gol	-	-	-	-	-	-	-	+		*Paratylenchus sp.* (18/200ml soil) *Criconemella sp.* (6/200 ml soil)
15	Gol Gol	Y	Y	-	+	-	-	-	+		
16	Gol Gol	-	-	-	-	-	-	-	+		
17	Gol Gol	-	-	-	-	-	-	-	+		*Paratylenchus sp.* (8/200 ml soil) *Tylenchus sp.* (12/10 g root)
18	Gol Gol	Y	-	-	-	-	+	-	+		
19	Gol Gol	-	-	-	-	-	-	-	+		*Paratylenchus sp.* (180/200 ml soil) *Criconemella sp.* (42/200 ml soil)
20	Gol Gol	Y	Y	-	+	-	-	-	+	*Pythium sp.*	*Paratylenchus sp.* (1566/200soil)
21	Pomona	-	-	-	+	-	-	-	+		
22	Pomona	Y	Y	-	-	-	-	-	+		
23	Pomona	Y	Y	+	-	-	+	-	+		*Paratylenchus sp.* (18/10 g root) *Heliocotylenchus sp.* (9/10g root)
24	Pomona	-	-	-	-	-	+	-	+		*Paratylenchus sp.* (6/200 ml soil)

Table 5. Summary of testing performed by Waite Diagnostics for seven viruses and phytoplasma. The vine numbers correspond to the same numbers given in table 4. Results 0 = not detected, 1 = detected, nt = not tested

Vine	LR1	LR3	LR4	RSPaV	GVA	Fleck	GFLV	GY
1	0	1	nt	1	0	0	0	0
2	0	0	nt	1	0	0	0	0
3	0	0	nt	1	0	0	0	1
4	0	0	nt	1	0	0	0	0
5	0	0	nt	1	0	0	0	0
6	0	0	nt	1	0	0	0	0
7	0	0	nt	1	0	0	0	1
8	0	0	nt	1	0	0	0	0
9	0	0	nt	1	0	0	0	0
10	0	0	nt	1	0	0	0	1
11	0	0	nt	1	0	1	0	0
12	0	0	nt	1	0	1	0	0
13	0	0	0	1	0	1	0	1
14	0	0	0	1	0	0	0	0
15	0	0	0	1	0	1(high)	0	0
16	0	0	0	1	0	0	0	0
17	0	0	0	1(low)	0	0	0	0
18	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	0	0	1	0	0	0	1
21	0	0	0	1	1	0	0	0
22	0	0	0	1	0	0	0	0
23	0	0	0	1	0	0	0	1
24	0	0	nt	1	0	0	0	0

Abbreviations: LR1, LR3 & LR4 = grapevine leafroll-associated virus types 1, 3 & 4. GY = grapevine yellows. RSPaV = *Rupestris* stem pitting associated virus. GFLV = grapevine fanleaf virus. GVA = grapevine virus A.

CONCLUSIONS

CHS Analysis Results

CHS found no pathogen that was consistently associated with the restricted growth affected vines and not associated with the "healthy" vine. CHS noted that based on the visual descriptions of the symptoms it was easy to gauge which plant was affected by restricted growth. The symptoms of decline and lack of thriftiness noted might be a result of ongoing problems, which have been building up over a number of years, and not as a result of anyone particular event within the last 12 months. This observation lead to the conclusion that the affected vines were in a less vigorous state than their healthy counterparts and, therefore, more likely to succumb to the restricted growth.

Waite Diagnostics virus testing

No virus was consistently detected in restricted growth affected vines and not in the "healthy" vines. These results indicate that it is unlikely that any of the viruses that were tested for contribute solely to the presence of restricted growth symptoms. However we did not test for several other known grapevine viruses and we cannot exclude the possibility that these other viruses may have had some role in causing restricted growth symptoms.

Phytoplasma

Of the vines that were selected for restricted growth symptoms in November, 67% were observed with AGY symptoms and phytoplasmas were isolated from most of these vines in January. No symptoms were observed in vines that were not affected with restricted growth symptoms and only one of these vines tested positive for phytoplasma. The results suggest that restricted growth symptoms may be associated with an AGY phytoplasma infection. These results are supported by the statistical analysis reported earlier in this report (Constable et al.), which shows a significant association between AGY and restricted growth. However, vines were sampled for PCR testing in January and we have no way of determining whether phytoplasma infection caused restricted growth or if, due to their less vigorous state the vines were more susceptible to new phytoplasma infection.

1.6 Yield trials from Australian Grapevine Yellows affected grapevines.

Fiona Constable, Yasmin Wilson, Michelle Kelly and Greg Buchanan

INTRODUCTION

One of the symptoms associated with Australian grapevine yellows (AGY) is bunch abortion or berry shrivels on bearing shoots affected with AGY symptoms. When the AGY disorder was first investigated in 1976 there was concern that it would be as severe as some of the European grapevine yellows diseases, such as Flavescence Doree, which can cause up to 100% yield loss (Caudwell, 1964). Early research in the Riverland suggested that yield losses up to 13% could occur in vineyards severely affected by AGY (Magarey and Wachtel 1986).

AGY symptoms are associated with a phytoplasma infection (Bonfiglioli et al. 1995, Padovan et al. 1995, Gibb et al., this report). To fully understand the effect of the AGY disorder and the associated phytoplasma on yield loss we compared the yields of affected and unaffected paired vines at three study sites in Sunraysia in 1997 and 1998. The results were analysed statistically to determine if there was any significant difference in yield between AGY affected vines and those that were unaffected.

METHODS AND MATERIALS.

Study sites

Three vineyards were chosen for this study, located in the Sunraysia district of Victoria (Vic) and New South Wales (NSW), at Gol Gol, NSW (site 1), Karadoc, Vic (site 2) and Wemen, Vic (site 3). The variety Chardonnay was grown at each site. The vineyard was established at site 1 in 1992 and 1993, at Site 2 in 1992 and at site 3 in 1993. The soil type at site 1 was a sandy clay loam except around rows eight to ten where the soil was a light to medium clay. At site 2, the soil type was heavy clay. At site 3 the soil type was deep red sand.

Vines

Yield trials were conducted in 1996/7 and 1997/8 at the three study sites. The vines selected for yield studies at site 1 and site 3 were grown on Ramsey rootstock and at site 2 all vines chosen for this study were on own roots.

At each site, 10 paired vines, one AGY affected and one unaffected in each pair, were chosen. The unaffected vines had no known history of AGY, restricted growth or late season leaf curl symptoms, as determined by mapping data collected over three years. The affected vines were chosen if they displayed AGY during that season but not restricted growth. In addition, one trial in 1997 was conducted where vines were selected at bud burst, if they displayed unlignified shoots and unaffected vines were selected if they did not display unlignified shoots.

On the day of harvest, symptoms were recorded for each vine. If vines displayed AGY symptoms on the day of harvest they were either included in the trial but placed into the affected group (1996/7) or they were dropped from the trial and another unaffected vine was chosen (1997/98). All bunches healthy enough for wine production were harvested from each vine and bunches that were shrivelled/dead were discarded.

The total yield for each vine was recorded and average yield *of* unaffected and affected vines from each site was calculated. To determine if there was a significant difference in the average yield between affected and unaffected vines a two-sample t-test, that assumed unequal variance, was performed. A 95% confidence interval was also calculated and those Intervals that do not include zero are indicative *of* a significant result.

RESULTS

Yield

Site 1 and site 3 showed no significant difference between yields *of* unaffected vines and those that displayed AGY symptoms. The difference at site 2 was moderately significant in both seasons. The unlignified wood trial at site 3 showed that there was significant difference in vines that had unlignified shoots at the beginning of the season and vines that did not. All the vines that had unlignified shoots displayed AGY symptoms and all but one vine displayed restricted growth symptoms.

DISCUSSION

Our results show that the presence of AGY symptoms alone does not significantly educe yield in most circumstances. AGY disorder is associated with bunch abortion or berry shrivels on affected shoots but often there are only a few affected shoots on a vine. At site 2 we showed that there was a moderately significant reduction in yield on affected vines, which may have been associated with the number of affected shoots on those vines. The moderately significant reduction in yield may also have been due to the interaction between environmental differences at this site, compared to other sites. The vines at site 2 were on their own roots and may be less vigorous in heavy clay. If vines are less vigorous or invaded by other pathogens, they may be more susceptible to the effects *of* a phytoplasma infection.

Vines displaying symptoms of both AGY and restricted growth did have a significant reduction in yield. This is an important result since it has been demonstrated that symptoms of AGY may be associated with restricted growth (See Chapter 1.2, this report).

PCR testing *of* vines harvested in 1998 showed that no unaffected vines tested positive for phytoplasma and all affected vines tested positive for phytoplasma (data not shown). In 1997, many of the affected vines did not test positive for phytoplasma using nested PCR, however these vines were tested at the end of February and early March when the ability *of* the nested PCR to detect phytoplasma in grapevines decreases. Since AGY symptoms are associated with phytoplasma (Bonfglioli et al. 1995, Padovan et al. 1995, Gibb et al. this report), it is likely that in some circumstances infection with phytoplasma may lead to yield reduction in affected Vines.

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Table 1 Average yield between unaffected and affected vines and the significance of the difference between them.

			1997					1998		
	N[1]	Av. Yield	SD[2]	p-value	95% CI	n	Av. Yield	SD	p-value	95% CI
site 1										
Unaffected	12	9.49	4.858	0.82	-5.31-4.27	10	20.62	5.948	0.43	-6.64 -11.10
AGY	7	10.01	4.549			10	18.39	4.489		
site 2										
Unaffected	9	11.7	6.459	0.06	-0.30-10.30	10	12.36	4.862	0.06	-5.56 - 16.54
AGY	11	6.7	3.908			10	6.87	4.66		
site 3										
Unaffected	10	16.8	7.983	0.115	-1.69-12.49	12	29.68	6.199	0.14	-1.36 -9.06
AGY	10	11.4	6.259			12	25.83	6.113		
site 3 US*										
Unaffected	12	12.6	6.033	0.004	2.39-11.21					
AGY	12	5.8	4.069							

1 number of test vines, 2 is standard deviation, *Unlignified shoot trial

1.7 Characterisation of phytoplasmas in Australian grapevines

Fiona Constable, Karen Gibb and Jane Moran.

INTRODUCTION

In recent years, molecular technology has developed rapidly enabling researchers to detect and distinguish between related organisms at the molecular level. These molecular detection and differentiation methods include polymerase chain reaction (PCR) methods m combination with restriction fragment length polymorphism (RFLP) analysis using nucleotide restriction enzymes and DNA sequencing of the PCR product. PCR provides a robust, rapid and sensitive technique for the detection of many plant pathogens including phytoplasmas (Ahrens and Seemüller, 1992; Deng and Hiruki 1991; Gundersen et al. 1994; Kirkpatrick et al.1994).

Detection of phytoplasmas is most often based on the 16S rRNA gene, which is highly conserved between most prokaryotic organisms. However this gene contains enough variable regions to enable taxonomic and phylogenetic studies of phytoplasmas (Schneider et al. 1995). Also of use in taxonomic and phylogenetic studies is the 23S rRNA gene, which is also highly conserved (Schneider and Seemuller, 1994), as well as the spacer region located between the 16S and 23S rRNA genes (Kirkpatrick et al. 1994, Smart et al.1996). The spacer region contains both conserved and highly variable regions (Kirkpatrick et al. 1994) and can be further used to differentiate phytoplasmas.

Using differences in the 16s-23s rRNA regions and the intervening spacer regions, phytoplasmas have been divided into specific taxonomic groups. In particular, these regions have been used to show that the various grapevine yellows phytoplasmas found overseas belong to different taxonomic groups (Daire et al. 1993, Seemuller et al. 1994). Flavescence doree phytoplasma has been assigned to the Elm Yellows group (Daire et al. 1993) and Bois Noir (BN, Daire et al. 1993) and Vergilbungskrankheit phytoplasma (VK) (Maixner 1994) to the stolbur group within the American aster yellows cluster.. Various other grapevine yellows phytoplasmas in Europe and the US have been classified Into the X-disease group (Chen et al. 1993, Prince et al. 1993).

Recent work conducted with the phytoplasma associated with Australian Grapevine Yellows (AGY) (Padovan et al. 1995, Padovan et al. 1996) has shown that this phytoplasma is genetically distinct from other grapevines yellows phytoplasmas but is most closely related to those in the stolbur group. Padovan et al. (1996) showed that the AGY phytoplasma DNA from the 16S and 23S rRNA genes and the intervening spacer regions had a 97.5% similarity when compared with the VK phytoplasma. Interestingly, AGY phytoplasma has been shown to be closely related to the phytoplasma associated with Papaya dieback in Queensland using both the 16S rRNA gene (Gibb et al. 1996) and the *tuf* gene (Padovan et al. 1996). Padovan et al. (1996) also showed that the AGY phytoplasma had a 99.5% similarity to the phormium yellow leaf phytoplasma (PYL) found in New Zealand. Davis et al. (1997) have proposed a new taxon, *'Candidatus* Phytoplasma australiense', associated with the Australian grapevine yellows phytoplasma.

The variability and relatedness of phytoplasmas is of particular interest as relationships can often help to elucidate the disease cycles associated with these organisms. In Australia, grapevine yellows symptoms have been observed in the majority of grapevine growing regions (Magarey and Wachtel 1986, Bonfiglioli et al. 1996) and phytoplasmas have been detected in several white and red grape varieties with symptoms of grapevine yellows (Bonfighoh et al. 1996). However, the phytoplasmas associated with grapevine yellows in Australia have not been well characterised. We report the results of a survey conducted to ascertain if more than one phytoplasma exists in Australian grapevines and the identity of those phytoplasmas.

MATERIALS AND METHODS

Sampling and Source of phytoplasmas

Tomato big bud (TBB) phytoplasma, used as a reference phytoplasma, was maintained in Periwinkle *(Caranthus roseus)* in glasshouse conditions at normal day length and at 18-23°C.

Samples were collected from three vineyards in Sunraysia. The variety Chardonnay was grown at each site. The vineyard was established at Gol Gol, in 1992 and 1993, at Karadoc in 1992 and at Wemen in 1993. At Gol Gol, vines were grown on a variety of rootstocks including Ramsay, KY3251, Schwarzmann, and Teliki5C. At Karadoc all vines were on own roots except for replanted vines, which were on Ramsay. At Wemen all vines were on Ramsay rootstock. In addition samples were collected from other vineyards in Sunraysia and from other grape growing regions. A small number of varieties other than Chardonnay were also tested. Samples were collected over the entire growing season, from October until May.

Shoots with symptoms typical of AGY were used as source material for phytoplasmas. Grapevines with symptoms of late season leaf curl and restricted growth were also used in phytoplasma experiments. Restricted growth is characterised by uneven and failed budburst. Shoot growth is stunted with shortened internodes and smaller than normal leaves. Shoots and cordons on affected vines may die back. Affected vines usually exhibit poor growth throughout the season. Leaves on late season leaf curl affected shoots remain green, curl backwards and remain on the vine after other leaves fall. The symptoms generally appear from early autumn onwards. DNA extraction and nested PCR was carried out according to the methods described previously in this report (Gibb et al.). Asymptomatic grapevine samples were also collected and tested.

Reference DNA from sweet potato with sweet potato little leaf (SPLL) disease was collected near Darwin, Northern Territory in 1991 and tomato with tomato big bud (TBB) was collected near Adelaide, South Australia in 1992. DNA of additional phytoplasmas, which have been grouped on the basis of their 16S ribosomal DNA restriction patterns and nucleotide sequence (Schneider et al. 1993; Seemuller et al. 1994) were also included. These were: Vergilbungskankheit (VK); Bios Noir (BN); Stolbur from France (StoIF); American Aster Yellows (AA Y); apple proliferation (AT); sunn-hemp witches broom (SunH); sesame phyllody from Thailand (Sept); phyllody in *Cleome viscosa* from Thailand (Clp); Crotalariaphyllody from Thailand (Crop); vaccinium witches broom (Vac); Green valley strain of western X disease (GVX); Blutenverkleinerung from Germany (BVK); Brinjallittle leaf (BLL).

Restriction fragment length polymorphism (RFLP) analysis

Five μl of the PCR products amplified in the nested PCR were digested using the restriction enzymes, *Alu* I, *Rsa* I, *Hpa* II and *Mse* I in the buffer supplied by the manufacturer (New England Biolabs, Beverly, MA, USA). Digestions were incubated overnight at 37°C and the fragments were separated by electrophoresis in an 8% polyacrylamide gel in 1 x TBE buffer and visualised by staining with ethidium bromide and photographed on a UV transilluminator.

Sequencing

Nested PCR products were sequenced by Micromon (Monash University, Microbiology Department, Clayton, Victoria) using the ABIPRISM system and compared to other phytoplasmas using LALIGN (Huang and Miller, 1991) through ANGIS (Australian National Genomic Information Centre, Sydney, N.S.W.). Sequences of various phytoplasmas were also analysed using the GCG 8.1.0 (Genetics Computer Group, Madison, WI, USA) program GAP (Gap weight 5.0 and Length weight 0.3) accessed through ANGIS.

RESULTS

Our results indicated the presence of three different phytoplasmas in Australian grapevines including the one identified as the AGY phytoplasma (Padovan et al' 1995, Padovan et al. 1996). One of the new phytoplasmas was often found in association with late season leaf curl symptoms. To determine the identity of the new phytoplasma, PCR products amplified by the nested PCR were subjected to RFLP analysis using the restriction enzymes, *Alu* I, *Rsa* I and *Mse* I. The new phytoplasma was compared to AGY and representatives of most of the major phytoplasma groups. For all three restriction enzymes, DNA patterns confirmed that AGY is closely related but not identical to VK and BN (Fig.1a, b, c). The new Australian grapevine phytoplasma was identical to the pattern of SUNH, a phytoplasma of *Crotalaria juncea* from Thailand, which is also indistinguishable from the Australian tomato big bud (TBB) phytoplasma (Fig. 1a,b, c).

Figure 1. Restriction digestion of nested PCR products amplified by the primers P1/P7 and RI6f2/m23sr using the restriction enzymes *Alu* I (fig 1a), *Mse* I (fig 1b) and *Rsa* I (Fig 1c). DNA markers are not shown but are indicated by the sizes given in base pairs. In each figure samples are: lane 1 = Brinjal little leaf (BLL); lane 2 = Blutenverkleinerung from Germany (BVK); lane 3 = Green valley strain of western X disease (GVX); lane 4 = vaccinium witches broom (Vac); lane 5 = Crotalaria phyllody from Thailand (Crop); lane 6 = phyllody in *Cleome viscosa* from Thailand (Clp); lane 7 = tomato big bud (TBB); lane 8 = sunn-hemp witches broom (SunHp); lane 9 = apple proliferation (AT); lane 10 = American Aster Yellows (AA Y); lane 11 = Stolbur from France (StolF);lane 12 = TBB type phytoplasma (GP-A) in Chardonnay; lane 13 = AGY in Chardonnay; lane 14 = Bios Noir (BN); lane 15 = Vergilbungskankheit (VK)

During the course of this study, grapevine samples showing AGY symptoms were collected from the Ovens Valley region in Victoria. Relatively small isolated pockets of Chardonnay are grown in this region, which is elevated and experiences cooler temperatures than Chardonnay grown in the Sunraysia district. The incidence of grapevine yellows symptoms in the spur pruned patch (8-year-old vines) from which our samples were collected, is 14%.

Figure 1a
Chapter 1.7
Alu I

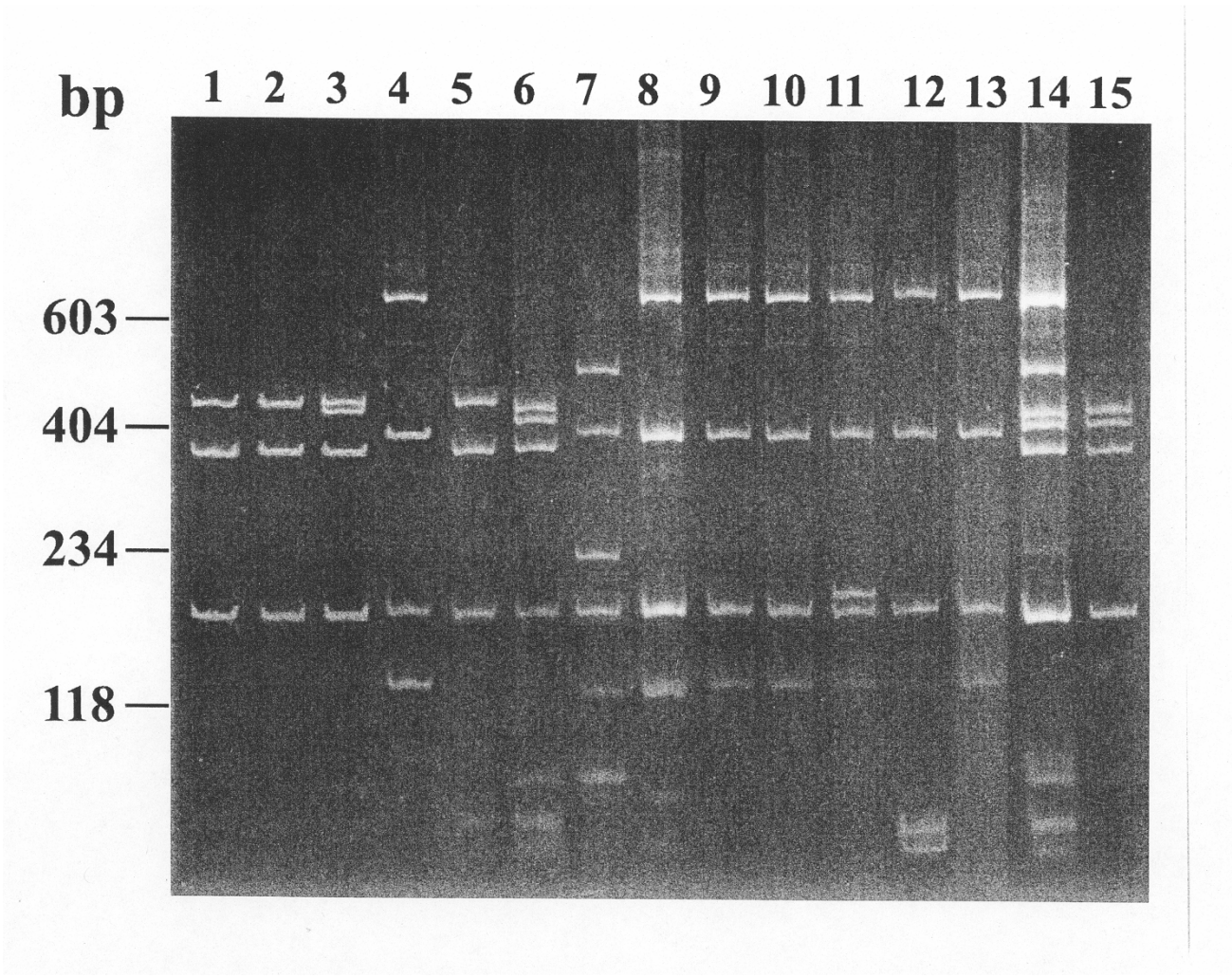
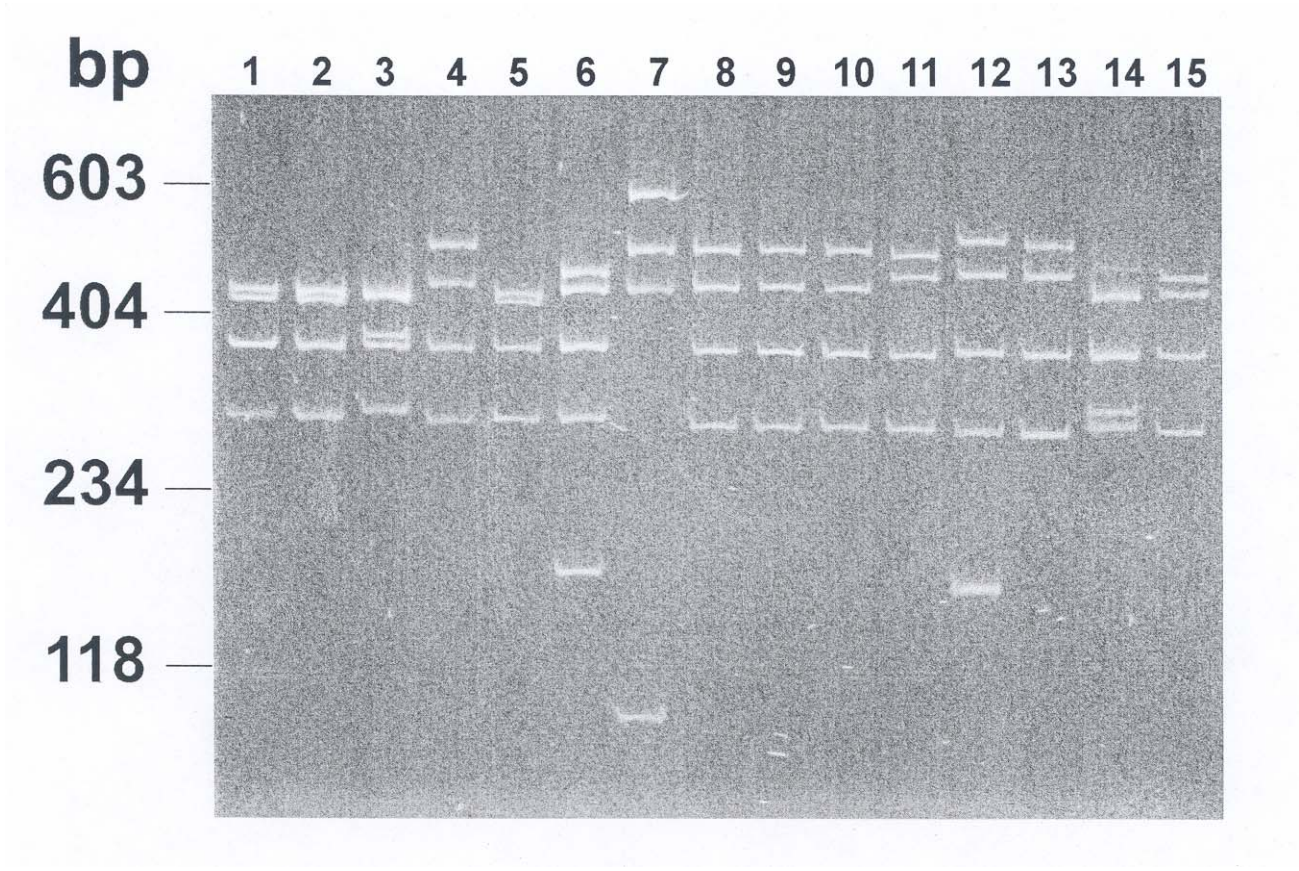


Figure 1b
Chapter 1.7
Mse I



Figure 1c
Chapter 1.7
Rsa I



In the caned prune patch (18-year-old vines) the percentage of incidence was 0.8%. Samples with AGY symptoms tested positive in the nested PCR and two representative samples were subjected to RFLP analysis using the restriction enzymes *Alu* I, *Hpa* II, *Mse* I and *Rsa* I. The Ovens Valley samples were compared to both the AGY phytoplasma and the grapevine phytoplasma indistinguishable from TBB.

Following digestion with *Alu* I, DNA patterns showed that the Ovens Valley AGY phytoplasma is very similar to the AGY phytoplasma with only minor changes in band sizes (Fig. 2). The differences are more marked when the enzymes *Hpa* II and *Mse* I are used (Fig. 2). With *Rsa* I some differences are also observed in band sizes (Fig. 2). Phytoplasma DNA from the two Ovens Valley samples were identical but were easily distinguished from the AGY phytoplasma using *Hpa* II and *Mse* I. Hereafter these phytoplasmas detected in the Ovens valley samples are refered to as the AGY vanant until identity can be further studied using sequence analysis.

Figure 2. Restriction enzyme digests of the phytoplasma DNA, amplified using nested PCR, from grapevines infected with AGY, TBB and ovens valley phytoplasmas. Restriction enzymes used form left to right are *Alu* I, *Hpa* II, *Mse* and *Rsa* I. bp = base pairs; lane 1 = grapevine with AGY phytoplasma; lane 2 = grapevine with TBB phytoplasma; lane 3 = grapevine 1 with Ovens Valley AGY phytoplasma; I lane 4 = grapevine 2 with Ovens valley AGY phytoplasma.

A preliminary sequence analysis comparing 1025 nucleotides of the 16Sr RNA gene showed that the AGY variant phytoplasma has a 95.3% similarity to the published sequence for the AGY phytoplasma. Also, a smaller part of the 16S rRNA gene sequence of the AGY variant phytoplasma compared with other phytoplasma sequences using GAP showed that the AGY variant was not closely related to FD or stolbur phytoplasmas.

Australian Grapevine Phytoplasmas and the Association with Various Disorders Studies on phytoplasmas in grapevine showed more than one phytoplasma was present, and infected grapevines may display more than one disorder. Grapevines with a range of disorders were tested between 1995 and 1998. Disorders were assessed as Australian grapevine yellows (AGY), late season leaf curl (LSLC) restricted growth RG) or a combination of disorders including AGY/RG,' AGY/LSLC, AGY/RG/LSLC, RG/LSLC and symptomatic (Table 1). When AGY symptoms were observed either alone or with restricted growth symptoms, approximately 60% of vines were phytoplasma positive and most of these were the AGY phytoplasma (Table 1). Interestingly, where vines had both AGY and late season leaf curl symptoms, half of the phytoplasmas detected were AGY and the other half were TBB. Of the 35 grapevines with both AGY and late season leaf curl symptoms, only 26% were phytoplasma positive, but this is significantly greater than for symptomatic vines (3%). Only 11 vines with all three disorders were tested, but of these 46% were phytoplasma positive with an almost equal split between AGY and TBB phytoplasmas. For vines with late season leaf curl symptoms, 10% were phytoplasma positive with twice as many being TBB than AGY. Only 5% of vines with restricted growth symptoms and 4% of vines with restricted growth and late season leaf curl symptoms were phytoplasma positive.

Figure 2
Chapter 1.7
Characterisation

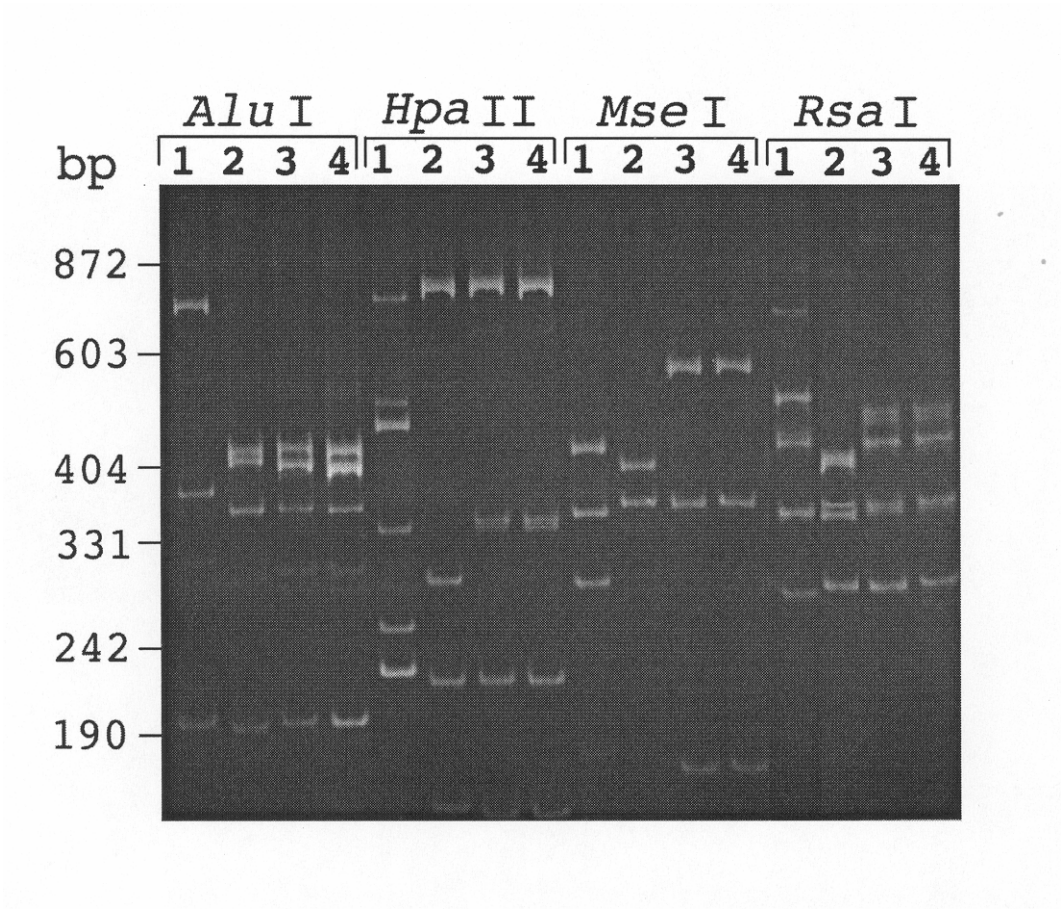


Table 1 Number of samples showing different disorders or that were asymptomatic, tested for phytoplasma using nested PCR, and the number that were positive and the distribution of different phytoplasmas amongst the nested PCR positive samples as determined by RFLP analysis using restriction enzymes (1995/96-1997/98).

Total number of samples tested and the number that were positive for phytoplasma using nested PCR			The distribution of different phytoplasmas amongst the total number of samples that were positive				
Disorder	Number tested	Number positive (%)	Number that were AGY (%)	Number that were TBB (%)	Number that were the AGY variant (%)	Number that were AGY and TBB together (%)	Number Unknown (%)
AGY	223	131 (59%)	94 (72%)	5 (4%)	6 (5%)	1 (>1%)	25 (19%)
LSLC	126	12 (10%)	4 (33%)	8 (67%)			
RG	203	11 (5%)	9 (82%)				2 (18%)
AGY/RG	66	40 (61%)	30 (75%)	3 (8%)	1 (2%)		6 (15%)
AGY/LSLC	35	9 (26%)	4 (44%)	4 (44%)			1 (12%)
AGY/RSG/LSLC	11	5 (45%)	3 (60%)	2 (40%)			
RSG/LSLC	28	1 (4%)		1 (100%)			
Asymptomatic	381	10 (3%)	5 (50%)	1 (10%)			4 (40%)

DISCUSSION

Our results show that more than one phytoplasma can be detected in Australian grapevines. The AGY phytoplasma is most often observed and commonly found in association with symptoms typical of AGY. Occasionally the AGY phytoplasma was found in association with restricted growth and late season leaf curl symptoms also.

A TBB type phytoplasma was also observed and often in association with late season leaf curl type symptoms but also with some AGY type symptoms. Our results (data not shown) suggest that the TBB phytoplasma can be detected more easily than the AGY phytoplasma in grapevines as a PCR product is often detected on the first round using P1/ P7 primers. Also in one vine from Karadoc, which was repeat sampled in the 1997/98 season we were able to detect phytoplasma in October and December through to March using nested PCR. In October and March we detected phytoplasma only in the nested round but in December to February the TBB type phytoplasma could be detected in the first and nested rounds. These results indicate that the TBB type phytoplasma may be present at a high titre in an infected grapevine throughout the season. More work needs to be conducted to determine the effect of this phytoplasma on grapevines.

We detected a new phytoplasma, the AGY variant, from the Ovens Valley, that is also associated with typical grapevine yellows symptoms. These included: irregular yellowing and backward curling of the leaves which drop from the vine early; necrosis of the yellow leaf tissue; overlapping of leaves on affected shoots; tip death of affected shoots; unlignifed, rubbery shoots; bunch abortion or berry shrivel. Our preliminary analysis, using both RFLP and sequence analyses, shows that this phytoplasma is distinct form AGY.

So far the AGY variant phytoplasma has only been found from the variety Chardonnay in the Ovens Valley and only in one vineyard. That this phytoplasma has not yet been detected elsewhere may indicate that the phytoplasma is unique to the Ovens valley region and in particular to the vineyard where it was detected. A survey of other vineyards in the Ovens valley region would be useful in determining the extent of this phytoplasma in Chardonnay and other varieties.

That more than one phytoplasma has been found infecting Australian grapevines has significant implications for management strategies to control the spread of these organisms. Like the grapevine yellows phytoplasmas found overseas (Caudwell et al. 1993; Maixner et al. 1995) it is likely that these different phytoplasmas have disease cycles involving different vectors and alternative hosts and that strategies for their management will also differ. Fortunately, much is known about TBB phytoplasma in Australia, including information about the vector *Orosius argentatus* occurring in vineyards (Osmelak et al. 1989, Kelly et al. this report) and alternative hosts (Davis et al. 1997; Schneider et al. 1998). We can use this knowledge about TBB phytoplasma to develop management strategies to control the spread of TBB type phytoplasmas in grapevines. We still do not understand the full disease cycle of AGY and it is possible that the AGY variant phytoplasma may have a different disease cycle to AGY. In the past we have used the relationship between the AGY phytoplasma and other phytoplasmas including PYL from New Zealand and VK in Germany to propose management strategies while we continue research to understand the AGY disease cycle. We need also to determine the relationship between the AGY variant phytoplasma to put in place management strategies to also control its spread.

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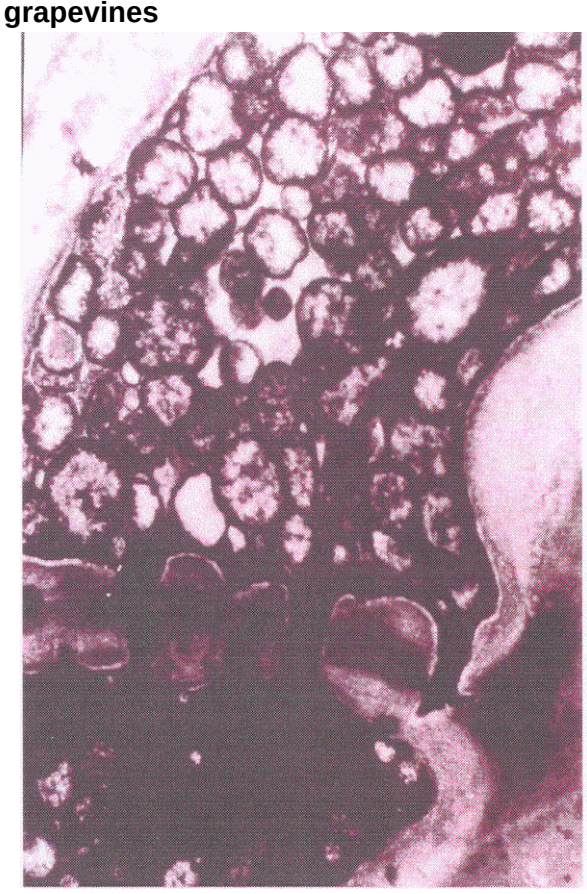
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Chapter 2 The development of diagnostic tools for the detection of phytoplasma DNA in Australian grapevines



2.1 Polymerase Chain Reaction for the detection of phytoplasmas in grapevines
Karen Gibb, Fiona Constable, Jane Moran and Anna Padovano

2.2 Polymerase Chain Reaction for the detection of phytoplasmas in grapevines
Rod Bonfiglioli

2.3 Optimising PCR Detection of AGY phytoplasma
Robert Faggian and Rebecca Mills

2.4 The development of ELISA for the detection of phytoplasma in grapevines
Fiona Constable and Rod Bonfiglioli

Chapter 2 Development of diagnostic tools for the detection of phytoplasma DNA in Australian Grapevines

2.1 Development of a phytoplasma detection method based on the polymerase chain reaction.

Karen Gibb, Fiona Constable, Anna Padovan and Jane Moran.

INTRODUCTION

The Polymerase Chain Reaction (PCR) is a process that is used to amplify a specific segment of DNA of known length, which lies between two regions of known nucleotide sequence. The known nucleotide sequences provide a region from which primers can be designed and it is between and including these regions that the amplification of DNA occurs. PCR occurs in a series of cycles and in each cycle the number of copies of target DNA increases exponentially. After the series of cycles is completed the target DNA is usually present in large enough concentration that it can be easily visualised after gel electrophoresis. A positive result is given when a band of DNA of the correct size is observed.

PCR provides a robust, rapid and sensitive technique for the detection of many plant pathogens Including phytoplasmas (Ahrens and Seemuller, 1992; Deng and HirukI, 1991; Gundersen et al. 1994; Kirkpatrick et al. 1994) and in particular Vergilbungskankheit (Maixner et al. 1995) and flavescence doree (Seemuller et al. 1994) associated phytoplasmas. PCR has also been used to detect phytoplasmas in Australian grapevines with AGY symptoms (padqvan et al. 1995).

The 16S rRNA gene is highly conserved between most prokaryotic organisms but contains enough variable regions to enable taxonomic and phylogenetic studies of phytoplasmas (Schneider et al. 1995). The 23S rRNA gene is also apparently highly conserved (Schneider and Seemuller, 1994). The spacer region located between the 16S and 23S rRNA genes contains both conserved and highly variable regions for many phytoplasmas (Kirkpatrick et al. 1994), which can be further used to differentiate phytoplasmas. PCR tests for phytoplasmas have been developed using the 16S rRNA gene, and, occasionally, the 23S rRNA gene and the spacer region between the 16S and 23S rRNA genes. Using these genes, universal and specific primers for the detection of phytoplasmas have been developed. We report the development of both a universal and specific test for AGY phytoplasma that also utilises the 16S and 23S rRNA genes.

MATERIALS AND METHODS

Source of phytoplasmas

Grapevines with suspected phytoplasma diseases were collected from three vineyards in the Sunraysia district of Victoria (Vic) and NSW (NSW) over three years form spring 1995 to autumn 1998.

The variety Chardonnay was grown at each site. The vineyard at site 1 was established in 1992 and 1993, at site 2 in 1992 and at site 3 in 1993. At site 1, vines were grown on a variety of rootstocks including Ramsey for seven rows, KY3251 for three rows, Schwarzmann for two rows, Ramsey for two rows and Teleki5C for four rows. At site 2 all vines were on their own roots except for replanted vines which were on Ramsey.

At site 3 all vines were on Ramsey rootstock.

The soil type at site was a sandy clay loam except around rows eight to ten where the soil was a light to medium clay. At site 2, the soil type was heavy clay. At site 3 the soil type was deep red sand.

In addition, samples were collected from other vineyards and from other grape growing regions. Leaves, petioles and young stems from the grapevine variety Chardonnay, with symptoms typical of AGY, were used as source material for phytoplasmas.

Reference DNA from sweet potato with sweet potato little leaf (SPLL) disease was collected near Darwin, Northern Territory in 1991 and tomato with tomato big bud (TBB) was collected near Adelaide, South Australia in 1992. DNA of additional phytoplasmas, which have been grouped on the basis of their 16S ribosomal DNA restriction patterns and nucleotide sequences (Schneider et al. 1993; Seemuller et al. 1994) were also included. These were: Vergilbungskankheit (VK); Bios Noir (BN); Stolbur from France (StoIF); American Aster Yellows (AA Y); apple proliferation (AT); sunn-hemp witches broom (SunH); sesame phyllody from Thailand (Sept); phyllody in *Cleome viscosa* from Thailand (Clp); Crotalaria phyllody from Thailand (Crop); vaccinium witches broom (Vac); Green valley strain of western X disease (GVX); Blutenverkleinerung from Germany (BVK); and Brinjallittle leaf (BLL).

Extraction of DNA

DNA was isolated from field-collected samples according to the phytoplasma enrichment procedure of Gibb and Padovan (1994). The nucleic acid pellet was resuspended in 50 ml TE buffer (1OmM Tris-HCI, ImM EDT A pH 8.0) and 3 ml was subjected to electrophoresis in a 1% agarose gel using 0.5x TBE running buffer. Products in gels were stained with ethidium bromide and visualised by UV Tran illumination. The quality and quantity of DNA was estimated from the gel and the nucleic acid was used as template for PCR either undiluted or after a 1/10 dilution.

PCR primers for the detection of phytoplasma DNA in grapevine

The PCR primers used to detect phytoplasma DNA in grapevines are listed in Table 1. A nested PCR procedure was used with the first primer pair P1 and P7 and the second primer pair R16F2 and rn23sr. These primers are specific for a region of the 16s rDNA and 23S rDNA regions in all known phytoplasmas.

A single round PCR test with the primers f/rStol was used to amplify a region of the 16S rRNA and 16S-23S spacer region, specifically from the stolbur group of phytoplasmas, to which the AGY phytoplasma is closely related. This potentially AGY -specific test, using f/rStol primers, was compared with another single round PCR test using the primer pair fStol /AGY2, which also amplified regions of the 16S rRNA and 16S-23S spacer region. The primer, AGY 2, was designed as a result of comparisons of the 16S-23S spacer region sequences of the AGY phytoplasmas with sequences of other phytoplasmas and is specific for the AGY phytoplasma (Gibb et al. 1998).

Table 1. PCR primers used to detect phytoplasmas in grapevines

Primer name (direction)	Primer sequence	Reference
P1 (forward)	5' AAG AGT TTG ATC CTG GCT CAG GAT T 3'	Deng and Hiruki (1991)
P7 (reverse)	5' CGT CCT TCA TCG GCT CTT 3'	Schneider et al. (1995)
R16F2 (forward)	5' ACG ACT GCT GCT AAG ACT GG 3'	Lee et al. (1993).
m23SR (reverse)	5' TAG TGC CAA GGC ATC CAC TGT G 3'	Padovan et al. (1995)
fStol (forward)	5' GCC ATC ATT AAG TTG GGG A 3'	Maixner et al. (1995)
rStol (reverse)	5' AGA TGT GAC CTA TTT TGG TGG 3'	Maixner et al. (1995)
AGY 2 (reverse)	5' GAT GTG ACC TAT TTT ATT TG 3'	Gibb et al. (1998)

Polymerase chain reaction (PCR)

For PCR, each reaction contained 0.2mM of each dNTP, 0.4 µM of each primer, 1 xDNA polymerase buffer supplied with the enzyme and 1.3 U thermostable DNA polymerase (Bresatec, Adelaide, Australia). For first round or single PCR, 1µl of undiluted or 1/10 diluted nucleic acid sample was added to the PCR cocktail mix. For nested PCR, 1µl of the first round reaction mix was added to the PCR cocktail mix containing the second primer pair. The total reaction volume was 50 µl in a Corbett FTS-1 thermocycler (Corbett Research, Mortlake, NSW, Australia). For the first round of the nested PCR test with primer pair Pl/P7, a manual hot start at 94°C *for* one minute was followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1.5 minute. PCR test conditions for the second round of nested PCR with primer pair R16F2/m23sr, or for the single round PCR test using the primer pair f/rStol were the same except that annealing temperature was 58°C. Similarly, single PCR tests with primer pairs P1/ AGY 2 or fStol /AGY 2 were the same except that the annealing temperature was 53°C.

After amplification, 5 µl from each sample was subjected to electrophoresis in a 1% agarose gel using 0.5xTBE running buffer. Products in gels were stained with ethidium bromide and visualised by UV transillumination. Total nucleic acid extracted from a symptomatic plants were subjected to the PCR as a negative control and in some experiments water controls were included, in which no plant nucleic acid was added to the PCR mix.

RESULTS

Based on nested PCR, and results from testing over three seasons, most positive signals were obtained from samples taken January (Table 2).

Table 2. The number of samples with AGY symptoms tested for the presence of phytoplasma using nested PCR and the percentage that were positive for phytoplasma during each month*

Month	Number tested	% positive
October	5	0
November	11	73
December	63	54
January	75	84
February	92	63
March	44	20
April	18	11
May	7	43

*samples were collected over three seasons from 1995/96 to 1997/98

Also, repeat sampling and testing using nested PCR of 20 grapevines from Gol Gol shows that the optimum sampling time was in February in the 1995/96 season and January in the 1996/97 season. (Table 3). Of the 14 grapevines found to be positive in February 1996, 10 were also positive in January and 6 were positive in March. Three vines were not positive on consecutive sampling dates. Only two of the 14 grapevines were only positive on the optimum sample date of February. One sample was negative on the optimum sample date but positive in January.

In 1996/97, 16 grapevines tested positive for phytoplasma and only one of these did not test positive in January. Of the 15 samples that tested positive in January, six samples did not test positive on consecutive sample dates. One sample tested positive only in January and one sample tested positive on two sampling dates only prior to January. Two grapevines did not test positive for phytoplasma in either season and no symptoms were recorded on these vines.

Table 3. Repeat nested PCR testing for detection of phytoplasma infection in 20 grapevines from one study site at Gol Gol (Vic) in 1995/96 and 1996/97

	1995/96 season				1996/97 season							
vine	Dec	Jan	Feb	Mar	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
1	-	+	+	-	-	+	-	+	+	-	-	-
2	-	+	+	-	-	+	+	-	-	-	-	-
3	-	-	+	-	-	+	+	+	+	-	-	-
4	-	-	+	+	-	+	+	+	-	-	-	-
5	-	-	-	-	-	-	+	+	+	-	-	-
6	-	+	+	+	-	-	+	+	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	+	+	+	-	-	-
9	-	+	+	+	-	-	-	+	-	-	-	-
10	-	-	+	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	+	-	+	-	-	-	-
12	-	+	+	-	-	+	+	+	+	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	-	+	-	-	-	-	+	+	-	-	-	-
15	-	+	+	+	-	-	-	-	-	-	-	-
16	-	+	+	+	-	+	-	+	-	-	-	-
17	-	-	+	-	-	+	-	+	-	-	-	-
18	-	+	+	+	-	-	+	+	-	-	-	-
19	-	-	+	-	-	+	-	+	+	-	-	-
20	-	+	+	-	+	-	-	+	+	-	-	-

In the 1995/96 season the nested PCR test and specific single round PCR test, using f/rStol, were compared at each sample time for 20 grapevines selected from the study site at Gol Gol (Fig. 1). From these results the optimum sample time was early February and at that time the same number of samples were found to be positive by both tests. Except for two samples, the same vines were found to be positive for phytoplasma (Fig. 1). In February when symptoms on the vines were less obvious phytoplasmas could only be detected using nested PCR.

Sensitivity of PCR tests

DNA from two phytoplasma positive grapevine samples was quantified using a DNA Mass Ladder (Life Technologies). The DNA was then diluted and subjected to four different PCR tests using the nested primers, f/rStol, PI/AGY2 or fstol /AGY2. For one sample the nested PCR amplified products down to 0.04 ng total plant DNA but the result was inconsistent in that no product was amplified when 0.08 ng DNA was used and 2 ng appeared to inhibit the reaction (Fig. 2). For the second sample, detection was down to 0.08 ng DNA. Single round PCR using f/rStol primers amplified phytoplasma DNA at all concentrations of total plant DNA (Fig. 2). Single round PCR using the P1/AGY2 primers was least sensitive in that detection was only down to 0.4 ng DNA with possible inhibition at higher concentrations (Fig. 2). Single round PCR using the fStol /AGY2 primers permitted detection down to 0.08 ng DNA (Fig. 2).

Other grapevine phytoplasmas
To test the specificity of the various PCR tests, grapevine phytoplasma DNA from Germany (VK) and France (BN) as well as AGY and a new Australian grapevine phytoplasma (GP-A) related to TBB phytoplasma (Gibb et al. 1996, Constable et al. this report), were subjected to the nested PCR test or to single round PCR tests using either the f/rStol, fStol /AGY2 or P1/AGY2. As expected, all phytoplasma DNAs were amplified using the universal nested PCR primers (Fig. 3). The PCR tests using f/rStol primers, which were designed to amplify the Stolbur grapevine phytoplasma DNA, amplified VK, BN and AGY DNA but not GP-A phytoplasma DNA (Fig. 3). The AGY specific single round PCR tests using the primer pair fStol / AGY2 or the primer pair P1/AGY2 were AGY specific as neither VK nor BN DNAs were amplified (Fig. 3).

A range of phytoplasma DNAs was also subjected to the nested PCR test to show that it was indeed universal. The Nested PCR test was able to detect StolF, AA Y, AT, SunH, Sept, Clp, Crop, Vac, GVX, BVK and BLL.

DISCUSSION
Utilising primers developed by other researchers we developed universal PCR tests to detect all phytoplasmas that might be found in Australian Grapevines. The nested PCR was used to detect phytoplasma DNA from most phylogenetic groups and, while we did not specifically test flavescence doree (FD) phytoplasma DNA, the P1/P7 primers were able to detect (FD) phytoplasma DNA (Padovan et al. 1996). Specific single round PCR test was also developed which could detect stolbur related phytoplasmas infecting grapevines including AGY, VK and BN. We also developed an AGY specific single round PCR test to detect AGY phytoplasmas only.

The comparison between the nested PCR and single round PCR test using f/rStol in the first season showed that the advantage in using the nested PCR test was that it could be used for detection of phytoplasmas later in the season when symptoms on the vines were less obvious. Since nested PCR could be used to detect most phytoplasmas and it was more sensitive over an entire season we used nested PCR for researching phytoplasmas in grapevines in subsequent years following development in 1995/96. Repeat testing and sampling of grapevines over three years showed that the best time for PCR testing to detect phytoplasma in Australian grapevines is in January and February.

Smart et al. (1996) showed that the spacer region between 16S and 23S rRNA genes to be a useful region for the development of group-specific phytoplasma PCR primers.
We developed a specific single round PCR test using the fStoVAGY 2 primer pair that could be used to detect AGY phytoplasma only. These primers have also been used to detect Papaya Dieback phytoplasmas (Gibb et al. 1998), which so far is indistinguishable from AGY phytoplasmas when comparing the *tuf (elongation* factor) gene and the 16S-23S genes and the spacer region.

The AGY2 reverse primer was designed from the AGY phytoplasma spacer region sequence data in a region that differed from the other stoibur-like phytoplasmas examined. The fStol / AGY 2 primer set is evidently more specific than the primers used for the nested PCR test because the latter will amplify gene products from most, if not all, phytoplasmas found so far in Australian grapevines. The comparison between the universal and specific tests could show if more than one phytoplasma was involved.

If a universal PCR test is used, restriction fragment length polymorphism (RFLP) analysis using restriction enzymes to digest the PCR product is required to determine the identity of the phytoplasmas.

As a result of this work two diagnostic tests are now available, one allows the detection of all phytoplasmas, which might be found in Australian grapevines, while the other is an AGY specific test. The AGY specific test can be used to screen plant and insect samples using a single round PCR without the extra time required for further RFLP analysis. This will greatly facilitate the screening of large numbers of samples m epidemiology studies and IS the only means of detecting mixed phytoplasma infections, which is important when trying to accurately monitor the incidence of disease in the field. However it is not as sensitive as nested PCR and can only be used for grapevine material in January or February when it has the best chance to detect phytoplasma DNA.

Because of its sensitivity and that it was universal for all phytoplasmas, the nested PCR test was used for the duration of the research project to study phytoplasmas in Australian grapevines, conducted at the Institute for Horticultural Development (IHD, Knoxfield, Victoria), from 1995 to 1998. The nested PCR test has also been adopted by Crop Health Services (IHD) for the detection of phytoplasmas in any crop.

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APPENDIX
Extraction methods and PCR protocols for phytoplasmas in Grapevines.

Perform all operations on ice unless otherwise specified. Use sterile glassware.

1. Cut up 1-2 grams of symptomatic, non-necrotic plant material into small pieces using a razor blade or scissors. Place the material in a mortar containing 7ml of cold phytoplasma grinding buffer. Let the tissue plasmolyse for 10-20 minutes before grinding. Grind thoroughly, add 6ml of fresh buffer and grind again. Transfer to a cold corex tube (or other centrifuge tube, plastic is OK). Grinding will be easier using an abrasive powder (glass, powder or sand).

2. Centrifuge in an SS34 rotor for 5 minutes at 3000rpm at 4°C. Pour the supernatant into a clean centrifugation tubes. Discard pellet.

3. Centrifuge the supernatant at 11000-13000 rpm for 25 minutes at 4°C. Discard the supernatant, drain briefly by inverting the tube and put it on a paper towel.

4. Re-suspend the pellet in 1ml of CTAB buffer (prewarmed to 60°C, avoid air bubbles) and transfer to a 2ml microcentrifugation tube. Incubate at 60°C for 30 minutes. Mix gently several times during incubation. Spin shortly before opening tubes.

5. Add 0.8-1 ml of chloroform isoamyl alcohol and mix thoroughly but gently. Centrifuge in a microfuge at room temperature for 5 minutes at 13000rpm.

6. Transfer the epiphase into a new 2ml micro centrifuge tube and add an equal volume of isopropanol (stored at -20°C). Mix immediately. Centrifuge for 5 minute at 13000rpm. Discard the supernatant and wash the pellet twice with 70% ethanol.

7. Dry the pellet under vacuum and re-suspend in 50µ1 of water. Run a 1 % agarose gel to check DNA quality and quantity. See over page for gel recipe.

Solutions for phytoplasma DNA enrichment procedure.

Phytoplasma grinding buffer	for 1 litre
K2HPO4-3H20	21.7 g (anhydrous = 16.7g)
KH2PO4	4.1 g
Sucrose	100 g
Polyvinylpyrrolidine (PVP-10)	20 g

Use sterile distilled water and filter sterilise. Can store in 50 ml aliquots at -20°C. Make up 100mM stocks of ascorbic acid (0. 176giml water) and store in 500 µl aliquots at -20°C for up to two weeks.

Just before using the grinding buffer, add ascorbic acid at 200µ1/20ml grinding buffer. Also add 0.03g/20ml-grinding buffer of bovine serum albumin (BSA) fraction V (0.15%).

Adjust pH to 7.6 after adding ascorbic acid and BSA.

CTAB buffer

CTAB	2%
NaCl	1.4M
Tris	100mM
EDTA	20mM
PVP-40	1%
Mercaptoethanol	0.2% (added at the last minute just before use in required volume of buffer)

Check pH is 8.0.

Gel electrophoretic analysis of DNA

1. Prepare a 1% agarose gel in 0.5 x TBE buffer. Heat to dissolve.

2. When the gel solution is cool enough to be held, add 5 µl of 10mg/ml ethidium bromide to 20 ml 1% agarose and mix. A void bubbles -do not stir vigorously.

3. Tape both ends of gel tray and pour gel, remove any bubbles present, add comb and leave to set.

4. Remove tape and place gel in gel tank. Add 0.5 x TBE buffer until the gel is just covered.

5. Take 2µ1 of DNA sample and place in appropriately labelled microfuge tube.

6. Add 1µl loading buffer to each tube and spin down contents for a few seconds in a microfuge.

7. Make a well plan including standards and load samples in slots accordingly. Use 2 µ1 of either spp-1 or ØX174/HaeIII + 1µl loading buffer as markers.

8. Run gel at 80V for 60 minutes.

9. View under UV illumination.

PCR protocols

PCR consumables are aliquoted in volumes for 10 reactions.

Solutions

1.Reaction buffer -50µl of 10Xbuffer and 3ul of 75mM $MgC1_2$ aliquoted into each **BLUE** tube and store at -20°C. These are provided with Bresatec Taq

2. dNTPs -stock dNTPs are ordered from Promega Corporation as 100mM stocks. Make up a 1mM working solution:

dATP 10µl
dGTP 10µl
dCTP 10µl
dTTP 10µl
SDW 960µl (SDW = sterile distilled water)

Total volume = 1ml.
Aliquot 50µl into **ORANGE** tubes and store at -20°C.

3. Primers -make up to a 4µM working solution and aliquot 50µ1 into each labelled **YELLOW** tube and store at -20°C.

4. Taq DNA polymerase. -Bresatec Taq .Aliquot 1.3µ1 into each GREEN tube and store at -20°C.

PCR reaction mix assembly

Stock	Amount required for 10 reactions	Final concentration
SDW	285.7µl	
Reaction buffer (plus 3µl 25mM Mg C12	53µl	1X (1.5mM MgC12
1mM dNTPs	50µl	0.1mM
4µM forward primer	50µl	0.4µM
4µM reverse primer	50µl	0.4µM
10U/µl Tag (Bresatec, Adelaide, SA)	1.3µl	1.3U/rxn
Total volume	**490mL**	

Aliquot 49µ1 into each thin wall PCR tube and if required overlay each reaction with mineral oil. Add 1µl of DNA (diluted or undiluted) to the reaction mix.

PCR cycling conditions and Primers:

1. Nested Test P1/P7: r16f2/m23sr

The cycling conditions for P1/P7 primers are:

Cycle	Step	Temp	Time
1-35	1	95°C	1min
	2	55°C	1min
	3	72°C	1.5min
36	1	4°C	hold

P1 (forward) = 5' AAG AGT TTG ATC CTG GCT CAG GAT T 3'
P7 (reverse) = 5' CGT CCT TCA TCG GCT CTT 3'

For the nested PCR take 1µl of product from the first round PCR and add it to the

PCR mix containing R16F2 (Lee et al. (1993). Phytopathology **83**, 834-842.) and m23SR (Padovan et al. (1995) Aust. J. of Grape and Wine Research **1,** 25-31.

R16F2 (forward) = 5' ACG ACT GCT GCT AAG ACT GG 3'
m23SR (reverse) = 5' TAG TGC CAA GGC ATC CAC TGT G 3'

The following cycling procedure was used for nested PCR:

Cycle	Step	Temp	Time
1-35	1	95°C	1 min
	2	57°C	1 min
	3	72°C	1.5 min
36	1	4°C	hold

PCR products *(*5µl PCR product and 2µL loading buffer) are run on a 1 % agarose gel in 0.5X TBE as mentioned earlier, stained with ethidium bromide and viewed under UV illumination To observe results from PCR:

1. Prepare a 1 % agarose gel in 0.5 x TBE buffer. Heat to dissolve.

2. When the gel solution is cool enough to be held, add 5µl of 10mg/ml ethidium bromide to 20 ml % agarose and mix. Avoid bubbles -do not stir vigorously.

3. Tape both ends of gel tray and pour gel, remove any bubbles present, and add comb and leave to set.

4. Remove tape and place gel in gel tank. Add 0.5 x TBE buffer until the gel is just covered.

5. Take 5µl PCR product and mix with 2µl loading buffer in appropriately labelled microfuge tube.

6. Make a well plan including standards and load samples in slots accordingly. Use 2µl of either spp-1 or ØX174/HaeIII + 1 µl loading buffer as markers.

7. Run gel at 80V for 60 minutes.

8. View under UV illumination.

Chapter 2.1 figure legends

Figure 1. Amplification of phytoplasma DNA by nested polymerase chain reaction (PCR) using the primers P1/P7 and R16f2/m23sr, which amplifies a region of the 16S rRNA gene and the spacer region between the 16S rRNA and 238 rRNA genes (A) and by single PCR using the stolbur specific primers f/r8tol (B). Lanes 1-20 = cohort of 20 vines used for monthly samplings over two growing seasons with samples taken February 1996 for the PCR results shown here, lane 21 = AGY phytoplasma positive control, lane 22 = water negative control. DNA markers are not shown but DNA band size is indicated in base pairs (bp).

Figure 2. Testing detection limits for four different primer sets. Tow samples of total DNA from phytoplasma positive samples were diluted and subjected to nested PCR using the primers Pl/P7; R16F2/m23sr (A), single round PCR using f/rStol (B), Pl/AGY2 (C) and fStol /AGY2 (d). DNA markers are not shown but DNA band size is indicated in base pairs (bp).

Figure 3. Comparison of the specificity of each polymerase chain reaction (PCR) test developed using phytoplasma DNAs from Germany (VK) and France (BN) as well as AGY and DNA from a new Australian grapevine phytoplasma (GP-A) that is related to TBB phytoplasma. Primer combinations were (A) Pl/P7, Rl6f2/m23sr, (B) f/r8tol, (C) P1/AGY2 and (D) fStoVAGY2. DNA markers are not shown but DNA band size is indicated in base pairs (bp).

Figure 1
Chapter 2.1

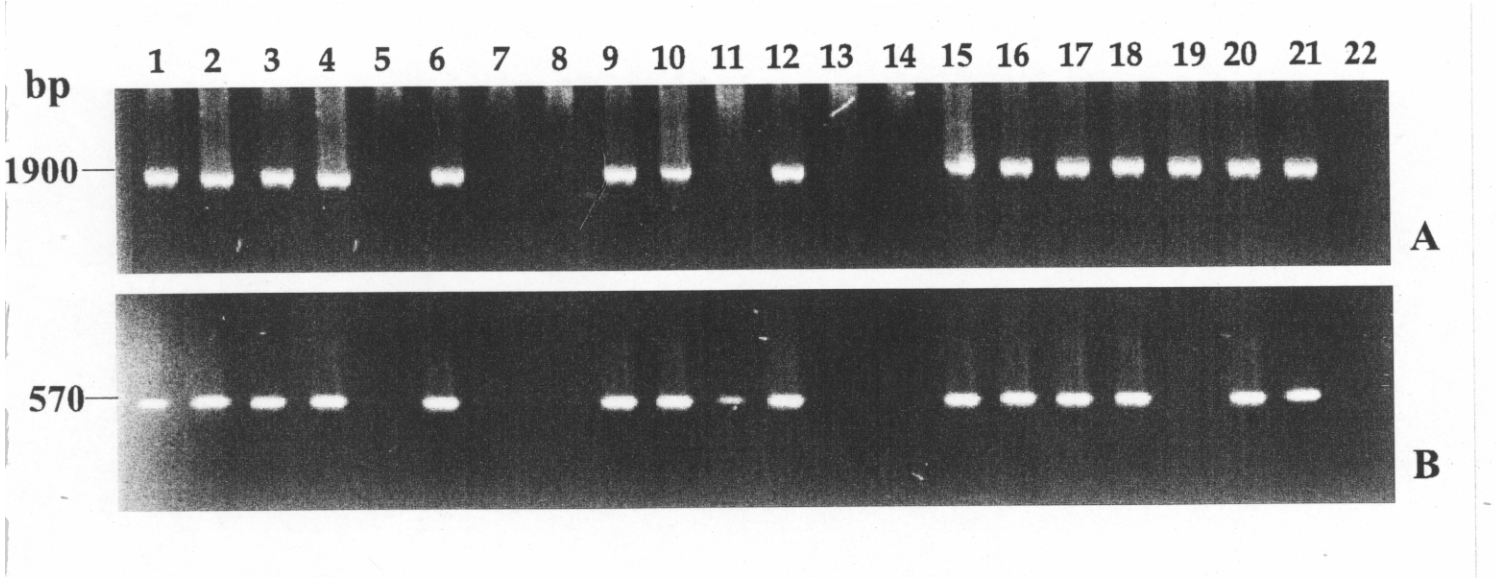


Figure 2
Chapter 2.1

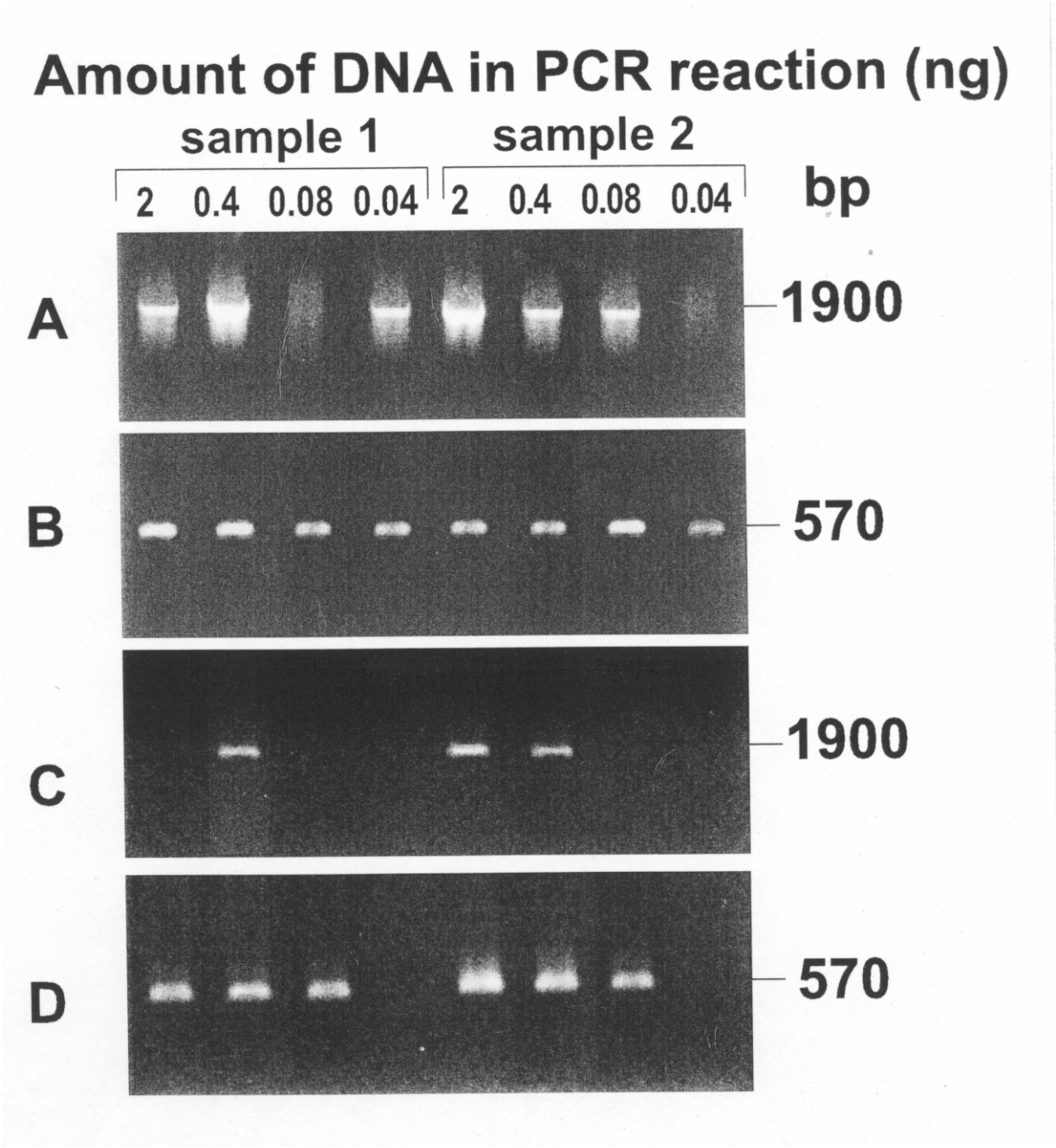
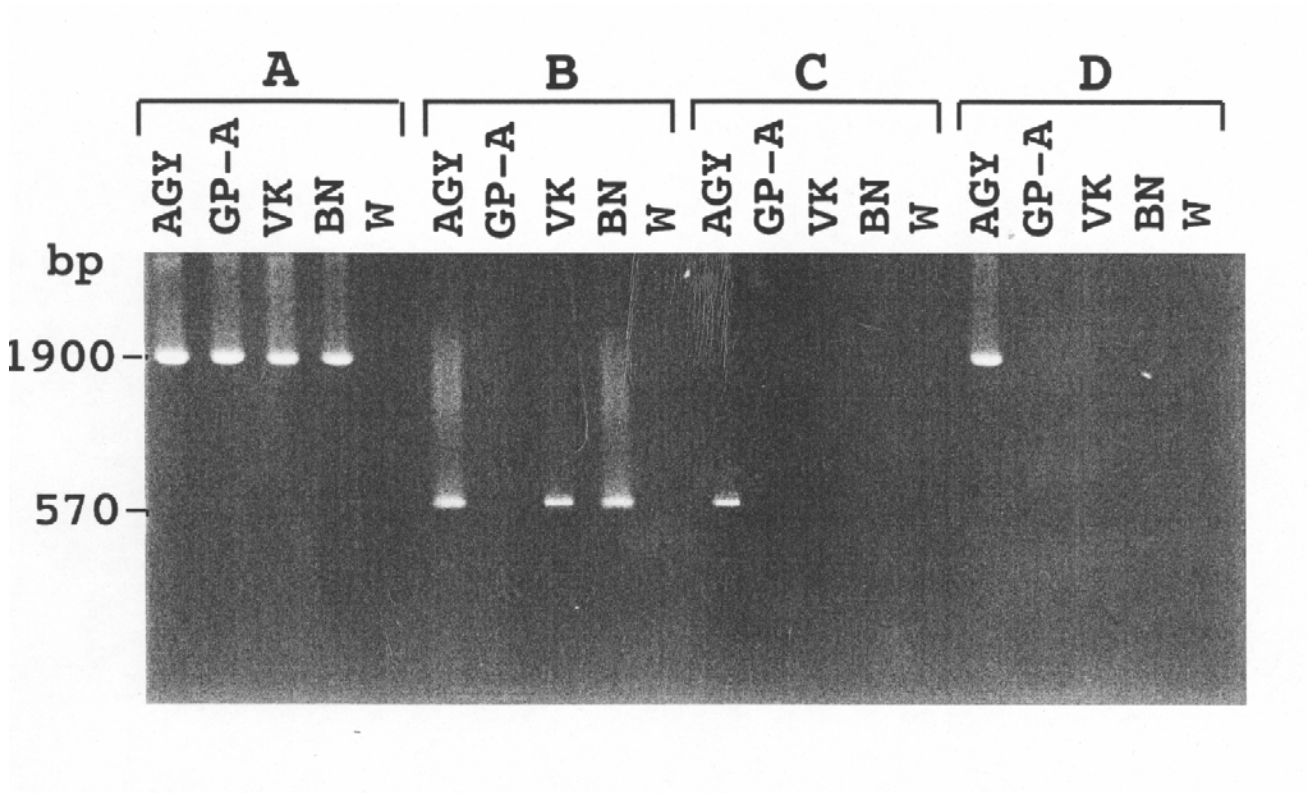


Figure 3
Chapter 2.1



2.2 Polymerase chain reaction for the detection of phytoplasmas in grapevines

Rod Bonfiglioli

The development of an appropriate, user-friendly and sensitive protocol for the detection of phytoplasma, based on the use of PCR was considered a high priority. A range of PCR techniques were investigated and the method now used is very sensitive for strongly symptomatic material (approximately 85-90%), but not as sensitive for a symptomatic grapevine material. As the full symptom cycle for grapevine phytoplasmas is not known, it is very difficult to tell exactly how sensitive the PCR test is for material that is either non-symptomatic or only mildly symptomatic.
A further difficulty in the use of PCR is that the technique relies upon the use of "universal" primers for the first round of amplification of target DNA. There is a strong suggestion from the results that some of the Universal primers (there are several pairs of these) actually preferentially amplify certain phytoplasmas. Because we occasionally observed the amplification of AGY in preference to TBB in our reactions, we now work with a single step procedure that distinguishes between AGY and TBB without seeming to favour either phytoplasma in the amplification process.

Nested PCR has been described as too sensitive to contamination resulting in false positives. We accept this criticism of the technique and worked within these constraints. Samples were tested in duplicate and appropriate controls were used. One advantage of the nested PCR process is the ability to verify its own results. We find that at times we get bands visible on the gels after the first round of the PCR process that are of the correct size range, but do not amplify during the second round of PCR. Our investigation of these bands has shown them to be bacterial 16S products that can co-amplify with phytoplasma targets. These bacterial products are eliminated in the second round of PCR.

2.3 Optimising PCR detection of AGY

Rob Faggian and Rebecca Mills

INTRODUCTION

Polymerase chain reaction (PCR) detection and characterisation of phytoplasmas using 'generic' primers is a well-established technique, and has resulted in the successful identification of a number of insect-vector species (Maixner et al. 1995; Liefting et al., 1997). Research at IHD has focused on finding the Insect vector and alternate hosts of the phytoplasma responsible for Australian Grapevine Yellows (AGY). In the first three years of the AGY project (CRCV 95/2), a PCR protocol with primer-pairs PI (Deng & Hiruki 1991) / P7 (Kirkpatrick et al. 1994) and nested primers R16F2 (Lee et al. 1993) / M23SR (Padovan et al.1995) (Table 1) was used to detect phytoplasmas in grapevines and insects. This procedure was followed by characterisation using restriction fragment length polymorphism (RFLP) analysis of the amplified products. However, this process was hampered by frequent smearing of PCR products (Fig 3 lanes 7 & 8) and poor reproducibility within and between PCRs.

The existing primer sequences were examined with respect to melting temperature, self-complementarily, primer-primer homology and GC content. Primers were re-designed to improve the efficiency with which they directed the amplification of phytoplasma DNA. The altered primers were based on an extensive sequence alignment containing the rDNA regions of more than fifty organisms, including thirty- six phytoplasmas. The amplified region was not significantly changed, allowing previous restriction patterns to be used for comparison. The new primers were used successfully for six months to continue testing possible insect vectors, alternate hosts and grapevine samples.

However, repeated PCR problems within the testing laboratory, not confined to the AGY protocol, severely disrupted and eventually curtailed the processing of samples. PCR' s continually failed for no apparent reason. Several power failures and multiple batches of contaminated reagents that disrupted the processing of samples for a six- month period accompanied these failures. It was therefore necessary to bulk samples according to species and collection date/site in order to process them before the end of the project. A list of the methods used to circumvent the PCR problems is given.

Table 1. Sequences and T_m's (melting temperatures) of all primer pairs.

Primer	Sequence	T_m	
P1	5′-AAGAGTTTGATCCTGGCTCAGGATT-3′	64.6	]
P7	5′-CGTCCTTCATCGGCTCTT-3′	58.6	
M23SR	5′-TAGTGCCAAGGCATCCACTGTG-3′	65.6	]
R16F2n	5′-ACGACTGCTAAGACTGG-3′	48.1	
P1n	5′-TCCTGGCTCAGGATTAACG-3′	58.5	]
P7n	5′-CGTCCTTCATCGGCTCTTAG-3′	59.8	
IHD4	5′-TAGTGCCAAGGCATCCACTG-3′	62.0	]
IHD5	5′-TGGAAACGACTGCTAAGACTGG-3′	61.6	

MATERIALS AND METHODS

Primer Design

Primer melting temperature (T_M) was calculated as described by Breslauer et al. (1986). Primer self-complimentary (hairpins produced when primers fold upon themselves) and primer-primer homology (dimmers- when two copies of a particular primer bind to each other; and cross-dimers- when a two different primers bind to each other) were examined using the Net Primer package (Premier Biosoft International, 1998). New primers (P1n, P7n, IHD4 and IHD5) were designed with the Primer3 package (Rozen & Skaletsky 1998) targeting conserved regions as identified by multiple sequence alignments. Multiple sequence alignments were performed using the GCG package (Program manual for the Wisconsin Version 8. 1994. Genetics Computer Group, 575 Science Drive, Madison, Wisconsin USA 53711) with 36 different phytoplasma sequences from Genebank, 4 mycoplasmas, a spiroplasma, an Acholeplasma, 5 chloroplast sequences from different plant or algal species, E. Coli, Salmonella, and 2 pseudomomads (Fig 1)

DNA Extractions

Leafhoppers

DNA was extracted from all species of leafhoppers using the method of Wang et al. (1993). Frozen leafhoppers (up to ten, depending on size) were placed in Eppendorf tubes with 300µl 0.5M NaOH and a small quantity of acid washed sand. The insects were ground thoroughly with a tissue homogenisation pestle. The homogenate was centrified at maximum speed in a micro centrifuge for 5minutes and 5 µL of the supernatant was removed and transferred to a fresh Eppendorf tube containing 500 µL TE. This crude DNA preparation was stored at – 20°C until needed.

Planthoppers

DNA was extracted from individual cixiid planthoppers using a modified version of the Goodwin et al. (1994) method. Frozen leafhoppers were placed in an Epperndorf tube with200µl CTAB extraction buffer and a small quantity of acid washed sand. The insect was ground thoroughly with a tissue homogenisation pestle, vortexed briefly and incubated at 65°C for 5minutes. One volume of chloroform/isoamyl alcohol (24:1, v/v) was added, and the mixture shaken vigorously before being centrified at a maximum speed for 5 minutes. The aqueous phase was removed and transferred to a fresh tube where 25µL sodium acetate (3 M, pH 5.2) and 2 volumes of ice-cold absolute ethanol were added. The mixture was incubated at –20C for 20minutes then centrifuged at maximum speed for 20 minutes. The supernatant was discarded and the DNA pellet washed twice with 70% ethanol before vacuum drying. The DNA was resuspended in 50µL TE and stored at –20°C until needed.

Planthoppers were extracted using the method of Goodwin et al. (1994) because higher yield of better quality DNA can be obtained than with the method of Wang et al. (1993). A cixiid plant hopper has been identified as the vector of Phorium yellows disease in New Zealand. It is caused by a phytoplasma that is believed to be closely related to the one associated with Australian Grapevine Yellows. Cixiid planthoppers were the main suspect in this study, and therefore it was deemed important to obtain good quality DNA to maximise the likelihood of successful PCR amplification. However, the method is quite time-consuming and thus could not be used for all insects.

Plant Tissue

DNA was extracted from plant tissue using a modified version of the Dellaporta et al. (1983) method. Vascular tissue from symptomatic, non-necrotic leaf material (up to 2 grams) was excised with a razor blade or scalpel. The tissue was placed in a mortar containing 7 mL cold phytoplasma grinding buffer and left to plasmolyse for 10-20 minutes. It was then ground thoroughly with a pestle and 6 mL of fresh buffer added before grinding again. The homogenate was transferred to a cold oak-ridge tube and centrifuged for 5 minutes at 3000 rpm in an SS34 rotor at 4°c. The supernatant was poured into a fresh oak-ridge be and centrifuged again at l3000 rpm for 25 minutes at 4DC. The supernatant was discarded and the tube drained briefly on a paper towel. The pellet was resuspended in 1 mL of CTAB buffer (pre-warmed to 60°C), transferred to an Eppendorf tube and incubated at 60°C for 30 minutes with gentle mixing. One mL of chloroform/isoamyl alcohol was added with gentle mixing, and the solution was centrifuged in a micro centrifuge for 5 minutes at max speed. The aqueous phase was transferred to a fresh Eppendorf tube and an equal volume of ice- cold isopropanol was added. The solution was mixed gently by inversion, incubated at -20°C for 20 minutes and centrifuged for 5 minutes at max speed. The supernatant was discarded, and the pellet washed twice with 70% ethanol before drying under vacuum. The pellet (DNA) was then resuspend in 50-100 µL sterile DNase free water or TE.

PCR Reaction Conditions

PCR reactions were carried out in a 50 µL volume with the following components: 1mM dNTP's (Promega), 0.65 U Taq DNA Polymerase (Bresatec, Adelaide), 1.5 mM MgCb, 1 x Buffer, 50 ng of forward and reverse primer (synthesized at Micromon, Monash University or Geneworks, Adelaide). Thermal cycling was performed in either a Hybaid PCR Express Thermal Cycler, a Corbett Research FTS-960 Thermal Sequencer or a Perkin Elmer Gene Amp PCR System 9600. The following thermal cycling regime was used: 35 cycles of 94 DC for 45 seconds, 56 DC for 45 seconds and 72 DC for 90 seconds for first round PCR's and 30 cycles of 94 DC for 30 seconds, 60DC for 30 seconds and 72 DC for 30 seconds for nested PCR' s.

RESULTS AND DISCUSSION

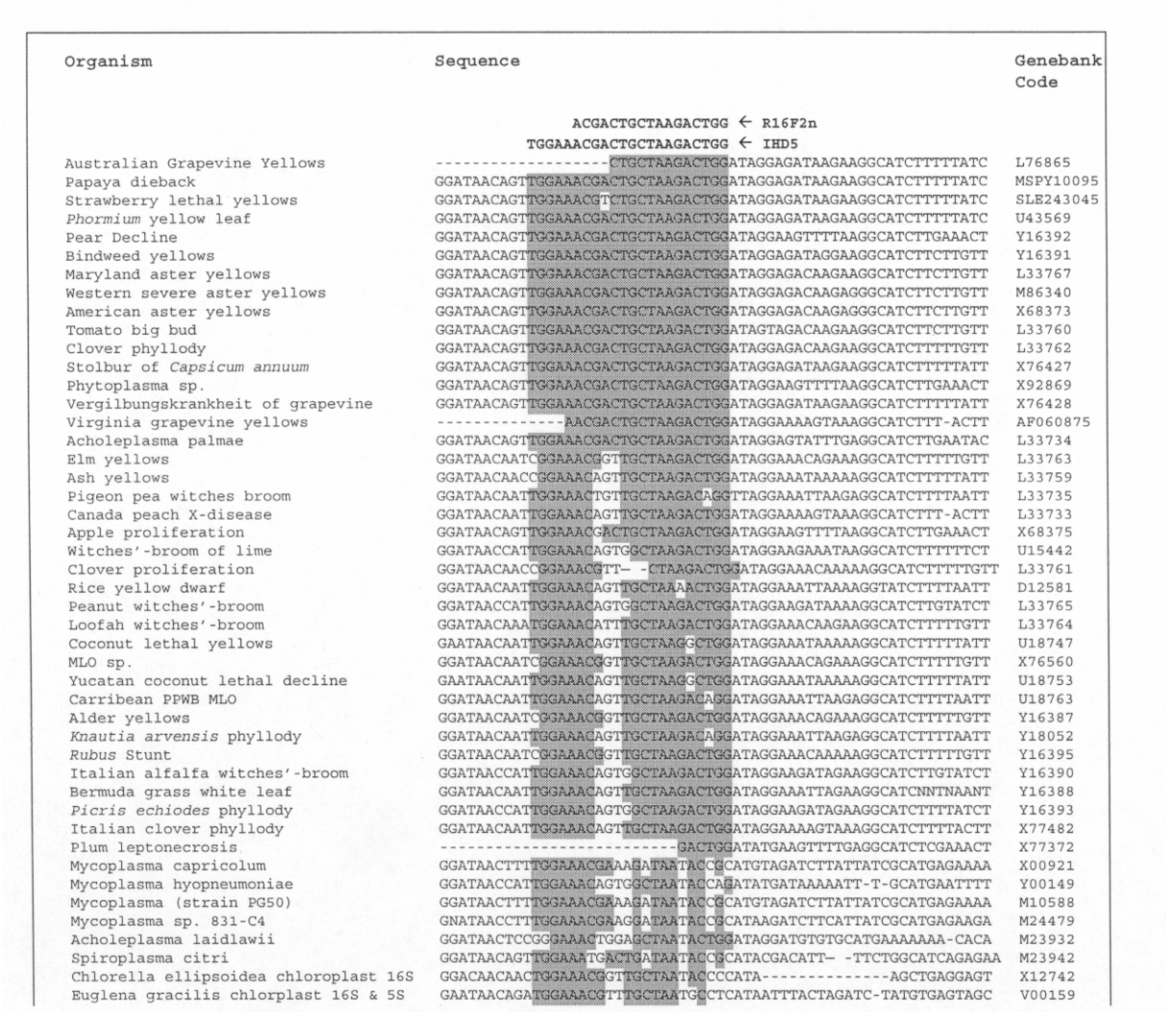
Primer Design

The existing primer pairs had large differences in Tm (Table 1), particularly the nested pair M23SR/R16F2n. Also, P1(Figure 2) and M23SR formed hairpin structures (significant self-complementarity), P1 and M23SR formed self-dimers, and primer- pairs P1/P7 and M23SR/R16F2n formed cross-dimer structures.

The alignment of Genebank phytoplasma sequences revealed that not all of the sequences extended the length of the target gene. Therefore, in some cases fewer sequences were used to design new primers. Also, it was necessary to assume that the conserved nature of the ribosomal RNA gene resulted in 100% sequence homology at the primer positions. For instance, when re-designing R16F2n (see Fig. 1), that particular region of the Australian grapevine yellows phytoplasma DNA sequence did not exist. However, based on the high sequence similarity between the AGY phytoplasma and related phytoplasmas (Papaya dieback, Strawberry lethal yellows and *Phormium* yellow leaf phytoplasmas) it is highly likely that the sequences are identical in this region of the rRNA gene. In fact, the original primer, R16F2n, also extended beyond the known sequence. Additionally, it is not vital that the homology between the 3' end of the primer and its target sequence be 100%, as opposed to the 5' end. Conserved regions were chosen in order to detect as many different phytoplasmas as possible, with the intention of performing RFLP analyses on the amplified product to identify the phytoplasma.

Using the newly designed primer-pairs, which did not form hairpins, dimers or cross dimers, smearing was largely eliminated and sensitivity increased with 100% reproducibility within and between PCRs (Fig. 3).

The combined effects of melting temperature differences, hairpin, dimer and cross- dimer formation are likely to be the cause of the smearing and inconsistent results obtained with P1/P7 and M23SR/R16F2. All these factors result in one or more primers' dropping out' of the reaction, creating concentration imbalances, which decrease the efficiency of PCR.



Organism	Sequence	Genebank Code
	ACGACTGCTAAGACTGG ← R16F2n	
	TGGAAACGACTGCTAAGACTGG ← IHD5	
Australian Grapevine Yellows	----------CTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTTTTATC	L76865
Papaya dieback	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTTTTATC	MSPY10095
Strawberry lethal yellows	GGATAACAGTTGGAAACGTCTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTTTTATC	SLE243045
Phormium yellow leaf	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTTTTATC	U43569
Pear Decline	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAAGTTTTAAGGCATCTTGAAACT	Y16392
Bindweed yellows	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGATAGGAAGGCATCTTCTTGTT	Y16391
Maryland aster yellows	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGACAAGAAGGCATCTTCTTGTT	L33767
Western severe aster yellows	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGACAAGAGGGCATCTTCTTGTT	M86340
American aster yellows	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGACAAGAGGGCATCTTCTTGTT	X68373
Tomato big bud	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGTAGACAAGAAGGCATCTTCTTGTT	L33760
Clover phyllody	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGACAAGAAGGCATCTTTTTGTT	L33762
Stolbur of *Capsicum annuum*	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTTTTATT	X76427
Phytoplasma sp.	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAAGTTTTAAGGCATCTTGAAACT	X92869
Vergilbungskrankheit of grapevine	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTTTTATT	X76428
Virginia grapevine yellows	----------AACGACTGCTAAGACTGGATAGGAAAAGTAAAGGCATCTTT-ACTT	AF060875
Acholeplasma palmae	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGTATTTGAGGCATCTTGAATAC	L33734
Elm yellows	GGATAACAATCGGAAACGGTTGCTAAGACTGGATAGGAAACAGAAAGGCATCTTTTTGTT	L33763
Ash yellows	GGATAACAACCGGAAACAGTTGCTAAGACTGGATAGGAAATAAAAAGGCATCTTTTTATT	L33759
Pigeon pea witches broom	GGATAACAATTGGAAACTGTTGCTAAGACAGGTTAGGAAATTAAGAGGCATCTTTTAATT	L33735
Canada peach X-disease	GGATAACAATTGGAAACAGTTGCTAAGACTGGATAGGAAAAGTAAAGGCATCTTT-ACTT	L33733
Apple proliferation	GGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAAGTTTTAAGGCATCTTGAAACT	X68375
Witches'-broom of lime	GGATAACCATTGGAAACAGTGGCTAAGACTGGATAGGAAGAAATAAGGCATCTTTTTTCT	U15442
Clover proliferation	GGATAACAACCGGAAACGTT- -CTAAGACTGGATAGGAAACAAAAAGGCATCTTTTTGTT	L33761
Rice yellow dwarf	GGATAACAATTGGAAACAGTTGCTAAACTGGATAGGAAATTAAAAGGTATCTTTTAATT	D12581
Peanut witches'-broom	GGATAACCATTGGAAACAGTGGCTAAGACTGGATAGGAAGATAAAAGGCATCTTGTATCT	L33765
Loofah witches'-broom	GGATAACAAATGGAAACATTTGCTAAGACTGGATAGGAAACAAGAAGGCATCTTTTTGTT	L33764
Coconut lethal yellows	GAATAACAATTGGAAACAGTTGCTAAGGCTGGATAGGAAATAAAAAGGCATCTTTTTATT	U18747
MLO sp.	GGATAACAATCGGAAACGGTTGCTAAGACTGGATAGGAAACAGAAAGGCATCTTTTTGTT	X76560
Yucatan coconut lethal decline	GAATAACAATTGGAAACAGTTGCTAAGGCTGGATAGGAAATAAAAAGGCATCTTTTTATT	U18753
Carribean PPWB MLO	GGATAACAATTGGAAACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATT	U18763
Alder yellows	GGATAACAATCGGAAACGGTTGCTAAGACTGGATAGGAAACAGAAAGGCATCTTTTTGTT	Y16387
Knautia arvensis phyllody	GGATAACAATTGGAAACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATT	Y18052
Rubus Stunt	GGATAACAATCGGAAACGGTTGCTAAGACTGGATAGGAAACAAAAAGGCATCTTTTTGTT	Y16395
Italian alfalfa witches'-broom	GGATAACCATTGGAAACAGTGGCTAAGACTGGATAGGAAGATAGAAGGCATCTTGTATCT	Y16390
Bermuda grass white leaf	GGATAACAATTGGAAACAGTTGCTAAGACTGGATAGGAAATTAGAAGGCATCNNTNAANT	Y16388
Picris echiodes phyllody	GGATAACCATTGGAAACAGTGGCTAAGACTGGATAGGAAGATAGAAGGCATCTTTTATCT	Y16393
Italian clover phyllody	GGATAACAATTGGAAACAGTTGCTAAGACTGGATAGGAAAAGTAAAGGCATCTTTTACTT	X77482
Plum leptonecrosis	----------GACTGGATATGAAGTTTTGAGGCATCTCGAAACT	X77372
Mycoplasma capricolum	GGATAACTTTTGGAAACGAAAGATAATACCGCATGTAGATCTTATTATCGCATGAGAAAA	X00921
Mycoplasma hyopneumoniae	GGATAACCATTGGAAACAGTGGCTAATACCAGATATGATAAAAATT-T-GCATGAATTTT	Y00149
Mycoplasma (strain PG50)	GGATAACTTTTGGAAACGAAAGATAATACCGCATGTAGATCTTATTATCGCATGAGAAAA	M10588
Mycoplasma sp. 831-C4	GNATAACCTTTGGAAACGAAGGATAATACCGCATAAGATCTTCATTATCGCATGAGAAGA	M24479
Acholeplasma laidlawii	GGATAACTCCGGGAAACTGGAGCTAATACTGGATAGGATGTGTGCATGAAAAAAA-CACA	M23932
Spiroplasma citri	GGATAACAGTTGGAAATGACTGATAATACCGCATACGACATT- -TTCTGGCATCAGAGAA	M23942
Chlorella ellipsoidea chloroplast 16S	GGACAACAACTGGAAACGGTTGCTAATACCCCATA----------AGCTGAGGAGT	X12742
Euglena gracilis chlorplast 16S & 5S	GAATAACAGATGGAAACGTTTGCTAATGCCTCATAATTTACTAGATC-TATGTGAGTAGC	V00159

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Tobacco chloroplast 16S                  GAACAACAGCTGGAAACGGCTGCTAATACCCCGTA------------GGCTGAGGAGC   V00165
Escherichia coli (MC4100)                GGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGAC-CAAAGAGGGGGA   X80732
Salmonella typhimurium                   GGATAACTACTGGAAACGGTGGCTAATACCGCATAACGTCGCAAGAC-CAAAGAGGGGGA   Z49264
Azoarcus evansii                         GGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTGAGGGGGAAAGCGGGGGAT   X77679
Chlamydomonas reinhardtii chloroplast    GGATAACATTGGGAAACTGTTGCTAATACCCCATAC------------AGCTGAGGAGT   X03269
16S
```

Fig. 1. Sample of sequence alignment with primer position highlighted.

Secondary structures of sense (or forward) primer:

Hairpins:

1. dG = -5.47 kcal/mol (3' Hairpin)

```
     GGTCCTAGTTTGAGAA-5'
   C ||||||
    TCAGGATT-3'
```

Dimers:

1. dG = -10.67 kcal/mol (3' Dimer)

```
   5'-AAGAGTTTGATCCTGGCTCAGGATT-3'
              ||||||    :::::
         3'-TTAGGACTCGGTCCTAGTTTGAGAA-5'
```

Cross Dimers:

1. dG = -7.71 kcal/mol (3' Cross dimer)

```
   5'-AAGAGTTTGATCCTGGCTCAGGATT-3'  (P1)
      |||||          | |
   3'-TTCTCGGCTACTTCCTGC-5'  (P7)
```

Fig. 2. Sample of output from NetPrimer analysis of P1.

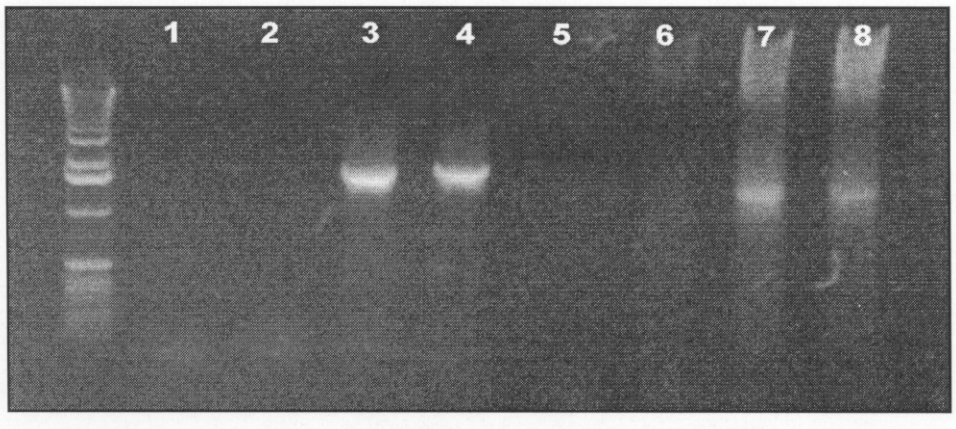


Fig. 3. Tomato Big Bud (TBB) phytoplasma amplified with both sets of primers. Negative control with new primers (Lanes 1 & 2); TBB amplified with new primers

(Lanes 3 & 4); Negative control with original primers (Lanes 5 & 6); TBB amplified with original primers (Lanes 7 & 8).

In the original protocol, annealing temperatures of 55° and 57°C were used for primer- pairs P1/P7 and M23SR/R16F2n respectively. However, in the nested step, R16F2 has a melting temperature lower than the annealing temperature. Therefore, not all of the primer is available for binding to the target DNA and is still denatured in solution. The result is smeared product, due to extension from only one bound primer.

This brief study demonstrates not only the importance of careful primer design, but also highlights the fact that primers are not always compatible. Therefore, they cannot be chosen freely from different sources for use as pairs in PCR.

The new primers, combined with the adjusted thermal cycling programs and new DNA extraction procedures for leafhoppers, produced consistent results from their introduction in March until October 1999. However, in late October, PCR's began to fail and continued to do so for six months despite various attempts to solve the problem. The areas most likely to result in PCR failures were identified (see below), and the appropriate steps taken to eliminate the problem:

1. **PCR Reagents.** All PCR reagents were replaced (dNTP's, MgCI2, 10 x Buffer, water and Taq) -in case the reagents had perished or been contaminated by a PCR inhibiting substance.

2. **DNA.** All extraction buffers were replaced and fresh extractions were performed to obtain positive control DNA -in case the DNA had perished or the buffers/extraction process was not efficiently extracting DNA from plant tissue and leafhoppers.

3. **Primers.** Primer stocks were replaced with fresh stocks, and a different supplier was used (Monash -Bresatec) -in case the primer stocks had perished or a manufacturing contaminant was inhibiting the PCR.

4. **Thermal Cycler.** PCR's were attempted in 4 different thermal cyclers at different stages of testing -in case one machine was faulty.

5. **Personnel.** Different people were recruited to set up the AGY PCR -in case the operator was inadvertently introducing errors into the process.

6. And finally, a number of molecular biology/PCR experts were consulted for their views.

Efforts to resolve the problem were frustrated by the fact that the PCR would occasionally work, giving the impression that a solution had been found, then inexplicably fail again several days later. Other protocols in the testing laboratory experienced short-term failures during the same six-month period, and another was severely disrupted for several months by contamination from an unknown source.

An example of how fickle the protocol became, and the illogical steps resorted to in order to find a solution follows. It is commonplace to create a 'master-mix' when setting up a PCR consisting of many tubes. A master-mix is a mixture containing the reagents necessary for all the individual PCR reactions in a given experiment, and is subsequently aliquoted out into individual PCR reaction tubes. It was found that by excluding the primers from the master-mix and adding them to each PCR tube individually (thus increasing both the handling time and number of manipulations per tube), that the PCR reactions worked. There is no theoretical explanation why this could result in improved reaction efficiency. PCR' s continued to work for about three weeks when set up in this fashion, until again failing for no apparent reason. In early May of 2000 the PCR' s once again began working

None of the listed points can be totally discounted, as they may have played some part in the PCR failures at various stages, as did power failures. However, a more likely cause is an airborne inhibitor. St. Pierre et al. (1994) experienced complete PCR failure during a three-month period in two consecutive years. Replacing all reagents and enzymes had no effect, and the problem was made more confounding when the PCR occasionally functioned for several days then abruptly failed again. Pollen was proposed as the inhibiting agent following high counts during the period of PCR failure. Experiments, including scanning electron microscopy, demonstrated that as little as 10 pollen grains per tube could result in a failed reaction. It was suggested that PCR assays be set up under completely sterile conditions to avoid false-negative results.

PCR failures have also resulted from glove powder (de Lomas et al. 1992). The use of powder free gloves or washing powdered gloves prior to use markedly reduced the frequency of unexplained false-negative results. In our experiments these measures did not prevent the AGY PCR from failing.

It is therefore suggested should the problem arise again, that the first action to be taken is to set up the entire PCR in a laminar flow or other sterile area. This will exclude any airborne particles that may float into open PCR tubes and inhibit the reaction.

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2.4 The development of diagnostic tools for the detection of phytoplasmas in Australian Grapevines: assessment of the protocol for enzyme linked immunosorbent assay (ELISA)

Fiona Constable and Rod Bonfiglioli

INTRODUCTION

The enzyme linked immunosorbent assay is an antibody-based method used to detect various pathogens in plants and animals. Most often antibodies are produced that have a specific affinity to proteins on the surrounding membrane of a pathogen. ELISA has been successfully used to detect FD in infected grapevines (Caudwell et al. 1990). In order to develop ELISA for AGY phytoplasma, the organism was to be transferred into periwinkle to raise the titre and provide an easy host from which it could be purified. Transmission of the AGY phytoplasma was achieved for three plants, but they died before ELISA development began.

ELISA has also been developed to detect the Australasian Tomato Big Bud (TBB) phytoplasma in various host plants (Minucci et al. 1996, Crossley and Clarke, 1996). A TBB type phytoplasma has been detected in Australian grapevines using molecular methods and was found to be indistinguishable from the Australasian TBB phytoplasma using RFLP analysis of the 16S and 23S rRNA genes and the intervening 168-23S spacer region (Gibb et al. 1996, Constable et al. this report). On the basis of this result we assessed a commercially available TBB ELISA to determine how valuable the ELISA test might be for detecting phytoplasmas in known infected grapevines.

MATERIALS AND METHODS

Source of phytoplasma

A TBB phytoplasma isolate, used as a positive control, was maintained in Periwinkle *(Caranthus rose us)* in a glasshouse with normal day length at 18-23°C.

Phytoplasma infected grapevine material was collected from the field in early February when the chance of detecting phytoplasmas is at its peak. The grapevines were assessed for phytoplasmas infection using a nested PCR test (Gibb et al. this report) and the type of phytoplasma present was determined by restriction fragment length polymorphism (RFLP) analysis using the restriction enzyme *Hpa* II (New England Biolabs,) according to the manufacturer's instructions.

Extraction of grapevine tissue for ELISA

Two methods for extracting plant material were assessed. F or both methods approximately two grams of grapevine material per sample, including veins, petioles and phloem scrapes from the main shoot, were ground in 10 ml of extraction buffer. To obtain phloem scrapes, bark was peeled from infected shoots and the vascular tissue was removed from the bark by scraping with a sterile scalpel blade. Only petioles and main veins from infected periwinkle were used.

The extraction buffer for Method one was phosphate buffered saline (0.14M NaCI, O.OIM Na2PO4-2H20, 0.0015M KH2PO4, 0.0027M KCI, pH 7.4) containing 0.05% v/v Tween 20 and 2%w/v Polyvinylpyrrolidone (MW 44,000). (PB8- Tween 20 - PVP). Each sample was ground with 10 ml PBS- Tween20-PVP in a mortar and pestle.

For Method two the extraction buffer was 0.2M Tris-HCI, 0.8% NaC1, 0.05% v/v Tween 2, and 2%w/v Polyvinylpyrrolidone (MW 44,000), pH 7.4. Each sample was placed in a separate plastic bag with 10 ml of buffer. The bags were sealed and each sample was macerated using a hammer.

Extracted samples, including healthy grapevine, periwinkle and TBB phytoplasma infected grapevine samples, were serially diluted with the appropriate extraction buffer to 1/40 (i.e. 1/10, 1/20, 1/40).

ELISA

The TBB ELISA (AGDEN Diagnostic Systems, Auchincruive, Scotland, UK) was carried out according to the manufacturer's instructions. As well as the samples, an extraction buffer control was loaded.

The results were viewed visually and read by a Titertek® ELISA plate reader at 405nm.

RESULTS

Using extraction Method. One, TBB phytoplasma could be detected in infected periwinkle but only at the initial 1/5 dilution TBB phytoplasma could not be detected in infected grapevines using this extraction method. Using Method Two TBB was not detected in any sample.

DISCUSSION

We had concerns about the sensitivity of ELISA to detect phytoplasma in grapevines.
This was supported when ELISA was unable to detect the presence of TBB phytoplasma in infected grapevine samples. It is likely that phytoplasmas do not exist in grapevines at high enough titers to be detected by ELISA.

A universal PCR test to detect phytoplasmas in grapevines has been developed and adopted as an alternative to an ELISA. TBB phytoplasmas can be detected in grapevines in the first round and nested amplifications. PCR tests for phytoplasma, have been adopted for commercial diagnosis by Crop Health Services, IHD, Victoria and Waite Diagnostics, University of Adelaide, South Australia.

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Chapter 3 The extent of AGY symptom distribution in Australian vineyards



3.1 AGY vineyard survey data
Peter Magarey, Malcolm Wachtel, Bob Emmett and Scott Gillett

3.2 Incidence and spread of AGY - analysis of mapping data from 1995 to 1998
F.E. Constable, K.S. Gibb, J.R. Moran and Y.M. Wilson.

3.3 Variety Survey
Rod Bonfiglioli

Chapter 3 The extent of AGY distribution in Australian vineyards

3.1 Overview of the AGY vineyard survey data set 1976-1985: Showing AGY levels in different vineyards and regions for up to 10 years

Peter Magarey, Malocolm Watchel, Bob Emmett and Scott Gillett

INTRODUCTION

In order to determine how AGY phytoplasma spreads in Australian vineyards and the speed of that spread, we surveyed the incidence and spatial arrangement of AGY, a new disease of unknown aetiology. The surveys began when AGY was first found in 1976/77. Individual vines were monitored for presence or absence of the disease symptoms (usually on each of the four arms of each vine in all years of survey). The majority of the data was collected from Riverland vineyards, but others in SA and Sunraysia, Vic, and Griffith, NSW were surveyed for fewer years.

In addition, a number of experiments were also undertaken in attempt to diagnose the disease. For example, the antibiotic Terramycin® (oxytetraclyine hydrochloride) was pressure-injected into vines and the subsequent levels of AGY were assessed by vineyard surveys. The vineyards and years for which data have been collected in early years, are listed in Table 1.

Table 1. List of vineyards surveyed for symptoms of AGY infection

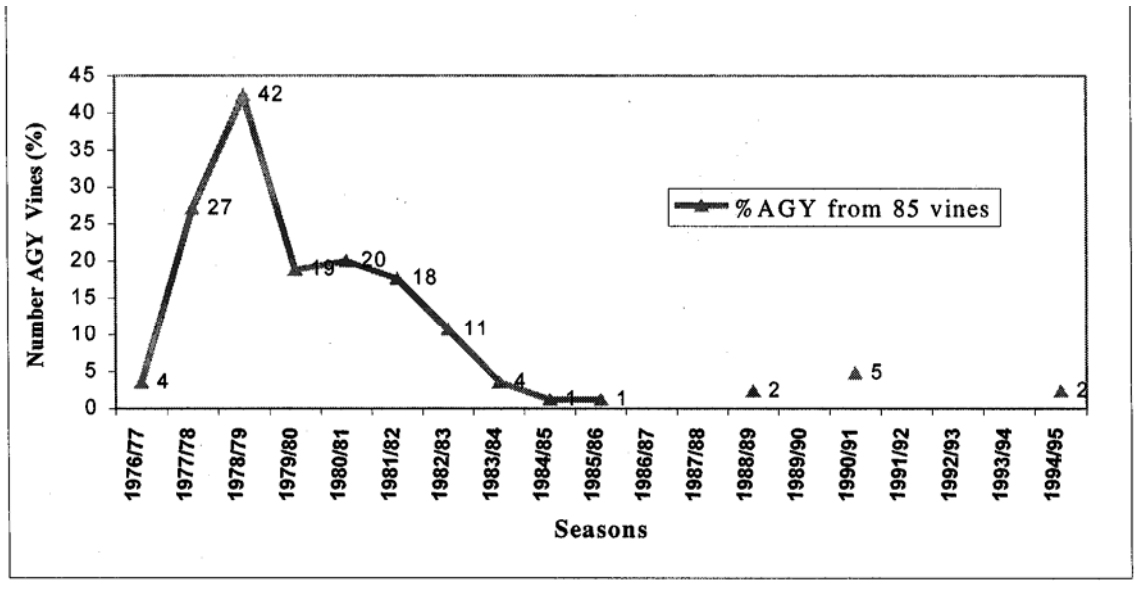
Name	Location /vineyard	Seasons	Data (rows x vines)	Vines
Riverland, SA				
cv. Riesling				
1. Block F	LRC, Loxton	1976/77 to 81/82, 85/86	2 x 44	84
2. Block B	LRC, Loxton	1977/78 to 81/82, 85/86	8 x 20	160
3. Halupka	Berri	1977/78 to 86/87	10 x 40	400
4. Klobucar Nth	Renmark	1977/78 to 86/87	6 x 56	336
5. Klobucar Sth	Renmark	1977/78 to 86/87	7 x 68	476
6. Perry	Renmark	1977/78 to 84/85	7 x 70	490
7. Ludas Nth	Renmark	1980/81 to 85/86	21 x 100	2100
8. Ludas Sth	Renmark	1981/82 to 82/83	16 x 70	1120
9. Schutz 1	Pyap	1979/80 to 82/83	9 x 130	1170
10. Schutz 2	Pyap	1980/81 to 82/83	8 x 102	816
11. Proud G	Loxton North	1979/80 to 1980/81	13 x 32	410
12. Proud F	Loxton North	1979/80 to 1980/81	6 x 50	269
13. Graetz	Loxton	1979/80 to 1980/81	33 x 32	1056
14. Fenwick	Berri	1997/98	20 x 64	1253
15. George	Loxton East	1980/81	39 x 114	4432

cv. chardonnay				
1. Schwarz North	Bookpurong	~1996/97 to 1997/98	10x90	900
2. Schwarz South	Bookpurong	~1996/97 to 1997/98	10x87	870
3. Broomwell	Paringa	~1981/82, 83/84,84/85	6x33	198
Victoria				
cv. Chardonnay				
1. Lindemann-North	Karadoc	1979/80 to 85/86	various	~500
2. Lindermann-South	Karadoc	1979/80 to 85/86	Various	~500
3. Caristo	Irymple	1981/82	6x52	271
New South Wales				
Cv. Chardonnay				
1. NSW Ag.	Griffith	1979/80 to 82/83	5x37	185
cv. Riesling				
2. NSW Ag.	Griffith	1979/80 to 82/83	4x37	144

AGY symptom mapping data from 1976 and 1984 were entered into computer files. Some of these data and later data were analysed by Patchy for tests of apatial aggregation on vineyards.
In 1995 and 1996, vineyards of cv Chardonnay in Buckland valley, Vic, were assessed for AGY symptoms.
The incidence and distribution of AGY symptoms were surveyed in one Victorian vineyard and in three South Australian vineyards from 1997 to 2000.

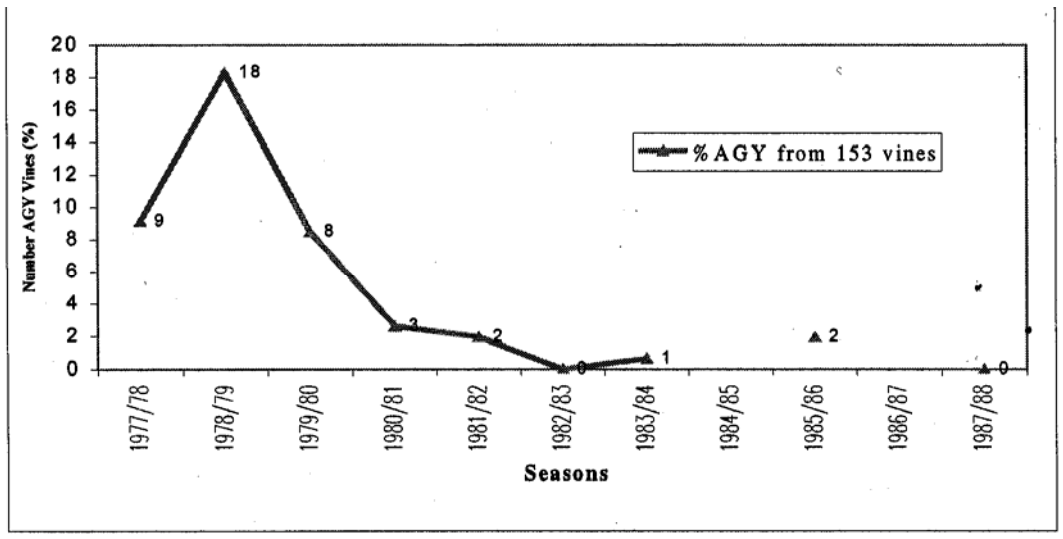
RESULTS
Representative samples of our data are presented

Figure 1. Season variation in incidence of AGY symptoms in cv Riesling at Block F, Loxton from 1976-1995.



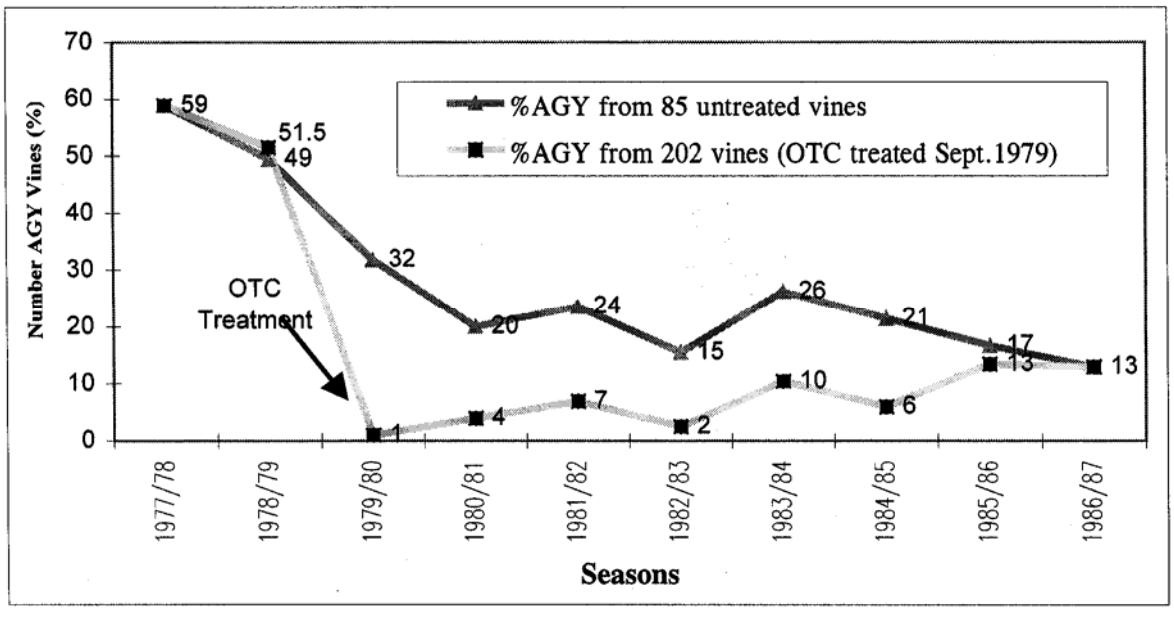
The changes in the incidence of AGY Block F, Loxton (Figure 1) are typical of the trends that occurred in each of the monitored Riverland vineyards. The incidence of AGY symptoms increased dramatically from 1976/77 to 1978/79; levels declined thereafter to minor incidences (for the years of our observation). In recent years the level of AGY has been much lower.

Figure 2. Seasonal variation in incidence of AGY symptoms in cv Riesling at Block B, Loxton 1978/1988 (note differences in scale of Y axis)



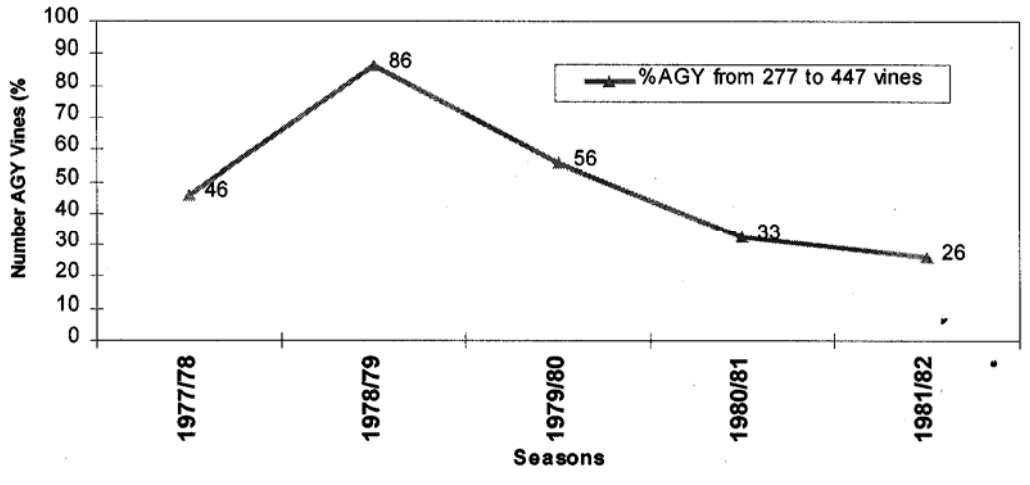
The incidence of AGY affected vines in Block B, Loxton (figure 2) increased and decreased at similar times as the incidence at other Riverland vineyards. The level of AGY symptoms was considerably lower in another block only 500m from Block B.

Figure 3. Seasonal variation in incidence of AGY symptoms in *cv Riesling* in Halpuka Renmark



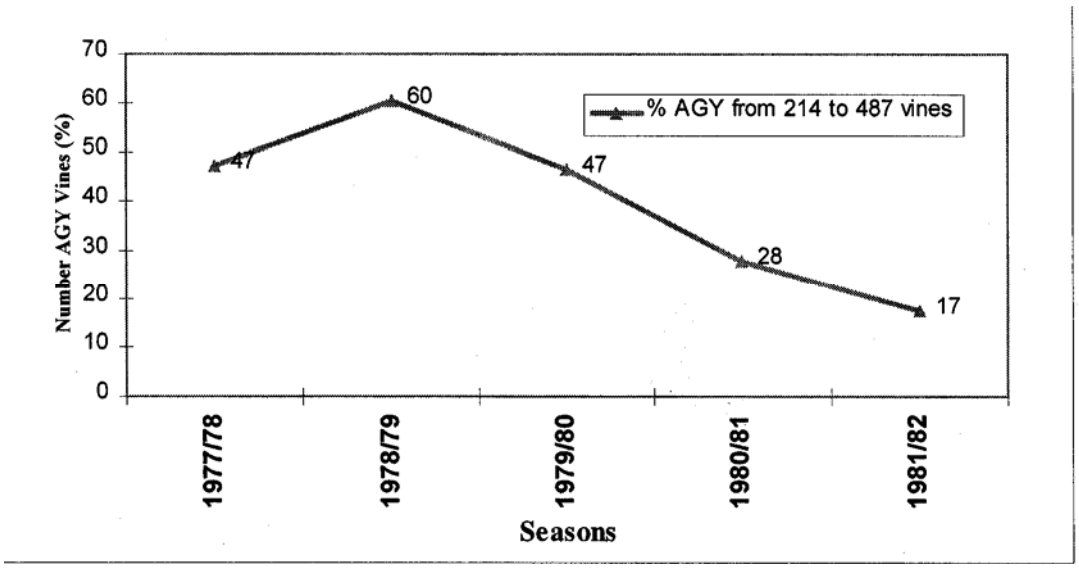
Vines that were treated once with antibiotic OTC in September 1979, showed a remarkable recovery. The control was 99% effective and lasted six years. Antibiotic reduction in disease incidence implicates a phytoplasma as the cause of AGY.

Figure 4. Season variation in incidence of AGY symptoms at Klobucar from1978-1982



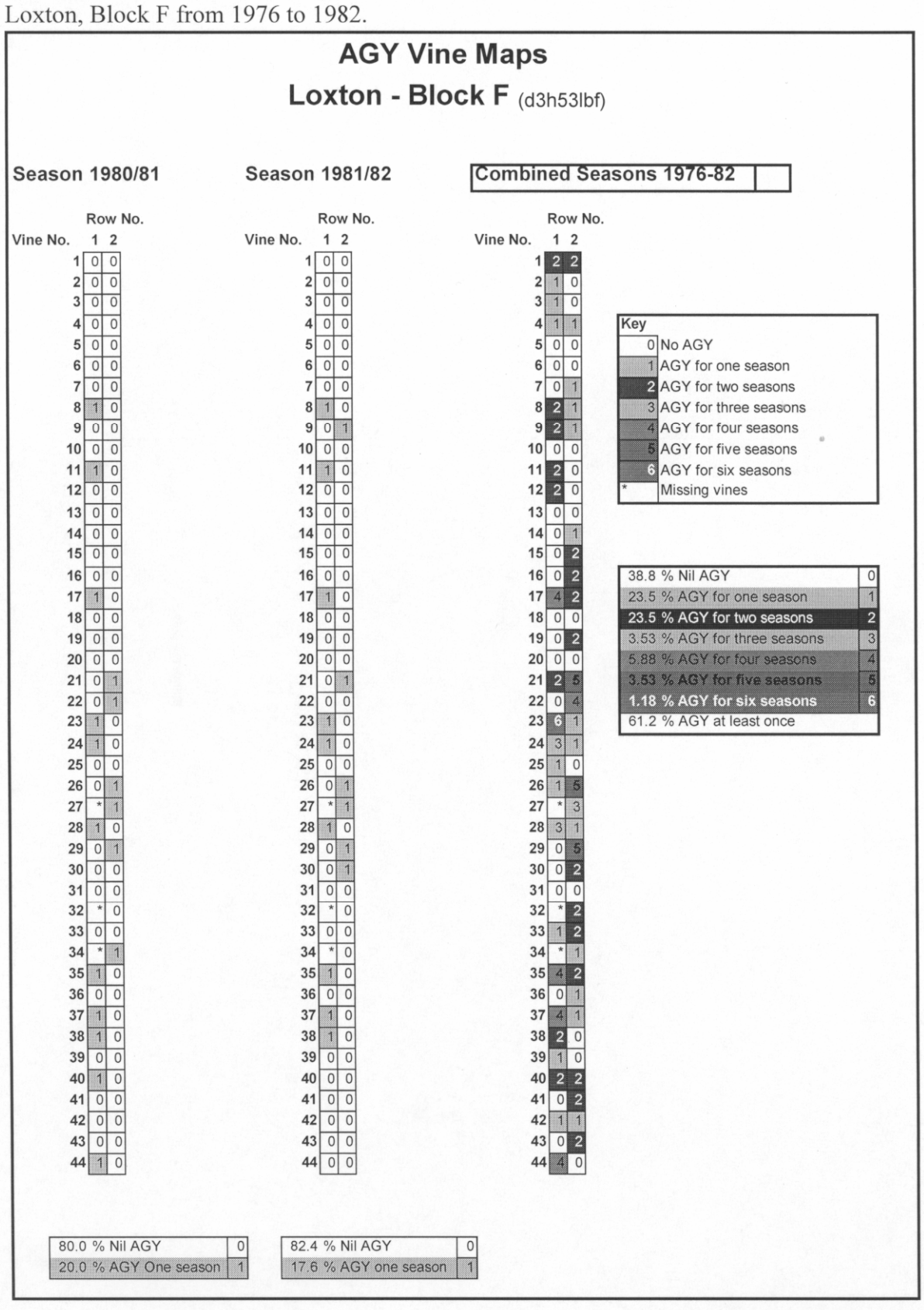
The incidence of AGY symptoms at Klobucar (figure 4) increased and decreased at similar times as the incidence at other Riverland vineyards.

Figure 5. Seasonal variation in incidence of AGY symptoms at Perry, Renmark from 1978-1982



The incidence of AGY symptoms at Perry (figure 5) increased and decreased at similar times as the incidence at other Riverland vineyards.

Figure 6. Maps of symptomless vines and vines with symptoms of AGY infection in Loxton, Block F from 1976 to 1982.



AGY symptoms are expressed on different vines in different years (Figure 6). Often only 3-5 % of vines were diseased for more than half the years we surveyed. Only one vine was diseased for all years. The disease is clustered in random groups.

Table 2. The incidence of AGY symptoms in vineyards of Riverland / Sunraysia.

Cultivar	Vineyard	1997/98	1998/99	1999/00
Riesling	Fenwick	14%	11%	49%
Riesling	Loxton B	<1%	<1%	<1%
Chardonnay	Gol Gol West	-	49%	32%
Chardonnay	Loxton 516	<1%	<1%	<1%

DISCUSSION

Symptoms of AGY are present in most viticultural areas of Australia but their incidence is highest in warmer inland regions. The incidence and severity of AGY differs from season to season. Two periods of (fairly rapid) increase in incidence have been followed by more gradual reductions. Levels of AGY in both Riverland and Sunraysia have appeared to rise and fall uniformly for most vineyards in each district. However in the late 1970's and early 1980's, AGY levels in the Riverland increased 2-3 years prior to a similar increases in Sunraysia, while in 1998/99 to 1999/00 the increase appeared earlier in Sunraysia. Some vineyards regularly have relatively high incidence of AGY *(eg* at Gol Gol and Fenwick at Berri); some vineyards regularly have low levels of AGY *(eg* Block B, LRC). This variation in incidence of symptoms may suggest the importance of adjacent vegetation as a reservoir of the AGY phytoplasma.

The increase in the incidence of AGY symptoms from 1976/77 (Figures 1 and 2) may be due to a change in the seasons from the very wet years of the mid-1970's. Changes in climate may have led to a build up of an insect vector. For instance, in the dry years inoculative insects may have moved into the irrigated and relatively greener vineyards to feed. The lack of AGY in the 1980's and '90's may have been due to a lack of insect vector activity, suggesting that an insect vector may be only an occasional feeder of grapevine. Spread patterns In AGY symptoms, over time and space, In the years prior to 1976/77 might provide clues as to the location and or nature of an insect vector.

The effectiveness of the antibiotic treatment in the Halupka vineyard may have been due to a reduction in phytoplasma levels (titre) within the vine. The phytoplasma levels may have remained low because there was no insect vector activity, at least until 1885/86 when an increase in incidence of symptoms was observed. The increase in incidence of symptoms was only observed in OTC treated vines. This increase may be because titre levels were reduced below expression threshold but not eliminated in treated vines. If that were the case then the titre may have taken a few years to increase to above the symptom threshold level. Antibiotic treatment could possibly used as a long-term control of AGY phytoplasma in foundation vine plantings.

In 1996 there was an abnormally high level of AGY (~7% vines affected) in a *cv* Chardonnay vineyard in the Buckland Valley. In comparatively cool regions elsewhere in Australia there are generally low levels of AGY *(eg* less than 1 % of vines). Samples of AGY affected vines were subsequently PCR tested by Fiona Constable, (Waite University) and found to contain a variant of the AGY phytoplasma. In determining the taxonomy of this pathogen and in tracing its relative origin, an understanding of the epidemiology of AGY may be gained.

Initial analyses of the spatial distribution of AGY affected vines (as undertaken by Michael Maixner using Patchy TM) appear to indicate the absence of radial patterns of AGY spread in a vineyard and a lack of significant edge effects. It is possible that mild edge effects are present in one of 8 vineyards tested to date. More frequently, AGY appears to occur in random clusters, consistent with the spread pattern associated with long-distance airborne movement of an inoculum source. This is the type of pattern to be expected from an insect vector that is originating some distance from the vineyard. To test these hypotheses, more detailed analyses are needed.

Using data from the maps (figures 6 and 7) levels of disease in the vineyard for each year have been calculated. The variation in incidence of AGY in individual vines and individual arms, over time and space show progress and patterns of spread that might provide clues as to the nature and source of an insect vector. The patterns may give further indication as to the (lack of) spread of disease through propagation.

The samples of the available data indicate some of the potential benefits from further analyses. The analysis of mapping data could provide the best understanding of the source of inoculum of AGY in vineyards and provide the best clues as to where to look for a possible insect vector. In addition, analysis could help to determine the role of spread of disease by cuttings at planting and provide information toward the development of a commercial control of AGY.

Acknowledgment

- The present collation of the data was supported in part by the Riverland Vine Improvement Committee (RVIC), and the CRC(V) National Task force on AGY.
  We are grateful for the assistance.

- Thanks to Malcolm Wachtel, Michael Maixner, Scott Gillett, Michelle Woidt, John Whiting, Jane Moran, and Rod Bonfiglioli

3.2 Incidence and spread of AGY -analysis of mapping data from 1995 to 1998.

Fiona Constable, Karen Gibb, Jane Moran and Yasmin Wilson.

INTRODUCTION

Australian Grapevine Yellows (AGY) was first described by Magarey and Wachtel (1983, 1986a, 1986b) and the disease has since been confirmed throughout Australia with a greater prevalence in the warmer irrigated districts of Sunraysia (Vic), the Riverland (SA) and the Murrumbidgee Irrigation Area (NSW). Research has identified a phytoplasma associated with symptoms of AGY (Bonfiglioliet al. 1995, Padovan et al. 1995, Gibb et al. this report). Studies have shown that the AGY phytoplasma is genetically distinct from other grapevine yellows phytoplasma found in Europe (Padovan et al 1995, Padovan et al. 1996).

Symptoms of AGY typically include:

- Irregular yellowing and backward curling of the leaves, which drop from the vine early.
- Possible necrosis of yellow leaf tissue.
- Overlapping of leaves on an affected shoot.
- Possible death of tips of affected shoots.
- Affected shoots fail to harden off and remain rubbery.
- Flowers on affected shoots may abort or berries may shrivel.

Usually only a few shoots on a vine are affected. The disease is most often noted in the varieties Chardonnay and Riesling, although it has been observed on other white and red varieties.

Chardonnay has become a popular variety in recent years. In 1994/95 the total area of Chardonnay in Australia was 9846 hectares (ha) -only sultana was planted over a larger area (15,412 ha) (Winemakers' Federation of Australia Incorporated, 1996). Van Hilst et al. (1995) projected the 1994/95 area would be 7290 ha, smaller than that actually planted, and would increase by 49% to 10880 ha in 1997/98. Although the actual planting figures for Chardonnay were not available at the time this report was written it is believed that an increase in the area planted with Chardonnay did occur. Chardonnay is considered to be highly susceptible to phytoplasma infection (Magarey and Wachtel, 1986b) and this cultivar is most often observed with AGY symptoms. With increased numbers of Chardonnay plantings it is likely that more AGY will be seen and we still have no full understanding of the effects of phytoplasma infections on Australian grapevines

Statistical spatial analysis of the incidence of disease, such as AGY, within a planting can help to determine the nature of spread of disease and its causal pathogen. By determining the nature of spread of disease, targeted vector and alternative host studies can be established for the eventual development of management strategies to control spread of the associated pathogen. Even without the knowledge of vectors and alternative hosts some control measures can be given, since certain spatial distributions indicate possible sources of the causal pathogen, for example clustering of disease associated with a pathogen indicates spread within the planting.

We report the incidence of AGY in three vineyards over three seasons. We also analysed the data to observe the spatial distribution of AGY to assist our understanding of the nature of spread of this disease.

MATERIALS AND METHODS

STUDY SITES

Three vineyards were chosen for this study, site 1 located at Gol Gol (NSW), site 2 at Karadoc (Vic) and site 3 at Wemen, (Vic). At each of sites 1 and 2 an area of 18 rows with 50 vines was selected. At the site 3, three areas each often rows with ten vines and four areas of 10 rows by 25 vines at each comer were selected.

The variety Chardonnay was grown at each site. The vineyard was established at site 1 in 1992 and 1993, at Site 2 in 1992 and at site 3 in 1993. A small amount of replanting had occurred at each site where vines had died and the age of those replants was difficult to assess. However, those replants were included if present in 1995 when the study began.

At site 1, vines were grown on a variety of rootstocks, which were Ramsay (rows 1 - 7), KY3251 (rows 8 -10), Schwarzmann (rows 11-12), Ramsay (rows13 -14) and Teliki5C (rows 15 -18). At site 2 all vines were on their own roots except for replanted vines, which were on Ramsay. At site 3 all vines were on Ramsay rootstock.

The soil type at site 1 was a sandy clay loam except around rows eight to ten where the soil was a light to medium clay. At site 2, the soil type was heavy clay. At site 3 the soil type was deep red sand.

DISEASE MAPPING

The study sites were mapped for AGY on a monthly basis from January through April in 1995/96, October through May in 1996/97 and in October, December, late January and April in 1997/98. A vine was recorded with AGY if one or more shoots displayed irregular veinal and interveinal yellowing and downward rolling of leaves, which overlaid one another in a shingled arrangement. (Fig 1.)

FIGURE 1. A Chardonnay vine with shoots displaying symptoms of AGY including irregular veinal and interveinal yellowing and downward rolling of leaves, which overlaid one another in a shingled arrangement.

ANALYSIS OF MAPPING DATA

Mapping data from sites 1 and 2 were analysed using the spatial analysis package, "Patchy" (Maixner, 1993) to study distribution of AGY. The aggregation of symptoms was assessed using fixed grid analysis in which a variance to mean ratio (VIM) and Morisita's index of dispersion (Id = n[S(x^2)-Sx]/[(Sx)2-Sx) were calculated. The fixed grid analysis lays a contiguous grid of subunits over the plot and all subunits, except those exceeding the tolerance level for missing plants, are used for calculation. Both calculations test for significance of aggregation of symptomatic plants or the absence of random distribution.

RESULTS

Incidence of Disease

The incidence of disease is given in Table 1. In the 1996/97 season, incidence of AGY symptoms increased by more than 20% at site 1 and site 3 then declined by 33% at site 1 in 1997/98 with no significant change at site 3. At site 2 incidence of AGY was low, however there was a slight but steady increase in incidence over three seasons.

Table 1. Incidence (%) of AGY in the 1995/96, 1996/97 and 1997/98 seasons at three study sites in Sunraysia

Study site	1995/96	1996/97	1997/98
Site 1	20%	54%	19%
Site 2	2%	4%	6%
Site 3	0.7%	24%	21%

The fixed grid analysis of data from site 1 showed a significant clustering pattern of vines with AGY in 1995/96 and 1997/98 but not for 1996/97 (Table 2.) Some significant clustering was observed at site 2 in 1995/96 and 1996/97 but not in 1997/98. No edge effect was observed in either season (Table 3.).



Figure 1: A Chardonnay vine with shoots displaying symptoms of AGY including irregular veinal and interveinal yellowing and downward rolling of leaves which overlay one another in a 'shingled' appearance.

Table 2. Results from the fixed grid analysis for AGY at site 1 in each season.

		1996					1997					1998				
Row	Column	n	m	v	V/M	I□	n	m	v	V/M	I□	n	m	v	V/M	I□
3	3	54	2.17	5.08	2.347***	1.615***	50	4.82	4.84	1.005	1.001	72	1.83	2.62	1.429*	1.232*
3	4	22	3.73	7.35	1.972**	1.252**	20	7.50	3.11	0.414	0.925	47	2.04	2.65	1.298	1.144
3	5	17	5.53	9.01	1.630	1.108	17	9.82	3.15	0.321	0.935	30	2.80	4.86	1.734**	1.256**
3	6	17	6.00	11.50	1.917*	1.145*	17	10.82	6.03	0.557	0.961	28	3.82	8.00	2.094***	1.279***
3	7	n<15					n<15					24	3.38	6.16	1.824**	1.237**
4	3	38	2.74	8.96	3.272***	1.816***	36	6.33	6.34	1.002	1.000	48	2.52	4.47	1.772***	1.302***
4	4	n<15					n<15					31	2.94	5.86	1.997***	1.332***
4	5	16	7.19	17.63	2.453**	1.191**	15	12.73	4.35	0.342	0.952	30	3.80	8.92	2.348***	1.346***
4	6	15	7.60	22.69	2.985***	1.246***	n<15					27	5.19	14.00	2.701***	1.318***
4	7	n<15					n<15					16	4.31	8.63	2.001*	1.221*
4	8	n<15					n<15					15	4.93	9.35	1.896*	1.172*
4	9	n<15					n<15					15	6.00	14.14	2.357**	1.213**
5	3	29	3.72	13.14	3.527***	1.661***	26	8.35	9.44	1.131	1.015	39	3.31	6.06	1.832**	1.247**
5	4	17	5.59	20.38	3.647***	1.451***	15	11.87	8.12	0.685	0.975	34	3.689	8.23	2.237***	1.329***
5	5	n<15					n<15					24	4.96	11.87	2.393***	1.272***
5	6	n<15					n<15					20	7.10	20.94	2.949***	1.263***
5	7	n<15					n<15					n<15				
5	8	n<15					n<15					16	7.25	21.13	2.915***	1.250***
5	9	n<15					n<15					16	8.94	33.80	3.781***	1.294***
6	3	21	4.48	17.26	3.856***	1.614***	21	9.81	12.56	1.281	1.027	28	3.96	7.81	1.971**	1.238**
6	4	n<15					n<15					26	4.38	11.61	2.647***	1.364***
6	5	n<15					n<15					17	6.24	19.07	3.058***	1.314***
6	6	n<15					n<15					16	8.31	29.16	2.895***	1.285***
6	7	n<15					n<15					n<15				
6	8	n<15					n<15					n<15				
6	9	n<15					n<15					n<15				
7	3	22	5.09	21.99	4.320***	1.628***	21	11.00	21.50	1.955**	1.083**	35	4.29	9.92	2.314***	1.300***
7	4	n<15					n<15					22	5.32	12.99	2.442***	1.261***
7	5	n<15					n<15					15	7.27	23.21	3.194***	1.284***
7	6	n<15					n<15					15	8.87	36.70	4.139***	1.333***
8	3	19	5.74	27.76	4.839***	1.640***	18	12.61	21.19	1.680*	1.051*	29	4.55	13.33	2.928***	1.412***
8	4	n<15					n<15					18	5.61	19.08	3.400***	1.408***
8	5	n<15					n<15					n<15				
8	6	n<15					n<15					n<15				
9	3	16	7.13	39.18	5.499***	1.597***	15	14.33	35.95	2.508**	1.099**	24	5.54	16.87	3.044***	1.356***
9	4	n<15					n<15					n<15				

n = number of subunits for each grid(the subunit size = number of pairs of rows x the number of columns.
M = mean number of symptomatic plants
V = variance of the number of symptomatic plants
V/M = mean to variance ratio. Significance, indicating aggregation, is indicated by * (P<0.05), **(P<0.01) and ***(P<0.001)
I□ = Morisita's index of dispersion. A significant deviation of I□ away from 1(random distribution), indicating aggregation, is indicated by * (P<0.05), **(P<0.01) and ***(P<0.001)

Table 3. Results from the fixed grid analysis for AGY at site 2 in each season.

		1996					1997					1998				
Row	Column	n	m	V	V/M	Iδ	n	m	V	V/M	Iδ	n	m	V	V/M	Iδ
3	3	86	0.16	0.23	1.425**	3.780**	81	0.38	0.49	1.278*	1.742*	79	0.53	0.46	0.860	0.734
3	4	55	0.16	0.21	1.305	3.056	51	0.53	0.61	1.160	1.308	47	0.66	0.71	1.073	1.112
3	5	40	0.20	0.22	1.077	1.429	35	0.74	0.78	1.057	1.077	33	0.85	1.01	1.188	1.222
3	6	38	0.37	0.46	1.236	1.167	33	0.85	1.13	1.335	1.397	31	1.19	0.96	0.805	0.838
3	7	31	0.26	0.26	1.025	1.107	31	0.81	0.89	1.109	1.137	29	1.14	1.27	1.113	1.098
4	3	63	0.22	0.30	1.371*	2.769*	59	0.53	0.81	1.533**	2.030**	55	0.73	0.65	0.889	0.846
4	4	40	0,25	0.29	1.179	1.778	37	0.70	0.88	1.254	1.366	33	0.91	1.27	1.400	1.441
4	5	34	0.24	0.25	1.045	1.214	34	0.88	1.08	1.220	1.251	33	1.09	1.46	1.339	1.310
4	6	33	0.39	0.50	1.260	1.692	34	1.06	1.57	1.485*	1.457*	33	1.45	1.44	0.992	0.995
4	7	22	0.32	0.42	1.313	2.095	21	1.05	1.25	1.191	1.182	21	1.52	2.36	1.550	1.355
4	8	22	0.41	0.54	1.317	1.833	20	1.35	1.50	1.113	1.083	19	1.89	2.65	1.401	1.206
4	9	21	0.48	0.66	1.390	1.867	20	1.55	2.16	1.390	1.247	19	2.26	3.20	1.416	1.178
5	3	47	0.28	0.47	1.682**	3.615**	41	0.66	1.08	1.641**	1.986**	39	0.97	0.76	0.783	0.777
5	4	36	0.28	0.32	1.154	1.600	35	0.80	1.46	1.824**	2.037**	35	1.20	1.64	1.363	1.301
5	5	26	0.31	0.30	0.980	0.929	27	1.07	1.61	1.499*	1.463*	24	1.38	2.07	1.506	1.364
5	6	26	0.54	0.74	1.371	1.714	27	1.30	2.37	1.829**	1.634**	24	1.83	1.62	0.885	0.939
5	7	18	0.39	0.49	1.252	1.714	17	1.35	1.49	1.103	1.182	15	2.07	2.92	1.415	1.194
5	8	18	0.56	0.61	1.106	1.200	18	1.67	2.00	1.200	1.083	18	2.33	3.18	1.361	1.150
5	9	18	0.78	1.24	1.597	1.780	18	1.94	3.11	1.602	1.247	18	2.78	3.36	1.209	1.073
6	3	38	0.37	0.46	1.236	1.670	36	078	1.38	1.771**	2.000**	33	1.15	1.01	0.875	0.892
6	4	31	0.35	0.37	1.042	1.127	29	1.00	1.57	1.571*	1.571*	29	1.38	1.53	1.109	1.078
6	5	22	0.41	0.35	0.852	0.611	21	1.29	1.71	1.333	1.256	19	1.58	2.37	1.500	1.310
6	6	21	0.67	0.63	0.950	0.923	21	1.52	3.06	2.009**	1.651**	19	2.16	1.81	0.837	0.927
6	7	16	0.50	0.67	1.333	1.714	15	1.60	1.54	0.964	0.978	15	2.47	3.12	1.266	1.104
6	8	16	0.69	0.76	1.109	1.164	15	1.93	1.78	0.921	0.961	n<15				
6	9	16	0.94	1.13	1.204	1.219	15	2.27	3.64	1.605	1.257	n<15				
7	3	37	0.35	0.40	1.141	1.423	35	0.89	1.34	1.512*	1.581*	33	1.36	1.24	0.908	0.933
7	4	24	0.38	0.42	1.116	1.333	23	1.17	1.88	1.599*	1.507*	33	1.36	1.24	0.908	0.933
7	5	17	0.41	0.51	1.232	1,619	16	1.56	1.73	1.107	1.067	22	1.73	1.92	1.113	1.064
7	6	18	0.72	0.80	1.109	1.154	18	1.78	2.89	1.625*	1.343*	15	2.07	2.64	1.276	1.129
8	3	32	0.44	0.77	1.760**	2.813**	34	1.00	1.58	1.576*	1.576*	31	1.48	0.92	0.623	0.749
8	4	21	0.43	0.46	1.067	1.167	21	1.24	1.79	1.446	1.357	19	2.11	2.43	1.156	1.072
8	5	18	0.50	0.62	1.235	1.500	18	1.67	1.53	0.918	0.952	17	2.18	2.03	0.932	0.970
8	6	18	0.83	1.32	1.588	1.714	18	2.00	2.71	1.353	1.171	17	2.82	2.90	1.029	1.010
9	3	28	0.54	0.70	1.311	1.600	27	1.15	2.21	1.923**	1.800**	25	1.68	1.48	0.879	0.929
9	4	18	0.61	0.72	1.182	1.309	17	1.47	3.26	2.220**	1.813**	15	2.40	3.54	1.476	1.190
9	5	n<15					15	1.73	2.92	1.687	1.385	n<15				
9	6	n<15					15	2.13	5.55	2.603***	1.724***	n<15				

n = number of subunits for each grid(the subunit size = number of pairs of rows x the number of columns.
M = mean number of symptomatic plants
V = variance of the number of symptomatic plants
V/M = mean to variance ratio. Significance, indicating aggregation, is indicated by * (P<0.05), **(P<0.01) and ***(P<0.001)
I□ = Morisita's index of dispersion. A significant deviation of I□ away from 1(random distribution), indicating aggregation, is indicated by * (P<0.05), **(P<0.01) and ***(P<0.001)

DISCUSSION

The spread of plant disease depends on three basic components -the host plant, the vector and the plant pathogen itself (Irwin and Ruesink, 1986). Any variation in the incidence of these components at each site is likely to lead to variable results in I incidence of disease.

The survey results indicate that incidence of AOY at each vineyard is variable. There was a significant increase in incidence at both Gol Gol and Wemen from 1995/96 to 1996/97. Incidence declined dramatically at Gol Gol the following year but stabilised at Wemen. Like the data presented by Magarey et al. (this report) the results indicate that the incidence of AOY may be epidemic, that is, the incidence of disease may be temporarily high.

Our results indicate that AOY is spreading within each vineyard, especially since incidence increased from 1995/96 to 1996/97 at each vineyard. The drop in incidence at Gol Gol may be explained by the remission of disease in affected vines, that is not all vines are visibly affected by AOY from year to year. Remission is also evident at the other study sites and has also been described by Osler et al. (1993) for a grapevine yellows disease in Northern Italy. Osler et al. (1993) also noted that, in one region where 15 vineyards were surveyed, the incidence of disease declined but continued to spread and was characterised by "recoveries" (or remissions) and new infections. We also noted that at each study site some vines show symptoms from year to year while others went into remission (Data not shown). However statistical analysis presented later in this report (Constable et al.) shows that vines affected previously with AOY I have a greater risk of being diseased in subsequent years.

The spatial analysis of the distribution of AOY showed disease was mostly clustered and no edge effect was observed in any season. This result is typical of a disease that spreads within the planted area either host-to-host or alternative host to host by an insect vector. This type of spread is typical for other grapevine yellows phytoplasma overseas, in particular for Vergilbungskrankheit (Maixner, 1995), which is closely related to the AOY phytoplasma (Padovan et al., 1995). If we assume that spread of AOY is occurring from vine to vine via an insect vector then the number of host plants is not a limiting factor since large areas of vines are usually planted in Australia. However, other plant species may be an important factor in the disease cycle of AOY as a host for the vector and/or pathogen.

Most phytoplasma diseases are spread by an insect vector and mostly by leafhoppers or planthoppers and are not transmitted mechanically through pruning or harvesting equipment. The presence and abundance of an insect vector is most likely to have an influence on the incidence of AOY within a vineyard; without a vector there is little chance that the disease will spread. If the vector is not prevalent at Karadoc this may explain the low level of infection at that site compared to Wemen and Gol Gol. It is unlikely that the low level of AOY is a limiting factor at Karadoc when extreme increases in incidence of the disease have been observed elsewhere.

The AOY phytoplasma is closely related to the phytoplasma associated with papaya dieback (PDB), which occurs in Northern Australia (Padovan et al. 1996). Experiments conducted by researchers in QLD, where papaya plants were screened with insect proof nets, have shown that PDB is insect vectored and that disease is

spread from outside the plantation (Denis Persley, pers. comm.). Like AGY, the vector for PDB phytoplasma has not been identified and the phytoplasma of both diseases may be transmitted by the same insect species.

Since our spatial analysis of the distribution of AGY at two vineyards indicated aggregation, and the incidence of AGY, was high at site 1 and site 3, we carried out targeted surveys for alternative hosts and insect vectors in the vineyards at site 1 and site 3 in 1997/98.

We recommend control of broad leaf weeds within affected vineyards because the distribution of AGY at two vineyards showed aggregation indicating potential spread within a vineyard from an alternative host. Spread of disease from an alternate host has been observed for the closely related phytoplasma Vergilbungskrankheit in Europe (Maixner 1995). Aggregation of AGY may also indicate spread from vine to vine, and the removal of affected shoots or affected vines, as they appear, may also prove useful in controlling spread of AGY.

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3.3 Variety Survey

Rod Bonfiglioli, U. of Adelaide, Waite Campus, Glen Osmond, South Australia

Many different grapevine cultivars are susceptible to Australian grapevine yellows phytoplasma. The red varieties, especially Merlot, may even be quite symptomatic at times. Generally, the red varieties do not show such strong symptoms, and some close observations may be required to suggest phytoplasma infection. In Merlot, the canes remain unlignified and the sections of leaves delineated by leaf veins and the veins may be red. Fruit, on affected vines, falls early after shrivelling.

Phytoplasmas have been detected in many grapevine cultivars in Australia:

Chardonnay
Riesling
Semillon
Traminer
Viognier
Sauvignon Blanc
Pinot Gris
Verdelho
Shiraz
Cabernet Sauvignon
Merlot
Pinot Noir
Ramsay rootstock

Chapter 4 Identification of vectors and alternative hosts for AGY phytoplasma



4.1 Field surveys of the incidence and abundance of candidate insect vectors of the AGY phytoplasma in Sunraysia
LeAnn Beanland, Michelle Kelly and Linda Pollack

4.2 Surveys of candidate leafhopper and planthopper vector species in South Australian vineyards.
John McFarlane and Peter Magarey

4.3 Identification of potential alternative hosts for the AGY phytoplasma
Michelle Kelly, Fiona Constable and Mali Malipatil

4.4 Tomato big bud transmission trials at IHD

a. Culturing of Orosius argentatus
John McFarlane and Fran Richardson

b. Handling and storage factors influencing the detection of Tomato Big Bud phytoplasma in leafhopper Orosius argentatus
John McFarlane, Fran Richardson and Rebecca Mills

c. Transmission of TBB phytoplasma from tomato to grapevine
John McFarlane, Fran Richardson and Rebecca Mills

4.5 a. Establishing colonies of leafhopper and planthopper species found in Sunraysia vineyards
LeAnn Beanland, Linda Pollock and Michelle Kelly

b. Experimental Transmission of AGY phytoplasma by test leafhoppers
LeAnn Beanland, Linda Pollock and Michelle Kelly

4.6 Use of bait plants to determine AGY phytoplasma inoculation period of feral insect vectors
LeAnn Beanland, Linda Pollock and Michelle Kelly

4.7 Propagation of AGY phytoplasma infected grapevine material LeAnn Beanland, Linda Pollock, Michelle Kelly, Graeme Fletcher and Peter Magarey

4.8 Experimental Transmission of AGY phytoplasma from grapevine to Dodder
Peter Magarey

4.1 Field surveys of the incidence and abundance of candidate insect vectors of the AGY phytoplasma in Sunraysia

LeAnn Beanland, Michelle Kelly and Linda Pollock

INTRODUCTION

Magarey and Wachtel first described Australian Grapevine Yellows (AGY) in 1978. AGY affected grapevines have yellow leaves that typically roll backward in an overlapping "shingling" pattern. Developing grape bunches shrivel and abort, and later in the season infected shoots may die (Magarey and Wachtel 1986, Constable et al. 1998). The disease has been found in several Australian states but is prevalent in warmer regions (Magarey and Wachtel 1986, Bonfiglioli et al. 1996).

The causal agent of Australian grapevine yellows is thought to be a phytoplasma given the name *Candidatus* Phytoplasma australiense (Davis et al. 1997). In other phytoplasma disease systems, phloem-feeding insects have been identified as the vectors responsible for transmitting the pathogens from infected to healthy host plants. Leafhoppers, planthoppers, psyllids and froghoppers are phloem feeding insects that have been implicated as vectors of plant diseases caused by phytoplasmas. In Europe, two grapevine yellows diseases are transmitted by phloem feeding insects. German grapevine yellows or Vergilbungskrankheit (VK) is transmitted by the planthopper *Hyalesthes obsoletus* Signoret (Maixner 1994) and, in France, flavescence doree is transmitted by the leafhopper *Scaphoideus titan us* Ball (Lefol et al. 1993). According to Liefting et al. (1998) the AGY phytoplasma is the same pathogen as that which causes *Phormium* (flax) yellows in New Zealand, as well as papaya dieback in Northern Australia. The insect vector of *Phormium* yellows is the planthopper, *Oliarus atkinsoni* (Cumber 1953, Liefting et al. 1997). Another planthopper in the genus *Oliarus* has been found in and around vineyards and efforts have been made optimise captures of this insect. In efforts to identify the insect vector of the Australian grapevine yellows, the search has been focussed on phloem-feeding insects in and around infected vineyards. Identification of the insect vector of the AGY phytoplasma is critical for the successful management of this important disease in vineyards. The following is a continuation of investigations initiated by Kelly et al. (1998).

Using a laboratory procedure, the polymerase chain reaction (PCR), the presence of the AGY phytoplasma in grapevines, insects and alternative plant hosts can be detected (Padovan et al. 1995). If an insect tests positive for phytoplasma by PCR, this provides evidence that the insect may be a vector. However phytoplasma positive insects may have fed on an infected plant but be incapable of transmitting the pathogen. In addition, non-vector leafhoppers that have recently fed upon a phytoplasma-infected plant may ingest phytoplasma particles and, if analysed, test positive for the pathogen (Purcell et al. 1981).

MATERIALS AND METHODS

A variety of techniques have been implemented to target and capture potential vectors of AGY. Fieldwork commenced m 1996 when preliminary data was collected in Chardonnay vineyards in Sunraysia. Test vineyards were located at Gol Gol NSW, Karadoc Vic, and Wemen Vic. A Black and Decker® leaf blower was converted into a vacuum by placing the cylinder from the fan end of the blower to the air intake opening. A fine mesh net was inserted into the suction end of the cylinder into which insects would be collected when vacuum sampling. Captured insects were transferred to a vial and transported to the laboratory and frozen at -20°C. Insects were identified to species level, when possible, and shipped to the Institute for Horticultural Development (IHD) at Knoxfield, Victoria, where they were tested for phytoplasma by PCR analysis.

1996/1997

In the first year preliminary data was collected for a survey of insects that were present in and around the vineyard. Every two weeks, vacuum samples were taken in the canopy of Chardonnay vineyards at Gol Gol, Karadoc and Wemen. The vacuum samples were not taken in a consistent way so that only total leafhopper numbers over the I sampling period are presented rather than mean insects per sample.

1997/1998

Vacuum samples were collected every two weeks from September 1997 to May 1998 at Wemen and Gol Gol. At the Wemen vineyard samples were collected from sites consisting of three vines in each of four blocks. Two of the blocks consisted of 240 vines in a 10 by 24 vine array and the remaining two had 100 vines in a 10 by 10 vine array. There is a single block at Gol Gol with 12 sites, made up of three vines each. Biweekly vacuum samples were collected from each of the 12 sites.

1998/1999

Vacuum samples were collected every two weeks at the Gol Gol and Wemen vineyards. In addition, pan traps were used at Gol Gol and in a Chardonnay block at the Sunraysia Horticulture Centre (SHC), Mildura. Pan traps were constructed from yellow pet food bowls mounted to vineyard posts and filled with approximately 500 ml of anti-freeze. Fifteen traps were mounted to posts and arranged within the block in an X -shaped pattern at SHC and 12 were mounted at the Gol Gol vineyard in a similar arrangement.

Sticky trapping was incorporating into the field component of the project in the 1998/99 season since they are useful and often more efficient at capturing target insects (Bosco et al. 1997). Yellow sticky traps (purchased from Biological Services, Loxton, SA) were placed in and around study vineyards. SHC and Gol Gol sticky traps were placed in the vine canopy adjacent to the pan trap sites. In addition, 11 traps were placed in the scrub adjacent to the vineyard at Gol Gol. Another 12 sticky traps were also placed in a 65 by 87 vine block in the Wemen Chardonnay block, in a 16 by 60 vine block of Chardonnay at Wingara (Vic), and 13 in a 50 by 18 vine block of Chardonnay at Karadoc. Sticky traps were collected and replaced weekly or fortnightly.

Because *Austroasca viridigrisea* is not a phloem feeder, but rather feeds on vascular bundles and *Zygina zealandica* feeds on the parenchyma of host plants (Murray Fletcher, pers.com), these species were excluded from our analyses beginning in 1998. Only phloem feeding insects were considered.

Comparison of sticky trap Sites for the Capture of *Orosius argentatus*

The number of o. *argentatus* captured on Gol Gol scrub and vineyard sticky traps was compared by trap site to determine if some traps were in positions that were better suited for the capture of leafhoppers. The habitat in which the most insects are captured may give clues as the host plants upon which the candidate insect feeds. Information about the possible host plants of insect species maybe useful for identifying which species are important, or at least abundant, In the environment of a vineyard. In addition, if a vector species is identified, knowing its preferred habitat and alternative hosts will help to target management strategies for the control of that vector.
The number of O. *argentatus* captured on all sticky traps at all sites was subject to analysis of variance to determine in which trap sites more leafhoppers could be found.

Comparison of number of leafhoppers captured on sticky traps above and in canopy at Gol Gol And Karadoc

Because no candidate vector species live on grapevines, it is believed that visits to the vineyard by inoculative vector insects may be random. Many leafhoppers can be found 2 to 10 meters above the ground in wind currents (Teraguchi 1986). When the wind speed diminishes, migrating insects fall to the ground or commence localised flight. This described behaviour may explain how vectors arrive at a vineyard. To determine if there are vector candidates either flying above the canopy, or being carried by winds, traps were placed approximately 50-60 cm above the foliage of grapevines.

At Gol Gol and at Karadoc, brackets were attached to vineyard posts adjacent to sticky trap sites. An eye-screw was placed approximately 6 cm above the bracket. Sticky traps were wired to one end of bamboo stakes (75cm), which were slipped through the eye screw so that the bottom of the stake rested on the bracket. The eye screw secured the stakes and allowed the erection of sticky traps above the canopy of test vines. Therefore, each sticky trap site had two traps: one above and one in the canopy. Captures in canopy traps were compared with those in traps placed above the canopy and analysed by ANOV A.

Comparison of daytime and crepuscular vacuum samples

Vacuum samples were collected around dusk on 4 dates: 16/2,23/2,26/2, and 1/3/99 to determine if there were diurnal patterns in the behaviour of leafhoppers that would result in greater capture or captures of previously uncaptured Hemiptera species during crepuscular periods. Samples were collected from areas in the scrub at sites where daytime vacuum samples were collected. Captured insects were identified to species, frozen and sent to IHD for PCR analysis to determine phytoplasma infection status. The number of O. *argentatus* captured in evening vacuum samples was compared to the captures of the species in daytime vacuum samples in order to determine the best period of sampling for capturing this leafhopper.

Identifying developmental hosts of *Oliarus* sp.

Beginning on 12/3/99, weed samples were collected from the vineyard and the scrub areas in and near the Gol Gol vineyard. Plants were carefully dug up so that as much of the roots as possible were recovered. Uprooted plant samples were placed in plastic bags, And transported to the laboratory in an insulated container. In the laboratory, the weed roots were inspected under a dissecting microscope for the presence of Oliarus nymphs.

Table 1. Weed samples that were inspected for Oliarus nymphs

DATE	PLANT	# PLANTS
12/3/99	Paspalum	3
(Gol Gol)	Onion Weed	2
	Summer Grass	3
10/6/99	Paspalum	9
(Gol Gol)	Onion Weed	4
	Summer Grass	4
16/6/99	Paspalum	4
(Gol Gol)	Summer Grass	7
	Onion Weed	1
29/6/99	Digitaria	3
(Gol Gol)	Paspalum	2
	Onion Weed	2
	Summer Grass	3
6/7/99 (Karadoc)	Paspalum	3
	Summer Grass	3
	Red Dock	1
	Prostrate Blue Bush	1
	Salt Bush	1
	Upright Blue Bush	1
19/7/99	Wild Oats	6
(Gol Gol)	Paspalum	4
	Winter Grass	1
	Perennial Grass	2
	Red Dock	1
	Onion Weed	1
21/7/99 (Karadoc)	Paspalum	2
	Winter Grass	2
	Perennial Grass	1
	Red Dock	1
	Wild Barley	2
16/9/99	Creeping Saltbush	1
(Gol Gol)	Wild Barley	3
	Wild Oats	3
	Clover	1
	Onion Weed	1
	Prairie Grass	1

On 01/07/99, 12 vines were randomly chosen from the Gol Gol test block of vines. The roots of these vines were inspected for developing *Oliarus* nymphs by carefully digging the soil from around the root zone to a depth of approximately 30cm. Two approximately 12.cm deep holes on either side of the vine were dug. Once the roots were inspected, the soil was carefully replaced so as not to damage the vines

Identifying alternative hosts of the AGY phytoplasma
In February, March, July and September 1999, several specimens of perennial weeds found in and around the Gol Gol vineyard were collected and sent to IHD for PCR analysis.

Table 2. Weed species collected from Gol Gol and tested for the AGY phytoplasma by PCR analysis.

Chenopodium ulicinum
Enchylaena tormentosa (Ruby saltbush)
Maireana sedifilia (Black bluebush)
Solanum nigrum L.
Cooba wattle foliage
Lycium ferocissimum (African Boxthorn)

1999/2000

Insect trapping
Field studies were concluded at all sites except at the Gol Gol vineyard. The number of vacuum sites at Gol Gol was reduced from 12 to 7. Weed and canopy vacuum samples were combined at each of the 7 sites. Collection by sticky traps continued and the 24 traps were collected and replaced each week. Above-canopy sticky traps were discontinued. Cixiid planthoppers were the only candidate vector species, captured on sticky traps, to be sent to IHD for PCR analysis. Additional field studies were initiated (See Bait Plant Study, Chapter 4.6).

Comparison of sticky trap sites for the capture of *O. argentatus*
The capture of O. *argentatus* at each trap site again was compared in 1999/2000.

Comparison of captures of *O. argentatus* on sticky traps placed on the trunk to those -placed in the canopy
In order to determine the optimum placement of sticky traps in the vineyard the captures of traps erected above the vineyard canopy were compared to those of traps placed in the vineyard canopy.

Arboreal beating sampling for live insects

In April and May of 2000, *Oliarus* sp. began to be captured by sticky traps in large numbers near Acacia trees (Cooba Wattles). In May, a new trapping method was initiated to capture live specimens of *Oliarus.* Many insects feign death by dropping off plants when they are jarred (Borror et al. 1989) and this behaviour is exploited in arboreal beating sampling. The collecting device for our arboreal beating sampling was a twin size white bed sheet placed under the branches of Cooba wattle trees. The branches hanging over the sheet were shaken to dislodge any resident insects. Fallen insects were collected from the sheet and introduced into tube cages housing oat plants. Tube cages were transferred to the laboratory and insects were placed by species into cages with a variety of plants.

RESULTS

1996/1997

More leafhopper species were captured on the vineyard floor or in adjacent paddocks than in the vineyard canopy (Figures 1 and 2). The 12 most abundant leafhopper species are listed in Table 3. *Zygina zealandica* was the most frequently captured species.

Numbers of most candidate species peaked in January. None of the insects tested by PCR were positive for AGY phytoplasma.

Table 3. Twelve leafhopper and one planthopper species most commonly found in and around test vineyards listed in order of their abundance.

Zygina zealandica (Myers)	*Nesoclutha pallida* (Evans)
Orosius argentatus (Evans)	*Orosius canberrensis* (Evans)
Austroasca viridigrisea (PaolI)	*Recilia hospes* (Kirkcaldy)
Balclutha saltuella (Kirschbaum)	*Arawa* sp.
Austrogallia torrida (Evans)	*Limotettix incertus* (Evans)
Batracomorphus angustatus (Osborn)	*Toya* sp. (a planthopper)
Exitianus nanus (Distant)	

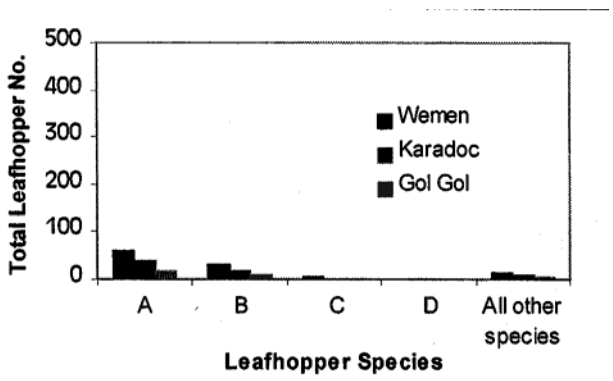


Figure 1. Total number of the four most abundant leafhopper species collected in vacuum samples from the vine canopy in three test vineyards in 1996/1997. A = *Orosius argentatus,* B = *Zygina zealandica,* C = *Austroasca viridigrisea,* D = *Balclutha saltuella*

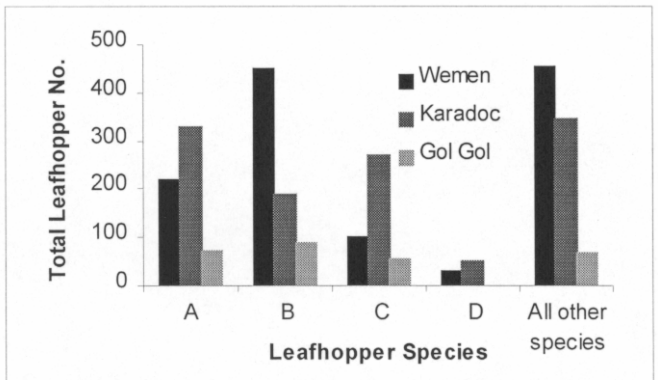


Figure 2. Total number of the four most abundant leafhopper species collected from weeds in and around three test vineyards in vacuum samples in 1996/1997. A = *Orosius argentatus*, B = *Zygina zealandica*, C = *Austroasca viridigrisea,* D = *Balcluthe saltuella*

Planthopper species in the family Delphacide were captured at all sites but n o more were collected from weed sites at Wemen and Karadoc than from other sites.

1997/98

The most abundant leafhopper in all samples from test vineyards was *O. argentatus*. As in 1996/97 fewer were captured in the vineyard (figure 3) than in the scrub near the vineyard (figure 4). Peak numbers of this leafhopper were observed in December and January. The only species besides. *O.Argentatus, Z. zealandica* and *A. viridigrisea*, captured in total seasonal numbers of greater than 50 were *Nesoclutha pallida* at Wemen (61) and *Austrogallia torrida* at Gol Gol (69)

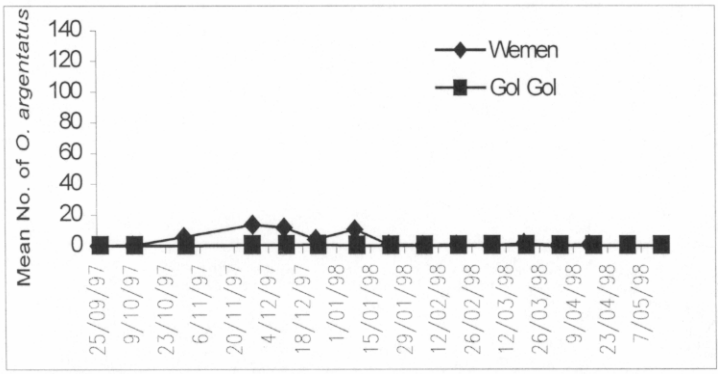


Figure 3. Mean number of *O. argentatus* captured in vineyard vacuum samples at Wemen and Gol Gol in 1997/1998.

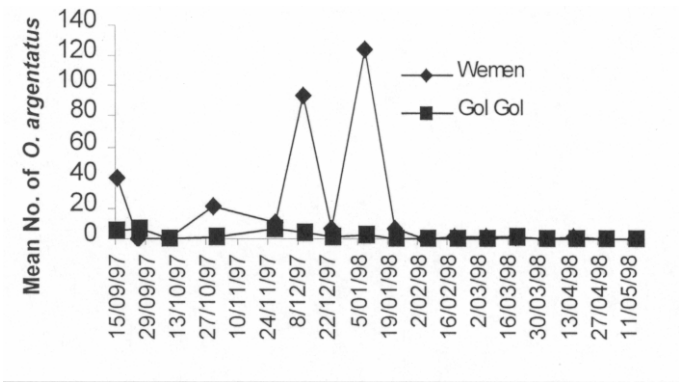


Figure 4. Mean number of *O. argentatus* captured in scrub vacuum samples at Wemen and Gol Gol in 1997/98.

1998/1999

Orosius argentatus was the most abundant leafhopper and fewer were captured in the vineyard (figure 5) than in the scrub (figure 6) on sticky traps and in vacuum samples (figure 7 and 8). A much smaller number of this species was captured in pan traps at Gol Gol (figure 9) and SHC (figure 11) and at Karadoc (figure 12) captured every few *O. argentatus. O. argentatus* numbers observed in pan traps and vacuum samples peaked initially in November. Numbers in sticky traps did not peak until January.
Blastopsyllids were abundant on sticky traps in 1998/99 and a peak was seen in July at all three vineyards (figure 13)
Nesoclutha pallida was more abundant in 1998/99 than in previous years with a total of 117 captured at Wemen. Only a total number of 19 were captured at Gol Gol.

Comparison of Sticky Trap Sites for the capture of *O. argentatus*
When the captures of *O. argentatus* between Gol Gol sticky traps were compared, the only significant difference was observed between two sticky traps in the scrub and all the other scrub and vineyard traps ($F_{35,609} = 4.72$; $P<0.00$). The two scrub trap sites captured more *O. argentatus* than other sites and one captured significantly more than the other. Both traps were under a canopy of Cooba wattles and situated close to blue saltbush.

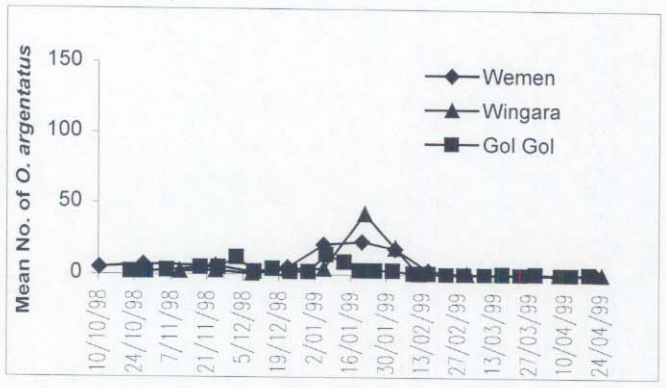


Figure 5. Mean number of *Orosius argentatus* captured on sticky traps in the vine canopy of Chardonnay vineyards at Wemen, Wingara and Gol Gol in 1998/99.

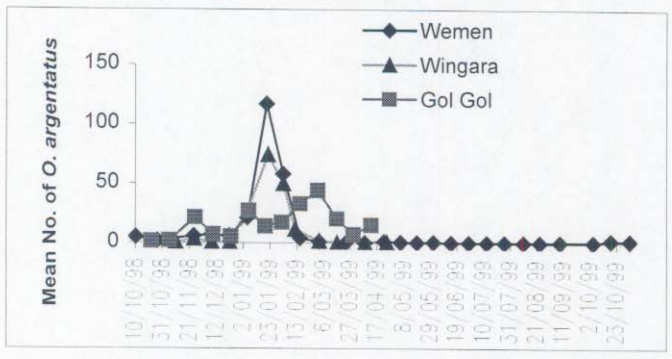


Figure 6. Mean number of *Orosius argentatus* captured on sticky traps in scrub near Chardonnay vineyards at Wemen, Wingara and Gol Gol in 1998/99.

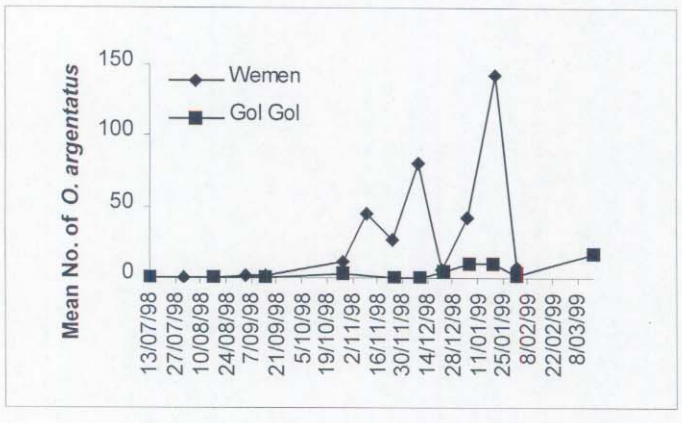


Figure 7. Mean Number of *Orosius argentatus* collected in scrub in vacuum samples near Wemen and Gol Gol vineyards in 1998/99.

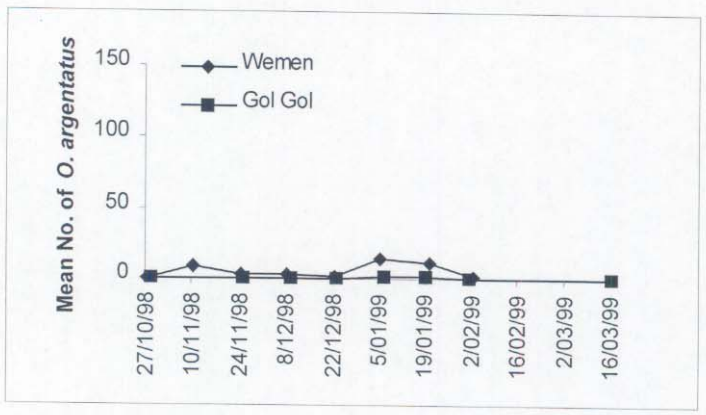


Figure 8. Mean Number of *Orosius argentatus* collected in vine canopy vacuum samples at Wemen and Gol Gol vineyards in 1998/99.

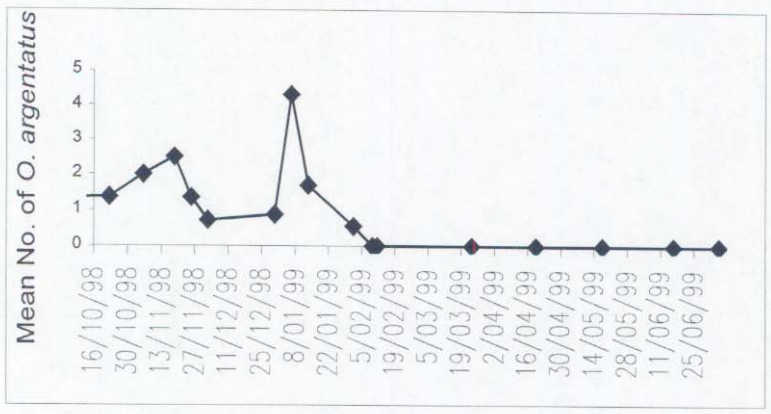


Figure 9. Mean number of *O. argentatus* captured in vineyard pan traps at Gol Gol in 1998/99.

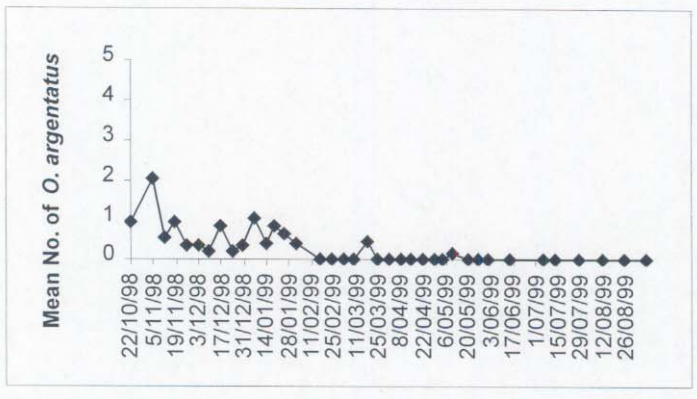


Figure 10. Mean number of *O. argentatus* captured in SHC pan traps in 1998/99.

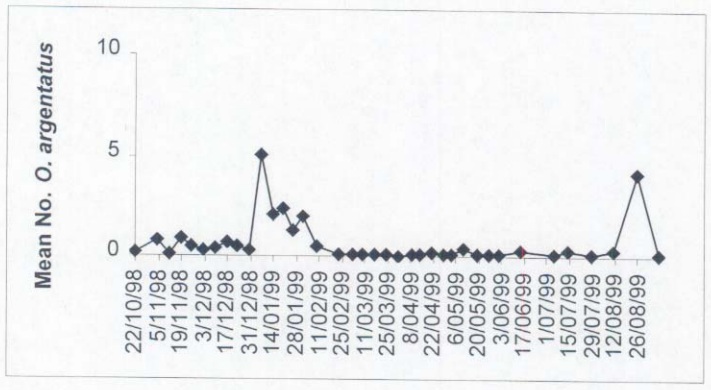


Figure 11. Mean number of *O. argentatus* captured on SHC sticky traps in 1998/99

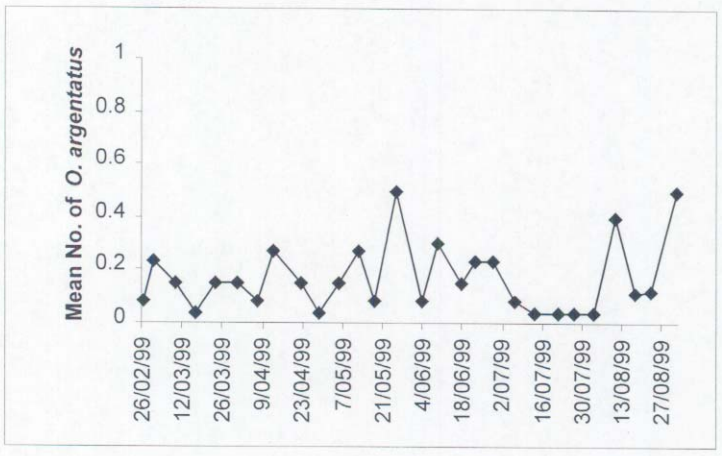


Figure 12. Mean number of *O. argentatus* captured on Karadoc sticky traps in 1998/99. Note the Y axis values.

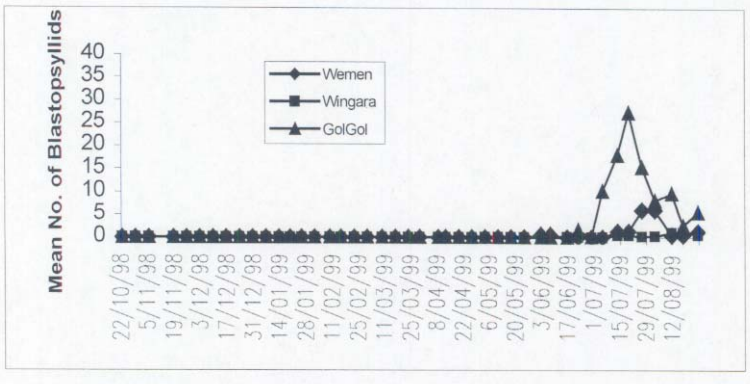


Figure 13. Mean No. of Blastopsyllids captured on Wemen, Wingara and Gol Gol sticky traps in 1998/99.

PCR Analysis of Insects

Of the hundreds of candidate insects analysed by PCR analysis for the AGY phytoplasma in 1998/99, only four bulked samples of leafhoppers of the species, *Orosius argentatus,* tested positive. All were captured in vacuum samples from Gol Gol. Two were collected from weeds in the vineyard and two were collected from scrub sites. Several O. *argentatus,* 1 Cixiid, 2 Rutherglen bugs, 1 *Zygina zealandica* and 2 *Austroasca viridigrisea* tested positive for the TBB phytoplasma. Five o. *argentatus* tested positive for other undescribed mollicutes.

Comparison of number of leafhoppers captured on sticky traps above and in the canopy at Gol Gol and Karadoc

There was no difference between the captures of sticky traps placed above the canopy and those traps placed in the canopy of grapevines *(F* 1, 1061 <00.0, *P* = 0.95).

Comparison of Daytime and crepuscular vacuum samples

There was no difference in the number of O. *argentatus* captured in evening vacuum samples and daytime vacuum samples *(F* 1,10 = 0.16, *P* = 0.7). No other candidate species were captured consistently enough in vacuum samples for a comparison of their numbers to be made. No new species were captured in crepuscular vacuum samples.

Identifying Developmental Hosts of *Oliarus* sp.

No Cixiid planthopper nymphs were found on the roots of inspected vines and weed plants.

Identifying Alternative Hosts of the AGY Phytoplasma

None of the weed specimens sent to IHD for PCR analysis tested positive for the AGY phytoplasma.

1999/2000

The largest peak of O. *argentatus* was observed in February, although smaller ones were observed in October and November. More were captured in vineyard sticky traps than on traps in the scrub (Figure 14).

Comparison of Sticky Trap Sites for the Capture of *o. argentatus* The captures of O. *argentatus* in the sticky traps at Gol Gol differed significantly *(F*32,980 = 5.81; *P<O.OO).* Two trap sites in the vineyard captured more O. *argentatus* than 92% of the other sites. A third site in the vineyard captured more than 87% of all the traps. Sticky traps at scrub sites did not capture more than traps in the vineyard sites.

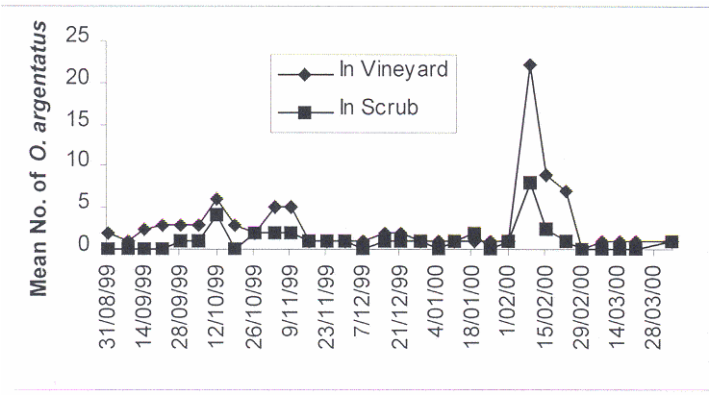


Figure 14. Mean number of *O. argentatus* captured on Gol Gol vineyard and scrub sticky traps in 1999/2000

Comparison of captures of O. argentatus on sticky traps placed on the trunk to those placed in the canopy

Sticky traps placed on the trunks of vines caught significantly more *O. argentatus* (mean = 304) than those traps placed in the vine canopy (mean =219) ($F_{1,521}$=17.26,P<0.00).

Unlike in previous years, Recilia hospes were captured in high numbers on sticky traps between January and April (figure 15). However, only 63 were captured in vacuum samples and these were sent to IHD for PCR analysis. None tested positive for AGY phytoplasma.

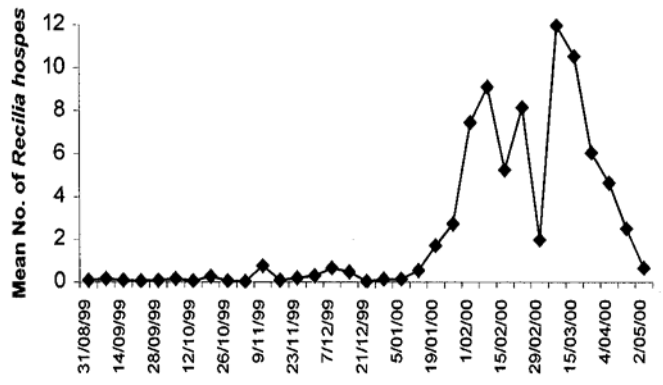


Figure 15. Mean number of *Recilia hospes* captured on Gol Gol sticky traps in 1999/2000

In 1999/2000 Cixiids were more abundant. The majority of these planthoppers were captured on two scrub sticky traps (figure 16).

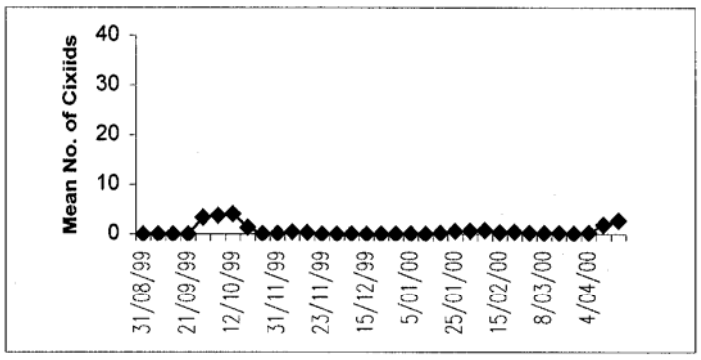


Figure 16. Mean No. of Cixiids captured on Gol Gol sticky traps in 1999/2000.

Small peaks in the number of Blastopsyllids were observed beginning in January (Figure 17).

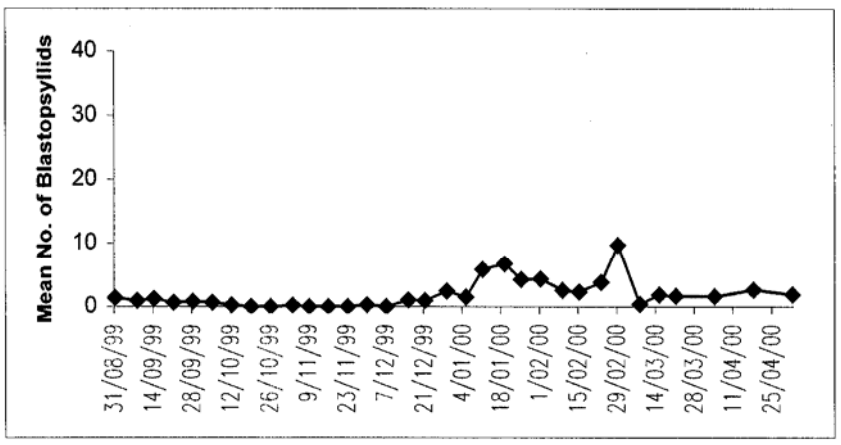


Figure 17. Mean No. of Blastopsyllids captured on Gol Gol sticky traps in 1999/2000.

Unlike vacuum sample captures in previous years, which peaked in January, peaks in the number of *O. argentatus* were observed in November in 1999/2000 vacuum samples (Figure 18).

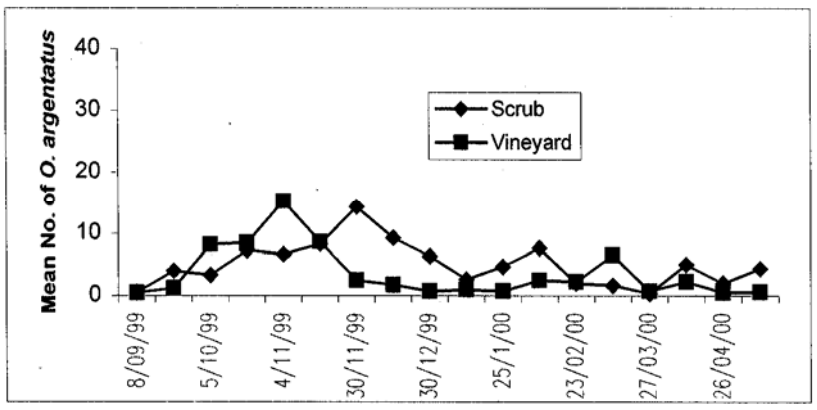


Figure 18. Mean number of *O. argentatus* captured in scrub and vineyard Gol Gol vacuum samples in 1999/2000.

Toya sp. were the most abundant candidate insects in vacuum samples (Figure 19). Their numbers were particularly high in January. Two other species were captured in numbers exceeding 100; *Balclutha saltuella* at 101 and *Nesoclutha pallid a* at 110. One *Oliarus* was captured in a vacuum sample on 9/05/00 in a scrub site.

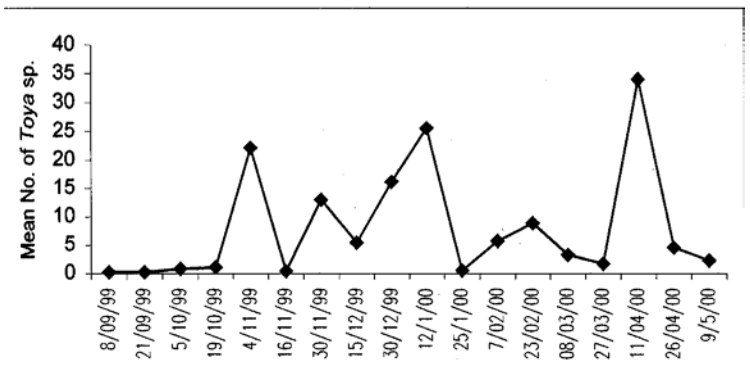


Figure 19. Mean number of *Toya* sp. Captured in Gol Gol vacuum samples in 1999/2000.

Arboreal beating sampling

Twelve *Oliarus* were captured on two dates from the branches of Cooba Wattles. One insect died and was sent to IHD for PCR analysis. It did not test positive for the AGY phytoplasma. Blastopsyllids were also collected but in larger numbers. Colonies of both species have been initiated from these collections.

PCR Analysis of collected leafhoppers

None of the leafhoppers analysed by PCR tested positive for the AGY phytoplasma. Four samples of *O. argentatus,* one of which was a bulked sample of 9 individuals tested positive for the TBB phytoplasma. Bulked samples of *Toya* sp, *Arawa* sp, *Recilia hospes* and *Orosius canberrensis* have also tested positive for the TBB phytoplasma. In addition, bulked samples (by species) of O. *argentatus, Austroasca viridigrisea, Toya* sp., *Balclutha saltuella, Eurymela distincta* (a membracid) and *Austroagallia torrid a* all tested positive for unidentified phytoplasmas.

DISCUSSION

Over the first three years of our field surveys, the largest numbers of candidate vectors were captured in vineyard weeds, adjacent paddocks and scrubby areas near test vineyards rather than in the vineyard canopies. However, on several occasions in 2000, more o. *argentatus* were captured in the vineyard than were captured on scrub traps. In 1999/2000 a cover crop was not planted in the vineyard and weeds may have been established in higher numbers than in previous seasons. Higher numbers of leafhoppers

are associated with weed cover in the vineyard. Generally, low leafhopper and

planthopper numbers in vine canopies suggest grapevines are unlikely to be a plant host for this potential AGY insect vector. Higher numbers of candidate insects in the weeds and cover crops on the vineyard floor suggest that the potential vector may prefer to live and feed on other plant hosts, unlike the leafhopper vector of flavescence doree, *Scaphoideus titanus that* develops on grapevines.

The common brown leafhopper, *Orosius argentatus* was generally the most abundant of ail phloem-feeding insects collected over the 4-year study despite higher numbers of *Zygina zealandica* captured in 1996/97 vacuum samples, and *Toya* sp. in 1999/2000 vacuum samples and Blastopsyllids in 1999/2000 sticky traps. Sticky trap captures are only a relative measure of abundance because they typically capture more active insects (Purcell and Suslow 1982). Our higher captures of Blastopsyllids on sticky traps may reflect their higher mobility rather than a greater abundance. *Austroasca viridigrisea* was also an abundant leafhopper captured in all years. *Zygina zealandica* is a parenchyma feeder and *Austroasca viridigrisea* feeds in the vascular bundles of host plants. Because neither feed on the phloem of their host plants, they are not considered vector candidates for the AGY phytoplasma. Because five *A. viridigrisea* leafhoppers have tested positive using universal primers, they are likely to be infected with an insect infecting mycoplasma rather than a phytoplasma. *O. argentatus* was also the only species to test positive for AGY phytoplasma. This leafhopper is the vector of Tomato Big Bud (TBB), another plant disease associated with a phytoplasma (Osmelak, 1987). The distribution of O. *argentatus* overlaps the areas where grapevines are grown and where AGY has been observed (Smart and Fletcher, 1996). Few O. *argentatus* have been captured in the vine canopy. Targeted collections made of this species for laboratory colonies are made in patches of mallow *(malva* sp), a plant also used as a developmental host (See Chapter 4.5a). The common brown leafhopper appears to be an important insect for consideration in our continued search for the vector of AGY. Our observations of peak numbers of O. *argentatus* around January are consistent with the findings of Osmelak et al. (1989). In contrast with our findings, these authors found *Batracomorphus angustanus* (Osborn), another possible vector, to be the second most abundant leafhopper captured in the vineyard.

Two planthoppers in the family Cixiidae, identified only by the genus name at present, *Oliarus* sp., have been captured on sticky traps at all test sites. Increasing the number of sticky traps in the scrub near the Gol Gol vineyards increased the number of *Oliarus* captured. Observations made of the capture of *Oliarus* made it possible to initiate alternate trapping methods (arboreal beating sampling) when their numbers were high. Attempts to live capture these insects at other times of the year have been unproductive. These insects are of particular interest because of their taxonomic relationship with the vector of New Zealand *Phormium* (flax) yellows phytoplasma, *Oliarus atkinsoni.* AGY and *Phormium* yellows phytoplasma are thought to be the same organism (Liefting et aI. 1998). However, none of the planthoppers tested for AGY phytoplasma have been positive. Transmission experiments using *Oliarus* have not been possible, as large numbers of live planthoppers were unavailable.

Conducting field surveys of the insects, in and around vineyards, is a critical first step in determining the suite of insects found. Testing candidate insects by PCR analysis for AGY phytoplasma is the second step in identifying potential vectors. PCR is a relatively new molecular technology that is still being refined, and recent improvements have enhanced our capacity to study the AGY phytoplasma. However, PCR analysis is not conclusive as non-vectors may test positive after feeding on an infected plant (Vega et al. 1993). Therefore transmission studies have been conducted on the most common phloem- feeding species found in our field surveys (See Chapter 4.5b).

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4.2 Survey of Candidate Leafhoppers and Planthopper Vector Species in South Australia
John McFarlane and Peter Magarey

INTRODUCTION

The incidence and abundance of candidate AGY phytoplasma vector species was measured at three vineyards in South Australia where AGY occurs. Although the region was not surveyed to the degree that Sunraysia has been, it is useful to compare the leafhoppers, planthoppers and psyllids that are found in both areas to help focus management efforts on a likely candidate vector.

MATERIALS AND METHODS

Sticky traps were first set up in 3 Riverland vineyards in October 1998. Eight traps were placed at various throughout Chardonnay vines in each of the vineyards and were mostly changed on a fortnightly basis until the end of March when hopper numbers had dropped to a low level. Trapping continued in similar positions in the same vineyards throughout the next season.

The most prevalent species was the leafhopper *Orosius argentatus* at each of the 3 vineyards in both seasons. This species occurred in much higher numbers than any other leafhopper or planthopper species. In both seasons this leafhopper reached peak numbers in January. The overall numbers were much lower in the second season.

Other leafhopper species that occurred in relatively large numbers throughout both seasons were *Austroagallia torrida, Austroasca viridigrisea, Batracomorphus angustatus* and *Zygina* sp. *A. torrida* and *B. angustatus* occurred in lower numbers in the second season but *A. viridigrisea* and *Zygina* sp. were more prevalent in the second season.

The leafhopper *Limotettix incertis* occurred in relatively large numbers in the early part of the first season but was rarely trapped in the second. *Kahaono* sp. was also trapped more in the first season than the second. Conversely, *Orosius canberrensis* and *Recilia hospes* were more prevalent in the second season. Other leafhopper species such as *Arawa pulchra, Balclutha saltuella, Exitianus nanus* and *Nesoclutha pallida* were rarely trapped during the two seasons.

The planthoppers *Oliarus* sp. and *Toya* sp. were also rarely trapped. The Cixiid *Oliarus* was found in October -November in the first season at only one vineyard and in November at a different vineyard in the second season. *Blastopsylia,* a psyllid, was rare in the first season was not found in the second season.

The eurymelid *Ipoides laeta* was found mostly at the same trap in one vineyard. It was more prevalent in the second season.

RESULTS AND DISCUSSION

The leafhoppers Orosius argentatus, Austroagallia torrida, Austroasca viridigrisea, Batracomorphus angustatus and Zygina sp. occurred in relatively large numbers

throughout both seasons in the 3 vineyards. *O. argentatus* was the most prevalent species. *O. argentatus*, *A. torrida,* and *B. angustatus* were trapped in larger numbers in the first season while *A. viridigrisea* and *Zygina* sp. were more abundant in the second season.

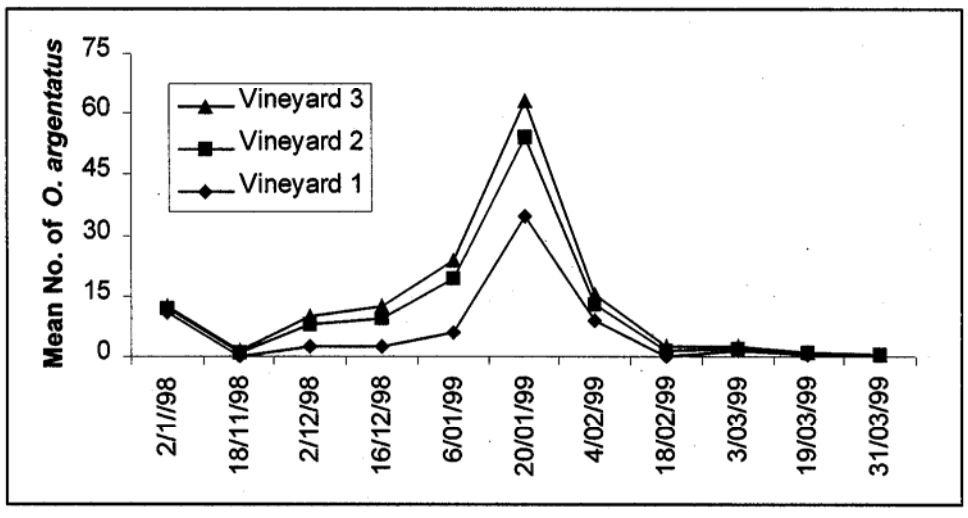


Figure 1. The mean number of O. argentatus captured on sticky traps at three vineyards in South Australia in 1998/99.

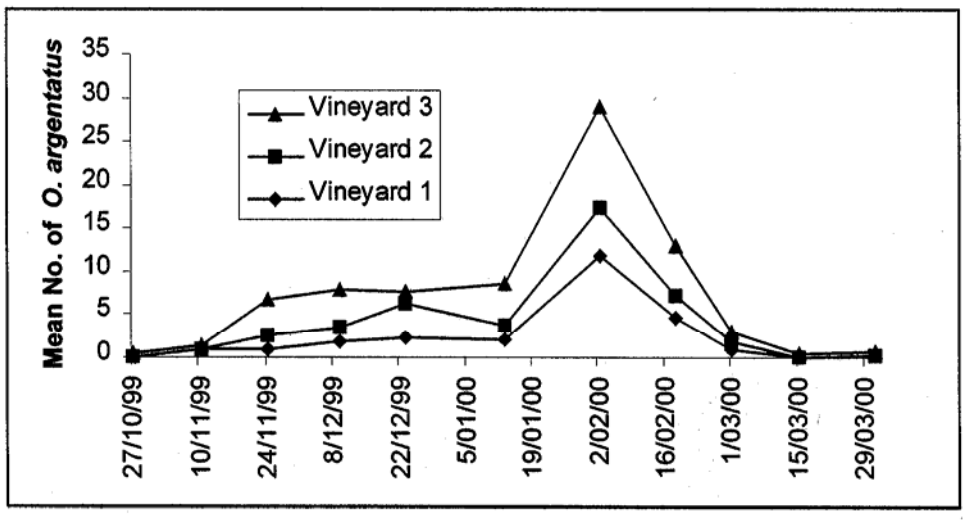


Figure 2. The mean number of O. argentatus captured on sticky traps at three vineyards in South Australia in 1999/00.

4.3 Identification of potential alternative hosts for the AGY phytoplasma.

Michelle Kelly, Fiona Constable, Mali Malipatil

MATERIALS AND METHODS

Every two weeks, while collecting leafhoppers and other candidate vector species, the plant species present in and around test vineyards were identified. The percentage abundance of each weed species, and the weed cover percentage in each site was recorded. Every six weeks plants were identified and collected from the vineyard floor. Each specimen was then inserted into a grip-lock plastic bag with a piece of absorption paper (to collect plant moisture and prevent the specimen from becoming mouldy), and labelled according to collection date, site and plant species. Plant specimens were shipped to IHD for PCR screening to determine the presence of phytoplasma.

Screening for phytoplasma

DNA extraction and nested PCR were the same as those described by Gibb et al (this report) for grapevines. Due to the large number of plants collected, up to five samples regardless of species, but from the same collection area at each vineyard, were bulked together if they were a symptomatic. DNA was extracted separately from plants that exhibited yellowing or unusual deformation that might be attributed to phytoplasma infection.

Restriction fragment length polymorphism (RFLP) analysis
Five µl of the PCR products amplified in the nested PCR were digested using the restriction enzyme *Hpa* II in the buffer supplied by the manufacturer (New England Biolabs, Beverly, MA, USA). Digestions were incubated overnight at 37°C and the fragments were separated by electrophoresis in a 1.5% agarose gel in 0.5 x TBE buffer and visualised by staining with ethidium bromide on a UV transilluminator.

RESULTS AND DISCUSSION

Nearly 40 different weed species from Vineyards A and B were identified (Table 8). Only 15% of the weed species identified were grasses, clovers or medics, the remainder were made up of broadleaf weeds. For most of the season, grasses, clovers and medics were present at low levels. No cover crops were sown.

In general, leafhopper numbers changed proportionally with the percentage of weed cover in vineyards A and B (Figures 7 and 8). The variety of weeds and the levels in Vineyard A were rarely high due to frequent herbicide applications. From September through to May paraquat and diquat (Spray Seed® was applied eight times, and Surflan® once. The range of plant species and incidence of weeds was often greater in Vineyard B where only one application of the herbicide glyphosate (Roundup® was applied in March.

Despite regular herbicide applications in Vineyard A, both vineyards experienced the same peaks and troughs in leafhopper numbers and percent weed coverage (Figures 7 and 8). The large drop in insect numbers on the 24th of December and the 23rd of January, may be associated with an extended period of moderate to high temperatures and low rainfall (Appendix 3). Alternatively, these troughs may be associated with the natural cycle of some leafhopper species. However, the dominant species' identified from the high leafhopper peaks (11/12/97 and 12/01/98) different vineyards. A substantial proportion of the insects collected from vineyard A were O. argentatus

(table 7). Vineyard B also experienced its peak in O, argentatus at this time, However, at least 65% of sap-sucking insects identified from these samples were Rutherglen bugs (table 5).

In vineyard A, the insecticide chlorpyrifos, applied for the management of light brown apple moth, appears to have had little or no impact on leafhopper populations (figure 7). This supports current recommendations in which use the insecticides to manage leafhoppers will not be prescribed until after the vector and its life cycle is known.

Vineyard A contained mainly broadleaf species until mid to late February. From mid to late February grass weed species were dominant and leafhopper numbers declined. The dominant weed species in vineyard A were turnip weed (observed throughout the season with a peak in September/ October), cape weed (present early in the season), skeleton weed (from November to mid December), and paddy melon (from late November to mid January). Medic species' were present in moderate levels only early in the season (September – October), although a peak in leafhopper numbers, predominantly *O. argentatus*, was also observed at this time.

Numbers of leafhoppers species *O. argentatus* were highest in Vineyards A and B early in mid season, when medic, cape weed, pigweed, wire weed and some turnip weed were recorded. *Z . zealandica* peaked during the middle of the season, particularly where turnip weed was high. Numbers of *Austroasca viridigisea* peaked from mid to late in the season, and were observed feeding on turnip weed and sow thistle.

There was a sharp rise in Rutherglen bug population size in sites M, N and 0 from the end of November, with an equally sharp fall in mid January (Table 4). All of these sites were located centrally within the Chardonnay block. During any other time of the season and in all other sites in vineyard B, the incidence of Rutherglen was low and infrequent.

Table 8: Identified Weed Species - Wemen and Gol Gol

Weed No	Botanical Name	Common Name
1	***Urtica urens*** L.	Stinging nettle
2	***Arctotheca calendula*** L.	Cape weed, Cape dandelion
3	***Polygonum aviculare*** L.	Wireweed, knotweed
4	***Emex australis*** Steinh.	Three cornered jack
5	***Medicago minima*** L.	Woolly burr medic
6	***Hordeum leporinum***	Barley grass, common foxtail
7	***Poa annua***	Winter grass, annual grass
8	***Malva parviflora*** L.	Small flowered mallow, marshmallow
9	***Brasicca tournefortii*** Gouan.	Wild turnip
10	***Papaver hybridum*** L.	Rough poppy, wild poppy
11	***Medicago trunculata*** Gaertn.	Barrel medic
12	***Cucumis myriocarpus*** Naudin.	Prickly paddy melon, paddy melon
13	***Vulpia myuros*** L.	Rats tail fescue
14	***Chondrilla juncea*** L.	Skeleton weed
15	***Trifolium tomentosum*** L.	Woolly clover
16	***Medicago polymorpha*** L.	Burr medic
17	***Citrullus lanatus*** Thunb.	wild melon, bitter melon
18	***Lactuca serriola*** L.	Prickly lettuce, wild lettuce, milk thistle
19	***Amaranthus*** **sp.**	
20	***Cenchrus longispinus***	Gentle annie
21	***Heliotropium europaeum*** L.	Common heliotrope
22	***Solanum nigrum*** L.	Black nightshade
23	***Sonchus oleraceus*** L.	Common sowthistle
24	***Portulaca oleracea*** L.	Common pigweed
25	***Tribulus terrestris***	Caltrop
26	***Solanum esuriale***	Tomato plant, potato plant, Quena
27	***Xanthium spinosum***	Bathurst burr
28	***Atriplex*** **sp.**	Saltbush
29	***Echinochloa cruspavonis***	Barnyard grass
30	***Digitaria ciliaris***	Summergrass
31	***Eragrostis falcata*** Gaudin	Lovegrass, sickle grass
32	***Stellaria media*** **(L.) Vill.**	Chickweed
33	***Enchylaena tomentosa*** **R. Br.**	Ruby saltbush or cotton bush
34	***Medicago sativa***	Small leaf burr medic
35	***Bassia*** **sp.**	Roly poly
36	***Fumaria*** **sp.**	Smoke weed, fumitory

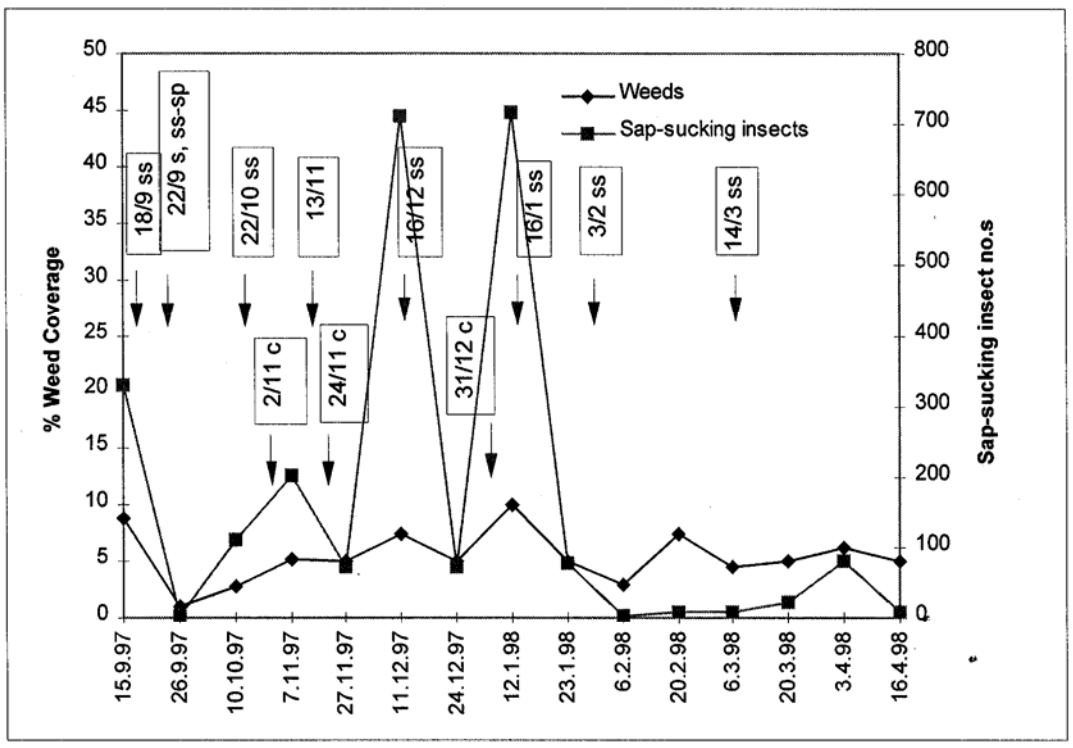


Figure 7. Sap-sucking insects numbers and weed incidence in Vineyard A

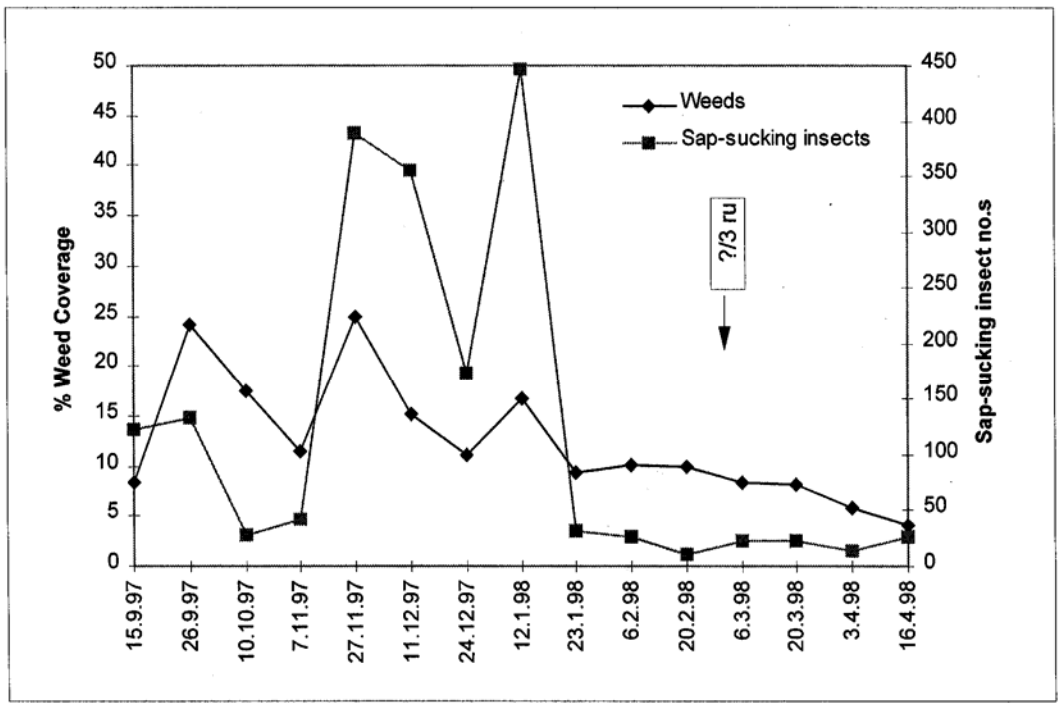


Figure 8. Sap-sucking insects numbers and weed incidence in Vineyard B

Key to Figures 7 and 8

ss = Sprayseed® ru = Roundup®

s = Surflan® c = chlorpyrifos

To see if there were any trends in the transience of insects from vineyard borders, graphs of leafhopper numbers and weed levels from edge and central sites were

plotted and compared (Figures 9 to 17).

In vineyard A, site A was located towards the centre of a chardonnay block, and sites C and D were located on opposite corners of the block (Appendix 1). Site D was closest to native scrub and a lucerne paddock, and, due to its hilltop location, was also most exposed. Grapevines were located along both edges of Site C. Site C also had the highest number of leafhoppers and weeds during the 1997/98 season, yet was furthest from native scrub and lucrence paddock. From our data, there was no obvious pattern of insect transience from one vineyard site to another, with insect levels rising and falling in unison between sites (Figures 9 to 11). The first collection samples (15/9/97) showed that insect numbers from sites C and D were similar, despite their different location with respect to pasture and native scrub.

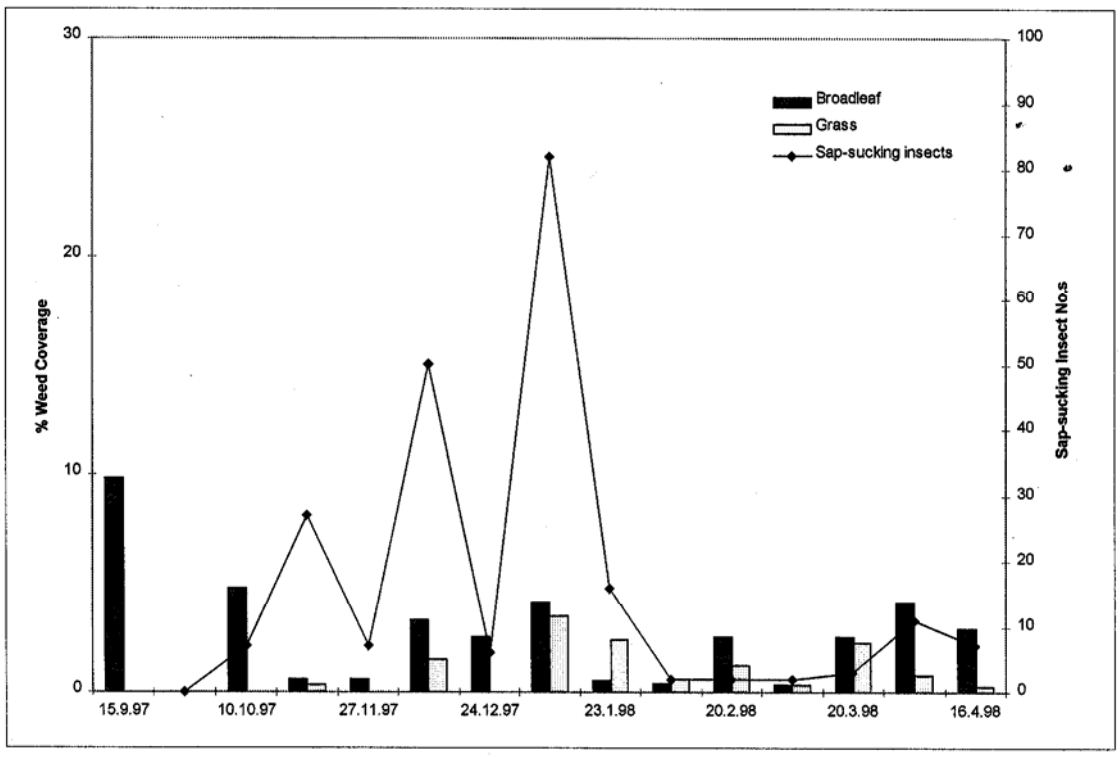


Figure 9 Site A - Sap-sucking insect numbers and incidence of broadleaf, grass and medic species

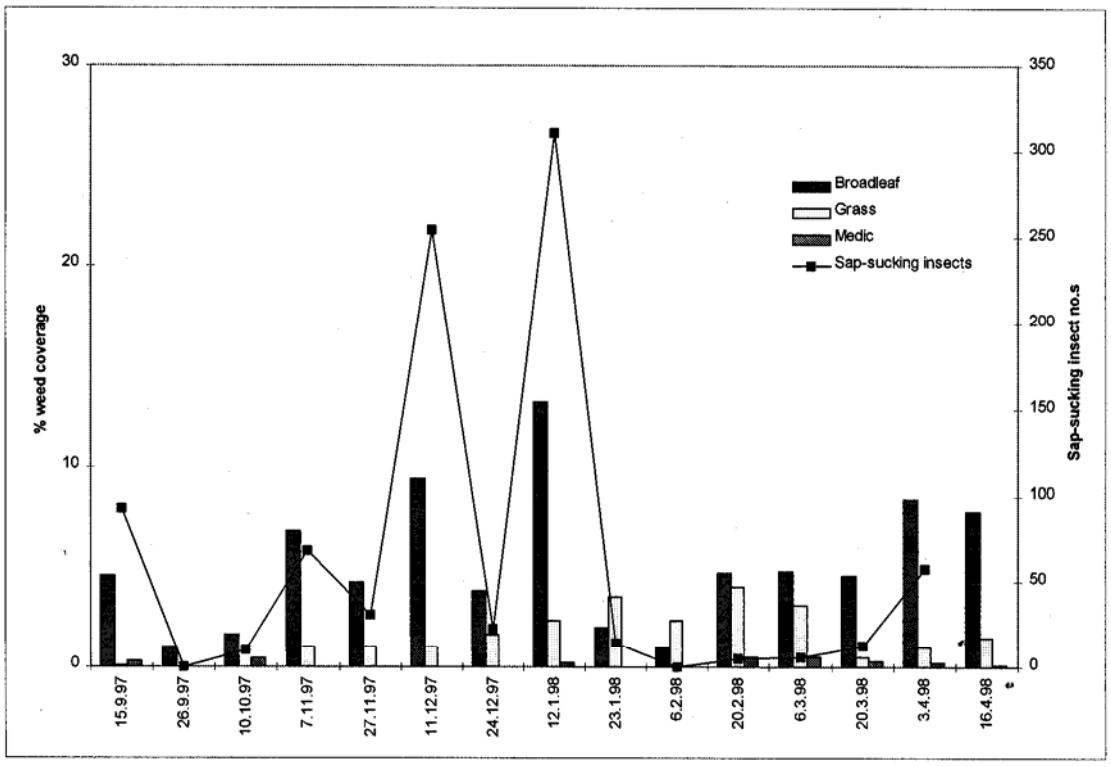


Figure 10. Site C - Sap-sucking insect numbers and incidence of broadleaf, grass and medic species

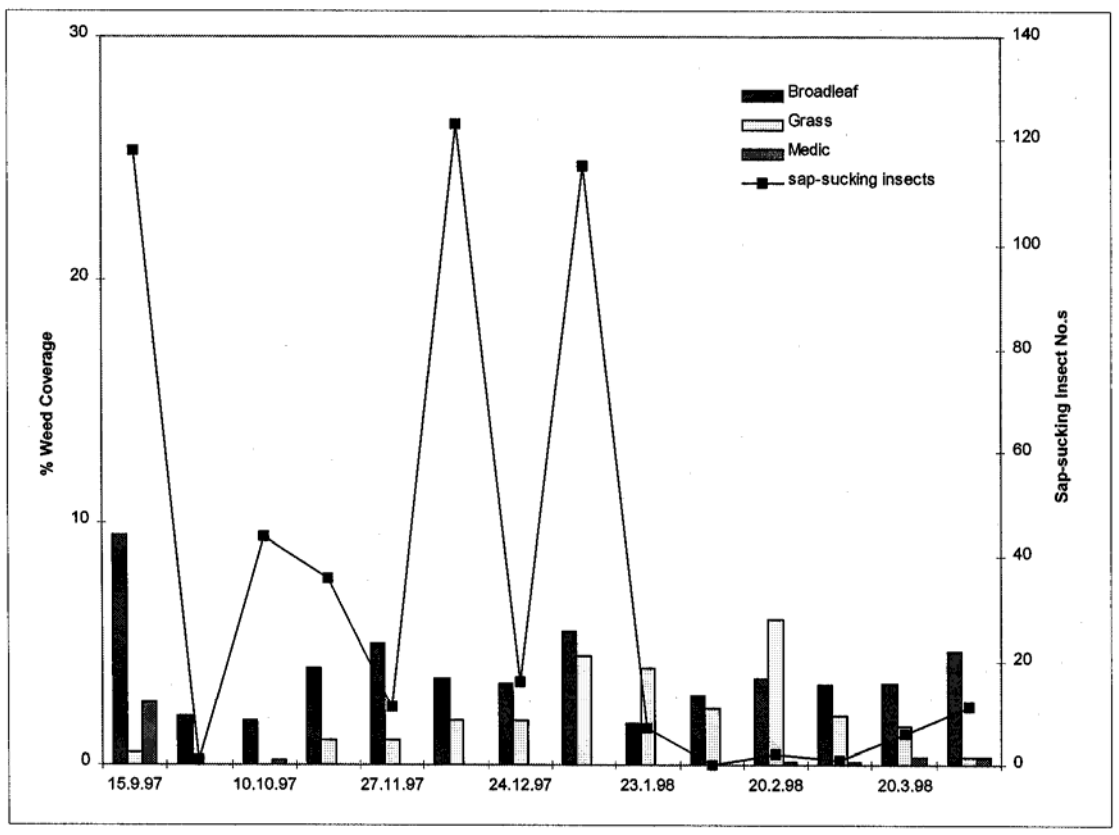


Figure 11. Site D - Sap-sucking insect numbers and incidence of broadleaf, grass and medic species

In Vineyard B, Site E was situated closest to native scrub and was also in close proximity to an open grass paddock (Appendix 2). It was also the only site from

which high numbers of sap-sucking insects were collected early in the season)Figure 12.) However, to follow any distribution of insects, more frequent collections are required. Site J, adjacent to the open grass paddock, also contained comparatively high leafhopper numbers over the season (Figure 13). The remaining central and edge sites generally followed the peaks of Site E for the remainder of the season. (Figures 14 to 17).

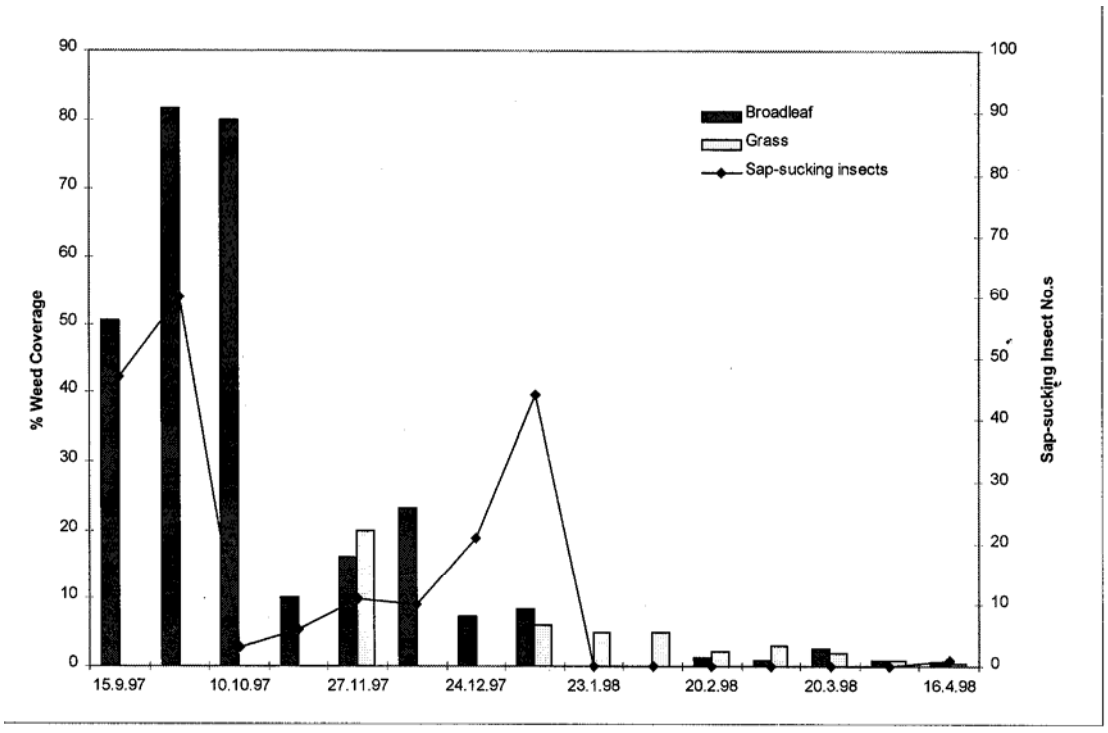


Figure 12. Site E - Sap-sucking insect numbers and incidence of broadleaf, grass and medic species

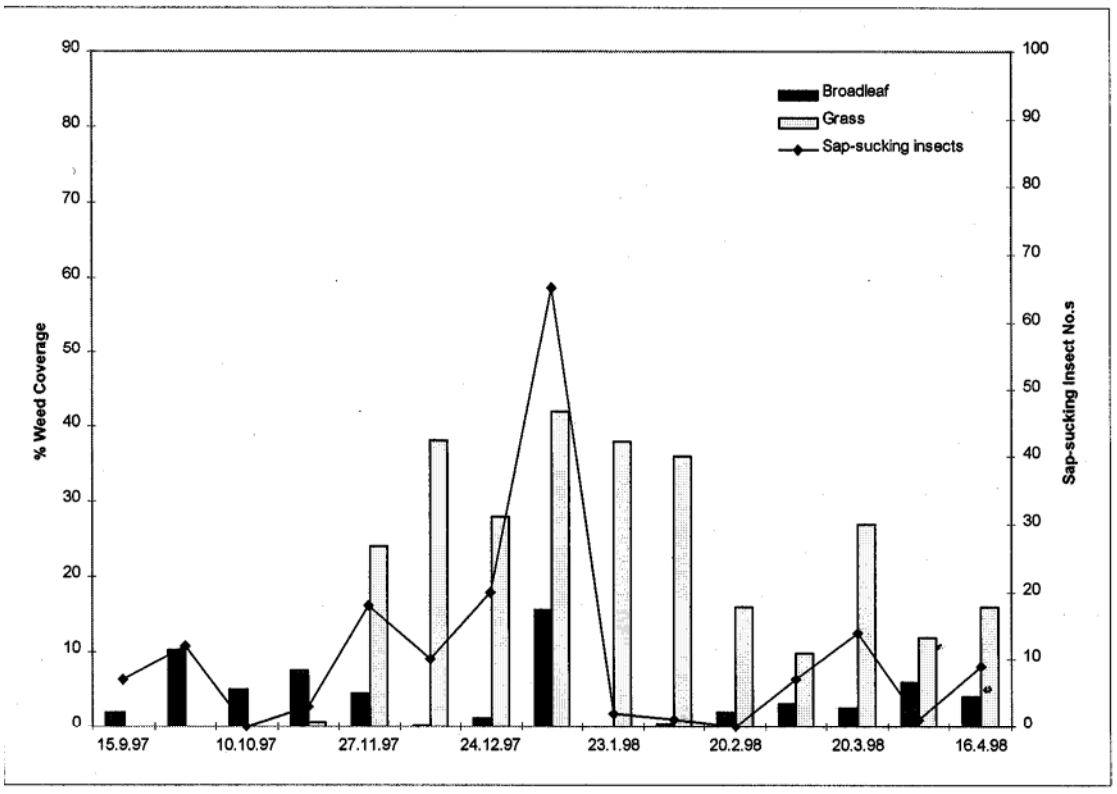


Figure 13. Site J - Sap-sucking insect numbers and incidence of broadleaf, grass and medic species

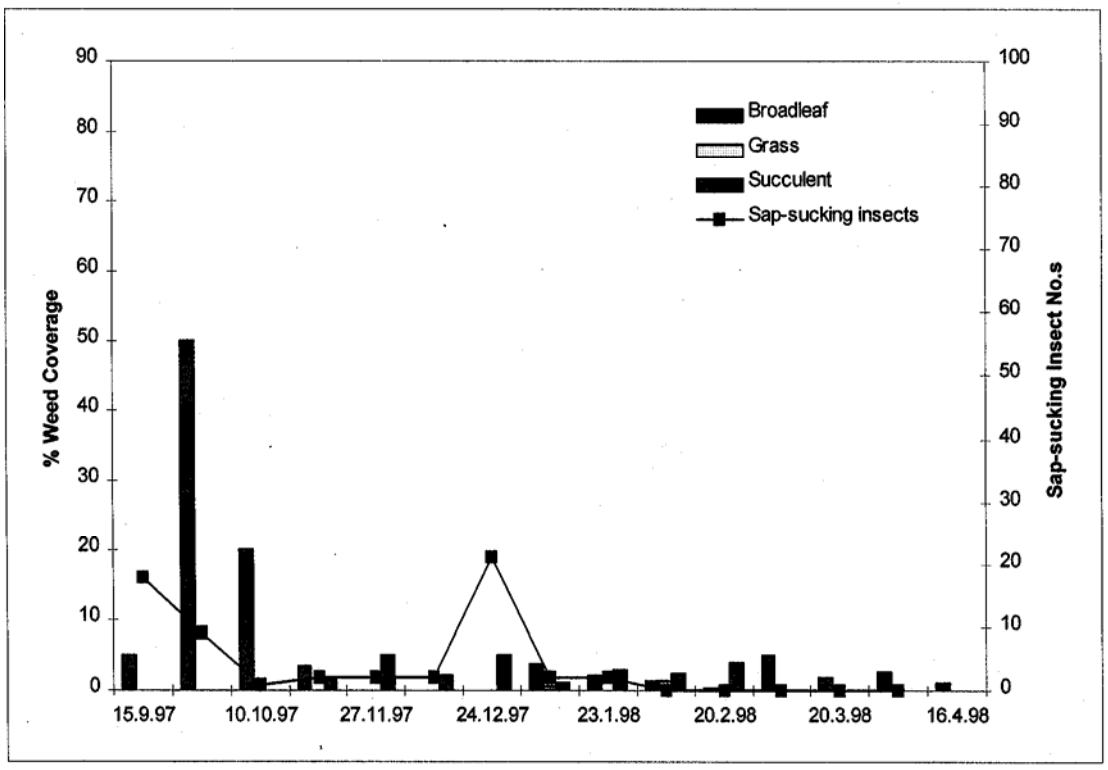


figure 14. Site G - Sap-sucking insect numbers and incidence of broadleaf, grass and medic species

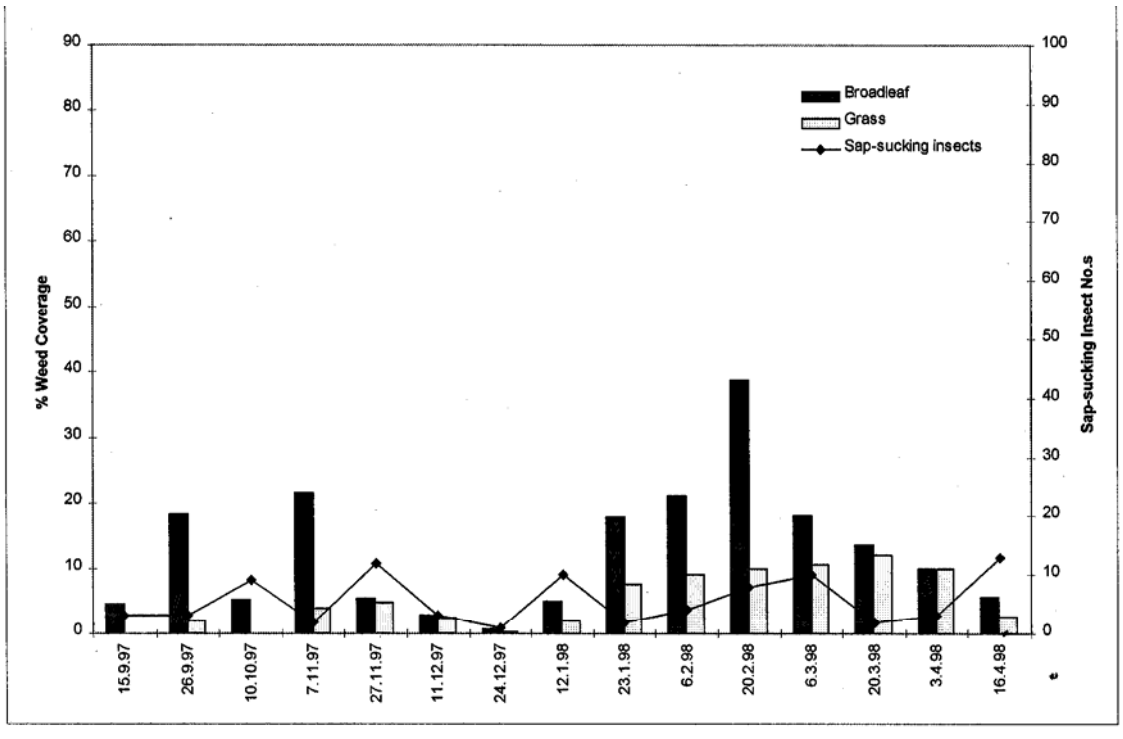


Figure 15. Site K - Sap-sucking insect numbers and incidence of broadleaf, grass and medic species

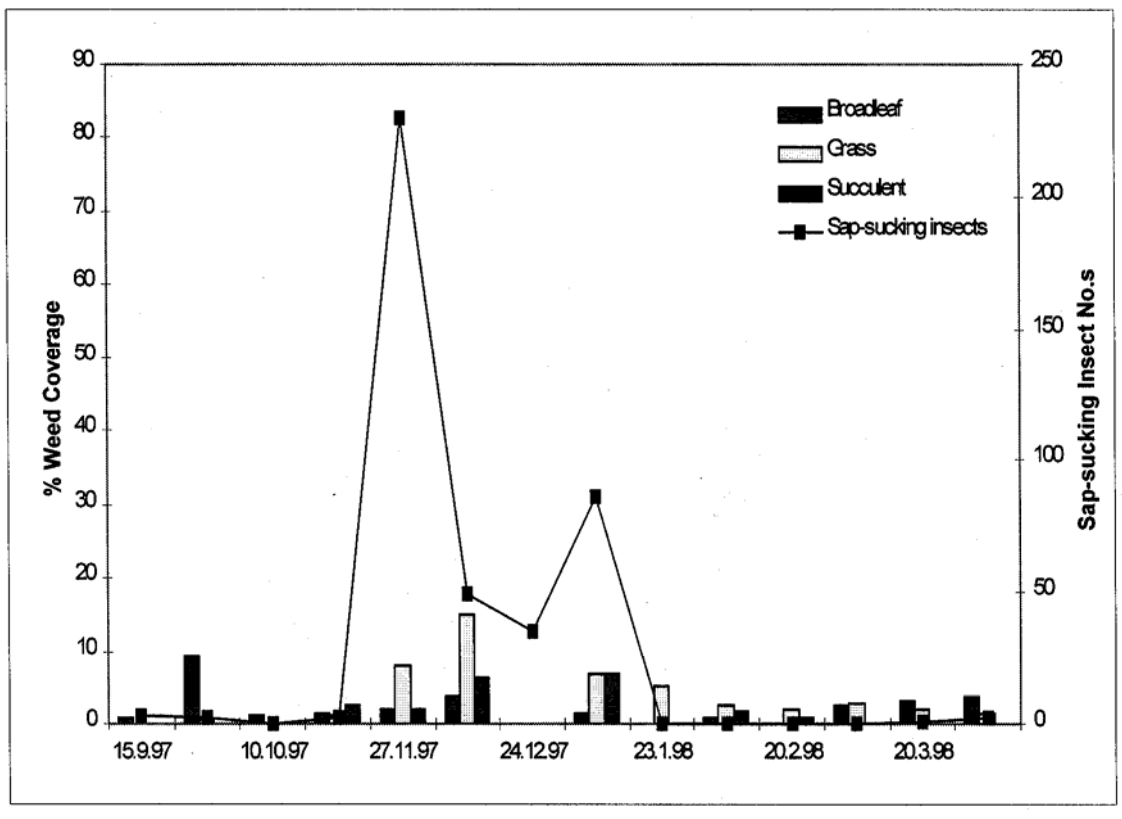


Figure 16. Site N - Sap-sucking insect numbers and incidence of broadleaf, grass and medic species

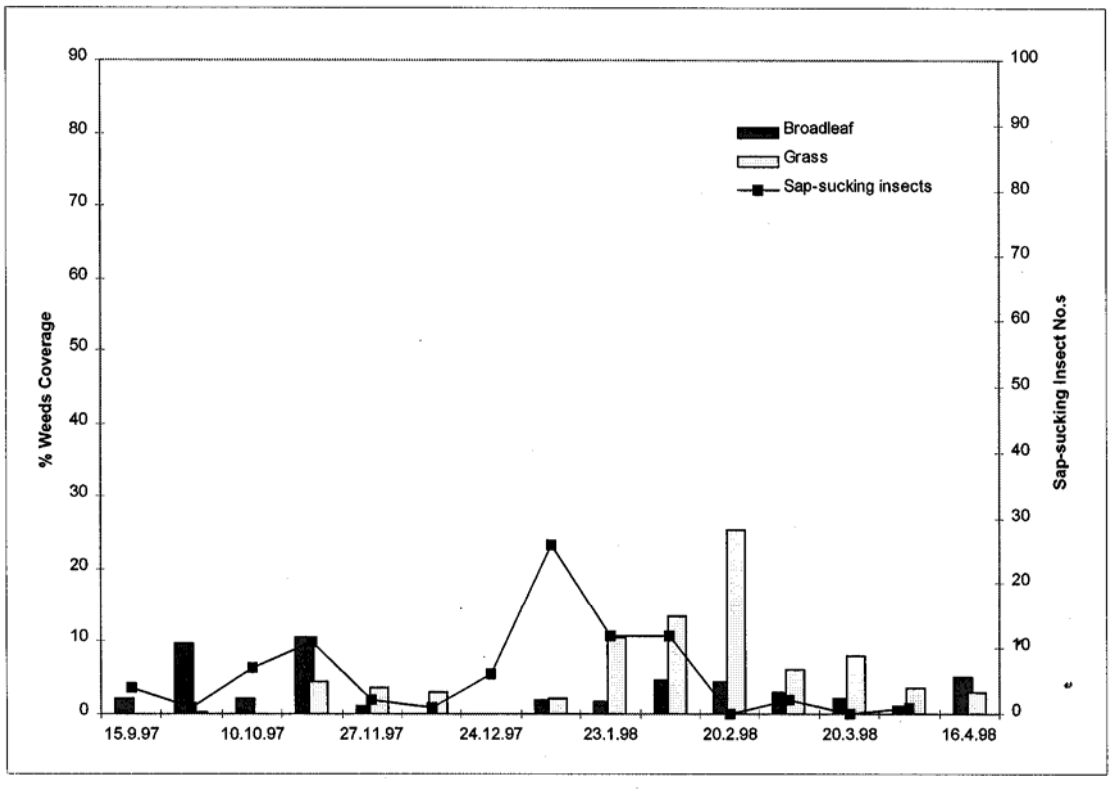


Figure 17. Site P - Sap-sucking insect numbers and incidence of broadleaf, grass and medic species

Screening by PCR

Phytoplasma was detected in several samples from both sites in samples of apparently unaffected plants and those with symptoms that may have been attributable to phytoplasma.

RFLP analysis revealed infection by TBB phytoplasma in the PCR positive samples. AGY phytoplasma was not detected.

SUMMARY

Only general assumptions can be made from the weed and leafhopper data. Overall, there appeared to be a rise and fall in leafhopper numbers with a corresponding rise and fall in weed levels, particularly in association with broadleaf weeds. More frequent insect collections and field examinations are required to determine the transience of leafhoppers.

In the meantime, we still do not know the insect vector or possible alternate hosts for the AGY phytoplasma. Control of broad leaf weeds in and around vineyards and use, of grass or cereal rather than broad leaf cover crops may help to reduce spread of AGY. However, application of insecticides to control these insects is probably of no use in the absence of information on the identity and seasonal abundance of the insect, vector. The peak periods of insect activity (October - February) that have been recorded also support the suggestion that desiccating the cover crop prior to the growing season (September) may help to reduce numbers vectors of AGY.

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4.4 a. Tomato Big Bud Trials: Culturing of *Orosius argentatus*

John MacFarlane and Fran Richardson

INTRODUCTION

Helson (1942) reared the leafhopper *Orosius argentatus* Evans on potted small- flowered mallow plants *(Malva parviflora* L.) enclosed in 7-in. lamp glasses with muslin tops. He studied the life-cycle in constant-temperature (CT) rooms with 1000- W. incandescent lights placed 2 feet above the plants. He concluded that the optimum temperature for o. *argentatus* development lay between 22°C and 28°C. Others (Harding and Teakle 1985, Moran et al. 1997) successfully reared *Orosius argentatus* on celery *(ApIum graveolens* L.) instead of mallow.

MATERIALS AND METHODS

A culture of *Orosius argentatus* was established at IHD Knoxfield in August 1998 from adults collected by sweep-netting weeds near Boort. The insects were fed on mallow in 45 x 45 x 60-cm cages with synthetic muslin sides. The cages were kept in a CT room at 22°C and 12-hrs light. o. *argentatus* breeding occurred on mallow but by December 1998, there were only a few nymphs and adults remaining on culture plants. The decline in numbers may be due to escapes through inadequately sealed cages, poor ventilation and lighting, poor condition of the potted mallow and a build- up of two-spotted mites and aphids.

Attempts were made to rectify these faults by sealing all cages, using a fan to improve air circulation, installing more "growth" fluoro tubes and removing mites and aphids from plants. By February, however, numbers were still too low to begin transmission work and the following changes were made:

- A new collection of 100 *O. argentatus* adults was obtained from weeds around IHD In mId-February. This resulted in the production of 750 nymphs for transmission experiments in March. The remainder (378) were the foundation of the present culture.

- Predatory mites *(Phytoseiulus persimilis)* were introduced to the cages and the glasshouse in early February and two fortnightly follow-up releases were made.
  This introduction considerably reduced two-spotted mite numbers and improved the vigour of the mallow. Later releases of predators were made in July, August -and November.

- The longevity of the mallow was also improved by using a clay loam soil in the pots. Potting mix was used only for the top few cms.

- Potted celery was provided as an additional food in -all cages.

- Two Philips 400W Son- T Agro high-pressure sodium lamps were installed at ceiling level in the CT room.

- Clear perspex tops, white "Contact" bottoms and new synthetic muslin sides were put on all cages in order to increase the light intensity to which plants and insects were exposed.

- The temperature in the CT room was raised to 25°C and the day length to 16 hrs light including a 1 hour "dusk" period of low light intensity.

- A release of the common brown lacewing, *Micromus tasmaniae,* was made in March to control aphids although predators did not establish. A culture of the aphid parasite *Aphidius* sp. was begun in May and this provided large numbers of wasps by November. Wasps and another release of lacewings in November provided improved control of aphids but did not eliminate them.

RESULTS AND DISCUSSION

Table 1. Starting dates and stages of introduced *O. argentatus* and the number of offspring they produced.

Cage #	Start Date	End Date	Insect No.	Stage	Source	Offspring
13	3-23/2/99	17-May	100	adults	field (IHD)	1128
4	1-4/3/99	15-Jul	68	nymphs	cage 13	270
5	4-18/3/99	3-Jun	181	nymphs	cage 13	27
10	13-30/4/99	11-Oct	181	nymphs	cage 13	51
1	18-26/5/99	11-Oct	97	Young adults	cage 4	146
8	1-8/6/99	27-Sep	101	Young adults	cage 4	157
13	12-21/7/99	8-Nov	47	Young adults	cages 4,10 & 1	131
14	5-6/8/99	8-Nov	53	nymphs	cages 8, 10 & 1	351
5	6-12/8/99	8-Nov	111	Young adults	cages 8, 10 & 1	48
11	8-10/9/99	29-Nov	90	Young adults	cages 13, 1 & 8	589
9	4/10/99	4-Jan	147	Young adults	cages 14 & 5	280
2	16-29/11/99	21-Feb	154	Young adults	cage 11	145
5	17-23/11/99	7-Feb	232	Young adults	cages 11 & 9	44
8	6-29/12/99	21-Feb	190	Young adults	cage 9	45
11	4-17/1/00	27-Mar	231	Young adults	cages 9, 2 & 5	486

Following the introduction of new culturing methods in February, a continuous *Orosius* colony was established. A tenfold production of offspring was achieved from the field-collected adults but this was not repeated in later generations. The numbers produced in were quite variable (Table 1). The best result was the production of 589 offspring from the introduction of 90 adults in to cages in September.

The main reason for the variability in the production of offspring appears to be the sporadic presence of aphids and the occurrence of thrips *(Thrips imaginis)* from November onwards. Large numbers of aphids reduced the quality of the mallow and celery food plants in the first half of the year and biological control agents introduced since were only been partly successful. Regular upsurges in aphid numbers led to the abandonment of several insect culture cages before the production of offspring. Thrips reduced the quality of the celery in particular since their onset in November. More success was obtained with biological control of two-spotted mite. Mite numbers were reduced to a low and consistent level by the regular introduction of predatory mites.

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4.4b Tomato Big Bud Trials: Handling and storage factors influencing the detection of Tomato Big Bud phytoplasma in the leafhopper *Orosius argentatus.*

John MacFarlane, Fran Richardson and Rebecca Mills

INTRODUCTION

A study of the effect of handling and storage factors on the preservation of phytoplasma in leafhoppers was undertaken as part of a project to identify the vector of the Australian Grapevine Yellows phytoplasma. For this project, various phloem feeding insects, especially leafhoppers and planthoppers, were collected in and around vineyards in North-western Victoria by vacuum sampler. They were sorted, frozen and then dispatched by courier to Melbourne for storage and the eventual analysis of the phytoplasma DNA using the PCR method.

In recent studies monitoring the incidence of phytoplasma in field populations of leafhoppers or planthoppers, captured insects have been stored at -20°C until DNA extraction (Goodwin et al. 1999; Weber and Maixner 1998). It has been suggested that while short-term (1 month) storage of insects at -20°C does not inhibit amplification of phytoplasma DNA, an inhibitory effect develops during long-term storage and degradation of phytoplasma DNA occurs (Marzachi et al. 1998; Vega et al. 1993).

Due to the large number of samples obtained in our work, it was necessary to store the samples for up to several months before DNA could be extracted from. The situation was further complicated by the transport of captured insects several hundred kilometres before storage and eventual DNA extraction. Because of these circumstances and the low percentage of insects found to contain phytoplasma (AGY or TBB), it was important to test the effect of the various handling and storage methods on the preservation of the phytoplasma within the sampled insects. *Orosius argentatus* was chosen as the test leafhopper, as it is the only insect found to carry the AGY phytoplasma. Because It has not been possible to maintain AGY -infected vines in the glasshouse, Tomato Big Bud TBB) phytoplasma was studied instead. It is one of several phytoplasmas known to be transmitted by O. *argentatus* (Grylls,1979).

MATERIALS AND METHODS

Production of infected leafhoppers

A colony of *O. argentatus* was established in February 1999 from field-collected leafhoppers. They were reared at 25°C on mallow *(Malva parviflora* L.) and celery *(Apium graveolens* L.). To produce phytoplasma-infected leafhoppers, young adult leafhoppers were caged on TBB-infected periwinkle *(Catharanthus roseus* L.) at 25°C for two days. They were then placed in individual microcentrifuge tubes prior to a range of treatments.

Handling and Storage Treatments
Three trials of various temperature and time regimes to which test insects were subjected.

Experiment 1.

1. Storage of tubes at -20°C and -75°C for 1 week, and 3 months.
2. 2. 20°C, thaw (each for one hour), then -20°C for 1 week
3. 3. 75°C, thaw (each for one hour), then -75°C for 1 week
4. 20°C, thaw, -20°C, thaw (each for one hour), then -20°C for 1 week
5. 5. 75°C, thaw, -75°C, thaw (each for one hour), then -75°C for 1 week

Experiment 2

1. Tubes held in an esky with cooler brick for 5 days (data logger showed first 15 hrs @ +12°C, next 15 hrs @ 12-20°C, rest of period @ +20°C.)
2. + 20°C for 5 days
3. -20°C for 5 days

Experiment 3Tubes held at -20°C for one week until extracted
2. 20°C for 6 hrs, thaw for 18 hrs, then -20°C for one week until extracted
3. 20°C for 6hrs, thaw for 18 hrs, -20°C for 6 hrs, thaw for 18 hrs, then -20°C for one week until extracted
4. +20°C for 2 days, then -20°C for one week until extracted

DNA Extraction & PCR
Following treatment, DNA was extracted from the insects using a modified Goodwin et al. (1994) method and then PCR analysed using a modified procedure (Gibb et al. 1996)

(See PCR Techniques Section).

RESULTS AND DISCUSSION

Table 3. PCR results following handling and storage treatments

Date	TBB source	Stage	Treatment Time	Treat. No	Treatment	No. Reps	PCR Result	PCR Result
Experiment 1							Extracted 18,19 Oct	Extracted 10 Jan
11/10/99	Old periwinkle	Young adult	13-Oct				No. of PCR positives	No. of PCR positives
	2 day acquisition period)		Treatments:	1	-75C	10	5	
			1hr for each step	2	-75C, thaw,-75C	10	7	
	CTAB DNA extraction		1hr for each step	3	-75C, thaw, -75C, thaw, -75C	10	5	
				4	-20C	10	10	
			1hr for each step	5	-20C ,thaw, -20C	10	7	
			1hr for each step	6	-20C, thaw, -20C, thaw, -20C	10	6	
				7a	-75C for 3 months	10		3
				7b	-75C for 6 months	10		
				8a	-20C for 3 months	10		4
				8b	-20C for 6 months	10		
Experiment 2							Extracted 25 Oct	Extracted 26 Oct
18/10/99	Old periwinkle	Young adult	20-Oct				PCR	PCR
	2 day acquisition period)		Treatments:					
				12	Held in esky 5 days	20	1	
	CTAB DNA extraction			13	20C 5 days	20	5	
				14	-20C for 5 days	20		6
Experiment 3							Extracted 15-18 Nov	
8/11/99	Young periwinkle	Young adult	10-Nov				PCR	
	(2 day acquisition period)		Treatments:	16	-20C	25	0	
			6h, (thaw) 18h	17	-20C, thaw, -20C	25	0	
	CTAB DNA extraction.		6h,18h,6h, 18h	18	-20C, thaw, -20C, thaw, -20C	25	0	
				19	20C for 2 days, -20C	25	0	

Experiment 1

The proportion of PCR positives was the highest in the -20C treatment with no thawing. -However, because the -75 treatment, with no thawing, had the lowest proportion of PCR positives (50%), it is not possible to determine the role of thawing in the degradation of DNA. In addition, the number of leafhoppers available for the trials was too small to distinguish between treatment effects on the DNA and the inherent variability in the rate of phytoplasma acquisition by leafhoppers. In another trial, the proportion of leafhoppers that tested positive, immediately after an acquisition access period and incubation period, was only 39%. Treatments 1 through 6 all showed a higher proportion of positives than 39%.

The low proportion of PCR positives, in the long storage treatments before the extraction step (mean 35%) compared to the treatments with no storage period (mean 75%), suggests that long storage periods may result in DNA degradation. There may be enzymes present in the insect body that can degrade DNA once the insect has died. Marzachi et al. (1998) observed a degradation in DNA stored for more than 1 month at - 120°C.

Experiment 2

Only 1 out of the 20 leafhoppers stored in the esky on a frozen brick tested positive for the DNA. The proportion of positives from this treatment is much lower than the 25% positive in the 20C treatment, which suggests that thawing and subsequent storage at room temperature may have been more deleterious to the quality of the DNA than a high storage temperature alone.

Experiment 3

None of the leafhoppers including control leafhoppers (exp. 16) tested negative, suggesting a problem with phytoplasma acquisition from the periwinkle or a problem with the PCR procedure. However, the positive controls in the PCR procedure did amplify and test positive, suggesting that the PCR worked properly. Young periwinkle appears to be a poor phytoplasma acquisition host for O. *argentatus.*

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4.4.c Transmission of TBB from tomato to grapevine

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INTRODUCTION

According to Gibb (et al. 1999) tomato big bud (TBB) phytoplasma has been detected in grapevines. The vector of TBB, O. *argentatus,* is commonly found in and around vineyards and have been captured and found to be positive for the TBB phytoplasma using PCR analysis. Evidence suggests that this leafhopper may transmit TBB phytoplasma to grapevines. Transmission trials were performed to determine if *O. argentatus* can transmit TBB phytoplasma to grapevine.

MATERIALS AND METHODS

Orosius argentatus colonies were housed in 45 x 45 x 60-cm cages with synthetic muslin sides. The cages were kept in a CT room at 25°C and 16 ills light. Young adults and nymphs were a two-day acquisition access period on cuttings from a TBB- infected tomato plant from Kerang. After the AAP mallow and celery were provided. A potted grape (indicator LN33) was introduced after 2-3 weeks and left in the cage until leaves were PCR analysed for phytoplasma at 8-10 weeks.

RESULTS AND DISCUSSION

Tomato big bud was successfully transmitted from infected tomatoes to uninfected tomato and celery. However, none of the test grapevines tested positive for the TBB phytoplasma. The inability to detect phytoplasma in test grapevines may indicate that *O. argentatus* can not transmit TBB to grapevines. However, the latent period of TBB in grapevine may not have been satisfied and the phytoplasma titer was not sufficiently high to be detectable. In a European grapevine yellows, the latent period is least five months and may be much longer (Osler et al. 1993). If this were the case, the vines would likely test positive at some period in the futures.

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4.5a Establishing colonies of leafhopper and planthopper species found in Sunraysia vineyards

LeAnn Beanland, Linda Pollock and Michelle Kelly

INTRODUCTION

Laboratory rearing of potential vectors is not only a useful first step in establishing whether an insect is a vector, but it can also provide important information for developing field management strategies when the vector is identified. One can determine which plants are suitable for both feeding by adults and development of nymphs. The life cycle of each species including the development and duration of each life stage -eggs, nymphs and adults can also be investigated. Most importantly, cultured leafhoppers can be used in transmission experiments to establish their potential to transmit the Australian Grapevine Yellows (AGY) phytoplasma.

The first step in determining the vector status of leafhopper and planthopper species is to have a consistent supply of ideally phytoplasma-free insects. In order to have access to enough test insects, colonies must be established. In the absence of another source of leafhoppers (another colony), feral insects must be captured. Field data collected since 1996, gave clues as to the species available and where and how to collect them. Vacuum collections were determined to be the most appropriate method for capturing candidate insect species.

Of particular interest were those known plant pathogen vectors captured at study sites. These include:

Family Cicadellidae

Subfamily Deltocephalinae
Balclutha saltuella -This species transmits cereal virus (Knight 1986).
Exitianus nanus -Another *Exitianus* species transmits maize chlorotic dwarf virus, which suggests that other congeners may also transmit plant pathogens.
Limotettix insertus- This species is a phloem feeder (Purcell 1985). According to Day (et al. 1952) because of the feeding behaviour of this insect, it is likely to be an efficient vector of phloem limited pathogens.
Orosius argentatus -This leafhopper is the vector of the Tomato Big Bud (TBB) phytoplasma and is the only field collected insect that has tested positive for the AGY phytoplasma using PCR analysis.
Nesoclutha pallida -This species is a vector of the cereal chlorotic mottle virus (Greber 1977), but may not be a phloem feeder (Purcell 1985) or is only an occasional phloem feeder. A phloem feeding habit is a prerequisite for a leafhopper to transmit phytoplasma. *Recilia hospes -R. hospes* is a vector of rice dwarf virus.

Subfamily Agallinae . *Austroagallia torrida* -This insect is a vector of rugose leaf curl virus in *Malva* (Gryllis 1979) and cereal chlorotic mottle virus (Greber 1977).

Family Cixiidae

Oliaris atkinsoni is a vector of the *Phormium* yellows phytoplasma in New Zealand. Other Cixiid species are vectors of certain European Grapevine Yellows.

MATERIALS AND METHODS

Capture of Insects

Leafhoppers were collected using vacuum sampling in and around a test vineyard in Gol Gol (NSW). In the field, captured insects were transferred to a large aluminium framed cage with an aluminium floor and organdy fabric covered walls. A sleeve covered the front of the cage, which allowed access and prevented escape of captured insects. The cage was brought to the laboratory where the leafhoppers were separated by species into smaller aluminium cages of similar construction.

Colony Maintenance

All colonies were kept in a constant temperature room at 25°C. Cages housing the insects were placed on shelves under grow lights on a 16:8h L:D schedule. Plants in cages were watered every day and new ones were added to cages as old plants declined. However, plants were left in cages until they died to ensure that developing leafhoppers could enclose before host plants are discarded. Humidifiers were used in colony rooms when the relative humidity drops below 65%.

Aphids, mites and thrips have been detected in all colony cages. Naturally occurring parasitoid wasps of aphids have reduced numbers of these pests to non-damaging levels. Predatory mites *(Phytoseiulus persimilis)* (purchased from Hawkesbury IPM, NSW) were introduced to cages fortnightly to manage the two spotted spider mite. Yellow sticky traps (purchased from Biological Services, Loxton, SA) were placed around colony cages to trap thrips and escaped leafhoppers.

RESULTS AND DISCUSSION

An *O. argentatus* (Super Family Cicadelloidea; Family Cicadellidae) colony was established in March 1999. Because O. *argentatus* will reproduce and feed upon mallow *(Malva)* Osmelak et al. (1989), they were placed in a cage with mallow as well as oats

(Avena sativa) celery *(Apium graveolens* L.) (Harding and Teakle 1985), tomato *(Lycopersicon esculentum* Mill) and faba beans. Cages were routinely inspected for the presence of nymphs and the plants upon which they were found was noted. *Malva* and faba beans are efficient developmental hosts for *O. argentatus.*

The first colony of *A. viridigrisea* (Super Family Cicadelloidea; Family Cicadellidae) was established in April 1999. Plants found to be efficient developmental hosts for the colony were faba bean, tomato (Page 1983) and celery.

In early May 2000, captured *Exitanus nanus* (Super Family Cicadelloidea; Family Cicadellidae) were brought back to the laboratory and introduced into cages to which oats, faba beans, strawberry and celery had been added. Feeding and developmental hosts I have not been determined. No nymphs as yet have been observed

In early May 2000 captured *R. hospes* (Super Family Cicadelloidea; Family Cicadellidae) were brought back to the lab and introduced into cages to which oats, faba beans, strawberry and celery had been added. Feeding and developmental hosts have not been determined. No nymphs as yet have been observed.

A colony of *Zygina zealandica* (Super Family Cicadelloidea; Family Cicadellidae) was established in April 1999. This abundant insect develops efficiently on oats and celery.

A colony of *Balclutha saltuella* (Super Family Cicadelloidea; Family Cicadellidae) was initiated in February 2000. Because this leafhopper prefers Graminae host plants (Knight 1986), oats were placed in cages with this colony. Large numbers of this species have not been produced and few nymphs have been observed.

A colony of *Austrogallia torrida* (Super Family Cicadelloidea; Family Cicadellidae) was established in January 2000. Oats and lucerne (Gryllis 1979) are used as feeding and developmental hosts for this species. Nymphs have been produced in consistent if not large numbers.

A colony of *Nesoclutha pallida* (Super Family Cicadelloidea; Family Cicadellidae) was established in May 1999. Because this insect is a grass feeder (Greber 1977), oats are used as feeding and developmental hosts for these colonies. Large numbers of this species have been produced.

A colony of a local *Oliaris,* for which a species name has not been assigned, (Super Family Fulgoroidea; Family Cixiidae) was initiated in May 2000. At that time sticky trap captures indicated that adults were abundant in the field. Several individuals were collected from the foliage of Cooba wattles by shaking the stems over a bed sheet placed under the trees. Planthoppers that fell on to the sheet were collected with an aspirator. Captured insects were brought back to the lab and introduced into cages to which oats, faba beans, strawberry and celery were added. Feeding and developmental hosts have not been determined. Sforza et al. 1999 successfully reared *Hyalesthes obsoletus,* another Cixiid and vector of a European grapevine yellows, on lavender. The survival of this colony is tentative until an appropriate developmental host has been found.

Several *Toya* (species unknown) (Super Family Fulgoroidea; Family Delphacidae) have been captured in vacuum samples. These insects have been placed in a cage to which oats, celery, clover, and tomato plants have been added. Appropriate feeding and developmental hosts have not yet been determined. The survival of this colony is tentative until all appropriate developmental host has been found.

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4.5b Experimental Transmission of AGY Phytoplasma by Test Leafhoppers

LeAnn Beanland, Linda Pollock and Michele Kelly

INTRODUCTION

Field collections and laboratory assays of captured insects have indicated that one species, *Orosius argentatus,* can harbour the AGY phytoplasma. Unfortunately, PCR analysis can only indicate that an insect has phytoplasma particles somewhere in its body or on its mouthparts. Non-vector leafhoppers that have recently fed upon a phytoplasma-infected plant may ingest phytoplasma particles and, if analysed, test positive for the pathogen (Purcell et al. 1981). In order to determine the vector status of insect candidates, transmission trials must be performed. Transmission studies involve confining candidate insects on a phytoplasma-infected plant for an acquisition access period (AAP). After the AAP, test insects must be given a latent period during which the phytoplasma infects and multiplies in the cells of the midgut. Phytoplasma particles move from the midgut to the hemolymph (insect blood) through which they are carried to the salivary glands. Phytoplasma particles enter and multiply in the cells of the salivary glands and afterward can be expelled in to a host plant when the leafhopper feeds. The latent period is typically 3 to 4 weeks in length. After the latent period has been satisfied, insects are inoculative and can transmit phytoplasma for the duration of their lives.

Objective: To determine which, if any, leafhopper species of which we have established colonies can acquire and transmit AGY phytoplasma from infected to uninfected plants.

MATERIALS AND METHODS

Test insect species included in transmission experiments were 1) *Orosius argentatus,* 2) *Austroasca viridigrisea,* 3) *Zygina zealandica,* 4) *Nesoclutha pallid* a and 5) *Austrogallia torrida.*

Testing survival of leafhopper species on grapevines

Preliminary experiments indicated that *O. argentatus,* A. *viridigrisea.* A. *zealandica,* N *pallid* a and B. *saltuella* could survive on grapevines at least 9 days. Although field surveys suggest that no leafhopper or planthopper species uses grapevine as a developmental host, survival on grapevines suggest that test species may feed on grapevine and potentially transmit pathogens while doing so.

Preliminary Transmission Studies

In March of 1999 two groups of approximately 30 individuals of the species *O. argentatus* were placed on AGY symptomatic shoots cut from Chardonnay vines at the SHC experimental vineyard and placed in flasks of water. A third group was placed in sleeves attached to intact symptomatic shoots on vines in the same vineyard. Test insects were allowed an acquisition access period (AAP) of two days. After the AAP test leafhoppers were given tomato plants upon which to feed for an estimated latent period. After approximately three weeks, dead leafhoppers were collected and sent to the Institute for Horticultural Development (IHD) in Knoxfield, for PCR

analysis to determine their AGY phytoplasma infection status. Living leafhoppers were allowed an extended latent period. All remaining leafhoppers were collected six weeks later and transported to IHD for PCR testing. A total of 48 O. *argentatus* were tested. Two groups of *Eurymela. Distincta,* a member of the closely related Eurymelldae family were also bio assayed. One group was given an AAP on cut vines and the second placed on intact vines (Chardonnay block at SHC). A total of 18 *E. distincta* were tested.

Transmission Studies

Transmission trials recommenced in January 2000. According to Gibb et al. (1999) phytoplasma is more easily detectable in January and February and enhanced detection may indicate a higher phytoplasma titer in the vines. Groups of 10 to 30 (depending on availability) test insects were introduced by species into small plastic cages enclosing a symptomatic leaf on a Chardonnay vine at SHC. Wet cotton wool was added to the end of each clip cage to prevent desiccation of test leafhoppers. As an extra precaution against escape, organdy sleeves were slipped over grapevine shoots upon which the clip cages were attached. Only symptomatic leaves were enclosed in sleeves so that escaped leafhoppers would feed on infected material only. The ends of the sleeves were secured with twist ties. Enclosed shoots were arranged so that they were in the shade of grapevine canopy and secured in position with twist ties. Test insects were originally allowed a 72-hour acquisition access period but mortality of test leafhoppers was unacceptably high. As a result, a 48-hour acquisition access period (AAP) was used for most trials. For each of the two acquisition access days, sleeves were monitored and a high humidity maintained by spraying water into the cages through the sleeves.

After the AAP, the grapevine leaf upon which the test insects fed was recovered and shipped to IHD for PCR analysis to insure infection by the AGY phytoplasma. Test insects of each species were collected from sleeves and clip cages and placed as a group on plants in tube cages for a latent period. O. *argentatus* and *A. viridigrisea* were placed on faba or garden beans, *A. torrida* was placed on lucerne, oats or celery, *N. pallid a* was placed on oats, and Z. *zealandica* was placed on oats or celery. After a 2 to 4 week period, surviving test insects were collected and each species was divided so that equal numbers were placed on papaya, strawberry, faba or garden bean, *Coprosma robusta,* grapevines, tomato or celery for an inoculation access period (lAP). The lAP was 3 days to 2 weeks in duration. Strawberry, papaya, garden bean, C. *robusta* and grapevines were chosen because they are all putative hosts of the AGY phytoplasma (Andersen et al. 1998; Leifting et al. 1998; Padovan et al. 1998; Schneider et a11999; Ross Beever pers. com.). Celery was chosen as a test plant because it is a host of the aster yellows phytoplasma and may also serve as a host of the AGY phytoplasma (Beanland et al. 2000). Tomato plants were used because they are a host of the Tomato Big Bud phytoplasma and may also be a host of the AGY phytoplasma (Osmelak 1987). After the first IAP test insects were transferred to a host of the same type for another 3 to 14-day lAP. Transfers continued until leafhoppers died. Recovered dead insects were placed in labelled cryovials and shipped on liquid nitrogen to IHD for PCR analysis. After the insects were transferred to a new host plant, the previous inoculation access test plants were placed in a glasshouse room where they were observed for symptoms of phytoplasma infection.

Leaf samples of symptomatic plants where sent to IHD for PCR analysis for determination of infection by AGY phytoplasma.

In tota1370 *Orosius argentatus,* 111 *Austroasca viridigrisea,* 126 *Austrogallia torrid a,* 68 *Nesoclutha pallid a,* and 45 *Zygina zealandica* were used in experiments, although many did not survive the AAP or the latent period.

TABLE 1. The number of each type of transmission test plant used for each species of leafhopper. In many cases more than one leafhopper was placed on any test plant.

Test Plant Type	*O.argentatus*	*A. viridgrisea*	*A. zealandica*	*A. torrida*	*N. pallida*
Grapevine	21	8	7	5	5
Strawberry	17	4	4	3	4
Papaya	13	2	2	4	1
Tomato	11	2	1	2	0
Celery	14	3	6	3	1
Beans	31	4	0	2	0
Coprosma robusta	11	2	0	0	0
Lettuce	5	0	3	0	0

Test plants were labelled, placed in a glasshouse and observed for expression of symptoms of phytoplasma. However, test grapevines were transferred to an insect proof hoop house so that they will experience a dormant period, which may be a prerequisite for symptom expression. If any test plant appeared to have symptoms of phytoplasma infection, leaf samples were collected from these plants and sent to IHD for PCR analysis.

Membrane Feeding

After a two-day AAP on symptomatic grapevines five leafhoppers of the species O. *argentatus* and two leafhoppers of the species *A. torrida* were tested in membrane feeding trials. 20ul of 5% sucrose TE solution was placed in the wells of microcentrfuge tubes caps that were then covered with a 1cm2 piece of parafilm. Test leafhoppers were individually placed in micro centrifuge tubes into the sides of each a small hole had been made. The filled and covered cap was placed on the microcentrifuge tube containing the single leafhoppers. Leafhoppers were allowed a 2 day lAP during which they could feed through the parafilm on the solution. Microcentrifuge tubes were places in a tube rack and positioned so that the tube caps faced a bright light source. Because leafhoppers are attracted to lights, it was hoped that orienting the cap end of the tube close to lights would increase the likelihood of test leafhoppers approaching and feeding on the TE solution. Inoculative leafhoppers would secrete saliva containing AGY phytoplasma particles into the test solution that could be detected by analysing the test solution by PCR. After the lAP, leafhoppers were returned to test plants for continued bioassays. The TE sucrose solution, upon which the test insects fed, was recovered from each tube and tested by PCR for the presence of phytoplasma.

RESULTS AND DISCUSSION

Preliminary transmission studies

Of the 48 *O. argentatus* tested from preliminary transmission trials, only two were found to be positive for the Tomato Big Bud phytoplasma by PCR analysis. Two of the 18 *Eurymela distincta* tested positive for another unidentified phytoplasma.

Transmission studies

Eighty-six *O. argentatus* have been recovered and sent to IHD for PCR analysis. Five bulked samples of 3 leafhoppers each tested positive for the TBB phytoplasma, but none tested positive for the AGY phytoplasma. None of the 35 *A. viridigrisea,* the 15 Z. *zealandica,* the 23 *A. torrida* or the 19 *N. pallid a* from transmission trials analysed have tested positive for AGY phytoplasma or any other phytoplasma.

Of the 11 PCR analysed acquisition grapevines, only one symptomatic grapevine that was used as an AAP plant has tested positive for AGY phytoplasma. However none of the test leafhoppers from trials using that grapevine tested positive for the AGY phytoplasma. In addition, none of the lAP test plants were infected with the AGY phytoplasma. One grapevine tested positive for the Tomato Big Bud phytoplasma.

In two transmission trials, the same AGY symptomatic grapevine was used as the acquisition test plant for O. *argentatus.* In one trial, the leaf upon which the o. *argentatus* fed tested positive for the TBB phytoplasma. In this trial test leafhoppers died before an adequate latent period had been satisfied and no phytoplasma was detected in the three recovered and PCR analysed leafhoppers. In the second trial, three of seven recovered and PCR analysed test leafhoppers were positive for the TBB phytoplasma. This is the first evidence that grapevines can serve as an acquisition host for the TBB phytoplasma for the O. *argentatus* vector.

Our colonies of *O. argentatus* were originally established from wild captured individuals. On occasions, after leafhoppers began reproducing in captivity, wild captured individuals were incorporated into colony cages. Hence, it is possible that test leafhoppers, positive for the TBB phytoplasma, were individuals who were already infected when captured. For example, in one trial, the acquisition grapevine leaf, upon which *test* o. *argentatus* fed, did not test positive for photoplasma, but an inoculation celery plant, upon which the same leafhoppers fed, was infected with the TBB phytoplasma. Two of the four O. *argentatus* were recovered and neither were infected with TBB phytoplasma. It is possible that the PCR analysis did not detect the phytoplasma in the grapevine leaf and the two non-recovered leafhoppers, from the trial, acquired the TBB phytoplasma from that leaf and transmitted TBB phytoplasma to the celery plant.

In another trial, a bulked sample of three o. *argentatus* tested positive for the TBB phytoplasma but the acquisition grapevine, upon which the test leafhoppers fed, did not. TBB phytoplasma infection in grapevine may be similar to the AGY phytoplasma in that it may be difficult to detect infection by PCR even from symptomatic leaves.

In one trial, two bulked samples of test O. *argentatus* were positive for the TBB phytoplasma but the acquisition grapevine leaf was not tested. In another trial, the incubation faba bean plant and three recovered test leafhoppers tested positive for TBB phytoplasma by PCR analysis but the acquisition grapevine was not tested.

Of the thousands of field collected O. *argentatus* that have been tested by PCR analysis in other trials, less than 1 % have tested positive for the TBB phytoplasma (3 individual leafhoppers and one bulked sample of nine leafhoppers, See Chapter 4.1). In our transmission experiments, five of the 52 samples (10%) of test O. *argentatus* have tested positive for the TBB phytoplasma. A higher number of TBB infected leafhoppers in transmission trials provides evidence that test O. *argentatus* were not previously infected with TBB phytoplasma but rather acquired the phytoplasma from experimental grapevines.

Only a small percentage of O. *argentatus* test leafhoppers were found to harbour phytoplasma. This is not surprising given the negative results from PCR tests of the acquisition grapevine hosts. Test leafhoppers may not have fed sufficiently long on vines during the AAP or did not acquire phytoplasma during feeding. Only symptomatic shoots were chosen for acquisition but many shoots may have had a very low phytoplasma titer. Additionally, shoots may have undergone heat therapy during the hotter periods of the experiment III which phytoplasma is killed by high ambient temperatures. There is also the possibility that grapevines are a dead-end host for the AGY phytoplasma. A dead-end host is one that can suffer from a phytoplasma infection but from which insects can not acquire the pathogen. Clearly, having other AGY infected plants is crucial for continued and successful transmission trials. No other test plant species have tested positive for the AGY phytoplasma.

Previous work also suggests that nymphs acquire phytoplasma at a greater rate than do adult insects (Beanland et al. 2000). Few O. *argentatus* nymphs were available for use in 1999/2000 transmission trials. It is suggested that future transmission trials should include a larger proportion of nymphs for phytoplasma acquisition.

Membrane Feeding Trials

Two of the five O. *argentatus* membrane solutions tested positive for phytoplasma but RFLP analysis indicated that the phytoplasma was not the AGY or TBB phytoplasma. The membrane solutions fed upon by *A. torrida* did not contain phytoplasma.

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4.6 Use of bait plants to determine AGY phytoplasma inoculation period by feral insect vectors

LeAnn Beanland, Linda Pollock and Michelle Kelly

INTRODUCTION

In order to determine if Australian Grapevine Yellows (AGY) phytoplasma inoculation events occur each year and at what time of the growing season these events occur, bait plant trials were performed. Bait plant trials also allow alternate hosts of *Candidatus* Phytoplasma australiense (Davis et al. 1997) to be identified and the acquisition of AGY phytoplasma in plant culture in the laboratory. Correlations of periods of inoculation(s) I with population trends of candidate vector species can also be determined.

MATERIALS AND METHODS

Preliminary Experiments

Twenty-four Chardonnay grapevines on Schwarzman rootstock were planted in 30-cm pots in a commercial potting mix. Each week, for five weeks beginning late February 1999, groups of four vines were placed in scrub near a Gol Gol vineyard for an acquisition access period (AAP) by any inoculative insect vectors present at that time of I year. Each potted vine was laced individually into a yellow plastic tray that held water to prevent the pots from drying out. The colour yellow is also attractive to leafhoppers and planthoppers. After the weeklong AAP, new vines were placed in the field and the original vines brought back to a glasshouse and observed for development of AGY symptoms. Four vines were used as controls and were kept in the glasshouse. Leaf samples of vines with unusual growth were sent to the Institute for Horticultural Developments (IHD), Knoxfield, to be tested by PCR analysis for the presence of AGY phytoplasma in July 1999. None tested positive. No new AGY symptoms developed and as all vines had severe powdery mildew they were discarded.

Full trials

A test vineyard in Gol Gol (NSW) was chosen which in the 1998/1999 growing season had 45% AGY occurrence.

Every two weeks beginning in early September 1999, two to eight pots of each bait plant, listed below, were placed in the scrub near the Gol Gol vineyard. Sites were chosen based on the captures of candidate insects by sticky traps at each test site in the scrub in the previous year.

Bait plants included:

Papaya- (var. *GD* x ER 643 from West fresh PTY LTD, Innisfail Queensland) Papaya dieback (PD) is thought to be caused by a phytoplasma that is indistinguishable from the AGY phytoplasma (Padovan et al. 1996). Although a different insect may transmit

Papaya Dieback phytoplasma, papaya may serve as a feeding and or developmental host plant for the insect vector of AGY phytoplasma (Padovan et al. 1998).

Strawberry- Strawberry Lethal Disease is caused by a phytoplasma also indistinguishable from the AGY phytoplasma. (Padovan et al. 1998; Andersen et al. 1998). Strawberry plants were obtained from cuttings from a Mildura garden. They were grown for more than 5 months in a glasshouse before use to ensure they were not infected with any diseases.

Grapevine- Chardonnay grapevines are an established host of AGY (Magarey and Wachtel 1978 and 1986; Smart and Fletcher 1996). Chardonnay rooted cuttings (10V5) were purchased from Boulevard Nurseries, Irymple, Vic.

Faba Beans *I* Garden Beans -AGY phytoplasma has been detected in garden beans in a papaya orchard in Queensland (Schneider et al. 1999). Faba bean seed was purchased at local nurseries and propagated in the glasshouse.

Flax -Flax plants were purchased from The Flaxman, Croydon, Vic.. *Phormium* (flax) Yellow Leaf (Cumber 1953) is associated with a phytoplasma indistinguishable from AGY phytoplasma (Andersen et al. 1998; Liefting et al. 1998).

Tomato -Tomato is the host of the Tomato Big Bug (TBB) phytoplasma that also has been detected in grapevines (Osmelak 1987). In addition, the vector of TBB, *Orosius argentatus* is a common visitor to vineyards (Osmelak et al. 1989).

Coprosma robusta -C. *robusta* is an indigenous woody shrub plant of New Zealand and host of a phytoplasma indistinguishable from *Phormium* Yellow leaf and AGY phytoplasma (Dr. Ross Beever, Landcare Research, Auckland N.Z. pers. com.) C. *robusta* seeds were obtained from Dr. Beever, and were plants were propagated in the glasshouse.

Celery -is one of several plant hosts of the aster yellows phytoplasma in the U. S. Plants were propagated in the glasshouse or were purchased from local nurseries. To ensure that purchased celery plants had not already been inoculated by a leafhopper, a sample of one or two plants from every punnet of purchased celery was maintained in the glasshouse and observed for symptoms of disease.

Seeds and transplants were planted in a potting mix made and sterilised on site. Potting mix was made of 5 parts peat moss, 10 river sand and 1 part vermiculite and perlite. Glasshouse plants were fertilised with liquid fertiliser (MaxiCrop®) following label instructions on a biweekly basis. Established grapevines were fertilised with Nuetrog® every 10-12 weeks. Spider mites were managed with biweekly releases of predatory mites (Hawksbury IPM) and aphids were managed by the introduction of field-collected lady beetles and purchased lacewings (Hawkesbury IPM) into glasshouse sections. Insecticides were only used when beneficial insects were ineffective. Insecticide treated

plants were not used for several weeks after application of insecticide. Seed propagated test plants were transplanted to 10cm pots and transplanted to larger pots as needed.

Groups of test plants (each containing every test plant type) were placed at each of three field sites and set in yellow trays. Site 1 was in an open area surrounded by native and weedy herbaceous and woody plants located adjacent to the first row of the vineyard. Site 2 was in a scrubby area adjacent to sticky trap sites where planthopper species were commonly captured. Site 3 was located between two citrus trees adjacent to the vineyard on the opposite side of the vineyard to Site 1.

Table 1. Number of each plant species used in Bait Trials.

	Number of Plants		
Plant Type	Site 1	Site 2	Site 3
Strawberry	8	8	4
C. robusta	4	4	2
Garden Bean[a]	4	4	2
Grapevine	3	3	1
Flax	3	3	1
Papaya[b]	3	3	2
Tomato	3	3	1
Celery	2	2	1

a. In the first week, only 5 garden bean plants were set out in the field (2,2,and 1 at each site).
b. The first and second batches of papaya had only 1 plant placed at Site 3.

Situating plants in the yellow trays may enhance their attractiveness to phloem feeding insects and the trays also served as reservoirs of water to prevent desiccation of test plants. At each site a wire fence was constructed around each plant group to protect them I from rabbits. Test plants were watered and inspected for damage every day. At the conclusion of the two-week-long acquisition access period, plants were collected, replaced with new plants and brought to SHC where they were sprayed with an insecticide and placed in a hoop house covered with insect proof material.

The abundance of previously surveyed candidate species- those that are phloem feeders that may be vectors of AGY -were determined by sticky traps and vacuum sampling (See Chapter 1). By comparing sticky trap and vacuum collections with inoculation events it is I possible to identify possible vector species.

To provide statistical evidence that glasshouses are free from inoculative vectors of AGY, control test plants were kept in the glasshouse over the course of the season long experiment. In the glasshouse the insect populations were monitored with yellow sticky traps.

Test plants in the hoop house were inspected regularly for symptoms of AGY. If symptoms appeared in a test plant, a leaf sample from that plant was collected and sent to IHD, Knoxfield for PCR analysis for AGY phytoplasma. To determine whether the hoop house could be a site of infection, yellow sticky traps were placed inside to monitor insect populations therein.

RESULTS AND DISCUSSION

Wind and sun damaged many test plants in the field. However, none of these plants died

and many recovered completely. None of the test plants tested by PCR were infected with the AGY phytoplasma.

Several batches of tomato plants had a condition of unknown aetiology and as a result were not useful for bioassays. Changes in the fertilisation programs alleviated the syndrome. Of the 7 sent to IHD, one has tested positive for phytoplasma, but the phytoplasma could not be characterised.

Several *Coprosma robusta* plants showed symptoms that could be caused by phytoplasma infection but none of the 3 samples sent to IHD for PCR analysis tested positive for the AGY phytoplasma. The incubation period for phytoplasma in C. *robusta* is unknown and the plants have been kept for future observations. The roots of several C. *robusta* plants were damaged when they were moved in the hoop house to make space for new plants. As a result two plants died before any symptoms could have developed.

Many of the grapevines developed powdery mildew and also developed yellowing and leaf curling. Of the 3 samples tested, none were positive for the AGY phytoplasma. It is suspected that the incubation period has not been satisfied and infected vines may not *show* symptoms until the growing season of2000/2001. According to Osler et al. (1993) a minimum incubation period of 5 months is necessary before symptoms of an Italian grapevine yellows are expressed.

The incubation period for *Phormium* yellows in flax is 7 months (Liefting et al. 1998). The earliest date of expected AGY symptom expression in test flax plants, assuming a similar incubation period, was in April 2000. Although several test flax plants did develop yellowing, AGY phytoplasma has not been detected in the 4 leaf samples analysed by PCR. Test flax plants have not been discarded and have been kept in the insect proof *hoop* house.

Ten leaf samples of papaya that had become chlorotic and deformed were sent to IHD for PCR analysis. Of those 10 leaves, one tested positive for phytoplasma, although the phytoplasma could not be characterised. The period of time, from exposure to inoculative insects until expression of symptoms of papaya dieback, is estimated to be 5 weeks

(Guthrie et al. 1998). Using this estimate it would be expected that all of the test papaya would have expressed symptoms by the conclusion of the trial.

According to Anna Padovan (pers.com.) symptoms of Strawberry Lethal Disease can be seen in June and July in runners that were planted in March or April. She suggested allowing 3 to 4 months from the period of exposure to the development of the symptoms. Six samples were collected from strawberry plants with yellowed or reddened leaves. None have tested positive for the AGY phytoplasma and all test strawberry plants were discarded.

There is no estimate of the incubation period for AGY phytoplasma in garden bean plants, as only one infected bean plant has ever been found. (Schneider et al. 1999). Several bean plants became yellowed or had unusual growth patterns. Samples from 8 plants were tested and 3 were positive for the Tomato Big Bud (TBB) phytoplasma. The other 5 beans did not test positive for phytoplasma.

Many of the test celery plants developed unusual growth patterns, including yellowing and stems and leaves with a twisted or curled appearance. Several also displayed a white streaking on the stems and leaves. Leaf samples from 7 celery plants were sent to IHD for PCR analysis. One tested positive for an unidentified phytoplasma.

All the test plants that tested positive by PCR were in the field and exposed to insect vectors in November. Inoculative insects were apparently active and feeding during this period. Interestingly, peaks in the number of *O.argentatus* captured on sticky traps and in vacuum samples can be seen in November. However, a large peak is seen on sticky traps in February. There is a small peak in the number of *Recilia hospes* captured on sticky traps in early November and many *Toya sp*. were captured in vacuum samples around the same time. There was no peak in the numbers of *blastopsylla sp*. Captured during November.

Symptoms may not appear fro several months in many of the remaining test plant species. Those plants that are suspect to have a long incubation period are maintained on the hoop house and inspections will continue until after November 2000, when AGY phytoplasma infected field grown grapevines begin to express symptoms. Suspected phytoplasma infections will be confirmed by PVR analysis.

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4.7 Propagation of Australian grapevine yellows phytoplasma infected grapevine material

LeAnn Beanland, Linda Pollock, Michelle Kelly, Graeme Fletcher and Peter Magarey

INTRODUCTION

Mapping data of AGY disease incidence suggest that symptoms of phytoplasma infection can be expressed in two or more years in the same vine (Gibb et al. 1999). In addition, Constable and Symons (1998) provided experimental proof of the persistence of AGY phytoplasma from one season to the next when they detected AGY phytoplasma in the wood of dormant vines. It is not clear why symptoms occur in two to more years in some vines but disappear after one year in others. Magarey and Wachtel (1986) described a remission of AGY symptoms to explain the sporadic incidence of the disease from year to year. They proposed that high temperatures kill Phytoplasma or reduce the titer to below that necessary to cause symptom expression. The distribution of phytoplasma in the host vine may also influence the year- to- year survival of the phytoplasma. A common symptom associated with phytoplasma infection is the inhibition of host wood lignification. If phytoplasma is confined to a single shoot that does not survive low winter temperatures then the surviving vine will be infection-free the following season. Pruning may also remove infected tissue leaving the remaining wood free from phytoplasma infection (Bouden-Padieu 1999).

Early attempts to propagate AGY phytoplasma infected wood have been unsuccessful (Magarey pers. com.). It appears that infected wood typically does not survive dormancy. However, to ensure that propagation material does not harbour AGY phytoplasma, as well as other diseases, hot water treatment of propagation material is recommended.

In order to be able to perform yearlong transmission experiments, several attempts have been made to propagate AGY phytoplasma infected plant material in the laboratory. In addition, it was possible to test various techniques for propagating infected material and provide clues to the risk associated with the introduction of infected propagation material into commercial vineyards.

MATERIALS AND METHODS

Propagation of 1.5 year old grapevine shoots

Because it is often only shoots that are symptomatic in infected grapevines, it is reasonable to assume that these infected shoots arose from buds in which the phytoplasma over-wintered, in the absence of an inoculation event by a vector. Phytoplasma infected shoots do not lignify properly and therefore often cannot survive low winter temperatures. In an attempt to avoid survival problems, older lignified wood, from which symptomatic tissue arose, was propagated to obtain AGY phytoplasma infected material for use in the lab. By propagating older lignified wood from AGY infected vines in the glasshouse, it was anticipated that survival would be enhanced and buds on this material had a reasonable chance of harbouring phytoplasma.

During the growing season of 1998/1999 symptomatic shoots at a Gol Gol (NSW) test vineyard were painted with white paint so that they could be identified in the winter. None of the shoots were tested by PCR analysis and were chosen on the basis of symptoms only. In June 1999 these painted shoots were cut from the vine. Symptomatic (youngest) wood was removed from the cuttings leaving buds on the 1997/1998 spurs. Diagonal cuts were made in the wood, proximal to the spurs, and rooting hormone was added to these cuts to encourage root production at these sites. Approximately 25 cuttings were planted in potting mix in 18 X 30 X 45 cm polystyrene boxes. Cuttings were planted at a 45° angle and arranged so that the buds on the 1997/1998 spur were above the soil surface and the cuts made in the wood were buried. The polystyrene box was buried to approximately half their height in moist sand in a heat bed (26°C). Moisture was maintained in the planting boxes to encourage root and shoot growth.

Bud grafting of AGY infected material

According to Tirtza Zahavi, Israeli viticulturalist, (pers. com.), scientists in Israel and, elsewhere have had success in obtaining phytoplasma-infected material by grafting buds of infected vines onto uninfected vines. This technique was incorporated into the trials to enhance the chances of obtaining AGY phytoplasma infected material for use in the laboratory.

In early March 2000, buds were cut from AGY symptomatic shoots collected from the Sunraysia Horticultural Centre (SHC) experimental Chardonnay block. Buds were placed in cuts made in the trunk of four potted Chardonnay vines so that phloem tissue on buds and potted vines was continuous. Tissue connections were secured with parafilm wrapped around the bud and the vine. Potted vines were placed in a greenhouse and observed for symptoms of phytoplasma infection.

Propagation of green grapevine material

In early March AGY symptomatic cuttings were collected from a vineyard in Mildura. On five days in March, cuttings were taken from another heavily infected vineyard in Merbein (Vic). Diagonal cuts were made to each shoot to obtain cuttings with 3 buds each. The proximal end of each cutting was dipped in rooting hormone and planted in potting mix in plastic bags. Four cuttings were planted in each bag and oriented so that two buds were below and one bud above soil surface. Plastic potting bags were placed on moist sand in a heat bed (26°C). Potting bags were covered with inverted clear plastic bags to maintain moisture. The clear plastic bags were removed from potting bags as roots developed on cuttings. Cuttings that did not survive were discarded. Fungicide was applied to the soil to prevent fungal diseases. Chicken manure fertiliser was added to each potting bag. The heat bed was covered with clear plastic sheeting in which a space heater was placed to maintain ambient moisture and warmth. Grow lights were suspended above the pots that were timed to come on at dusk and remain on until dawn.

Tissue culture of AGY phytoplasma infected vines

According to Petrovic et al. (2000) phytoplasma can be more easily detected in infected tissue cultured plant material than in plants grown in soil. One possible explanation for this phenomenon is that the phytoplasma titer is higher in tissue cultured material. Alternately, the presence of PCR Inhibitory plant products is

reduced in *in vitro* grown plants enhancing the detection of phytoplasma by this method.

Successful propagation of AGY infected material allows for the continuation of transmission experiments during winter when field grapevines are dormant. Tissue cultured vines also may have reduced secondary chemistry that could interfere with insect feeding. Additionally, if the phytoplasma titer is higher in *in vitro* grapevines, then the likelihood of acquisition of phytoplasma by test insects is also enhanced. Tissue culture was employed in order to obtain AGY infected material for use in transmission experiments.

Four AGY symptomatic shoots (approximately 50 cm in length) were collected at the SHC experimental Chardonnay block in April 2000. Collected shoots were delivered to the Tissue Culture Lab at Boulevard Nurseries, Irymple, Vic. for *in vitro* culture. Buds from the shoots were divided into those cut from the proximal end of the shoot and those cut from the distal end. Six cultures from the proximal buds and seven cultures from the distal buds were successfully cultured using a standard mineral media (Kathy Mullins, Boulevard, pers. com.). In mid-May the proximal cultures were further subcultured into 21 cultures. The distal cultures had not been subcultured by 25 May.
Root and leaf samples from each of the six original proximal cultures were collected and sent to IHD for PCR analysis.

RESULTS AND DISCUSSION

Propagation of 1.5 year old grapevine shoots

Several of the cuttings had fully developed leaves by mid-August 1999. The eight vines that survived were transplanted into 30cm pots in late August. None of the vines had displayed symptoms of AGY infection by January 2000. They were inspected again for symptoms in mid-April. A leaf sample from one plant with curled, yellowed leaves was sent to IHD for PCR analysis but did not test positive for the AGY phytoplasma. Test vines have not been discarded and observations of them will continue. Test vines may require another dormant period before they express symptoms. Only one third of the cuttings survived. AGY infections may have contributed to reduced survival of propagation material and non-infected or wood with a low titer of phytoplasma may well have been selected for in these trials.

Bud grafting of AGY infected material

None of the 4 test vines had expressed symptoms of AGY phytoplasma infection by August 2000. Vines have not been discarded and observations will continue.

Propagation of green grapevine material

Many of the cuttings began to produce roots within 4 weeks of planting. Approximately 30% of all cuttings survived and developed vigorous shoots and another 10% had developed roots only by late May 2000. To date, none of the leaves have displayed symptoms of AGY infection.

Tissue culture of AGY phytoplasma infected vines

The 7 *in vitro* cultures from distal shoot tissue were greener and healthier than the proximal shoot cultures. One of the proximal cultures did not produce roots and the leaves were malformed and yellowed. The PCR analysis indicated that none of the tissue-cultured samples were positive for the AGY phytoplasma. Because the cuttings were taken late in the season after long periods of temperatures in the upper 30s and lower 40s, any phytoplasma present in test shoots may have already been killed or had the titer reduced before cuttings were collected. Collection of cuttings earlier in the season would reduce the likelihood of phytoplasma kill and enhance the chances of obtaining AGY infected material for use in transmission experiments.

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4.8 Experimental Transmission of AGY phytoplasma from grapevine to dodder

Peter Magarey

INTRODUCTION

Experiments were undertaken in an attempt to transmit AGY phytoplasma to an alternate host or use in insect vector studies at SHC.

MATERIALS AND METHODS

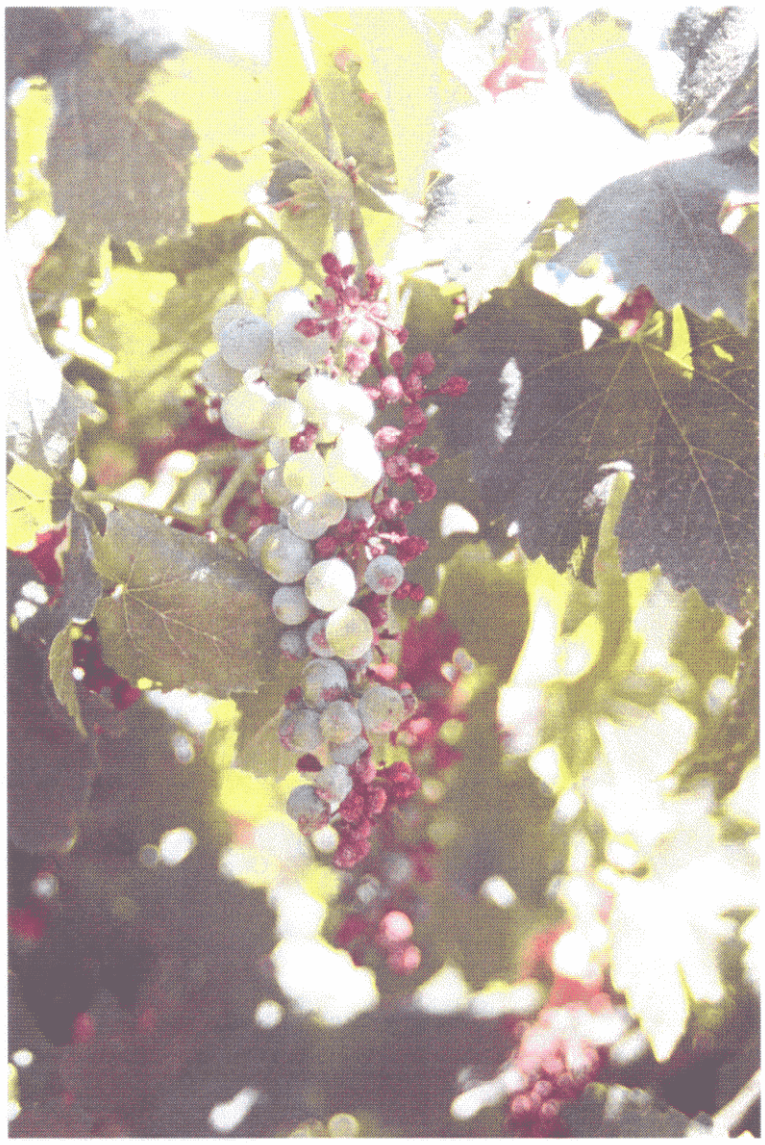
In 99/00, three vines of cv Riesling with clear symptoms of AGY were selected as sources of the AGY phytoplasma. Parasitic plant dodder *(Cuscuta campestris),* cultured on potted broad bean *(Viciafaba),* was twisted around affected vines. A shades house was erected over the test vines and they were maintained in this position for ~2 weeks. The apical portions of dodder growing from attachments on the grapevine shoots were removed and cultured in the glasshouse.
Attempts were then made to culture the dodder on to broad bean. PCR analyses were performed on the AGY affected grapevine material and on the dodder cultures.

RESULTS AND DISCUSSION

The dodder attached well to vines in about two weeks. PCR analysis of this dodder showed it was AGY positive, thus transmission to dodder from grapevine was successful. This advice came after the dodder culture had died. Before the dodder died, several attempts were made unsuccessfully to establish the infected dodder on freshly grown broad bean. There were no temperatures above 40°C during the experimental period that might have killed the phytoplasma.

Thanks to Karen Gibb, Scott Gillett, Michelle Wbidt, and to Robert Faggian at Knox IHD for PCR

Chapter 5 Control and management options for spread of phytoplasma



5.1 Hot water treatment for the control of spread of phytoplasma in Australian Grapevines.
Yasmin Wilson, Graeme Fletcher and Fiona Constable

5.2 The need for phytosanitary protocols to restrict the introduction and spread of phytoplasmas from other countries.
Fiona Constable

5.3 The role of propagation material in spread of AGY phytoplasma
Peter Magarey

5.4 Possible management strategies for AGY disease in grapevines
Peter Magarey

5.1 Transmission of phytoplasmas through grapevine propagation material.

Yasmin Wilson, Graeme Fletcher and Fiona Constable

INTRODUCTION

Hot water treatment can be used for controlling or eliminating various pathogens from dormant grapevine cutting material. Overseas, hot water treatment is used to control *flavesence doree* phytoplasma by heat treating at 50°C for 45 minutes (Caudwell, 1990). In Australia, many commercial nurseries and Vine Improvement Associations hot water treat grapevine cutting material at 50°C for 30 minutes to control *Agrobacterium tumefaciens* (Ophel et al. 1990) and 50°C for 5 minutes to control grape phylloxera (S.A. Plant Quarantine Standard).

Objectives

1. Test for transmission of phytoplasma through vegetative propagation material.

2. To determine if hot water treatments affect the incidence of phytoplasma in vine propagules.

MATERIALS AND METHODS

Dormant cuttings were collected from Chardonnay vines with a known history of phytoplasma symptoms from Sunraysia in July 1996 (Table 1.).

Each cane was labelled according to source vine, cane number and disease status, before being placed into plastic bags and cool stored according to VAMVVIA practice. Approximately 2000 two bud cuttings were taken from the material and labelled according to bud position, cane number, vine number and property location. The cuttings were randomly assigned to four hot water treatments, which had been replicated five times (Table 2.). Approximately 100 cuttings were placed into each plot.

All the cuttings were soaked overnight in a cool water bath at room temperature before being heat-treated. The temperature and time regimes were chosen to encompass those currently being used to eliminate or control many pathogens from grapevine planting material ego phylloxera 55°C for 5 minutes (Windle 1994), crown gall 50°C for 30 minutes (Ophel et al. 1990) and *flavescence doree* 50°C for 45 minutes (Caudewell, 1990).

The hot water treatments were performed in a fibreglass dipping tank (1.00m x 1.00m x 0.85m). The tank was fitted with a thermostatically controlled water heater accurate to 0.5°C. To ensure uniform heating throughout the water, a circulating pump and hose was used to distribute the hot water evenly across the tank, thus minimising the possibility of "hot spots" forming in certain areas.

Following the hot water treatment, the cuttings were then propagated in pots containing a sterilised potting mix of perlite, sand and peat moss. The pots were randomly placed sand beds in a glasshouse at the Sunraysia Horticultural Centre, Irymple. The glasshouse was not insect proof. The cuttings were managed under VAMVVIA practices.

Table 1: Sunraysia vineyards used for cutting selection.

Property Location & Property	Row	Vine	Tag
Gol Gol (Camerons)	3	42	
Gol Gol (Camerons)	5	47	
Gol Gol (Camerons)	1	49	
Karadoc (Sos)	12	5	
Karadoc (Sos)		M42	
Karadoc (Sos)		M43	
Karadoc (Lindemans)	3	1	
Karadoc (Lindemans)	3	2	
Karadoc (Lindemans)	3	9	
Karadoc (Lindemans)	3	14	
Karadoc (Lindemans)	3	15	
Karadoc (Lindemans)	3	20	
Karadoc (Lindemans)	3	48	
Karadoc (Lindemans)	4	5	
Karadoc (Lindemans)	4	8	
Karadoc (Lindemans)	4	12	
Karadoc (Lindemans)	4	14	
Karadoc (Lindemans)	4	24	
Karadoc (Lindemans)	4	50	
Karadoc (Lindemans)	4	52	
Wemen (Carey)	139	2E	94
Wemen (Carey)	137	31E	65
Wemen (Carey)	137	34E	64
Wemen (Carey)	136	2E	88
Wemen (Carey)	103	10W	3
Wemen (Carey)	142	2E	?
Wemen (Carey)	134	31W	79
Wemen (Carey)	133	6E	85
Wemen (Carey)	133	2E	82
Wemen (Carey)	134	3E	86
Wemen (Carey)	133	4W	96
Wemen (Carey)	138	?W	63

Table 2. Hot water treatments applied to the cuttings

Temperature (°C)	Time (minutes)
55	5
50	20
50	30
Control (no dip)	Control (no dip)

In March 1997 the cuttings were visually assessed for (1) cutting mortality and (2) symptoms, which may be associated with phytoplasmas. The surviving cuttings were then transplanted to individual pots before being relocated to a shade house. Once hardened off, the cuttings were then planted in the field in spring, 1997. Observations for symptoms, that may be associated with phytoplasmas, were taken in January 1998.

RESULTS

In March 1997, the cuttings were visually assessed for mortality levels and any unusual symptoms ie. Leafrolling AGY symptoms (Table 3.).

Table 3. Visual assessment results for March 1997.

Treatment	Dead	Alive	Leaf rolling symptom
Control	72	428	21
55°C/5mins.	118	369	41
55°C/20mins.	66	425	23
55°C/30mins.	90	395	23
TOTAL	**346**	***1617**	**108**

* This figure represents the total number of cuttings alive.

In January 1998, when the cuttings had been growing in the field for approximately 6 months, another visual assessment was performed to detect any AGY -like-symptoms.
PCR tests were conducted for phytoplasma on random samples that showed unusual shingling and backward curling of leaves. None of the samples tested using nested PCR were positive for phytoplasma (Table 4).

Table 4. The number of samples from each hot water treatment that displayed similar shingling and backward curling of leaves and the result from PCR testing for phytoplasma

	No. samples	PCR result
Control	2	-
55°C/5	1	-
50°C/20	3	-
50°C/30	3	-

DISCUSSION

Initial assessments of cutting mortality showed a slight increase in mortality of cuttings dipped at 55°C for 5 minutes (Table 3.). Some cuttings displayed a leaf- rolling symptom that is not treatment specific, and is probably due to inadequate drainage in some pots. This symptom disappeared when the cuttings were transferred to the field in 1998.

No typical AGY symptoms were observed, although some shingling and backward curling of leaves was observed. No phytoplasma was detected by nested PCR in any of the samples selected that displayed shingling and backward curling of leaves in the 1997/98 season.

In respect to hot water treatment, there have been standard protocols developed and adopted commercially by VAMVIA for all grape varieties. These standard protocols are being documented for incorporation into AVIA vine accreditation practices. Vine improvement associations have introduced twice-yearly inspections of source vines for phytoplasmas, and other disorders. Assistance to inspectors and staff has been provided through technical advice, training workshops and field days.

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5.2 The need for phytosanitary protocols to restrict the introduction and spread of phytoplasmas from other countries.

Fiona Constable

INTRODUCTION
The Australian Quarantine & Inspection Service (AQIS) has phytosanitary protocols in place to minimise the introduction of grapevine quarantinable pests via imported planting material. Quarantine protocols differ depending on the source and type (tissue culture or scion wood) of material imported. Grapevines imported from AQIS accredited sources do not require pathogen testing while non-accredited material requires extensive disease screening. A list of procedures that minimise the introduction of phytoplasmas is given in Table 1. All AQIS protocols mentioned in this discussion are taken from the AQIS Booklet on indexing -Grapevines.

> The International Plant Protection Commission of the World Trade Organisation defines a quarantine pest as "a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled. [1]

The phytoplasma associated with flavescence doree (FD) is the only grapevine yellows phytoplasma registered as quarantinable by AQIS. FD is considered an economically important phytoplasma disease of grapevines in France and has been studied since the mid 1950's. Consequently the aetiology and epidemiology of FD is better understood than most other grapevine yellows phytoplasmas.

The natural vector for FD is the leafhopper, *Scaphoideus titan* Ball, however, the disease has also been transmitted experimentally to broad bean by *Euscalidius variagatus* Kirschbaum, indicating the potential for more than one vector for this disease (Lefol et al. 1994). Neither leafhopper has been recorded in Australia (pers. comm. Malipatil). In Germany the FD phytoplasma has recently been recorded in the absence of *S.titanus* and it is suggested that the phytoplasma either arrived via cuttings or, if the disease is endemic, another vector exists (Maixner et al. 1995). In the light of this information and since we do not know if a vector also exists in Australia that might spread FD, it is important that protocols remain in place to I continue to minimise the likelihood of an FD introduction.

Other grapevine yellows diseases, with symptoms similar to FD, have also been reported from many countries but their aetiologys are not well understood. Even so, evidence suggested that they are also associated with a prokaryotic organism (Magarey 1986). It is now known that there are distinct phytoplasmas associated with grapevine yellows diseases found overseas including Vergilbungskrankheit (Maixner 1994) and Bois Noir (Daire et al. 1994). These phytoplasmas have not yet been incorporated into the AQIS list of quarantinable grapevine pathogens. Research into

So far, none of the phytoplasmas that infect grapevines overseas have been detected in Australia, indicating that our quarantine protocols have been effective (Mark Wattam

(Pers. Comm.). It would be prudent to review the AQIS list of quarantinable diseases of grapevines and add any known phytoplasmas associated with grapevine yellows diseases overseas to continue to limit their introduction.

Table 1. AQIS protocols that minimise the introduction of phytoplasmas associated with grapevine yellows diseases overseas as given in the AQIS Booklet on Indexing.

	Approved source		Non-approved source	
Treatment	Tissue culture	Other than tissue culture	Tissue culture	Other than tissue culture
Hot water treatment	**Yes**	**Yes**	**Yes**	**Yes**
Growth in a closed Government quarantine facility - year 1	**Yes**	**Yes**	**Yes**	**Yes**
Growth in the open - year 2	**Yes**	**Yes**	**Yes**	**Yes**
Visual inspection for disease	**Yes**	**Yes**	**Yes**	**Yes**
Woody indexing – LN33 rootstock	**No**	**No**	**Yes**	**Yes**

Establishment, growth and visual inspection.

Grapevines remain in quarantine for a minimum of two years so that the imported vines can be established and visually inspected for evidence of various pathogens including phytoplasma. While this period of quarantine is likely to allow for the development of phytoplasma associated symptoms, it has been found that different cultivars have varying degrees of sensitivity to phytoplasma infection (Magarey 1986; Caudwell et ill. 1993). It is possible that symptom less infections may occur in some varieties and rootstocks (Credi, 1989; Daire et al. 1993).

In association with visual inspection for symptoms, detection methods for phytoplasmas of various crops include indexing onto susceptible hosts and electron and fluorescent microscopy. The recent development of molecular tests, including the polymerase chain reaction (PCR), has aided the detection of these organisms. The PCR method is exceptionally sensitive and may be used to screen actively and rapidly large sample numbers.

The use of PCR methods in association with visual inspections may improve detection of phytoplasma infections in grapevines. Active testing with PCR methods may also reduce the amount of time a variety is required to remain in quarantine although other pathogens including fungi and bacteria may still require the two year post entry quarantine period for expression. Research is required to determine whether screening

by methods such as PCR should be carried out prior to or after establishment of grapevine material. Overseas research suggests that phytoplasmas can be detected in shoots and leaves from the beginning of the annual growth cycle and the ability to detect phytoplasma peaks at berry ripening (Serrone and Barba 1996). No research has yet been conducted to determine if the phytoplasma can be detected in dormant wood.

Hot water treatment

All imported grapevines undergo hot water treatment at 50°C for not less than 20 minutes. The grapevine material is immersed in a hot water bath that is capable of maintaining an evenly dispersed and constant temperature. After treatment the material is immediately plunged into a cold water bath or cold running water and dried as quickly as possible. Hot water treatment has been shown to be an effective treatment for the eradication of FD phytoplasma and eggs from the FD vector *Scaphoideus titanus* (Caudwell et al. 1993; Boidron and Grenan 1992). The recommended treatment is 50°C for 45 minutes and it has been shown that this treatment does not harm vine material in controlled situations (Caudwell et al. 1993). Hot water treatment at 50°C for anywhere between 20 to 60 minutes is effective (Caudwell et al. 1993). It is likely that the longer treatment ensures effective eradication of the FD phytoplasma while not harming the vine material. The minimum of 20 minutes is close to the point where hot water treatment is insufficient (Caudwell et al. 1993) and may pose a risk of survival of phytoplasmas In Infected material.

Woody Indexing

Woody indicators and visual inspections are used for screening for FD phytoplasma. The grapevine indicator LN33, is double chip budded with buds from the candidate vine material either as dormant winter budding or late spring budding. A minimum of two indicator plants is used for woody indexing. A non-inoculated indicator is also used for a comparison of symptoms. If both buds fail after 28 days, when budding tape is removed, budding is repeated. Indicators are maintained in a healthy, vigorous and pest free states to ensure symptoms are not obscured.

Due to the uneven distribution of phytoplasmas through the vine, grafting is likely to give a greater chance of detecting phytoplasma than visual inspections or microscopy techniques. However, research in Italy and France suggests that grafting may not be the best method for determining infection. In the case of FD it has been found that transmission of phytoplasma through grafting is difficult (Caudwell and Larrue 1979). Detection is improved by grafting the LN33 indicator into potentially diseased shoot tissue rather than the reverse (Credi et al. 1990). However, it is likely that phytoplasmas will not always be transmitted when being screened by woody indexing. Cuttings from infected grapevine material are more likely to fail and the risk of disease via cuttings is minimal (Credi et al. 1990). However, there is a risk that transmission will occur which may not be evident while the plants are being established and observed under quarantine conditions. The use of PCR in association with woody indexing may improve detection of phytoplasma infections in grapevines and will also be useful in confirming phytoplasma if symptoms are suspected.

CONCLUSION

The importation of quality planting material and effective quarantine protocols may have reduced the chance of grapevine phytoplasma entering Australia. However, a risk does exist and it is recommended that:

- the list of quarantinable grapevine pathogens be reviewed to include other grapevine yellows phytoplasma found overseas.
- imported material continues to be established and visually inspected for symptoms.
- woody indexing to continue but that the indicator be grafted into the variety being screened.
- active testing using recently developed PCR techniques be incorporated.
- encourage the importation of quality grapevine material from accredited sources where possible (also review overseas sources for accreditation).
- review the hot water treatment protocols.

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Daire, X, Clair, D, Larrue, J, Boudin-Padieu, E and Caudwell, A 1993. Diversity among mycoplasma-like organisms inducing grapevine yellows in France. *Vilis* 32(3), 159-63.

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Maixner, M, Rudel, M, Daire, X, and Boudin-Padieu, E. 1995. Diversity of grapevine yellows in Germany. *Vitis* 34(4), 235-6.

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5.3 The Role of Propagation Material in Spread of AGY

Peter Magarey

INTRODUCTION

The objective of these experiments was to assess the risk of propagation material spreading AGY phytoplasma from vineyard to vineyard.

MATERIALS AND METHODS

Trial series 1.

During the 1970's and 80's, Bob Emmett and Peter Magarey at Loxton, undertook some 600 -700 separate attempts to transmit AGY via propagation material collected from AGY affected vines. Propagation was made in the glasshouse by chip budding, grafting (dormant and green-grafting and wedge and patch grafting) and by direct propagation of either hardwood or greenwood cuttings, the latter in a heat-bed with overhead misting. Subsequent growth of the potted vines was assessed the next season.

Trial series 2.

In 1997/98 at Bookpurnong, in the Riverland, SA, 1200 cuttings were harvested from arms of *cv* Chardonnay vineyard. Three groups of 400 cuttings each were taken from: a). grapevine arms recorded with AGY symptoms in the previous season; b). arms without symptoms on AGY affected vines; and c). Symptom less vines.

The cuttings were propagated according to standard commercial procedures at Riverland Vine Improvement Committee's plantings at Monash, SA. Cuttings were then planted in the field and grown for 12 months.

RESULTS

Trial series 1.

No symptoms typical of AGY developed on any potted vine. Some vines showed minor downward curling or yellowing of leaves but PCR analyses were not available to show presence or absence of AGY phytoplasma

Trial series 2.

No AGY symptoms were observed on any test vine after one season.

DISCUSSION

Our results suggest that AGY is not readily transmitted by usual means of propagating grapevine material, however, assessment after a longer period of time is desirable since symptoms may take longer time to express.

Trial Series 1: Thanks to Bob Emmett, Graeme Fletcher, Miriam Pywell, Malcolm Wachtel
Trial Series 2: Thanks to David Nitschke and team at RVIC, Malcolm Wachtel.

5.4 Possible management strategies for AGY disease in grapevine

Peter Magarey

INTRODUCTION
Experiments were performed to test possible management strategies for AGY in grapevines

MATERIALS AND METHODS

Developing a commercial control
The incidence and distribution of AGY on vines in Year 1 was mapped. Diseased arms of affected vines were removed during winter pruning of vines. The missing arms were replaced with canes from either the same or adjacent vines. In Year 2, the presence or absence on individual arms was noted and recorded.

Investigating the epidemiology and aetiology of AGY
Vines were mapped for presence of AGY symptoms in 1977/78. In dormancy 1978, AGY affected vines were pressure injected with tetracycline antibiotic and they were mapped for presence of AGY for the next 8 seasons.

RESUL TS AND DISCUSSION

Developing a commercial control
The levels of AGY symptoms were significantly reduced on (approx.) 32 vines pruned to remove inherent AGY disease as compared with (approx.) 30 adjacent untreated vines. This treatment effect lasted for at least two seasons.

Conclusions:
The Inoculum of AGY phytoplasma can be reduced to a titre below threshold for symptom-expression by such treatment. A single antibiotic treatment lasted for 7 seasons in the period from 1978/79. This is the likely minimum time period between presumed leafhopper activity *ie* between inoculation events of significance in that vineyard. Vectos insects may infect a vineyard with a titre' above the threshold for symptom expression' only as rarely as once in 6 or 7 years. This interval is likely to vary with regions and with seasonal conditions

Investigating the epidemiology and aetiology of AGY

The incidence of AGY symptoms was reduced to zero in treated vines, as assessed in the season following treatment. Levels of AGY symptoms remained significantly less than untreated controls for the subsequent six seasons.

Conclusion:
It is conceivable that this method will form the basis of a commercial management strategy for AGY. Pruning treatment would only be effective for the duration of vector inactivity.

Appendices

1. Communications strategy
2. Publications and communications
3. *IPM Viticulture: Research to Practice*™ module for AGY



COMMUNICATION STRATEGY

for

The National Program for Management of Phytoplasmas in Australian Grapevines.

July 1996

BACKGROUND

Grapevine Yellows diseases are caused by small organisms called phytoplasmas. Australian Grapevine Yellows (AGY) was first detected in 1975 and has since been monitored closely, especially in the Sunraysia and the Riverland districts. It is widespread in Australia and has varied in incidence over time. Recently it has become more evident, especially in the warmer, inland grapegrowing regions.

In July 1995 the Australian Council of Viticulture convened a meeting from which a National Reference Group was established to address the issues relating to AGY. A Steering Committee with strong industry involvement oversees a collaborative research project which has been designed to address the issues relating to Grapevine Yellows in Australia.

This research project was initiated in October 1995 and is being conducted by scientists from:

- Cooperative Research Centre for Viticulture, (The University of Adelaide)
- Institute of Horticultural Development, Knoxfield, (Agriculture Victoria).
- Sunraysia Horticultural Centre (Agriculture Victoria)
- Primary Industries South Australia
- Northern Territory University.

The issue of Grapevine Yellows has generated a high level of industry interest and concern, this has led to a heavy demand on researchers for information dissemination by way of seminars, field days, presentations and written information.

Objectives of the Communication Plan:

- To provide identified stakeholders and the general public with suitable information from the research project in a timely and effective manner.
- Provide a mechanism for industry feedback into the project.

The Process:

The plan objectives were designed by the project leaders and the plan development took place in a group meeting held at Sunraysia Horticultural Centre on May 10 1996. A list of participants is attached as Appendix 1.

This document is by no means a static summary and it may be improved as the project develops.

1. F
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II. COMMUNICATION WITHIN REFERENCE GROUP

Information	Action	Time	Responsibility
Bimonthly progress reports	Deliver to CRCV and Steering Committee	2 Monthly commencing Aug. 96	R Hamilton / G Buchanan
Summary of bimonthly reports	Send hard copy to Reference Group	2 monthly	R Hamilton / G Buchanan
Press Release	Assess and modify reports from RH/GB & release to all stakeholders.	As required.	Steering Committee
Define Reference Group	Circulate list to all members	May 1996 (see appendix 2).	H Armstrong / G Wellman

III. COMMUNICATION TO GROWERS AND WIDER INDUSTRY

Information	Action	Time	Responsibility
Research Progress Report, Grapevine Phytoplasmas in context.	Technical Briefing Trial Run to Steering Committee.	August 1 1996 .	CRCV GY Research Project Team
Research Progress Report, Grapevine Phytoplasmas in context.	Technical Briefing (invitations to GY Reference Group, Stakeholders # 1, #3, #5, #7)	Late August 1996 Dec 1996 May 1997	CRCV GY Research Project Team
Technical Articles	Plan, prepare and publish for growers and wider industry.	As required (through Team Meetings).	CRCV GY Research Project Team

IV. COMMUNICATION WITH MEDIA

Information	**Action**	**Time**	**Responsibility**
GY in perspective 'Take home' messages from technical briefing.	Prepare Media standby statement.	August 1996.	HA GB/RH to coordinate more detailed responses.
Media Clippings	Maintain clippings file, collect all possible reports	Ongoing	Ref. Group → HA
Media training for R&D staff	Consider training course	?	RH/GB
Media referral	Refer interviews/stories	Ongoing	HA

APPENDIX I.

Participants in Communication Planning Meeting.

Name	Organisation
John Whiting	Agriculture Victoria
Fiona Constable	Agriculture Victoria, IHD
Jane Moran	Agriculture Victoria, IHD
Bob Emmett	Agriculture Victoria, SHC
Greg Buchanan	Agriculture Victoria, SHC
Robert Hayes	Agriculture Victoria, SHC
Yasmin Wilson	Agriculture Victoria, SHC
Hugh Armstrong	CRCV/GWRDC
Peter Clingeleffer	CSIRO
Graeme Wellman	Mildara Blass
Grieg Greenwood	MVWIDC
Peter Wilson	MVWIDC
Harry Creecy	New South Wales Agriculture
Tony Somers	New South Wales Agriculture
Karen Gibb	NTU
James MacDonald	PISA
Peter Magarey	PISA
Richard Hamilton	SARDI
Rob Gibson	Southcorp
Rod Bonfiglioli	University of Adelaide
Graeme Fletcher	VAMVVIA
Peter Murphy	VAMVVIA
Rhett Marlowe	WGCA

APPENDIX II.

Grapevine Yellows Reference Group

Name	Organisation	Role	Telephone	Fax
Jim Hardie	CRCV	Steering Committee	08 3039405	08 3039449
Peter Hayes	GWRDC	Steering Committee	08 3642688	08 3643315
Graeme Wellman	Mildara Blass	Steering Committee	050 252303	050 253300
Rob Gibson	Southcorp	Steering Committee	085 609389	085 609424
Paul Croxton	AVIA	Steering Committee	050 514578	050 514523
Rhett Marlowe	WGCA	Steering Committee	08 3643990	08 3640907
Greg Buchanan	Agriculture Victoria, SHC	Project Manager	050 514500	050 514523
Richard Hamilton	PISA/SARDI, Adelaide	Project Manager	08 3039577	08 3039403
Fiona Constable	Agriculture Victoria , IHD	Project Team	03 92109222	03 98003521
Graeme Fletcher	VAMVVIA	Project Team	050 514500	050 514523
Hugh Armstrong	CRCV/GWRDC	Project Team	08 3039405	08 3039449
Jane Moran	Agriculture Victoria, IHD	Project Team	03 92109222	03 98003521
Karen Gibb	Agriculture Victoria, NTU	Project Team	03 92109222	03 98003521
Peter Magarey	PISA	Project Team	085 959100	085 959199
Rod Bonfiglioli	University of Adelaide	Project Team	08 3037392	08 3037102
Yasmin Wilson	Agriculture Victoria, SHC	Project Team	050 514500	050 514523
Bill Panagiotopoulos	PISA	Cooperator	085 866761	085 865848
Bob Emmett	Agriculture Victoria, SHC	Cooperator	050 514500	050 514523
Bob Symons	University of Adelaide	Cooperator	08 3037423	08 3037102
Harry Creecy	New South Wales Agriculture	Cooperator	069 630555	069 630255
John Whiting	Agriculture Victoria	Cooperator	054 304444	054 484982
Peter Clingeleffer	CSIRO, Merbein	Cooperator	050 513100	050 513111
Peter Murphy	VAMVVIA	Cooperator	050 514500	050 514523
Robert Hayes	Agriculture Victoria, SHC	Cooperator	050 514500	050 514523
Tony Somers	New South Wales Agriculture	Cooperator	049 302403	049 302410
Jeremy Cass	MIAVIA	Cooperator	069 681202	
Mali Malipatil	Agriculture Victoria, IHD	Cooperator	03 92109222	03 98003521

APPENDIX III. Nursery Contacts

Appendix 2: Publications and communications

Conferences, seminars and Workshop presentations

Beanland, L. 2000. In search of the insect vector of the Australian Grapevine Yellows. XIIIth Meeting of the International Council for the Study of Viruses and Virus-like Diseases of the Grapevine. The University of Adelaide, SA 12-17 March. Poster

Beanland, L. 2000. In search of the insect vector of the Australian Grapevine Yellows Australian Entomological Society 31st AGM Scientific Conference, July

Bonfiglioli, RG 1997 Epidemiology of grapevine yellows in Australia. Department of Horticulture, Viticulture and Oenology seminar, The University of Adelaide, SA,
March.

Gibb, KS, Davis, R and Constable, FE 1996 Tomato big bud phytoplasm -a widespread and successful phytoplasm in Australia. Abstract, International Organisation for Mycoplasmas conference, Florida, USA, July.

Constable, FE, Gibb, KS and Wilson Y 1996 Australian grapevine yellows: phytoplasma diagnosis and identification, general display.

Wilson, Y and Constable, F 1996 Australian grapevine yellows: symptom identification, general display.

Refereed publications

Bonfiglioli, RG, Lherminier, J, Symons, RH and Boudon-Padieu, E 1997
Oligodeoxynucleotides as probes for in situ hybridisation with transmission electron microscopy to localise specific phytoplasmas in plant cells. *The Plant Journal* (submitted).

Gibb, K.S., F.E. Constable, J.R. Moran and A.C. Padovano 1999. Phytoplasmas III Australian grapevines-detection, differentiation and associated diseases. Vitis
38:107-114.

Addresses to industry conferences, seminars and workshops

Constable, FE 1996 Identification of potential insect vectors. Industry update, CSIRO, Merbein, Victoria, 13 November.
Constable, FE 1997 Australian grapevine yellows: Current research strategies. Mornington Peninsula Vignerons' Association, Victoria, 8 March.

Constable, FE 1997 Australian grapevine yellows symptoms and latest research findings. Industry update at the Australian Vine Improvement Association inspectors' information/training day, CSIRO, Merbein, Victoria, 13 March.

Constable, FE and Moran, J 1996 Australian grapevine yellows. Yarra Valley Wine Grape Growers' Association at Institute for Horticultural Development, Knoxfield, Victoria, 29 May.

Constable, FE, Gibb, KS and Moran, J 1996 Is that symptom caused by phytoplasma Grapevine yellows update seminar and field trip, Sunraysia Institute of TAPE, Mildura, 9 May.

Gibb, KS, Constable, FE, and Moran, J 1996Phytoplasma diagnostics and identification. Grapevine yellows update seminar and field trip, Sunraysia Institute of TAPE, Mildura, 9 May.

Moran, J 1995 Australian grapevine yellows. Sunraysia grower group at beginning of project, Victoria, November.

Moran, J. Constable, FE and Gibb, K 1996 Patterns of spread. Grapevine yellows update seminar and field trip, Sunraysia Institute of TAPE, Mildura, 9 May.

Moran, J 1996 Australian grapevine yellows. Mornington Peninsula Vignerons' Association, Victoria, 8 March.

Wilson.Y1996 the effects of hot water treatment on the viability of Chardonnay cuttings. AGY industry meeting seminar at CSIRO, Merbein, Victoria, 13 November.

Wilson, YM 1996 the effects of hot water treatment on the viability of Chardonnay cuttings. Seminar at Sunraysia Horticultural Centre, Irymple, Victoria, 27 November.

Wilson, YM 1997 Industry update at the Australian Vine Improvement Association inspectors' information/training day, CSIRO, Merbein, Victoria, 13 March.

Industry journal articles

Beanland, L., M. Kelly, R. Faggian, J. MacFarlane and D. Glenn. 1999 .In search of an insect vector of Australian grapevine yellows: species composition and abundance of potential vectors. GGWM Oct. 1999. 41-46.

Bonfiglioli, RG, Carey, CT, Schliefert, LF, Kinnear, AJ and Symons, RH 1997 Description and progression of symptoms associated with grapevine yellows

disease in young Chardonnay vine in the Sunraysia region of Australia. *Australian Grapegrower and Winemaker* (in press).

Bonfiglioli, RG, Guerrini, S and Symons, RH 1996 Cooperative Research Centre for Viticulture: sampling program for grapevine yellows diseases. *Australian Grapegrower and Winemaker* 394, 22-4.

Constable, FE and Moran, J 1997 *Australian Grapevine Yellows Newsletter No.2.*

Gibb, KS and Constable, FE 1996 Australian grapevine yellows -a mysterious dilemma. *Victorian Viticulture News* Winter, 21.

Hayes, R 1996 Grape growers need to consider irrigating. *Murray Valley Winegrape Growers Newsletter* June.

Giddings, J 1997 Post-harvest irrigation. *Murray Valley Winegrape Growers Newsletter* April.

Moran, J 1996 *Australian Grapevine Yellows Newsletter No.1.*

Symons, RH, Bonfiglioli, RG, Habili, N and Hamilton, RP 1997 It's not worth the gamble: the penalties of using infected planting material. *Australian Grapegrower and Winemaker* 396, 13-15.

Wilson, Y 1996 *Winegrape production in California.* A report on the Murray Valley Winegrape Study Tour (and discussions into winter chilling injury). Overseas report, Sunraysia Horticultural Centre, Irymple, Victoria.

Wilson, Y 1997 winter chilling injury. *Australian Grapegrower and Winemaker* Annual Technical Issue (in press)

Wilson, Y and Potts, A 1996 *the effects of hot water treatment on the viability of Chardonnay cuttings.* Interim report for the Australian Vine Improvement Association.

Training course contributions

Bonfiglioli, RG and Clark, M 1996 Plate capture PCR diagnostics workshop. Horticulture Research International East Malling, Kent, UK, November.

Constable, FE 1996 Viruses, viroids and phytoplasmas. Presentation as a part of the Farm Chemical Users course, Institute for Horticultural Development, Knoxfield, Victoria, January.

Constable, FE 1996 Diagnostic techniques -viruses/viroids/phytoplasmas. Plant protection course for Crop Health Services agents. Institute for Horticultural Development, Knoxfield, Victoria, 15 April and 22-23 August.

Constable, F 1996 Viruses, viroids and phytoplasmas. Two one-hour lectures to LEAP students employed at the Institute for Horticultural Development, Knoxfield, Victoria, May.

Constable, FE 1996 Australian grapevine yellows -an overview of research carried out at Institute for Horticultural Development, Knoxfield, Victoria. Presentation to Royal Melbourne Institute of Technology students, September.

Constable, FE 1996 Australian grapevine yellows -a case study in molecular epidemiology. Presentation for the Australian Centre for International Agricultural Research Master Class lectures, November.

Other communications

A harvest hint. The need for maintaining soil moisture. Sunraysia television April -June 1997.