# THE BIOLOGY AND EPIDEMIOLOGY OF AUSTRALIAN GRAPEVINE PHYTOPLASMAS

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A thesis submitted to the University of Adelaide in fulfilment of the requirements of the degree of Doctor of Philosophy

March 2002

## TABLE OF CONTENTS

## ABSTRACT

The distribution and persistence of phytoplasmas was determined in Australian grapevines. Phytoplasmas could be detected using polymerase chain reaction (PCR) from shoots, cordons, trunks and roots throughout the year and phytoplasmas appear to persistently infect Australian grapevines from year to year. Phytoplasmas were not always detected in samples from the same sampling area from one sampling period to the next. Phytoplasma detection by PCR was improved by sampling from shoots, cordons and trunks and October was the best time to test for phytoplasmas when these three tissue types were sampled.

Only Australian grapevines yellows phytoplasma (AGYp) and tomato big bud phytoplasma (TBBp) were detected by PCR and RFLP techniques from any grapevine sample used in the distribution and persistence studies. Genetic variability was detected within isolates of AGYp and between AGYp and the papaya dieback phytoplasma (PDBp) using heteroduplex mobility assay (HMA) of the *tuf* gene. AGYp variants and PDBp were indistinguishable when they were compared using HMA of the *tuf* gene.

The diseases expressed by grapevines used in the distribution and persistence studies were recorded. Australian grapevine yellows disease (AGYd) was expressed by 17/20 grapevines at sometime during the study. Only 4/20 grapevines expressed restricted growth disease (RGd). Late season leaf curl disease (LSLCd) affected 15/20 grapevines during the study. AGYd affected all grapevines with RGd and LSLCd. The three diseases were persistently expressed in some grapevines and remission of disease was observed in others. The results of PCR detection in the same grapevines indicated that phytoplasmas were more frequently detected in AGYd grapevines that also expressed RGd and LSLCd compared to grapevines expressing AGYd alone. Asymptomatic phytoplasma infections occurred. Phytoplasmas were detected less frequently in asymptomatic plant material compared to AGYd affected material.

RGd and LSLCd may be associated with AGYd in some grapevines. However all three diseases can occur independently.

Full length chromosomal DNA of TBBp was obtained from grapevine and digested with *Bss*HII. The digested TBBp chromosomal DNA was subjected to pulsed field gel electrophoresis (PFGE) and from this the chromosome was estimated to be 680 kb. This is the first report of isolation of an intact phytoplasma chromosome directly from naturally infected grapevine. Using PFGE and Southern hybridization, no significant difference in size was observed between full-length chromosomes of TBBp isolates from different regions and hosts. Some variation was observed after digestion of chromosomes of different TBBp isolates with *Bss*HII indicating that some genomic diversity exists amongst isolates of TBBp. AGYp chromosomal DNA was not detected using PFGE and Southern hybridization.

A new phytoplasma was detected in grapevines with grapevine yellows disease (GYd) from the Buckland Valley of Victoria ~~was characterised~~ & characterized. Buckland Valley grapevine yellows phytoplasma (BVGYp) could not be amplified by PCR using primers specific for AGYp and the stolbur group of phytoplasmas indicating that it was unlikely to be a stolbur group phytoplasma. BVGYp was amplified by PCR using primers specific for the aster yellows phytoplasma group, indicating that it may be more closely related to the Aster yellows group phytoplasmas. Sequence analysis of 16SrRNA gene sequences showed that BVGYp clustered with members of the Aster yellows group of phytoplasmas and had greatest sequence similarity with Clover phyllody phytoplasma (97.1%) of the Aster yellows group.

The associated disease, GYd, was surveyed in three blocks of Chardonnay over four years in one vineyard. GYd affected many grapevines and was characterised by remission of disease, some recurrence and occurrences in previously unaffected grapevines. A regional survey of the Buckland Valley indicated that GYd and BVGYp occurred in the

same restricted grape growing area. Within this area BVGYp was detected in two vineyards that had been established using planting material from different sources. One could therefore speculate that BVGYp was present in these grapevines as a result of aerial transmission and was not present in the original planting material.

## THESIS DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed:

Fiona Elizabeth Constable

Dated: 26/03/02.

**Acknowledgements**

Many thanks to all my supervisors:

Prof. Bob Symons for giving me the opportunity to do a PhD in his lab and for the lively discussions about grapevines and their pathogens.

Dr. Karen Gibb for her interest and ideas throughout my grapevine phytoplasma experience and especially for taking on my supervision for the writing of this thesis.

Prof. John Randles also for taking on my supervision for the writing of this thesis and all the energy he expended helping me to finish.

Prof. Peter Langridge for his assistance and support.

Thankyou to my husband, Jamie, for encouraging me and believing in my abilities.

Thanks to my parents who encouraged a good education in science.

Many thanks to all the members of the "Symons lab": Halina Bogcaz, Dr. Rod Davies, Dr. Helen Healy, Dr. Brendan King, Dr. Ying Hong Liu, Dr. Nuredin Habili, Dr. Nick Paltridge and Daryl Webb. It was good fun! Special thanks to Helen for looking after me in so many ways and for many words of wisdom.

None of the work could have been done without the growers, who allowed me to trek through their vineyards and take samples! In particular I would like to thank Lucky and Midge Cameron, Carmel and Grant Carey, Frank Sos, Adrian Lacey, Mr. Whiffen and the Michilini's.

To the people who helped organise field trips, gather data and collect samples, thankyou so much. Special thanks to Linda Pollock for keeping me company in the field and recording information, field trips would have taken so much longer without your help! Many thanks to John Whiting for organising and assisting me with the Buckland Valley field work. Thankyou to my good friend and colleague Yasmin Chalmers also for helping me in the field and especially for feeding and entertaining me after a hard days work in the Sunraysia vineyards.

Thanks to Ms Janine Jones and her colleagues from BiometricsSA who analysed the survey data.

Thanks to Dr. Michael Maixner for providing German grapevine yellows DNA and the "Patchy" program that was also used to analyse the survey data.

Thanks to Dr. Anna Padovan for teaching me pulsed field gel electrophoresis.

This PhD was funded through an "Australian Research Council Strategic Partnership with Industry - Research and Training" grant and the industry partners were SouthCorp Wines Pty Ltd, Chalmers Nurseries and Sunraysia Nurseries Three years of survey data, from 1995/96 to 1997/98, from Sunraysia was collected during a three year project entitled "National program for the management of phytoplasmas in Australian grapevines". That project was funded by the Grape and Wine Research and Development Corporation, the Dried Fruits Research and Development Council and the Department of Natural Resources and Environment in Victoria, through the Collaborative Research Centre for Viticulure. From that time I thank the my direct project supervisors Ms Jane Moran, Dr.Greg Buchanan and Dr. DeAnn Glenn and the grapevine yellows steering committee. Huge thanks to Tim Griffin for his exceptional technical assistance during that time.

## GLOSSARY

| | | | |
|---|---|---|---|
| A | adenine | HMA | Heteroduplex mobility assay |
| AGYd | Australian grapevine yellows disease | IPTG | isopropyl-β-D-thiogalactopyranoside |
| AGYp | Australian grapevine yellows phytoplasma | Ile | isoluecine |
| BNp | bois noir phytoplasma | kb | kilobases |
| bp | base pairs | km | kilometre |
| BSA | bovine serum albumin | LSLCd | late season leaf curl disease |
| BVGYp | Buckland Valley grapevine yellows phytoplasma | M | molar |
| C | cytosine | $MgCl_2$ | magnesium chloride |
| °C | degrees Celsius | NT | Northern Territory |
| CHEF | contour-clamped homogeneous-electric field | min | minute |
| cm | centimetres | mL | millilitre |
| CTAB | cetyltri-methylammonium bromide | MLO | mycoplasma-like organism |
| dATP | 2'-deoxyadenosine 5'-triphosphate | mM | millimolar |
| dCTP | 2'-deoxycytidine 5'-triphosphate | NaCl | sodium chloride |
| DNA | deoxyribonucleic acid | $Na_2HPO_4$ | disodium hydrogen phosphate |
| dNTP | deoxynucleotide triphosphates | $NaH_2PO_4$ | sodium dihydrogen phosphate |
| DTT | dithiothreitol | NaOAc | sodium acetate |
| EDTA | ethylenediaminetetraacetic acid | NaOH | sodium hydroxide |
| FDp | flavescence dorée phytoplasma | ng | nanogram |
| g | grams | NTU | Northern Territory University |
| *G* | centrifugal force | PAG(E) | polyacrylamide gel (electrophoresis) |
| G | guanine | PCR | polymerase chain reaction |
| GYd | grapevine yellows disease | PDBp | papaya dieback phytoplasma |
| h | hour | PFG(E) | pulsed field gel (electrophoresis) |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid | PIPES | piperazine-N,N'-bis(ethanesulfonic acid |

| | |
|---|---|
| PVP-40 | polyvinyl pyrrolidine |
| PYLp | Phormium yellow leaf phytoplasma |
| rDNA | ribosomal DNA |
| RFLP | restricted length fragment polymorphism |
| RGd | restricted growth disease |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| s | second |
| SA | South Australia |
| Sarcosyl | n-lauryl sarcosine |
| SDS | sodium dodecyl sulphate |
| SPLL-V4p | sweet potato little leaf phytoplasma strain V4 |
| SSC | sodium chloride/sodium citrate |
| T | thymine |
| TBBp | tomato big bud phytoplasma |
| Tris | tris-(hydroxymethyl)aminomethane |
| tuf | elongation factor Tu gene |
| U | Unit |
| UV | Ultra violet |
| µCi | microcurie |
| µg | microgram |
| µL | microlitre |
| µM | micromolar |
| USA | United States of America |
| V | volts |
| Vic | Victoria |
| VKp | German grapevine yellows phytoplasma |
| $w/_v$ | weight per volume |
| water | sterile distilled water |
| X-Gal | 5-bromo-4-chloro-3-indoyl-β-D-galactoside |

## LIST OF FIGURES

## LIST OF TABLES

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 Background

Phytoplasmas were first observed in thin sections of phloem tissue from mulberry plants with a dwarf disease but not in unaffected mulberry plants (Doi *et al* 1967). Prior to this discovery, many diseases now known to be associated with phytoplasmas were attributed to viruses mainly because the associated symptoms could be induced in unaffected plants after they were grafted with affected material or were fed on by insect vectors that had fed on affected plants. Since their discovery, phytoplasmas have been associated with diseases of several hundred plant species worldwide (Seemüller *et al* 1998). Various symptoms have been associated with phytoplasma infections in plants including viresence, phyllody, yellowing, decline and dieback. Many phytoplasmas are spread to plants by insect vectors, most of which belong to the superfamilies Cicadelloidea (leafhoppers) and Fulgoroidea (planthoppers) (Lee *et al* 1998a).

Phytoplasmas were originally referred to as mycoplasma–like organisms (MLO) since morphologically and ultrastructurally they resemble animal mycoplasmas (*Mycoplasma sp.*), which belong in the class *Mollicutes* (common name: mollicutes) of the kingdom *Prokaryotae*. Like mycoplasmas, phytoplasmas lack a rigid cell wall, have a double membrane and are pleiomorphic. Also phytoplasmas are susceptible to antibiotics such as oxy-tetracycline but they are resistant to penicillin because they lack a cell wall (Razin and Freundt 1984). Like other members of the mollicutes, phytoplasma genomes have a low mole percent guanine plus cytosine content (mol % G+C) value compared to other organisms (Kollar and Seemüller, 1989; Sears *et al* 1989). Phytoplasmas differ from mycoplasmas in being obligate intracellular parasites, principally restricted to the phloem cells of infected plant hosts or the salivary

glands of their insect vectors (McCoy, 1984). Phytoplasmas have not been successfully cultured *in vitro* (Kirkpatrick, 1991).

**1.2 The phytoplasma genome**

The genome sizes of phytoplasmas are amongst the smallest known for cellular organisms. Full-length chromosomes have been isolated from more than 80 phytoplasmas representing 12 of the major phylogenetic groups and their genome sizes are between 530 kilobases (kb) to 1350kb (Firrao *et al* 1996; Gibb *et al* 1995; Marcone *et al* 1999; Neimark and Kirkpatrick 1993; Oshima *et al* 2001; Padovan *et al* 2000; Zriek *et al* 1995).

Physical and genetic maps have been produced for the chromosomes of western X-disease, apple proliferation, SPLL-V4 and European stone fruit yellows phytoplasmas (Firrao *et al* 1996; Lauer and Seemüller 2000; Marcone and Seemüller 2000; Padovan *et al* 2000). Such maps provide information on the arrangement of genes and how these organisms might function at the molecular level, perhaps providing information on how phytoplasmas might induce disease. Comparisons can be made between species and strains of phytoplasmas to identify areas of genomic conservation or alterations that might also provide clues as to function and plant-host or even vector-host interactions. For example a strain of onion yellows (OY-M) phytoplasma that only induces mild symptoms has a smaller genome size compared to the wild type line (OY-W) suggesting that some of the genes associated with pathogenicity are located on the chromosome of OY-W but have been deleted from OY-M (Oshima *et al* 2001).

Phytoplasma genomes have two ribosomal RNA (rRNA) operons, which contain the 16S rRNA, the intergenic $tRNA^{Ile}$ and 23S rRNA genes (Schneider and Seemüller 1994) and one of these rRNA operons is inverted on the sweet potato little leaf strain V4 phytoplasma (SPLL-V4p) chromosome (Padovan *et al* 2000). Sequence heterogeneity was observed between the 16S rRNA genes representing the two ribosomal operons of Phormium yellow

leaf strain of '*Candidatus* Phytoplasma australiense' (Liefting *et al* 1996). Recently a complete rRNA operon was constructed from Loofah witches' broom phytoplasma and the rRNA genes had an arrangement of 5'-16S-23S-5S-3' with a $tRNA^{Ile}$ in the intergenic spacer region and $tRNA^{Val}$ and $tRNA^{Asn}$ genes down stream of the 5S rRNA gene (Ho *et al* 2001). In some phytoplasmas the $tRNA^{Ile}$ gene is absent from one rRNA operon (Ho *et al* 2001; De la Rue *et al* 2001).

Several other phytoplasma genes have also been identified including membrane protein genes (Berg *et al* 1999; Kakizawa *et al* 2001), a DNA gyrase gene (Chuang and Lin, 2000) and a glucose-inhibited division protein gene (Padovan *et al*, 2000). The ribosomal protein gene *rps7* and the genes encoding for elongation factors Tu and G of phytoplasmas have a similar transcriptional organisation to *Escherichia coli* but a different organisation to the more closely related *Mycoplasma* species (Berg and Seemüller 1999). Currently researchers are attempting to sequence the entire genome of the western X-disease phytoplasma to determine the number and types of genes present on the phytoplasma genome (Liefting and Kirkpatrick, 2000).

Extrachromosomal DNAs have also been reported for several phytoplasmas (Chen *et al* 1992; Davis *et al* 1988a; Davis *et al* 1990; Denes and Sinah 1991; Harrison *et al* 1991; Kuboyama *et al* 1998; Kuske and Kirkpatrick 1990; Kuske *et al* 1991; Nakashima and Hayashi 1995; Nakashima and Hayashi 1997; Rekab *et al* 1999; Schneider *et al* 1992). The presence of extrachromosomal DNAs may be significant in the pathogenicity and virulence of phytoplasmas as this DNA may encode some biologically important genes. For example Kuboyama *et al* (1999) suggested that differences between plasmids of OY–W and OY-M phytoplasmas, notably the copy number of the plasmid, which is reduced in OY-M, and constructive heterogeneity between plasmids of OY-W and OY-M, may have also been associated with differences in pathogenicity between the two isolates of the phytoplasma.

Additionally, a potential translation product of extrachromosomal DNAs of the X-clade phytoplasmas shares a high sequence similarity to replication initiation proteins of geminiviruses (Rekab *et al* 1999).

**1.3 Taxonomy of phytoplasmas**

Phytoplasmas have not been cultured *in vitro* and many traditional methods for the classification of bacteria and bacteria like organisms that are based on morphological, biochemical, physiological, antigenic and pathogenic properties cannot been used to characterise them. Initially phytoplasmas were distinguished only by differences in host specificity, symptomatology and vector specificity (Kirkpatrick 1991; McCoy 1984). Phytoplasmas were grouped together taxonomically according to the similarity of the symptoms induced in host plants. For example, phytoplasmas causing yellows diseases were grouped together. After methods were devised to isolate intact phytoplasmas, antibodies were raised and used to differentiate and group some phytoplasmas according to their serological properties (Clark *et al.,* 1989a; Kirkpatrick, 1991).

With the development of various methodologies to study nucleic acid composition of organisms, the genetic relatedness of phytoplasmas to each other and to other mollicutes could be determined. Methods included comparisons of nucleic acid properties between organisms, such as mol % G+C and genome size (Johnson, 1984). Nucleic acid hybridisation/homology experiments were also used to determine the genetic relatedness between phytoplasmas (Ahrens *et al* 1993; Davis *et al* 1988b; Deng and Hiruki, 1991b; Lee and Davis 1988; Mäurer *et al* 1993; Schneider and Seemüller, 1994).

Most recently the classification of phytoplasmas has been based on direct comparisons of the nucleotide sequence of the 16S ribosomal ribonucleic acid (rRNA) gene. The 16S rRNA gene is present in all prokaryotic organisms and contains both variable and highly conserved regions, which make it suitable for phylogenetic classifications of many bacteria including

mollicutes (Stackebrandt 1991). On the basis of the sequence analysis of the 16S rRNA gene it has been shown that phytoplasmas form a distinct cluster within the class *Mollicutes* (Gundersen *et al* 1994; Kuske and Kirkpatrick 1992; Lee *et al* 1993b; Lim and Sears 1989; Seemüller *et al* 1994). "Phytoplasma" is now the accepted trivial name of this distinct group of mollicutes.

Various classification schemes have been proposed using sequence analysis and restriction site analysis of the 16S rRNA gene and the phytoplasma cluster has been divided into several major groups and subgroups (Gundersen *et al* 1994; Lee *et al* 1993b; Lee *et al* 1998b; Schneider *et al* 1993; Seemüller *et al* 1994). At the time of writing this thesis 15 major phytoplasma groups (Table 1.1), named for their type strain members, were recognised based on restriction site analysis of the 16SrRNA gene and ribosomal protein gene sequences (Lee *et al* 1998b; Montano *et al* 2001).

Other genes or DNA regions have also been used for the differentiation and classification of phytoplasmas and include the ribosomal protein genes *rpl22 and rps3* (Gundersen *et al* 1994; Lim and Sears 1992; Toth *et al* 1994), the 16S - 23S rRNA spacer region (Kirkpatrick *et al* 1994, Schneider *et al* 1995) and the elongation factor Tu (*tuf*) gene (Marcone *et al* 2000; Schneider *et al* 1997). Both the *rpl22 and rps3* ribosomal protein genes and 16S-23S spacer region are more than the 16S rRNA gene yet phylogenetic analysis of the ribosomal protein genes and the spacer region generally results in similar phylogenetic groupings of phytoplasmas found using the 16S rRNA gene. The *tuf* gene is also more variable in sequence compared to the 16S rRNA gene and is useful for further differentiating members of the aster yellows groups (Schneider *et al* 1997). Marcone *et al* (2000) have used the *tuf* gene to confirm an additional aster yellows subgroup identified after RFLP analysis of the combined 16S rRNA gene and 16S-23S spacer region sequence.

Table 1.1 The major phytoplasma groups as determined by restriction fragment length polymorphism analyses of 16S rRNA and ribosomal protein gene sequences (Lee *et al* 1998b; Montano *et al* 2001)

| 16Sr phytoplasma group | Group name |
|---|---|
| 16SrI | Aster Yellows group |
| 16SrII | Peanut witches' broom group |
| 16SrIII | X-disease group |
| 16SrIV | Coconut lethal yellows group |
| 16SrV | Elm yellows group |
| 16SrVI | Clover proliferation group |
| 16SrVII | Ash yellows group |
| 16SrVIII | Loofah witches' broom group |
| 16SrIX | Pigeon pea witches'-broom group |
| 16SrX | Apple proliferation group |
| 16SrXI | Rice yellow dwarf group |
| 16SrXII | Stolbur group |
| 16SrXIII | Mexican periwinkle viresence group |
| 16SrXIV | Bermuda grass white leaf group |
| 16SrXV | Hibiscus witches' broom group |

**1.4 Detection of phytoplasmas**

Several methods are used to detect phytoplasmas in plant hosts or insect vectors. Fluorescence microscopy using 4',6-diamidino-2-phenylindole (DAPI) staining techniques have been widely used to detect phytoplasma DNA in the phloem of infected plants (Cazelles 1978; Dale 1988; Hiruki and Deng 1992; Pastore *et al* 1995; Schaper and Converse 1985; Sinclair and Griffiths 1995; Sinclair *et al* 1989). Electron microscopy has been used to observe phytoplasma bodies in the phloem of infected plants (Andersen *et al* 1998; Gibb *et al* 1995; Greber and Gowanlock 1979; Plavsic-Banjac *et al* 1973; Seemüller and Lederer 1988). DNA

probes for *in situ* hybridisation in conjunction with light microscopy (Deng and Hiruki 1991a) and oligodeoxynucleotides as probes for *in situ* hybridisation in conjunction with transmission electron microscopy (Lherminier *et al* 1999) and confocal microscopy (Webb *et al* 1999) have been used to localise specific phytoplasmas in plant cells and insect vectors. Serological assays, using polyclonal or monoclonal antibodies raised against purified phytoplasmas, have also been developed to detect and identify phytoplasmas, including enzyme linked immunosorbent assays (ELISA), immunofluorescence microscopy, immunosorbent electron microscopy and gold label antibody decoration (Boudon-Padieu *et al* 1989; Chen and Jiang 1988; Clark *et al* 1989b; da Rocha *et al* 1986; Guo *et al* 1998; Hsu *et al* 1990; Lee *et al* 1993a; Milne *et al* 1995; Saeed *et al* 1993; Vera and Milne 1994). Cloned phytoplasma DNA has been used to generate random probes for the detection of phytoplasmas in plant and insect hosts by nucleic acid hybridisation (Ahrens *et al* 1993; Chen *et al* 1994; Davis *et al* 1988b; Deng and Hiruki 1990; Deng and Hiruki 1991b; Kirkpatrick *et al* 1987; Schneider and Seemüller 1994).

Polymerase chain reaction (PCR) techniques for the detection of phytoplasmas were first reported in 1990 (Deng and Hiruki 1990). PCR amplification of the 16S rRNA gene is currently the most widely used tool for phytoplasma detection and is sensitive, specific and rapid. "Universal" PCR techniques have been developed that allow amplification of the 16S rRNA gene of any known phytoplasma (Ahrens and Seemüller 1992; Deng and Hiruki 1991c; Gundersen and Lee 1996; Lee *et al* 1993b; Smart *et al* 1996). DNA sequence analysis of the PCR product can be used to confirm the identity of the phytoplasma detected by PCR. Digestion of the PCR amplification product with restriction endonucleases gives a DNA "fingerprint" and can also be used to identify the phytoplasma (Ahrens and Seemüller 1992; Lee *et al* 1998a). Additionally, specific diagnostic PCR tests have been developed to detect and identify members of groups of phytoplasmas and individual phytoplasmas (Davis and Lee

1993; Gibb *et al* 1999; Lee *et al* 1994; Smart *et al* 1996). PCR methods have also been used to amplify other regions of DNA for phytoplasma detection, including ribosomal protein genes (Lee *et al* 1998a; Lim and Sears 1992), the *tuf* gene (Schneider *et al* 1997) and the 23S rRNA gene (Guo *et al* 2000).

**1.5 Grapevine phytoplasmas**

Phytoplasmas have been associated with yellows diseases of grapevines in many viticultural regions worldwide. Molecular techniques have shown that there are several distinct grapevine yellows phytoplasmas from different regions that represent various phylogenetic groups and subgroups. However, the symptoms associated with phytoplasma infections of grapevines are similar in each region and include:

- Irregular yellowing and backward curling of the leaves, which drop from the vine early
- The yellow leaf tissue may become necrotic
- Overlapping of leaves on affected shoots
- Tips of affected shoots may die and shoots may die back
- Affected shoots fail to harden off and remain rubbery
- Flowers on affected shoots may abort or berries may shrivel

Significant reductions in yields have been reported for some grapevine yellows diseases (Caudwell 1964; Constable *et al* 2000b; Magarey and Wachtel 1986b).

The flavescence dorée disease (FDd) and the associated FD phytoplasma (FDp) have been detected in France, Spain, Italy and Germany (Batlle *et al* 1997; Daire *et al* 1993; Daire *et al* 1997; Kuszala *et al* 1993; Maixner *et al* 1995a; Prince *et al* 1993). FDp is a member of the Elm Yellows (EY, 16SrV) group of phytoplasmas (Daire *et al* 1993; Davis and Prince 1994; Prince *et al* 1993). Some genetic variation has been observed amongst isolates of FDp based on differences of the 16S rRNA gene sequence and other DNA fragments (Angelini *et al*

2001; Daire *et al* 1997; Davis and Dally 2001). In some cases the reported variability in FDp is associated with isolates from different countries.

The natural vector for FDp is the leafhopper *Scaphoideus titanus* (Ball), which lives and feeds on grapevines (Bonfils and Schvester 1960; Caudwell *et al* 1970). Subsequently FDp is spread via *S. titanus* from grapevine to grapevine (Schvester *et al* 1969). FDp has also been shown to spread through infected cuttings and rootstocks and a hot water treatment of cuttings is recommended to control spread of this phytoplasma (Caudwell *et al* 1994; Caudwell *et al* 1997; Pavan *et al* 1997). In Germany, a variant of FDp is found in the absence of *S. titanus* (Maixner *et al* 1995b).

Both Bois Noir (BNd) and Vergilbungskrankheit (VKd) diseases and the associated phytoplasmas, BNp and VKp respectively, belong to the Stolbur group (16SrXII) of phytoplasmas, and are very closely related (Daire *et al* 1994; Lee *et al* 1998b; Maixner *et al* 1995a). Both phytoplasmas are vectored by the planthopper *Hyalesthes obsoletus* Signoret (Maixner 1994; Sforza *et al* 1998). The alternative host bindweed (*Convulvulus arvensis*) has been identified for both BNp and VKp and is a primary host for *H. obsoletus* in vineyards (Maixner 1994; Sforza *et al* 1998). Additionally, several alternative hosts for a Stolbur phytoplasma, considered to be BNp, were identified in France including sweet cherry (*Prunus, sp.*) lilac (*Syringa vulgaris*) fig tree (*Ficus carica*), Elm (*Ulmus sp.*) and hoary cress (*Cardaria drabra*), also a host for *H. obsoletus* (Sforza *et al* 1998). Phytoplasmas of the Stolbur group, including BNp and VKp, have been detected in grapevines in France, Germany, Italy, Greece, Portugal, Israel, Croatia and Spain (Daire *et al* 1994; Davis *et al* 1997b; Lavina *et al* 1995; Maixner 1994; Prince *et al* 1993; Seruga *et al* 2000).

Phytoplasmas of the Western X group (16SrIII) have been identified from grapevines in Italy and Virginia in the United States of America (Prince *et al* 1993; Davis *et al* 1998). The phytoplasma found in Virginia is distinct from other members of the Western X group and has

been designated VGYIII and belongs in the subgroup III-I (Davis *et al* 1998). In addition a second grapevine phytoplasma was detected in Virginia (VGYI) and is a member of the Aster Yellows group (16SrI), sub group I-A and is distinct from other grapevine phytoplasmas (Davis *et al* 1998).

Several other phytoplasmas including clover phyllody phytoplasma (Chen *et al* 1994) and Italian periwinkle viresence phytoplasma (Alma *et al* 1996) have also been detected in grapevines. Phytoplasmas of the Aster yellows group have been detected in grapevines but not further identified in Italy (Arzone *et al* 1995; Bianco *et al,* 1993; Chen *et al* 1994; Davis and Prince 1994; Prince *et al* 1993). Grapevines with yellows symptoms have also been reported in Lebanon (Haidar *et al* 1996).

**1.6 Australian grapevine yellows and associated phytoplasmas**

Grapevine yellows symptoms were first reported in 1976 in Australia and the disease referred to as Australian Grapevine yellows (AGYd) (Magarey and Wachtel, 1983). A phytoplasma aetiology was inferred for AGYd because AGYd symptoms are similar to those of other grapevine yellows diseases that are also associated with phytoplasmas. Additionally, electron microscopy (Magarey *et al* 1988), antibiotics (Magarey and Wachtel 1986b) and PCR techniques (Bonfiglioli *et al* 1995; Gibb *et al* 1999; Padovan *et al* 1995) have confirmed an association between phytoplasmas and AGYd.

Early and recent surveys have shown that AGYd and phytoplasmas are found in most viticultural regions of Australia (Bonfiglioli *et al* 1996; Magarey and Wachtel 1986b). A particularly high incidence of AGYd occurs in the warmer inland districts of Sunraysia in New South Wales and Victoria, Murrimbidgee Irrigation Area in New South Wales and the Riverland in South Australia compared to other regions. Chardonnay and Riesling appear to be most often affected (Magarey and Wachtel 1986a) but phytoplasmas have been detected in other white and red varieties (Bonfiglioli *et al* 1996). Previous research has shown that the

incidence of AGYd may be temporarily high in one year in some Chardonnay vineyards (Magarey and Wachtel 1986b). At the time of writing this thesis Chardonnay is the most commonly planted white wine grape variety in Australia. In 2000 the area planted with Chardonnay for the entirety of Australia had increased from 9846 Ha in 1994/95 (Anon. 1996) to 18434 Ha (Anon. 2001). With the increased plantings of Chardonnay in Australia, particularly in the warmer inland districts, it is possible that AGYd has become more prevalent and the nature of the spread and expression of disease has also changed.

PCR for the detection of Australian grapevine phytoplasmas has been shown to be most reliable in January or February when AGYd affected shoots are sampled (Gibb *et al* 1999). However, the distribution of phytoplasmas within infected grapevine tissues, such as shoots cordons, trunk and roots, throughout a season is not known. Location and titre of phytoplasmas in various grapevine tissues may also influence the effectiveness of the PCR test to detect phytoplasmas, particularly if phytoplasmas are unevenly distributed throughout a grapevine or located in parts other than shoots.

Three phytoplasmas, differentiated by sequence analysis and restriction enzyme analysis of the 16S rRNA gene and the 16S-23S spacer region, have been detected in AGYd affected grapevines (Constable *et al* 1998; Gibb *et al,* 1999). These phytoplasmas include the AGY phytoplasma (AGYp), of the Stolbur group (16SrXII) of phytoplasmas, and the Australian tomato big bud phytoplasma (TBBp), of the peanut witches' broom group (16SrII) of phytoplasmas. The third phytoplasma, as yet uncharacterised, was detected by PCR and RFLP analysis in AGYd affected grapevine samples in one vineyard located in the Buckland Valley of Victoria (Gibb *et al* 1999). Of the three phytoplasmas, the AGY phytoplasma is most commonly detected in grapevines with AGYd (Gibb *et al* 1999).

AGYp has recently been designated the *Candidatus* species name '*Candidatus* Phytoplasma australiense' as analysis of the 16SrRNA gene sequence showed that the AGYp

is distinct from other members of the Stolbur group, which also includes the VK and BN phytoplasmas (Davis *et al* 1997). On the basis of the 16S rRNA gene sequence AGYp has a 99.7% sequence similarity with the papaya dieback phytoplasma (PDBp) found in northern Australia (Padovan *et al* 1996) and a 99.5% sequence similarity with *Phormium* yellow leaf phytoplasma (PYLp) in New Zealand (Liefting *et al* 1998). It has been suggested that all three phytoplasmas are strains of the one species of phytoplasma (Liefting *et al* 1998). No insect vector has been identified for AGYp, although AGYp has been detected in the common brown leafhopper, *Orosius argentatus* (Evans) using PCR techniques (Beanland *et al* 1999). Other phytoplasmas, closely related to AGYp, are transmitted by planthopper species from the family Cixiidae and PYLp is transmitted by *Oliarus atkinsonii* in New Zealand (Cumber 1953; Liefting *et al* 1997).

'*Candidatus* Phytoplasma australasia' is the designated *Candidatus* species name of TBBp (White *et al* 1998). TBBp has a broad plant host range (Davis *et al* 1997; Schneider *et al* 1999) and its detection in grapevines was not surprising. *O. argentatus* is a vector of TBBp in other crops (Hill 1943; Hosking and Danthanarayana 1988; Osmelak 1984; Osmelak 1988; Osmelak *et al* 1989). Recent studies have shown that TBBp can be acquired from grapevine by *O. argentatus* and subsequently transmitted to Faba bean (Beanland 2001) but the ability of the leafhopper to transmit TBBp back to grapevines has not been confirmed.

The transmission of phytoplasmas through grapevines cutting has not been demonstrated (Magarey 1986).

Restricted growth disease (RGd) also commonly affects Chardonnay grapevines in Sunraysia but the aetiology of the disease is not known (Bonfiglioli *et al* 1995; Padovan *et al* 1995). Symptoms of RGd include retarded growth resulting in shortened shoots and smaller leaves. The affected grapevines have an overall appearance of stunting or lack of vigour throughout the season compared to unaffected grapevines. Some grapevines with RGd may

have also displayed uneven or no bud development resulting in canes and cordons that were bare in places or entirely bare with little or no bunch development. The disease can cause yield loss. No significant association between phytoplasmas and RGd affected shoots has been shown (Bonfiglioli *et al* 1995; Padovan *et al* 1995; Gibb *et al* 1999), yet phytoplasmas are considered as one potential cause.

Late season leaf curl disease (LSLCd) can also affect Chardonnay grapevines in the Sunraysia district (Bonfiglioli *et al* 1995; Gibb *et al* 1999). Symptoms associated with LSLCd include the appearance of symptoms from late summer where leaves on affected shoots roll tightly downward, remain green, and overlay one another in a shingled appearance (Figure 2.3). The leaves are often tough, leathery and quite brittle. In one study phytoplasmas were detected in 48/59 LSLCd affected shoot samples (late AGY; Bonfiglioli *et al* 1995) but in another study phytoplasmas were detected in 12/126 shoot samples from LSLCd affected grapevines (Gibb *et al* 1999). Consequently the association between phytoplasmas and LSLCd is uncertain.

From observations in one vineyard, Bonfiglioli *et al* (1997) proposed that AGYd, RGd and LSLCd diseases were related and that LSLCd was followed by the presence of AGYd in the same grapevines in following years. It was also proposed that AGYd often led to the presence of RGd in subsequent years. Other research using an analysis of relative risk on the observations from three vineyards over three years also suggested that AGYd may be associated with RGd or LSLCd (Constable *et al* 2000a).

Remission of AGYd, RGd and LSLCd has been observed (Magarey and Wachtel 1986b; Constable *et al* 2000a). The cause of remission of each disease is not known but the decline in phytoplasma titre may contribute. "Heat curing" of AGYd after several days of hot weather has been observed (Magarey and Wachtel 1986b) and it has been speculated that this contributes to the decline in phytoplasma numbers and consequently remission of disease.

Remission or recovery from grapevine yellows symptoms, similar to AGYd, in affected vines in Europe is commonly observed including the grapevine yellows (GY) disease in Italy (Osler 1993) and FDd in France (Caudwell, 1961; Caudwell, 1964). However, statistically, once vines had been affected with AGYd, they were at greater risk of displaying the symptoms again in following years (Constable *et al* 2000a) indicating persistence of disease. Similarly once a vine has been affected by RGd or LSLCd it was more likely to display the disorder in following years (Constable *et al* 2000a).

Phytoplasmas and diseases with a real or possible association with phytoplasmas, including AGYd, RGd and LSLCd, are of considerable concern to the Australian viticulture industry because of reduced grapevine health and potential yield loss. Consequently research on Australian grapevine phytoplasmas has been intensive, particularly since 1995, and understanding of grapevine phytoplasmas and associated diseases has improved. The research presented in this thesis aimed to further the knowledge of the biology and epidemiology of Australian grapevines phytoplasmas.

### 1.7 Aims

The overall aims of the work presented in this thesis were:

1. **To determine the seasonal persistence and distribution of phytoplasmas in Australian grapevines**
2. **To determine the incidence and distribution of AGYd, RGd and LSLCd in Australian vineyards**
3. **To characterise phytoplasma chromosomes isolated directly from Australian grapevines**
4. **To characterise the new phytoplasma associated with grapevine yellows in the Buckland Valley of Victoria**

# CHAPTER 2

# THE SEASONAL DISTRIBUTION OF PHYTOPLASMAS IN AUSTRALIAN GRAPEVINES

## 2.1 Introduction

Australian grapevines yellows phytoplasma (AGYp) and tomato big bud (TBBp) phytoplasma are associated with Australian grapevine yellows disease (AGYd, Bonfiglioli *et al* 1995; Padovan *et al* 1995; Gibb *et al* 1999). Polymerase chain reaction (PCR) techniques used to detect phytoplasmas in AGYd affected shoots were most reliable in January/February, when the incidence of symptomatic grapevines is also highest. From March onwards, the reliability of the PCR test for phytoplasmas declines (Constable *et al* 1998; Gibb *et al* 1999). There are no reports that phytoplasmas can be detected in samples from cordons, trunks or roots of Australian grapevines. It is not known whether using these tissues can increase the reliability of the PCR test for phytoplasma detection, especially in spring, autumn and winter when shoots are less reliable.

Remission of AGYd has been observed (Magarey and Wachtel 1986b; Constable *et al* 2000a). However, to date there have been no reports which show that the remission of AGYd is due to the "disappearance" of phytoplasmas from grapevines. Persistent expression of AGYd can also occur (Constable *et al* 2000a). It is not known whether the persistence of AGYd from year to year is due to persistent infections of phytoplasmas or to new infection events.

Restricted growth disease (RGd) can be expressed in grapevines with AGYd (Bonfiglioli *et al* 1997; Constable *et al* 2000a). The aetiology of RGd is unknown. To determine whether phytoplasmas may be involved, shoots from grapevines with RGd have been tested by PCR but no association between phytoplasmas and RGd was shown (Bonfiglioli *et al* 1995; Padovan *et al* 1995; Gibb *et al*, 1999).

Late season leaf curl disease (LSLCd) can also affect grapevines with AGYd (Bonfiglioli *et al* 1997; Constable *et al* 2000a). Shoots from grapevines with LSLCd were also tested by PCR to determine whether phytoplasmas may be involved (Bonfiglioli *et al* 1995; Gibb *et al* 1999). The association between phytoplasmas and LSLCd is unclear as some researchers found a high association with phytoplasmas and LSLCd affected shoots (80%, Bonfiglioli *et al* 1995) and others found a low association (10%, Gibb *et al* 1999).

This chapter describes studies on the persistence and distribution of phytoplasmas in grapevines and the relationship between detection of phytoplasmas and the expression of RGd, AGYd and LSLCd. The relevance of these results to detection and identification of phytoplasmas in grapevines is discussed. Restriction fragment length polymorphism (RFLP) and heteroduplex mobility assays (HMA) have been used to determine if there was detectable variation amongst isolates of AGYp and TBBp in Australian grapevines.

## 2.2 Methods

### Source of infected plants

To determine the persistence and distribution of phytoplasmas within grapevines a cohort of 20 grapevines, var. Chardonnay, from three vineyards in Sunraysia were examined for the presence of phytoplasmas over two seasons. The vineyard location, the history of disease and phytoplasmas that were detected is listed in Table 2.1 for each of the 20 grapevines. Grapevines in group A were selected because they had a known history of disease and phytoplasma infection with AGYp or TBBp. Grapevines in group B were selected because they had no history of disease. Grapevines in group C were selected because they had no previous history of disease and displayed AGYd for the first time in December 1998.

For the heteroduplex mobility assay (HMA), additional AGYp and TBBp isolates were collected from Sunraysia from various grapevine varieties including Chardonnay, Riesling and a red table grape variety. AGYp and TBBp isolates were also collected from the

Riverland region of South Australia (SA), from the grapevine varieties Chardonnay and Shiraz, from the Barossa Valley, SA, in the varieties Chardonnay and Semillon and from Echuca, Victoria, in the grapevine variety Chardonnay. A TBBp isolate from *Hypochoeris radicata* was collected from a vineyard in McLaren Vale, SA.

**Disease descriptors**

Restricted growth disease (RGd) symptoms included retarded growth resulting in shortened shoots and smaller leaves. Affected grapevines have an overall appearance of stunting or lack of vigour throughout the season compared to unaffected grapevines (Figure 2.1). Some grapevines with RGd may have also displayed uneven or no bud development resulting in canes and cordons that were bare in places or entirely bare with little or no bunch development.

Australian grapevine yellows disease (AGYd) symptoms included one or more shoots showing irregular veinal and interveinal yellowing and downward rolling of leaves that overlaid one another in a shingled appearance (Figure 2.2). Shoots affected with AGYd may also have displayed abortion of the flowering bunches early in the season or berry shrivel later in the season. Affected shoots often displayed tip death followed by dieback of the shoots, node by node. Leaf blades on affected shoots tended to fall early. The petioles remained attached to the shoots for longer than the leaf blades but would eventually abscise from the shoot. The stem of affected shoots often developed a blue, waxy appearance and remained rubbery later in the season.

Late season leaf curl disease (LSLCd) was defined by the appearance of symptoms from late summer where leaves on affected shoots rolled tightly downward, remained green, and overlaid one another in a shingled appearance (Figure 2.3). The leaves were often tough, leathery and brittle.

Table 2.1. The vineyard location, date of planting, disease history and the phytoplasma species detected prior to 1998 for each of the 20 grapevines used in the study of persistence and distribution of phytoplasmas in grapevines.

| Group | Grapevine | Vineyard | Year planted | Disease history | | | Phytoplasma detected |
|---|---|---|---|---|---|---|---|
| | | | | 1995/96 | 1996/97 | 1997/98 | |
| A | 1 | Gol Gol | 1992 | AGYd | | RGd AGYd | AGYp |
| | 2 | Gol Gol | 1992 | | AGYd | RGd | AGYp |
| | 3 | Gol Gol | 1992 | AGYd | | RGd | AGYp |
| | 4 | Karadoc | 1993 | AGYd RGd LSLCd | RGd LSLCd | AGYd | TBBp |
| | 5 | Karadoc | 1993 | RGd LSLCd | RGd LSLCd | RGd LSLCd | TBBp |
| | 6 | Karadoc | 1993 | AGYd RGd LSLCd | RGd LSLCd | AGYd RGd LSLCd | TBBp |
| | 7 | Wemen | 1993 | | LSLCd | AGYd LSLCd | AGYp |
| | 8 | Wemen | 1993 | | | AGYd RGd LSLCd | AGYp |
| | 9 | Wemen | 1993 | | AGYd LSLCd | RGd LSLCd | AGYp |
| B | 10 | Gol Gol | 1992 | Asymptomatic | | | unknown |
| | 11 | Gol Gol | 1992 | Asymptomatic | | | unknown |
| | 12 | Karadoc | 1993 | Asymptomatic | | | unknown |
| | 13 | Karadoc | 1993 | Asymptomatic | | | unknown |
| | 14 | Wemen | 1993 | Asymptomatic | | | unknown |
| | 15 | Wemen | 1993 | Asymptomatic | | | unknown |
| C | 16 | Gol Gol | 1993 | Asymptomatic – displayed AGYd for the first time in December 1998 | | | unknown |
| | 17 | Gol Gol | 1993 | Asymptomatic – displayed AGYd for the first time in December 1998 | | | unknown |
| | 18 | Karadoc | 1993 | Asymptomatic – displayed AGYd for the first time in December 1998 | | | unknown |
| | 19 | Karadoc | 1993 | Asymptomatic – displayed AGYd for the first time in December 1998 | | | unknown |
| | 20 | Karadoc | 1993 | Asymptomatic – displayed AGYd for the first time in December 1998 | | | unknown |

a.

b. c.

Figure 2.1. Examples of RGd affected grapevines, var. Chardonnay.

a. An example of a grapevine affected with a severe form of RGd

b. and c. Milder forms of RGd. The arrows indicate the affected grapevines. Note that the canopy of each grapevine is not as well developed as other neighbouring grapevines that are considered to be 'healthy'.

Figure 2.2. Examples of shoots from grapevines, var. Chardonnay, affected by AGYd.

(a) Early symptoms of AGYd developing on a young shoot. Note the veinal chlorosis, the beginning of interveinal chlorosis and overlapping leaves, which are rolling downwards

(b) Downward curling and veinal chlorosis, with some necrosis, on AGYd affected Chardonnay leaves

(c) Irregular yellowing and some necrosis of leaves from a Chardonnay shoot with AGYd.

(d) Irregular yellowing and necrosis of leaves on a shoot affected by AGYd. The bunch attached to the shoot shows berry shrivel.

(e) Irregular reddening and backward curling of leaves from a Shiraz shoot associated with AGYd. This grapevine was positive for AGYp

(f) Irregular chlorosis and backward rolling of leaves from an AGYd affected Semillon shoot. This grapevine was positive for TBBp

a.

b

c.

d.

e.

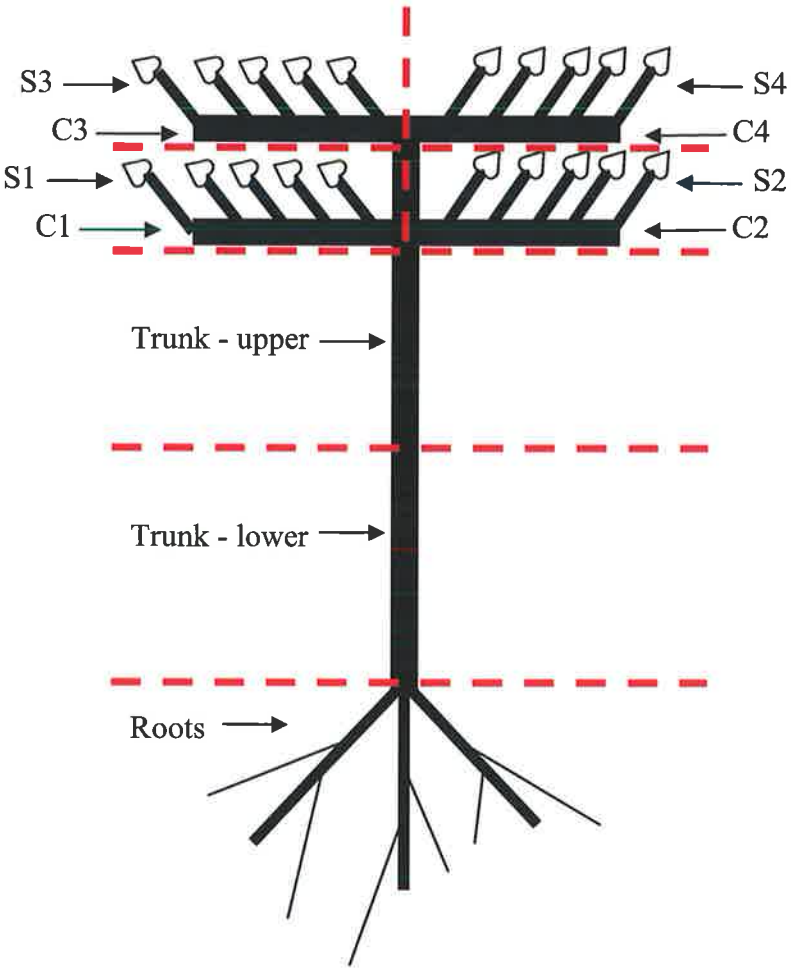
f

Figure 2.3. An example of an LSLCd affected shoot from the variety Chardonnay. Note that the leaves are rolled backwards tightly and overlapping one another.

**Sampling of grapevine tissues**

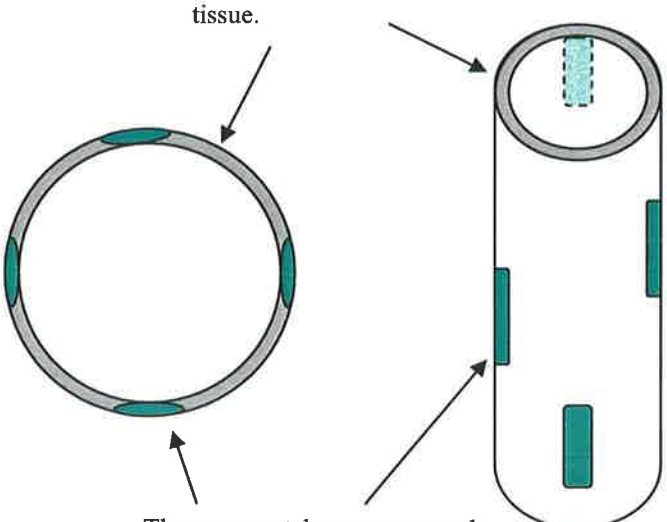
To determine the location of phytoplasmas within grapevines throughout the year and in subsequent years, vascular tissue was sampled from shoots, cordons, trunk and roots from each of the 20 grapevines. Figure 2.4a shows a schematic representation of a grapevine, illustrating the 11 sampling sites for shoots, cordons, trunk and roots. For each grapevine, the canopy was divided into four sections and a shoot and a cordon sample were taken from each section. Three shoots were selected from each section of the canopy and shoots with obvious AGY symptoms were selected in preference to asymptomatic shoots. From each cordon, four to five sections, approximately 1cm wide and 2 cm long, of vascular tissue, were randomly selected and pooled into one sample. The trunk of each grapevine was sampled separately from the upper and lower sections. From each section of the trunk, four sections, approximately 1.5cm wide and 3cm long, of vascular tissue were randomly selected and pooled into one sample (Figure 2.4b). Five to six pieces of fine and thick roots were randomly selected within a 60cm radius and a 60 cm depth of base of the trunk and pooled into one sample for each grapevine.

Grapevines in group A and B (Table 2.1) were selected and sampled in October1998 and were sampled again in January, April, July and October of 1999 and in January, April and July of 2000. Grapevines in group C (Table 2.1) were selected and sampled in December 1998 and then at the same time as the other grapevines.




a.

The grey ring represents the inner bark, which contains vascular tissue.



b.

The green patches represent where vascular tissue was sampled from the inner bark of trunk and cordons at each sampling period

Figure 2.4. A diagrammatic representation of the areas from which the 20 grapevines were sampled.

a. Each grapevine was sampled from 11 locations at each sampling period including roots, lower trunk, upper trunk, cordons and shoots. The canopy was divided into four sections and separate cordons (C1-C4) and shoots (S1-S4) samples were taken from each section.

(b) A schematic representation of how phloem was collected from the cordons and trunks of each grapevine during each sampling period. From each cordon, four to five sections, approximately 1cm wide and 2 cm long, of vascular tissue that contained phloem, were randomly selected and pooled into one sample. Similarly four sections, approximately 1.5cm×3cm, of vascular tissue were randomly selected and pooled into one sample from the upper area and the lower area of the trunk of each grapevine. Five to six pieces of fine and thick roots were selected from each grapevine and pooled into one sample.

**Extraction of DNA from grapevine**

Leaf veins, petioles and scrapings of vascular tissue from stems were used for DNA extractions from shoots. Vascular tissue was scraped from the inner bark of cordon, trunk and root samples and used for DNA extraction. A separate sterile scalpel blade was used for each sample to avoid contamination between samples. DNA was extracted from each sample using a DNeasy plant DNA extraction kit (Qiagen, Inc. Mississauga, ON, USA) according to the method of Green *et al* (1999).

**Primers for amplification of phytoplasma DNA in grapevine**

The PCR primers used to amplify phytoplasma DNA in grapevines are listed in Table 2.2. A nested PCR procedure was used with the first primer pair fP1 and rP7 and the nested primer pair R16F2n and m23sr for universal detection of phytoplasmas in grapevines. These primers are specific for a region of the 16S rRNA and 23S rRNA genes in all known phytoplasmas. For specific detection of AGYp the second primer pair AUSGYF1 and AUSGYF2 was used instead of the generic nested phytoplasma primers. To specifically detect the TBBp the nested primer pair fU5 and rSPLL was used. The primer pair fP3 and m23SR was used to amplify the spacer region between the 16S and 23S rRNA genes (16S-23S SR DNA) from AGYp and TBBp. The primer pair ftufAY and rtufAY were used to amplify the gene coding for the elongation factor Tu (*tuf*) of AGYp

Table 2.2. PCR primers used to amplify phytoplasma DNA in grapevines

| Primer pair | Primer name | Gene location | Position | Nucleotide sequence | Use in PCR and expected product size (kbp) | Reference |
|---|---|---|---|---|---|---|
| fP1/rP7 | fP1 | 16S | 6-30 | 5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3' | First round – universal 1.75 kbp | Deng and Hiruki (1991c) |
| | rP7 | 23S | 68-51 | 5'-CGT CCT TCA TCG GCT CTT-3' | | Schneider *et al* (1995b) |
| R16F2n/m23SR | R16F2n | 16S | 152-171 | 5'-GAA ACG ACT GCT AAG ACT GG-3' | Nested - universal 1.6 kbp | Gundersen and Lee (1996) |
| | m23SR | 23S | 51-20 | 5'-TAG TGC CAA GGC ATC CAC TGT G-3' | | Padovan *et al* (1995) |
| fP3/m23SR | fP3 | 16S | 1518-1534 | 5'-GGA TGG ATC ACC TCC TT-3' | Nested - universal 0.35 kbp | Schneider *et al* (1995b) |
| AUSGYF1/ AUSGYR2 | AUSGYF1 | 16S | 194-214 | 5'-ATC TTT AAA AGA CCT CGC AAG-3' | Nested - AGYp specific 0.64 kbp | Davis *et al* (1997) |
| | AUSGYR2 | 16S | 838-814 | 5'-AGT TTT ACC CAA TGT TTA GTA CTC-3' | | Davis *et al* (1997) |
| fU5/rSPLL | fU5 | 16S | 369-386 | 5'-CGG CAA TGG AGG AAA CT-3' | Nested - TBBp specific 0.9 kbp | Lorenz *et al* 1995 |
| | rSPLL | 16S-23S spacer region | 36-18 | 5'-AGA TGA TTG ATT TTA TTG G-3' | | Schneider and Gibb 1997 |
| ftufAY/ rtufAY | ftufAY | *tuf* | 24-41 | 5'-AAA AGT AGA GCT TAT GA-3' | Single round - Aster yellows/Stolbur group specific 0.94 kbp | Schneider *et al* (1997) |
| | rtufAY | *tuf* | 969-950 | 5'-CGT TGT CAC CTG GCA TTA CC-3' | | Schneider *et al* (1997) |

**Polymerase chain reaction (PCR)**

For PCR, each reaction contained 0.2mM of each dNTP, 0.625 µM of each primer, 1.5mM $MgCl_2$, 1xDNA polymerase buffer supplied with the enzyme and 2.5 U thermostable *Taq* DNA polymerase (Gibco/BRL, Life Technologies, Rockville MD, USA). For the first round or single PCR, 1 µL of undiluted or 1/10 diluted nucleic acid sample was added to the PCR mixture. For the nested PCR, 0.5 µL of the first round PCR reaction mixture was added to the PCR mixture containing the second primer pair. The total reaction volume was 20 µL in an MJM PTC-100 thermocycler (Geneworks, Adelaide, SA, Australia). For the first round of the nested PCR test with primer pair P1/P7, a hot start at 94°C for one minute was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1.5 min. PCR test conditions were the same for the second round of nested PCR with the generic primer pairs R16F2n/m23SR and fP3/m23SR and the specific primer pairs fAUSGY/rAUSGY and fU5/rSPLL were also the same. Single PCR test conditions with primer pair ftufAY/rtufAY were also the same except that the annealing temperature was 52°C.

After amplification, 5 µL from each sample was subjected to electrophoresis in a 1% agarose gel using 0.5×TBE (0.045M Tris-borate, 1mM EDTA, pH 8.0) running buffer. Products in gels were stained with ethidium bromide and visualised by UV transillumination. Total nucleic acid extracted from asymptomatic plants was used in the PCR as a negative control and water controls, in which no plant nucleic acid was added to the PCR mix, were also included. DNA markers were pUC19/*Hpa*II and SPP-1 Phage DNA/*Eco* RI markers (GeneWorks).

**Restriction fragment length polymorphism (RFLP) analysis**

RFLP analysis using restriction endonucleases was used to further elucidate the phytoplasma species that were detected in grapevines by universal nested PCR. Five-8 µL of the PCR product, amplified in the nested PCR, were digested using the restriction

endonucleases *Alu* I or *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 1.2% agarose gel in 0.5 ×TBE buffer and visualised by staining with ethidium bromide and photographed on a UV transilluminator.

**Heteroduplex mobility assay (HMA)**

HMA was used to detect variation amongst isolates of the same phytoplasma. HMA experiments were done with DNA fragments amplified by nested PCR using the primer pair fP3 and m23SR to amplify 16S-23S spacer region for both AGYp and TBBp. The *tuf* gene of AGYp was amplified using single PCR with the primer pair ftufAY and rtufAY and also used for HMA experiments. After PCR amplification the PCR products were run on a 1% agarose gel in 0.5× TBE and gel purified using an UltraClean™ Gelspin DNA purification kit (Mo Bio Laboratories, Inc., Solana Beach CA, USA) according to the manufacturer's instructions.

HMA was adapted from the method of Cousin *et al* (1998) and Bowyer *et al* (2000). Fifteen µL of PCR product from a phytoplasma standard was mixed with 15 µL of each purified PCR product, used as a test sample. Two µL of 10× annealing buffer (1 M NaCl, 100 mM Tris-Cl, pH 7.8 and 20 mM EDTA) was added to the mixture, which was then overlaid with mineral oil. The mixture was denatured at 98°C for 3 minutes then allowed to anneal at room temperature for 1 h. The 16S-23S rDNA samples were analysed by HMA using 6% polyacrylamide gels in 1×TBE buffer that were cast and run in a Hoefer SE 400 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA, USA). Before loading the samples, the gels were run for 20 min at 70 V. The samples were run on the gels for 5 h at 150 V and at room temperature. The *tuf* gene samples were analysed by HMA using 5% polyacrylamide, 4M urea gels in 1×TBE buffer in the same electrophoresis system. Before loading the samples, the gels were run for 20 min at 70 volts. The samples were run on the gels for 3.5 hours at 250 volts and at room temperature. After

electrophoresis, gels were stained with ethidium bromide (5ug/ml) for 20 minutes then destained with distilled water for 20 minutes and viewed using a UV transilluminator. Alternatively, the gels were stained using a Silver Stain Plus (BioRad Laboratories, Hercules, CA, USA) kit according to the manufacturers directions. DNA markers were pUC19/*Hpa*II and SPP-1 Phage DNA/*Eco* RI markers.

The AGYp standard for HMA was an arbitrarily selected isolate from a Gol Gol vineyard in the Sunraysia region of Victoria. The TBBp standard for HMA and DNA from an isolate of papaya dieback phytoplasma (PDBp) were provided by Dr. K. Gibb, Northern Territory University.

**2.3 Results**

**Detection and identity of phytoplasmas**

By July 2000 (the last sampling period) phytoplasmas were detected by PCR techniques in all 20 grapevines selected for the distribution and persistence studies (Table 2.3). Not all grapevines tested positive in any one sampling period. For example, in July 2000 the largest proportion of grapevines, 18/20, tested positive for phytoplasmas and in January 2000 the lowest proportion of grapevines, 8/19, tested positive for phytoplasmas. Grapevines 1, 4, 5 and 20 were the only grapevines that were positive for phytoplasmas for all sampling periods that they were tested.

AGYp and TBBp specific PCR and/or RFLP analysis identified the presence of either AGYp or TBBp in the twenty grapevines (Table 2.3). TBBp was detected in four grapevines from the Karadoc vineyard. AGYp alone was detected in the remaining 16/20 grapevines. Both AGYp and TBBp were detected in grapevines 5, 6 and 20. TBBp was detected every time grapevine 5 was tested for phytoplasmas and AGYp was only detected in April 2000. In grapevines 6 and 20 TBBp and AGYp were detected in three sampling periods, TBBp alone was detected in one sampling period and AGYp was detected in three

and four sampling periods respectively. The pattern of TBBp and AGYp detection in the dually infected grapevines over the two year study was different

Table 2.3. The phytoplasmas detected in each grapevine at each sampling time.

| Grapevine | Oct-98* | Dec-98 | Jan-99 | Apr-99 | Jul - 99 | Oct-99 | Jan-00 | Apr-00 | Jul-00 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | | A | A | A | A | A | A | A |
| 2 | A | | A | A | A | A | | A | A |
| 3 | | | | A | A | A | A | | A |
| 4 | T | | T | T | T | T | T | T | T |
| 5 | T | | T | T | T | T | nt | T+A | T |
| 6 | T+A | | T+A | A | A | A | | T | T+A |
| 7 | A | | A | A | | | | A | A |
| 8 | | | A | | A | | | A | A |
| 9 | A | | A | A | A | A | A | | A |
| 10 | A | | A | A | A | A | A | | A |
| 11 | | | A | A | A | | | A | A |
| 12 | | | | | | | | A | A |
| 13 | nt | | | | | | | A | A |
| 14 | A | | A | A | A | A | | | |
| 15 | A | | | | | | | | |
| 16 | | A | | A | A | A | A | A | A |
| 17 | | A | A | A | A | A | A | | A |
| 18 | | A | A | A | A | A | | A | A |
| 19 | | A | A | A | A | A | | A | A |
| 20 | | T+A | T+A | T+A | A | A | T | A | A |

* A = Australian grapevine yellows phytoplasma, AGYp;
T = tomato big bud phytoplasma, TBBp; nt = not tested

**The distribution of phytoplasmas in grapevine tissues**

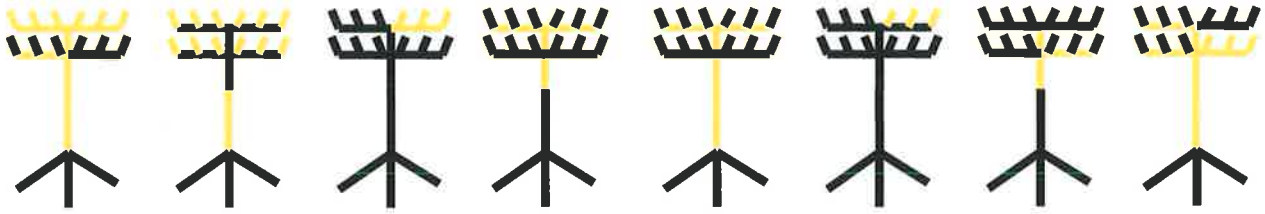
Few grapevines were phytoplasma positive in every tissue type in one sampling period (Table 2.4). Phytoplasmas were not always detected from the same tissue types from one sampling period to the next. Figure 2.5 is a diagrammatic representation of the distribution of phytoplasmas in three of the grapevines over time as determined by PCR. This diagram shows PCR detectable phytoplasmas are not systemically distributed through grapevines in any sampling period.

Table 2.4. The location of phytoplasmas within shoots, cordons, trunks and roots of each grapevine at each sampling period.

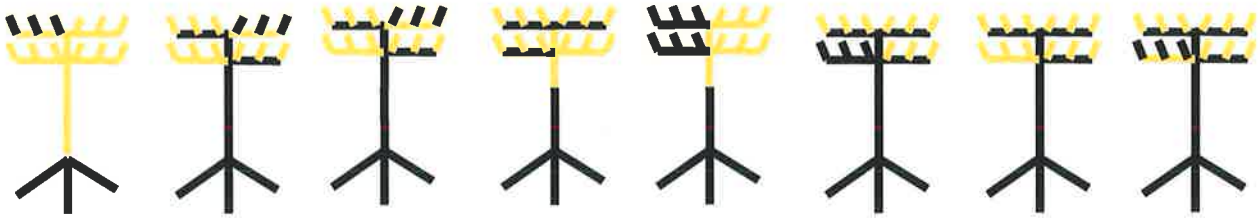
| Grapevine | Oct-98* | Dec-98 | Jan-99 | Apr-99 | Jul - 99 | Oct-99 | Jan-00 | Apr-00 | Jul-00 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | SCT | | ST | SC | CT | CT | S | CT | SCT |
| 2 | SCT | | SCT | SCR | CT | SCT | | ST | SCT |
| 3 | | | | CR | CT | CTR | SCR | | T |
| 4 | SCT | | SC | SC | SCT | SCT | S | SC | SC |
| 5 | SCT | | SCTR | S | SC | C | nt | SCT | SCT |
| 6 | SCT | | SR | C | SCT | SCT | | SCR | SCT |
| 7 | TR | | CR | C | | | | S | CT |
| 8 | | | S | | SC | | | CT | CT |
| 9 | CT | | S | T | SCT | C | CT | | CT |
| 10 | SCT | | S | T | SCTR | ST | S | | T |
| 11 | | | ST | | CT | | | CT | CT |
| 12 | | | | | | | | CT | CT |
| 13 | nt | | | | | | | C | T |
| 14 | SCT | | SC | TR | CT | T | | | |
| 15 | C | | | | | | | | |
| 16 | | SCT | | CT | CT | CT | S | SC | ST |
| 17 | | SCT | S | C | C | SCT | S | | CT |
| 18 | | SCT | S | SCT | SCT | CT | | T | T |
| 19 | | ST | SC | CT | CT | CT | | SCT | CT |
| 20 | | SCT | SCR | S | S | T | S | T | T |

*S= shoots; C = cordons; T = trunk; R = roots; nt = not tested

Grapevine 1 - Previously tested positive for AGYp and with a history of AGYd.



Grapevine 4 - Previously tested positive for TBBp and with a history of AGYd



Grapevine 11 - No previous history of disease, AGYd affected this grapevine in December 1999.


Figure 2.5. A diagrammatic representation of the distribution of detectable phytoplasmas in three grapevines over two years. The areas of the grapevines that are coloured yellow indicate where phytoplasmas were detected.

**The expression of AGYd, RGd and LSLCd with time**

At each sampling period, the 20 grapevines were assessed for the presence of RGd, AGYd and LSLCd. Table 2.5 lists the diseases affecting each of the grapevines in both seasons.

Table 2.5. The expression of restricted growth (RGd), Australia grapevine yellows (AGYd) and late season leaf curl (LSLCd) diseases by each grapevine in each season.

| Group | Grapevine | 1998/99 | | | 1999/00 | | |
|---|---|---|---|---|---|---|---|
| A | 1 | RGd | AGYd | LSLCd | | AGYd | |
| | 2 | RGd | AGYd | LSLCd | | AGYd | LSLCd |
| | 3 | | | LSLCd | | AGYd | |
| | 4 | RGd | AGYd | LSLCd | | AGYd | LSLCd |
| | 5 | | AGYd | LSLCd | | AGYd | LSLCd |
| | 6 | RGd | AGYd | LSLCd | RGd | | LSLCd |
| | 7 | | AGYd | | | AGYd | |
| | 8 | | AGYd | LSLCd | | | LSLCd |
| | 9 | | AGYd | LSLCd | | AGYd | |
| B | 10 | | AGYd | LSLCd | | AGYd | |
| | 11 | | | | | (AGYd)* | |
| | 12 | | | | | | |
| | 13 | | | | | | |
| | 14 | | AGYd | LSLCd | | | |
| | 15 | | | | | | |
| C | 16 | | AGYd | LSLCd | | AGYd | LSLCd |
| | 17 | | AGYd | LSLCd | | AGYd | LSLCd |
| | 18 | | AGYd | LSLCd | | | |
| | 19 | | AGYd | LSLCd | | | |
| | 20 | | AGYd | LSLCd | | AGYd | LSLCd |

*AGYd was observed in December 1999 but was not apparent in January 2000

The nine grapevines in group A had a previous history of AGYd, RGd and/or LSLCd at some time in the three years preceding this study. They also had a history of AGYp or TBBp infection (Table 2.1).

During the study period 1/9 grapevines were affected by AGYd only, 4/9 grapevines were affected by LSLCd and AGYd and 4/9 were affected by RGd, AGYd and LSLCd (Table 2.5). RGd was displayed by 1/4 grapevines in both seasons. AGYd was displayed

by 7/8 grapevines in both seasons. LSLCd was displayed by 5/8 grapevines in both seasons.

The six grapevines in group B had no history of disease in the three seasons preceding this study (Table 2.1). Three grapevines remained asymptomatic during the two-year study period (Table 2.5). Two grapevines displayed AGYd and LSLCd in 1998/99 and one of these had AGYd in the following season while the other was asymptomatic. One grapevine displayed AGYd in 1999/00

The five grapevines in group C had no history of disease in the preceding three seasons but displayed AGYd in December 1998 (Table 2.1). In 1998/99 all of these grapevines also displayed LSLCd (Table 2.5). In 1999/00 three grapevines displayed AGYd and LSLCd while the other two were asymptomatic.

**The frequency of phytoplasma detection in grapevine tissue samples in each season**

The results presented are the combined data for AGYp and TBBp detection. The combined samples from all tissue types gave a higher proportion of positives in each season than each individual tissue type considered on its own for diseased and asymptomatic grapevines (Table 2.6).

In both seasons diseased grapevines, with one or a combination of RGd, AGYd and LSLCd, gave a higher proportion of PCR positive samples compared to asymptomatic plants from individual tissue types or when the samples from the different tissue types were combined (Table 2.6).

The proportion of phytoplasma positive samples decreased for asymptomatic and diseased grapevines from October until April in the 1999/2000 season and then increased in July 1999 (Table 2.6). The proportion of phytoplasma positive samples decreased again from October 1999 to January 2000 and increased until April 2000. After April 2000 the proportion of phytoplasma positive samples from diseased grapevines increased again in July 2000 and the proportion of phytoplasma positive samples decreased in asymptomatic plants.

Table 2.6. The proportion (%) of samples from shoots, cordons, trunks or roots only and from the combined data in which phytoplasmas were detected by PCR at each sampling period from grapevines that were asymptomatic (H) or diseased (D) with one or a combination of restricted growth, Australian grapevine yellows or late season leaf curl diseases.

| Sampling period | Oct-98 | | Dec-98 | Jan-99 | | Apr-99 | | Jul-99 | | Oct-99 | | Jan-00 | | Apr-00 | | Jul-00 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Diseased or Healthy | D | H | D | D | H | D | H | D | H | D | H | D | H | D | H | D | H |
| Number of vines in each class | 11 | 3 | 5 | 16 | 4 | 16 | 4 | 16 | 4 | 14 | 6 | 13 | 6 | 14 | 6 | 14 | 6 |
| Total number of samples tested | 121 | 33 | 55 | 174 | 44 | 174 | 44 | 174 | 44 | 152 | 66 | 141 | 66 | 152 | 66 | 152 | 66 |
| Shoots (S) | 14 | 0 | 21.8 | 16.7 | 2.3 | 4.6 | 0 | 6.9 | 0 | 5.3 | 0 | 9.9 | 0 | 7.9 | 3.0 | 7.2 | 0 |
| Cordons (C) | 20.7 | 6 | 5.5 | 6.3 | 0 | 10.3 | 0 | 17.2 | 2.3 | 15.8 | 6.1 | 1.4 | 0 | 6.6 | 6.1 | 9.2 | 3.0 |
| Trunk (T) | 13.2 | 0 | 5.5 | 1.7 | 2.3 | 5.7 | 0 | 8.6 | 0 | 6.6 | 6.1 | 0.7 | 0 | 6.6 | 6.1 | 11.8 | 10.6 |
| Roots (R) | 0.8 | 0 | 0 | 2.3 | 0 | 1.7 | 0 | 0.6 | 0 | 0.7 | 0 | 0.7 | 0 | 0.7 | 0.0 | 0.7 | 0.0 |
| S + C | 34.7 | 6 | 27.3 | 23 | 2.3 | 14.9 | 0 | 24.1 | 2.3 | 21.1 | 6.1 | 11.3 | 0 | 14.5 | 9.1 | 16.4 | 3 |
| S + T | 27.3 | 0 | 27.3 | 18.4 | 4.5 | 10.3 | 0 | 15.5 | 0 | 11.8 | 6.1 | 10.6 | 0 | 14.5 | 9.1 | 19.1 | 10.6 |
| S + R | 14.9 | 0 | 21.8 | 19 | 2.3 | 6.3 | 0 | 7.5 | 0 | 5.9 | 0 | 10.6 | 0 | 8.6 | 3.0 | 7.9 | 0 |
| C + T | 33.9 | 6 | 10.7 | 8 | 2.3 | 16.1 | 0 | 25.9 | 2.3 | 22.4 | 12.1 | 2.1 | 0 | 13.2 | 12.1 | 21.1 | 13.6 |
| C + R | 21.5 | 6 | 5.5 | 8.6 | 0 | 12.1 | 0 | 17.8 | 2.3 | 16.4 | 6.1 | 2.1 | 0 | 7.2 | 6.1 | 9.9 | 3 |
| T + R | 14 | 0 | 5.5 | 4 | 2.3 | 7.5 | 0 | 9.2 | 0 | 7.2 | 6.1 | 1.4 | 0 | 7.2 | 6.1 | 12.5 | 10.6 |
| S + C + T | 47.9 | 6 | 32.7 | 24.7 | 4.5 | 20.7 | 0 | 32.8 | 2.3 | 27.6 | 12.1 | 12.1 | 0 | 21.1 | 15.2 | 28.3 | 13.6 |
| S + C + R | 35.5 | 6 | 27.3 | 25.3 | 2.3 | 16.7 | 0 | 24.7 | 2.3 | 21.7 | 6.1 | 12.1 | 0 | 15.1 | 9.1 | 17.1 | 3 |
| S + T + R | 28.1 | 0 | 27.3 | 20.7 | 4.5 | 12.1 | 0 | 16.1 | 0 | 12.5 | 6.1 | 11.3 | 0 | 15.1 | 9.1 | 19.7 | 10.6 |
| C + T + R | 34.7 | 6 | 10.7 | 10.3 | 2.3 | 17.8 | 0 | 26.4 | 2.3 | 23 | 12.1 | 2.8 | 0 | 13.8 | 12.1 | 21.7 | 13.6 |
| S + C + T + R | 48.7 | 6 | 32.7 | 27 | 4.5 | 22.4 | 0 | 33.3 | 2.3 | 28.3 | 12.1 | 12.8 | 0 | 21.7 | 15.2 | 28.9 | 13.6 |

Phytoplasmas were less frequently detected in diseased grapevines in October, January, April or July in 1999/2000 compared to the corresponding months 1998/99 (Table 2.6). In October 1999 and April and July 2000 phytoplasmas were more frequently detected in samples from asymptomatic grapevines compared to the corresponding moths in the previous season. Phytoplasmas were not detected in asymptomatic grapevines in April 1999 or January 2000.

Phytoplasmas were most frequently detected in cordons from diseased grapevines in October 1998 and April, July and October 1999, in shoots in December 1998 and January 1999 and 2000, and in trunks in April and July 2000 (Table 2.6). Phytoplasmas were most frequently detected in the cordons of asymptomatic grapevines in October 1998 and April 2000, in shoots in July 1999 and trunks in July 2000. An equal proportion of phytoplasma positive samples was detected in shoots and trunks of asymptomatic grapevines in January 1999 and in cordons and trunks in October 1999.

In December 1998, 20 separate shoot samples were collected from the five grapevines with AGYd and 11/20 samples contained AGYd symptomatic shoots and 9/20 samples were asymptomatic. Phytoplasmas were detected in 10/11 AGYd symptomatic samples and 2/9 asymptomatic samples.

In January 1999, 59 separate shoot samples were collected from the 15 AGYd affected grapevines and 37/59 samples contained AGYd symptomatic shoots while 22/59 samples were asymptomatic. Phytoplasmas were detected in 29/37 AGYd symptomatic samples only.

In January 2000, 39 separate shoot samples were collected from the ten AGYd affected grapevines and 18/39 samples contained symptomatic shoots while 21/39 samples were asymptomatic. Phytoplasmas were detected in 13/18 AGYd symptomatic shoot samples and in 1/21 of the asymptomatic shoot samples.

In April 1999 59 separate shoot samples were collected from the 15 LSLCd affected grapevines and 36/59 samples contained LSLCd symptomatic shots and 23/59 samples were asymptomatic. Phytoplasmas were detected in 9/36 LSLCd symptomatic shoots.

In April 2000, 31separate shoots samples were collected from the eight LSLCd affected grapevines and 14/31 samples contained symptomatic shoots while 17/31 samples were asymptomatic. Phytoplasmas were detected in 6/14 LSLCd symptomatic shoots samples and in 7/17 asymptomatic shoots samples.

**The association between phytoplasmas and disease**

In 1998/99, four grapevines (grapevines 1, 2, 4 and 6) were affected by RGd and AGYd (Table 2.5) and phytoplasmas were detected in a larger proportion of samples from these grapevines compared to grapevines with AGYd alone in every sampling period (Table 2.7). In 1999/00 one grapevine (grapevine 6) had RGd, but not AGYd (Table 2.5). Phytoplasmas were detected in a larger proportion of samples from this grapevine in October 1999 and April 2000 compared to grapevines with AGYd alone (Table 2.7). Phytoplasmas were detected in a similar proportion of samples from RGd affected and AGYd affected grapevines in July 2000. Phytoplasmas were not detected in grapevine 6 in January 2000. Phytoplasmas were detected most frequently in the shoots of grapevines affected by RGd or AGYd in January 1999 and 2000. At other times phytoplasmas were detected frequently in various tissue types and there was no common pattern of detection in the various tissue types amongst the different disease classes of grapevines. Of the four grapevines with RGd during the two year study period, TBBp and AGYp were detected in 1/4, TBBp alone was detected in 1/4 and AGYp alone was detected in 2/4 (Table 2.3, Table 2.5).

In 1998/99 phytoplasmas were detected in a larger proportion of grapevines affected by LSLCd and AGYd compared to those affected by AGYd or LSLCd alone (Table 2.8). However only one grapevine had AGYd alone and one grapevine had LSLCd alone

compared to 14 grapevines with both diseases and this is likely to have influenced the results (Table 2.5). In 1999/00 phytoplasmas were detected in a larger proportion of grapevines affected by LSLCd alone or in grapevines affected by LSLCd and AGYd compared to grapevines with AGYd alone in each sampling period (Table 2.8). Phytoplasmas were detected most frequently in the shoots of grapevines affected by AGYd and LSLCd or AGYd alone in January 1999 and 2000. At other times phytoplasmas were detected frequently in various tissue types and there was no common pattern of detection amongst the different disease classes. TBBp was detected in four grapevines with LSLCd and AGYd and three of these grapevines were also infected with AGYp. AGYp was the only phytoplasmas detected in all other grapevines with LSLCd and/or AGYp (Table 2.3, Table 2.5).

Table 2.7. The number of samples from shoots, cordons trunks and roots and the total number of samples in which phytoplasmas were detected in grapevines with RGd or AGYd in each sampling period in 1998/99 and 1999/00. The proportion (%) of positive samples out of total number of samples that were tested is also given.

| | 1998/99 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sampling period | Oct-98 | | Jan-99 | | Apr-99 | | Jul-99 | |
| Diseased or Healthy* | R | A | R | A | R | A | R | A |
| Number of vines in each class | 4 | 6 | 4 | 11 | 4 | 11 | 4 | 11 |
| Total number of samples tested | 44 | 66 | 44 | 119 | 44 | 119 | 44 | 119 |
| Shoots | 11 | 6 | 12 | 17 | 5 | 4 | 5 | 7 |
| Cordons | 13 | 12 | 3 | 8 | 7 | 10 | 9 | 18 |
| Trunk | 8 | 8 | 2 | 1 | 0 | 10 | 5 | 9 |
| Roots | 0 | 0 | 1 | 3 | 1 | 1 | 0 | 1 |
| Total | 32 | 26 | 18 | 29 | 13 | 25 | 19 | 35 |
| % of the total number of samples that were positive | 72.7 | 39.4 | 40.9 | 24.4 | 29.5 | 21 | 43.2 | 29.4 |
| | 1999/00 | | | | | | | |
| Sampling period | Oct-99 | | Jan-00 | | Apr-00 | | Jul-00 | |
| Diseased or Healthy* | R | A | R | A | R | A | R | A |
| Number of vines in each class | 1 | 12 | 1 | 11 | 1 | 12 | 1 | 12 |
| Total number of samples tested | 11 | 130 | 11 | 119 | 11 | 130 | 11 | 130 |
| Shoots | 1 | 7 | 0 | 14 | 4 | 8 | 1 | 10 |
| Cordons | 3 | 21 | 0 | 2 | 2 | 6 | 1 | 12 |
| Trunk | 1 | 9 | 0 | 1 | 0 | 8 | 1 | 16 |
| Roots | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 |
| Total | 5 | 38 | 3 | 18 | 7 | 22 | 3 | 38 |
| % of the total number of samples that were positive | 45.5 | 29.2 | 0 | 15.1 | 63.6 | 16.9 | 27.3 | 29.2 |

* R+A = grapevines with RGd and AGYd; A = grapevines with AGYd only

Table 2.8. The number of samples from shoots, cordons trunks and roots and the total number of samples in which phytoplasmas were detected in grapevines with LSLCd and AGYd, LSLCd only or AGYd only in 1998/99 and 1999/00 in each sampling period. The proportion (%)of positive samples out of total number of samples that were tested is also given.

| 1998/99 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sampling period | Oct-98 | | | Jan-99 | | | Apr-99 | | | Jul-99 | | |
| Disease* | L+A | L | A | L+A | L | A | L+A | L | A | L+A | L | A |
| Number of vines in each class | 8 | 1 | 1 | 14 | 1 | 1 | 14 | 1 | 1 | 14 | 1 | 1 |
| Total number of samples tested | 88 | 11 | 11 | 152 | 11 | 11 | 152 | 11 | 11 | 152 | 11 | 11 |
| Shoots | 17 | 0 | 0 | 29 | 0 | 0 | 8 | 0 | 0 | 12 | 0 | 0 |
| Cordons | 25 | 0 | 0 | 10 | 0 | 1 | 16 | 1 | 1 | 27 | 3 | 0 |
| Trunk | 15 | 0 | 1 | 3 | 0 | 0 | 10 | 0 | 0 | 14 | 1 | 0 |
| Roots | 0 | 0 | 1 | 3 | 0 | 1 | 2 | 1 | 0 | 1 | 0 | 0 |
| Total | 57 | 0 | 2 | 45 | 0 | 2 | 36 | 1 | 1 | 54 | 4 | 0 |
| % of the total number of samples that were positive | 64.8 | 0 | 18.1 | 29.6 | 0 | 18.1 | 23.7 | 9.1 | 9.1 | 35.5 | 36.4 | 0 |
| 1999/00 | | | | | | | | | | | | |
| Sampling period | Oct-99 | | | Jan-00 | | | Apr-00 | | | Jul-00 | | |
| Disease | L+A | L | A | L+A | L | A | L+A | L | A | L+A | L | A |
| Number of vines in each class | 6 | 2 | 6 | 5 | 2 | 6 | 6 | 2 | 6 | 6 | 2 | 6 |
| Total number of samples tested | 64 | 22 | 66 | 53 | 22 | 66 | 64 | 22 | 66 | 64 | 22 | 66 |
| Shoots (S) | 5 | 3 | 2 | 9 | 3 | 5 | 7 | 0 | 1 | 9 | 1 | 1 |
| Cordons (C) | 13 | 4 | 8 | 0 | 0 | 2 | 4 | 4 | 2 | 5 | 3 | 7 |
| Trunk (T) | 5 | 2 | 4 | 0 | 0 | 1 | 5 | 2 | 3 | 8 | 3 | 8 |
| Roots (R) | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 |
| % of the total number of samples that were positive | 23 | 9 | 15 | 9 | 4 | 9 | 16 | 6 | 6 | 22 | 11 | 20 |
| % of the total number of samples that were positive | 35.9 | 40.9 | 22.7 | 17 | 18.2 | 13.6 | 25 | 27.3 | 9.1 | 34.4 | 50 | 30.3 |

* L+A = grapevines with LSLCd and AGYd; L= grapevines with LSLCd only; A = grapevines with AGYd only.

**Heteroduplex mobility assay**

A band of approximately 350bp corresponding to the homoduplex was observed when the isolate used as the standard was compared to itself and also in every test sample after HMA of the 16S-23S rDNA (data not shown). Also a band of approximately 1000bp corresponding to the homoduplex was observed after HMA of the *tuf* gene, when the isolate used as the standard was compared to itself and also in every test sample. In HMA of both the 16S-23S rDNA (data not shown) and the *tuf* gene, a band slightly larger than the homoduplex that corresponded to single stranded DNA, was observed from the standard and every test sample.

No heteroduplexes between the standard and isolates of TBBp were detected when HMA was done using the 16S-23S spacer region (data not shown). No heteroduplexes between the standard and isolates of AGYp were detected when HMA was done using the 16S-23S spacer region (data not shown).

When HMA was done using the *tuf* gene, heteroduplexes were formed between the AGYp standard and PDBp (Figure 2.6) and between the AGYp standard and two AGYp samples, C433 and C434, from one grapevine at Gol Gol in the same sampling period (Figure 2.7). Heteroduplexes were not observed between the standard and any other AGYp isolate using the tuf gene for HMA. When the C433, C434 and PDBp samples were tested against themselves using the *tuf* gene for HMA no heteroduplexes formed in the C433 and PDBp samples and a faint heteroduplex was observed in the C434 sample (Figure 2.8). When the C433 and C434 were tested against each other and against PDBp no heteroduplex was formed in the C433/PDBp mixture and a faint heteroduplex was formed in both the C434/PDBp and C433/C434 mixtures (Figure 2.10).

Occasionally bands were also observed at the top of the gels, just beneath the wells. Because these bands were not observed every time the same samples were tested and

because they were often observed when a sample was tested against itself they were not considered to be heteroduplex.

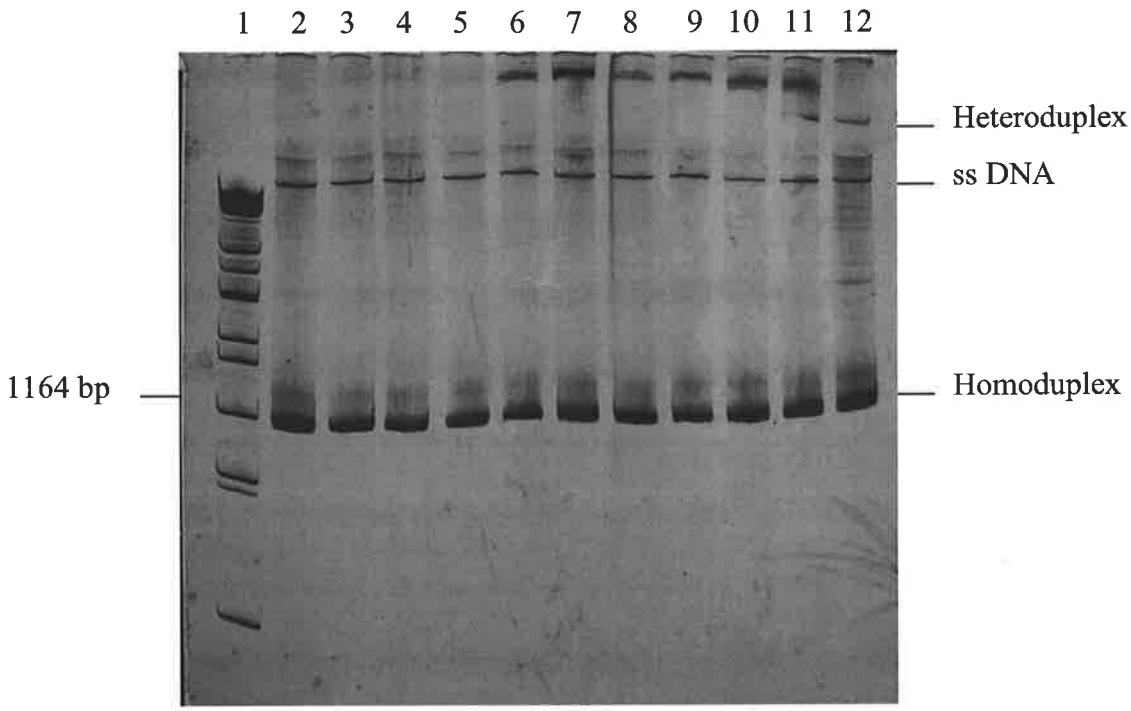



Figure 2.6. Detection of two variants of the AGY strain of '*Canditatus* P. australiense' by heteroduplex mobility assay (HMA) of the *tuf* gene. Lane 1 = SPP-1 marker, lane 2 = AGYp standard, to which all other samples were compared, Lane 3 - 12 = isolates of the AGYp from one grapevine that was sampled from different locations and different times.

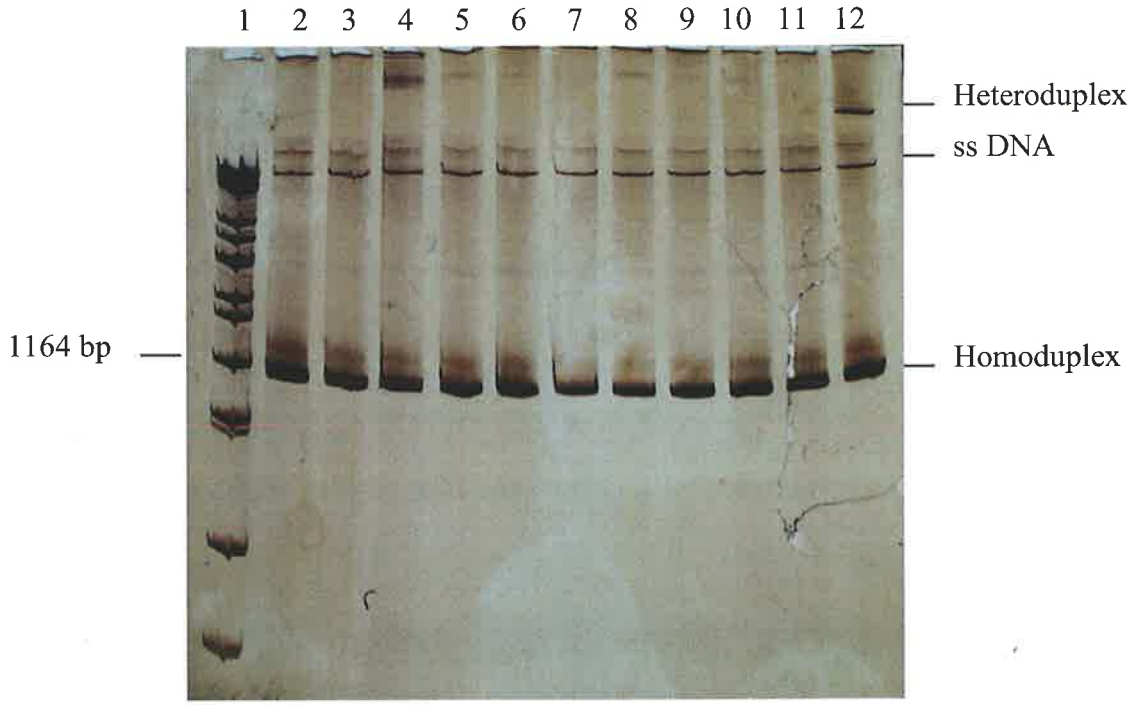



Figure 2.7. Heteroduplex mobility assay (HMA) of the *tuf* gene from AGYp from different grapevines and different grape growing regions and of the *tuf* gene from PDBp. Lane 1 = SPP-1 marker, lane 2 = AGYp standard, lanes 3= Riesling, Sunraysia, lane 4 = Shiraz, Riverland, lane 5 = Chardonnay, Echuca, lane 6 = red table grape variety, Sunraysia, lane 7 = Chardonnay, Gol Gol, Sunraysia, lane 8 = Chardonnay, Karadoc, Sunraysia, lane 9 = Chardonnay, Wemen, lane 10 = Chardonnay, Sunraysia, lane 11 = Chardonnay, Sunraysia and lane 12 = PDBp.

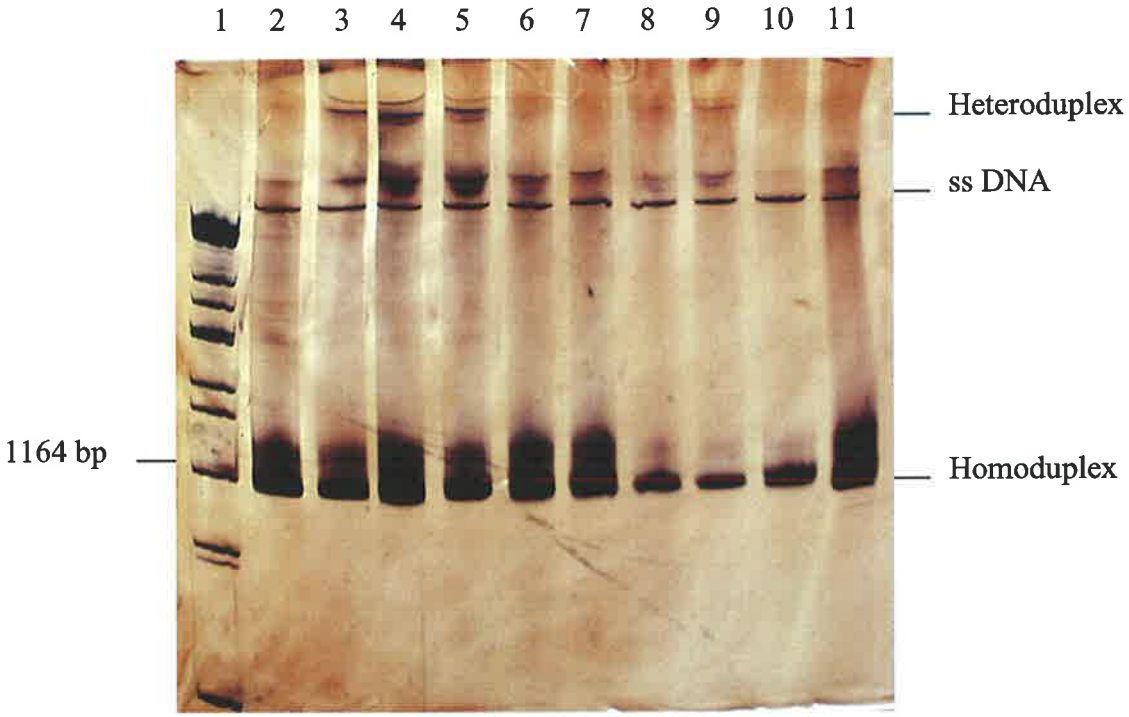



Figure 2.8. A comparison of the PDBp and AGYp strain variants with each other and to the '*Canditatus* P. australiense' standard by heteroduplex mobility assay (HMA) of the *tuf* gene. Lane 1 = SPP-1 marker, lane 2 = AGYp standard compared to itself, lane 3 = C434 (upper trunk sampled in July 2000) compared to AGYp, lane 4 = PDBp strain compared to AGYp, lane 5 = C 433 (lower trunk sampled in July 2000) compared to AGYp, lane 6 = PDBp compared with C434, lane 7 = PDBp compared with C433, lane 8 = C434 compared with C433, lane 9 = C434 compared with itself, lane 10 = C433 compared with itself and lane 11 = PDBp compared with itself.

## 2.4 Discussion

Phytoplasmas were detected in all of the selected grapevines at some time during the study period. The universal nested PCR test that was used in this study is expected to detect all known grapevine phytoplasma species. Only AGYp and TBBp were detected from any grapevine sample used for this study. Previous research has also shown that AGYp and TBBp infect Australian grapevines and that none of the phytoplasmas that infect grapevines overseas have been detected in Australia (Gibb *et al* 1999). The results presented here suggest that TBBp occurs less frequently in grapevines in Sunraysia as this species was only detected in grapevines from the same vineyard. AGYp was detected in most grapevines and mixed infections with AGYp and TBBp occurred in three grapevines. TBBp alone was detected in one grapevine. There was no seasonal influence on the detection of TBBp compared to AGYp, as the timing of detection of both phytoplasmas in dually infected individual grapevines was different.

Phytoplasmas could be detected in various grapevine tissues throughout the year. These results suggest that phytoplasmas may persistently infect Australian grapevines from year to year. This result was not unexpected as the sieve elements of grapevine phloem function for two or more years (Esau 1948) and may provide an environment in which phytoplasmas can reside and multiply through the dormant period. Also, other phytoplasma species have been detected in the dormant wood of grapevines in Italy (Del Serrone and Barba 1996). The dissemination of other phytoplasma species, in France and Italy, through dormant grapevine cuttings also indicates that phytoplasmas can persistently infect grapevines through winter (Credi 1994; Caudwell *et al* 1997; Bianco *et al* 2000).

Not all grapevines were positive in all sampling periods. The detectable levels of phytoplasmas in different tissues of individual grapevines fluctuated throughout the year and from season to season. Phytoplasmas were not always detected in samples from the same sampling area from one sampling period to the next. Phytoplasma detection by PCR

was improved by sampling from shoots, cordons and trunks and October was the best time to test for phytoplasmas when these three tissue types were sampled. Roots alone gave low detection frequency throughout the study and were unreliable for phytoplasma detection by PCR. AGYd symptomatic shoots were most reliable for phytoplasma detection in December and January. Cordons or trunks were more reliable at other times of the year.

AGYd affected most of the test grapevines at sometime during the study period. Ten grapevines displayed AGY symptoms in both seasons, indicating that the disease can persistently affect a grapevine from season to season. Phytoplasmas were detected in most AGYd affected grapevines in both sampling periods. Also phytoplasmas were detected more frequently in AGYd affected shoots in January of both seasons compared to asymptomatic shoots from the same grapevines and shoots from asymptomatic grapevines. Similar results showing an association between AGYd symptomatic shoots and the presence of phytoplasmas have been reported previously (Bonfiglioli *et al* 1995; Padovan *et al* 1995; Gibb *et al* 1999). Phytoplasmas are considered to be the most likely cause of AGYd and persistent expression of AGYd is likely to be associated with persistent phytoplasma infections although subsequent infections would also contribute to the occurrence of disease.

RGd affected four grapevines that were also affected by AGYd and it is possible that the two diseases are associated and phytoplasmas may contribute to the presence of RGd. Phytoplasmas were more frequently detected in grapevines that had both RGd and AGYd compared to grapevines with AGYd only and it is possible that the number of phytoplasmas influenced the expression of RGd in affected grapevines. More grapevines expressing one or a combination of RGd and AGYd need to be tested for phytoplasmas to confirm this result. RGd does not always occur in AGYd affected grapevines. RGd can also persistently affect grapevines from year and one grapevine was persistently affected during the study and all grapevines with RGd had the disease in a previous season.

LSLCd affected 15/20 grapevines during the study and eight of the grapevines displayed the disease in both season. Thus LSLCd can also persistently affect grapevines. All grapevines with LSLCd expressed AGYd at some time through the study and the two diseases may be associated. However AGYd can occur with or without LSLCd and LSLCd can occur with or without AGYd in the same season. Bonfiglioli *et al* (1997) proposed that LSLCd was a late and first expression of phytoplasma infection in grapevines in autumn, which was then followed by the development of AGYd in the next spring. Three grapevines used in this study that had no previous history of disease developed AGYd before developing LSLCd. Additionally, five grapevines were selected for this study because they displayed AGYd symptoms for the first time in 1998 and had no previous history of RGd, AGYd or LSLCd. These results suggest that if LSLCd is associated with the presence of phytoplasmas, it is not the first expression of disease associated with the pathogens. Phytoplasmas were more frequently detected in grapevines with LSLCd and AGYd or LSLCd only and it may be that the number of phytoplasmas affects the expression of LSLCd in a grapevine. More grapevines expressing one or a combination of LSLCd and AGYd need to be tested for phytoplasmas to confirm this result.

Asymptomatic phytoplasma infections occur in Australian grapevines.

Asymptomatic grapevines and grapevines affected by RGd, AGYd and/or LSLCd showed similar increases and decreases in the proportion of samples in which phytoplasmas were detected over the two year study period. Phytoplasmas were detected less frequently in asymptomatic plant material. Phytoplasmas were less frequently detected in diseased grapevines in 1999/00 compared to the previous season indicating a seasonal influence on detectable phytoplasma levels. In asymptomatic plants, phytoplasmas were detected more frequently in 1999/00 compared to 1998/00, except for January. However some grapevines that were asymptomatic in 1999/00 were diseased in the previous season

and it is likely that even though phytoplasmas had reached detectable levels they were too low in number to induce disease. This was also likely to be due to some seasonal influence.

No variability amongst the isolates of TBBp and AGYp was observed using HMA of the 16S-23S rDNA amplified by PCR. Genetic variability was observed amongst isolates of the AGYp using HMA of the *tuf* gene. Heteroduplexes were formed between the *tuf* gene PCR products of the AGYp standard and two AGYp isolates from one grapevine. HMA using the *tuf* gene also revealed that the PDBp isolate was different to the AGYp standard, even though these phytoplasmas are considered to be the same phytoplasma species, '*Candidatus* Phytoplasma australiense' (Liefting *et al* 1997). No heteroduplex was observed when the PDBp isolate was compared to one of the AGYp variants. These results suggest that the *tuf* gene sequence from the AGYp variant is indistinguishable from the PDBp *tuf* gene sequence. Sequence analysis of the *tuf* gene from the AGYp variant and PDBp was not done due to time constraints. A faint heteroduplex was detected when one of the AGYp variants was compared to itself and also with PDBp and the other AGYp variant. This result indicates a mixed infection of the common AGYp isolate and the variant in this sample.

In conclusion the results from this chapter show that phytoplasmas may persistently infect Australian grapevines from year to year. Phytoplasma detection by PCR is best when AGYD symptomatic shoots are sampled in January. When sampling from shoots, cordons and trunks, October was the best time to test for phytoplasmas by PCR. AGYd, RGd and LSLCd may be persistently expressed in some grapevines and remission of each disease was observed in others. The results of PCR detection in the same grapevines indicated that phytoplasmas were more frequently detected in AGYd grapevines that also expressed RGd or LSLCd compared to grapevines expressing AGYd alone. Asymptomatic phytoplasma infections occurred. Phytoplasmas were detected less frequently in asymptomatic plant

material. Genetic variability amongst isolates of the AGYp was observed using heteroduplex mobility assay (HMA) of the *tuf* gene.

# CHAPTER 3

# THE INCIDENCE, DISTRIBUTION AND EXPRESSION OF AUSTRALIAN GRAPEVINE YELLOWS, RESTRICTED GROWTH AND LATE SEASON LEAF CURL DISEASES IN SELECTED AUSTRALIAN VINEYARDS

## 3.1 Introduction

In Chapter 2, AGYd was shown to affect 17 of the 20 grapevines selected for the phytoplasma persistence and distribution studies. Only 4/20 grapevines expressed RGd. LSLCd affected 15/20 grapevines during the study. AGYd affected all grapevines with RGd and LSLCd. The three diseases were persistently expressed in some grapevines and remission of disease was observed in others.

In this Chapter the yearly and cumulative pattern of incidence of AGYd, RGd and LSLCd in several vineyards is reported. The temporal expression of each disease in individual grapevines was determined. The spatial distribution of each disease in each year was determined. The frequency of association between AGYd and RGd or AGYd and LSLCd was examined.

## 3.2 Materials and methods

### Study sites

Table 1 lists the vineyards used as study sites for the disease survey. The number and location of grapevines with AGYd, RGd and LSLCd was determined at each study site. RGd, AGYd and LSLCd are described in Chapter 2.

Table 3.1. The location, the variety grown, the year the vineyard was established, the size of the study area and the duration of the survey at each vineyard used in this study.

| Location | Variety | Year the vineyard was established | Size of the study area in the vineyard | Duration of the survey |
|---|---|---|---|---|
| Gol Gol, Sunraysia, New South Wales | Chardonnay | 1992 and 1993 | 18 × 50 grapevines | January 1996 – April 2001 |
| Karadoc, Sunraysia, Victoria | Chardonnay | 1992 | 18 ×50 grapevines | January 1996 – April 2001 |
| Wemen*, Sunraysia, Victoria | Chardonnay | 1993 | 3 blocks of 10 × 10 grapevines<br>4 blocks of 10 × 25 grapevines | January 1996 – April 2001<br>October 1996 – April 2001 |
| Colignan, Sunraysia, Victoria | Chardonnay | 1996 | 20 × 20 grapevines | October 1998 – April 2001 |
| Paringa, Riverland, NSW | Shiraz | 1998 | 2 blocks of 20 × 55 grapevines | April 1999 – April 2001 |
| Paringa, Riverland, NSW | Shiraz | 1999 | 8 × 55 grapevines | April 2000 |

*All areas surveyed at Wemen belong to one vineyard.

**Spatial analysis of disease mapping data**

The disease survey data for AGYd, RGd and LSLCd from Gol Gol, Karadoc, Colignan and Paringa were analysed using a fixed grid analysis available through the "Patchy" spatial analysis package (Maixner, 1993) to determine whether diseased grapevines were clustering. 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. The fixed grid analysis calculates the mean number of affected plants in each subunit of the grid and the variance amongst subunits across the plot. This information is used to calculate the variance to mean ratio (V/M; Madden, 1989). If the V/M equals or is not significantly different from 1 then the pattern of disease is random. If V/M is significantly greater than 1 clustering can be inferred. The size of the subunits tested for

clustering range from 2 × 2 (minimum allowed size) grapevines in rows and columns to row-length/2 × column-length/2 (maximum allowed size) grapevines. Each grid of a specific subunit size is tested in separate fixed grid analysis. Figure 3.1 provides an example of how the grid of subunits is laid over a plot.

Spatial autocorrelation used the "join-count" test statistic to test the hypothesis that the spatial distribution of AGYd, RGd or LSLCd affected plants in the vineyard is random (Jones, 2002). The join-count statistic was appropriate for this study because it tests the spatial autocorrelation of nominal level (presence/absence) spatial data. In this study the join-count statistic is based on counting the number of times diseased grapevines occupied adjoining locations in the study sites, either in the row and column direction (Rooks case) or in the diagonal direction (Bishops case).

**Temporal analysis of disease mapping data**

The number of grapevines at each study site that had RGd, AGYd and/or LSLCd either present or absent for each was counted. Log-linear models (McCullagh and Nelder, 1989) were used to determine if the presence of AGYd, RGd and LSLCd changed over time and if there was any interaction between the three diseases and time at each vineyard (Jones, 2002). The models assume a Poisson distribution with a logarithmic link function. Terms were dropped from the models by using backward elimination, which determines the importance of each term by assessing the change in residual deviance. Non-significant terms were omitted from the models.

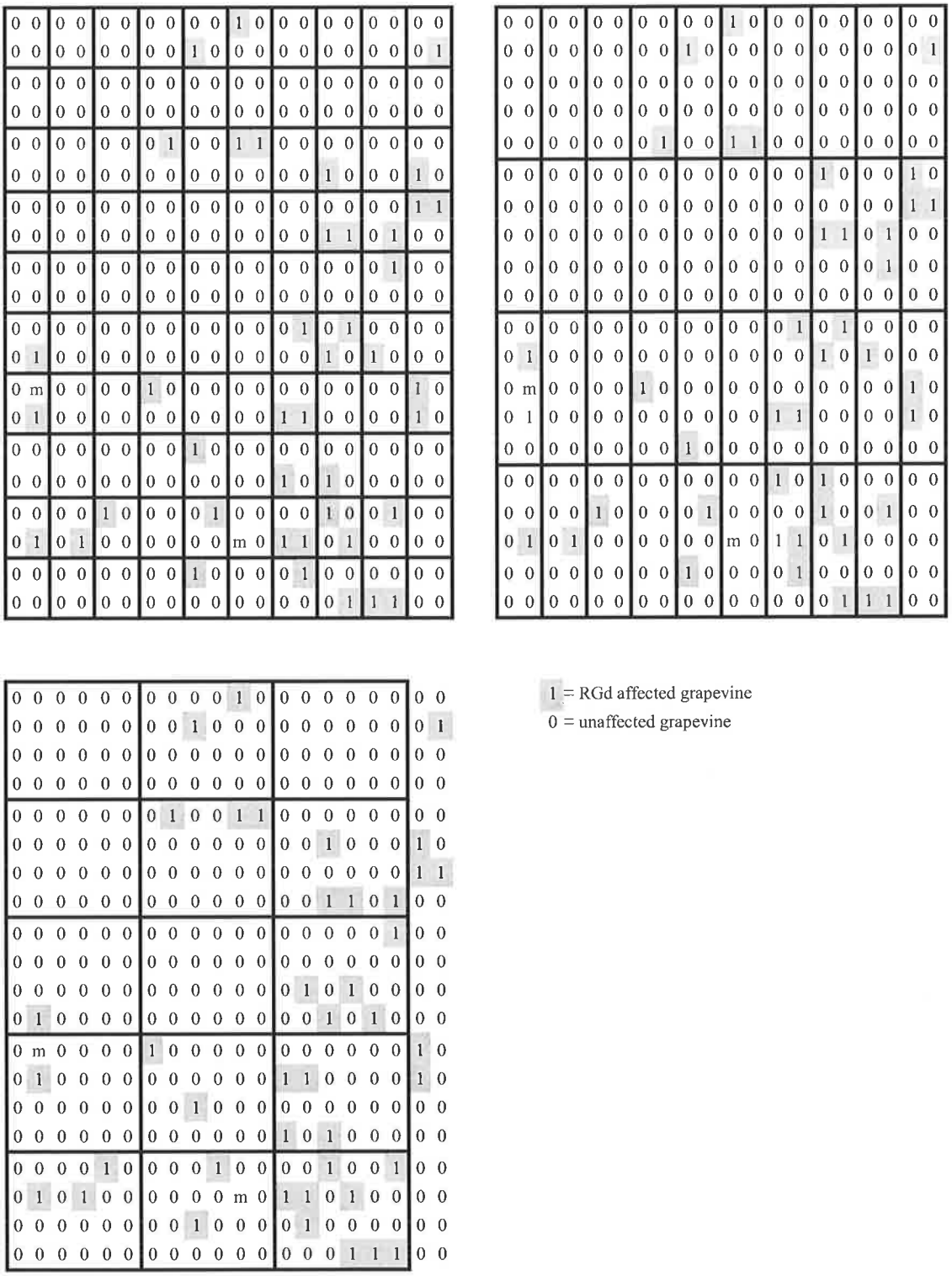



Figure 3.1. Three examples of a contiguous grid of subunits laid over the map of asymptomatic and RGd affected grapevines at Colignan in 1999/00. a. The subunit size in this example is 2 × 2 grapevines in columns and rows. b. The subunit size in this example is 2 × 5 grapevines in columns and rows. c. The subunit size in this example is 6 × 4 grapevines in columns and rows.

## 3.3 Results

### Vineyard surveys for disease incidence

The maps of grapevines affected with RGd, AGYd and LSLCd in each year are attached in Appendix 1. The yearly incidence (%) was determined for each disease from the number of grapevines that were affected by a disease in each year. To cumulative incidence (%) of each disease was determined by additing of new records of diseased grapevines in each year to records of diseased grapevines in previous years.

*AGYd* The incidence of AGYd in each year was different at each study site (Figure 3.1). The pattern of AGYd incidence from year to year was different between study sites. At Gol Gol the highest incidence of AGYd was 54.5% in 1996/97 and the lowest incidence was 19.6% in 1997/98 (Figure 3.1a). At Karadoc AGYd incidence increased in incidence each season from 1.8% in 1995/96 to 52.2% in 2000/01(Figure 3.1b). At Wemen (six years) the highest incidence of AGYd was 25.4% in 1997/98 and the lowest incidence was 0.7% in 1995/96 (Figure 3.1c). At Wemen (five years) the highest incidence of AGYd was 24.1% in 1996/97 and the lowest incidence was 12.5% in 1999/00 (Figure 3.1d). At Colignan the incidence increased from 13.8% in 1998/99 to 73.4% in 1999/2000 and then decreased to 48.4% in 2000/01(Figure 3.1e). At Paringa the incidence of AGYd decreased from 5.9 % in 1998/99 to 4.1% 1999/00 and AGYd was not observed in 2000/01(Figure 3.1f).

The cumulative incidence of AGYd in each vineyard increased from year to year over the survey period (Figures 3.1a – 3.1f). By 2000/01 the cumulative incidence of AGYd at each study site was: 77.3% at Gol Gol; 71.5% at Karadoc; 53.7% at Wemen (six years); 55.7% at Wemen (five years); 90.8% at Colignan; and 9.6% at Paringa.

New occurrences of AGYd were observed in grapevines that were planted during the survey. AGYd affected 48/151 replacement grapevines at Gol Gol, 5/9 replacement grapevines at Karadoc, 3/28 replacement grapevines at Colignan and 1/29 replacement

grapevines at Paringa. In 1999/00 AGYd was also observed in the new planting of Shiraz grapevines behind blocks 1 and 2 at Paringa but the incidence of affected grapevines was not recorded.

*RGd* The incidence of RGd in each year was different at each study site. The pattern of RGd incidence from year to year was different between study sites.

At Gol Gol the highest incidence of RGd was 44.8% in 1997/98 and the lowest incidence was 5.3% in 1995/96 (Figure 3.2a). At Karadoc the highest incidence of RGd was 43.9% in 1996/97and the lowest incidence was 13% in 2000/01(Figure 3.2b). At Wemen (six years) the highest incidence of RGd was 40.1% in 1998/99 and the lowest incidence was 1% in 2000/01(Figure 3.2c). At Wemen (five years) the highest incidence of RGd was 49.1% in 1996/97 and no RGd was observed 2000/01(Figure 3.2d). At Colignan the incidence of RGd increased from 6.3% in 1998/99 to 10.6% in 1999/00 and then decreased to 1% in 2000/01(Figure 3.2e). RGd was not observed at Paringa.

The cumulative incidence of RGd in each vineyard increased over the survey period (Figure 3.2a – 3.2e). By 2000/01 the cumulative incidence of RGd at each study site was: 68.9 % at Gol Gol; 73.1% at Karadoc; 76.7 at Wemen (six years); 54.7% at Wemen (five years); 16.8% at Colignan.

New occurrences of RGd were observed in grapevines that were planted during the survey. RGd affected 16/151 replacement grapevines at Gol Gol, 6/9 replacement grapevines at Karadoc and 1/7 replacement grapevines at Wemen.

*LSLCd* The incidence of LSLCd in each year was different at each study site. The pattern of LSLCd incidence from year to year was different between study sites.

At Gol Gol the highest incidence of LSLCd was 30.3% in 1996/97 and the lowest incidence was 9.4% in 1999/00 (Figure 3.3a). At Karadoc the highest incidence of LSLCd was 27.5% in 1998/99 and the lowest incidence was 14.8% in 1997/98 (Figure 3.3b). At Wemen (six years) the highest incidence of LSLCd was 14% in 1997/98 and the lowest

incidence was 2% in 1995/96 (Figure 3.3c). At Wemen (five years) the highest incidence of LSLCd was 18.7% in 1996/97 and the lowest incidence was 2.9% in 1999/00 (Figure 3.3d). At Colignan the incidence of LSLCd decreased from 41.2% in 1998/99 to 9% by 2000/01 (Figure 3.3e). LSLCd was not observed at Paringa.

The cumulative incidence of LSLCd in each vineyard increased over the survey period (Figure 3.3a – 3.3e). By 2000/01 the cumulative incidence of LSLCd at each study site was: 60.9% at Gol Gol; 61.9% at Karadoc; 36.7% at Wemen (six years); 34.7% at Wemen (five years); 54% at Colignan

New occurrences of LSLCd were observed in grapevines that were planted during the survey. LSLCd affected 32/151 replacement grapevines at Gol Gol, 6/9 replacement grapevines at Karadoc and 1/7 replacement grapevines at Wemen.

**Expression of AGYd in Shiraz grapevines at Paringa**

At Paringa AGYd was not observed in spring or early summer. AGYd was first observed in Shiraz grapevines at Paringa from late summer and increased in incidence until leaf fall. The symptoms were similar to those described for white varieties except that reddening was observed instead of yellowing (Chapter 2; Figure 2.1e; Magarey, 1986; Magarey and Wachtel, 1983; Magarey and Wachtel 1986a). Bunches were not affected.

**Disease expression with time**

Table 3.2 shows the proportion of grapevines affected with AGYd, RGd and LSLCd that were observed for the entire survey period at each site. The proportion of grapevines displaying AGYd decreased at Karadoc, Wemen and Colignan as the number of years for which grapevines were affected by each disease increased. At Gol Gol the proportion of AGYd affected grapevines was similar for one year and up to 4 years and then the proportion decreased when grapevines displayed the disease for five or six years. At all vineyards the proportion of grapevines displaying RGd or LSLCd decreased as the number of years for which grapevines were affected by each disease increased.

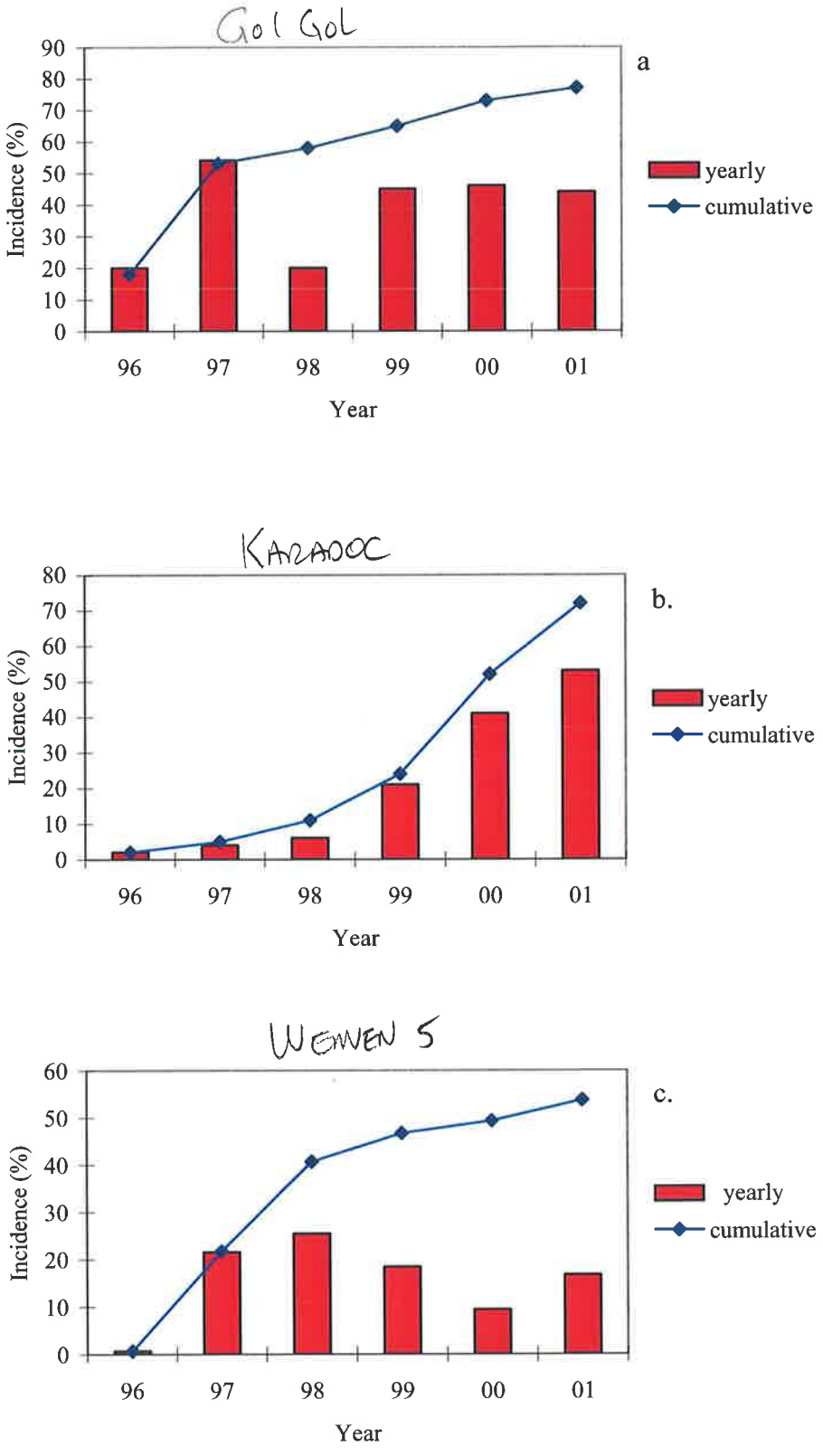



Figure 3.2.The yearly and cumulative incidence (%) of grapevines affected with AGYd over six seasons at Gol Gol (a), Karadoc (b) and Wemen (c), five years at Wemen (d) and three years at Colignan (e) and Paringa (f).

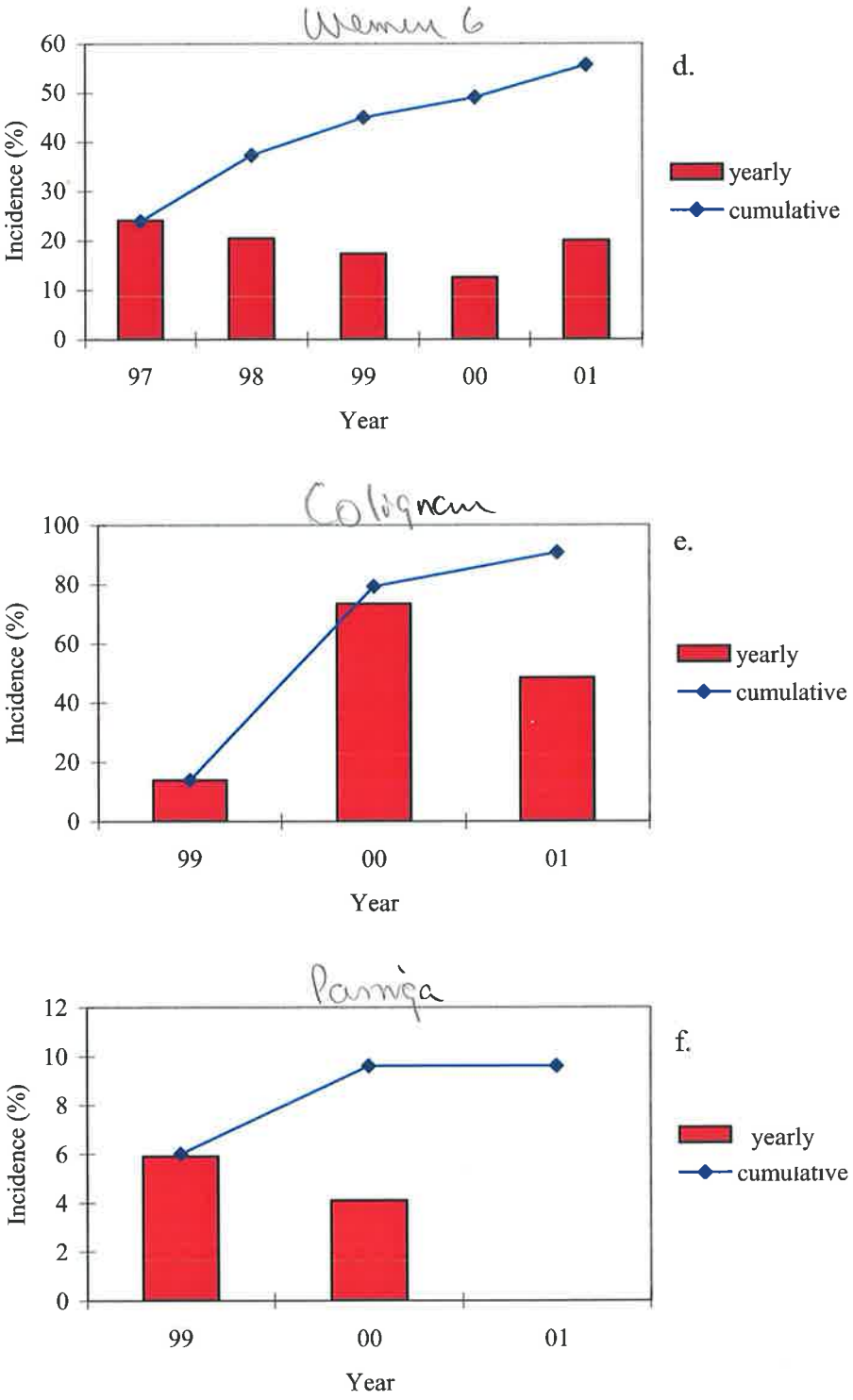



Figure 3.2 continued..

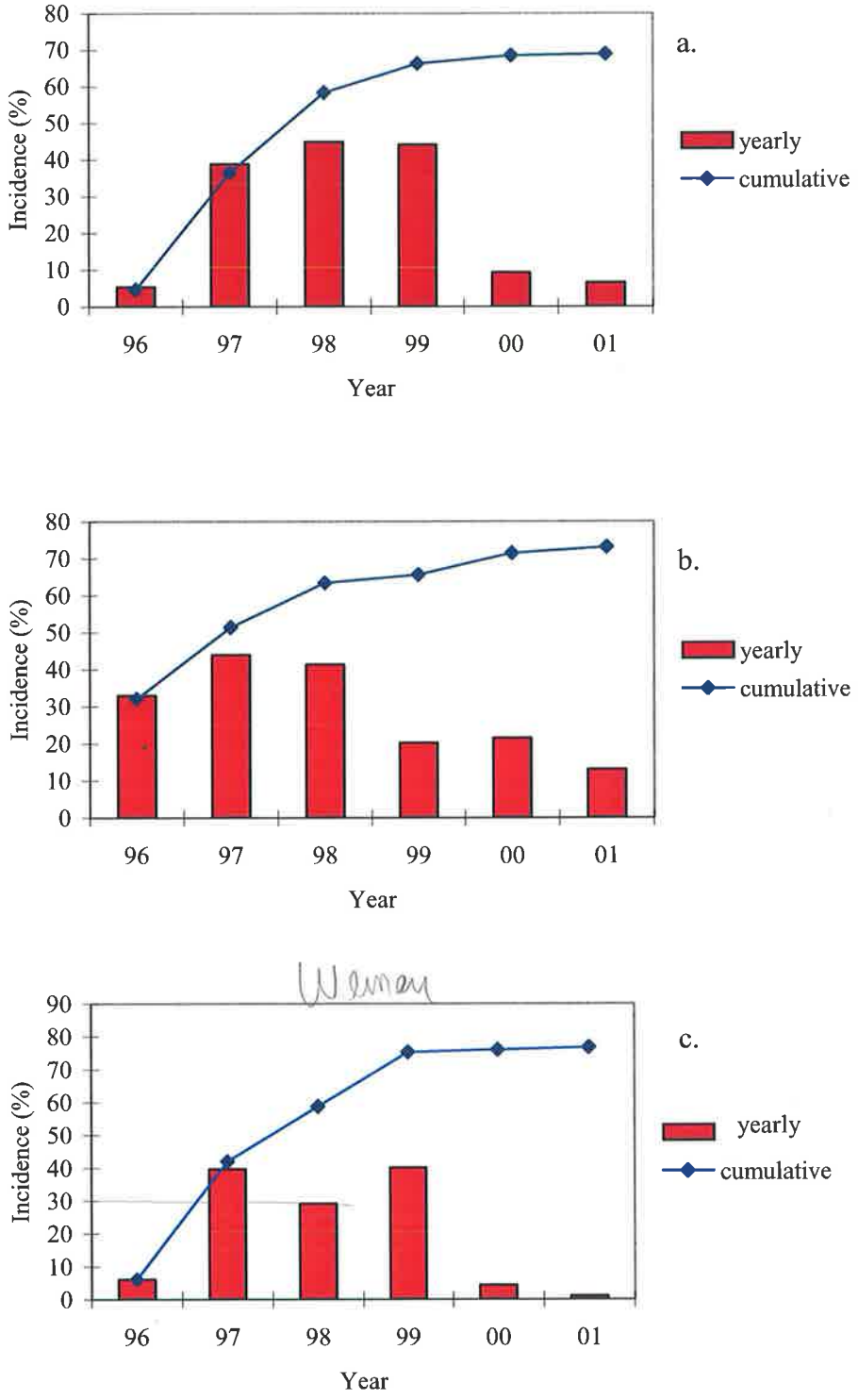



Figure 3.3. The yearly and cumulative incidence (%) of grapevines affected by RGd over six seasons at Gol Gol (a), Karadoc (b) and Wemen (c), five years at Wemen (d) and three years at Colignan (e).

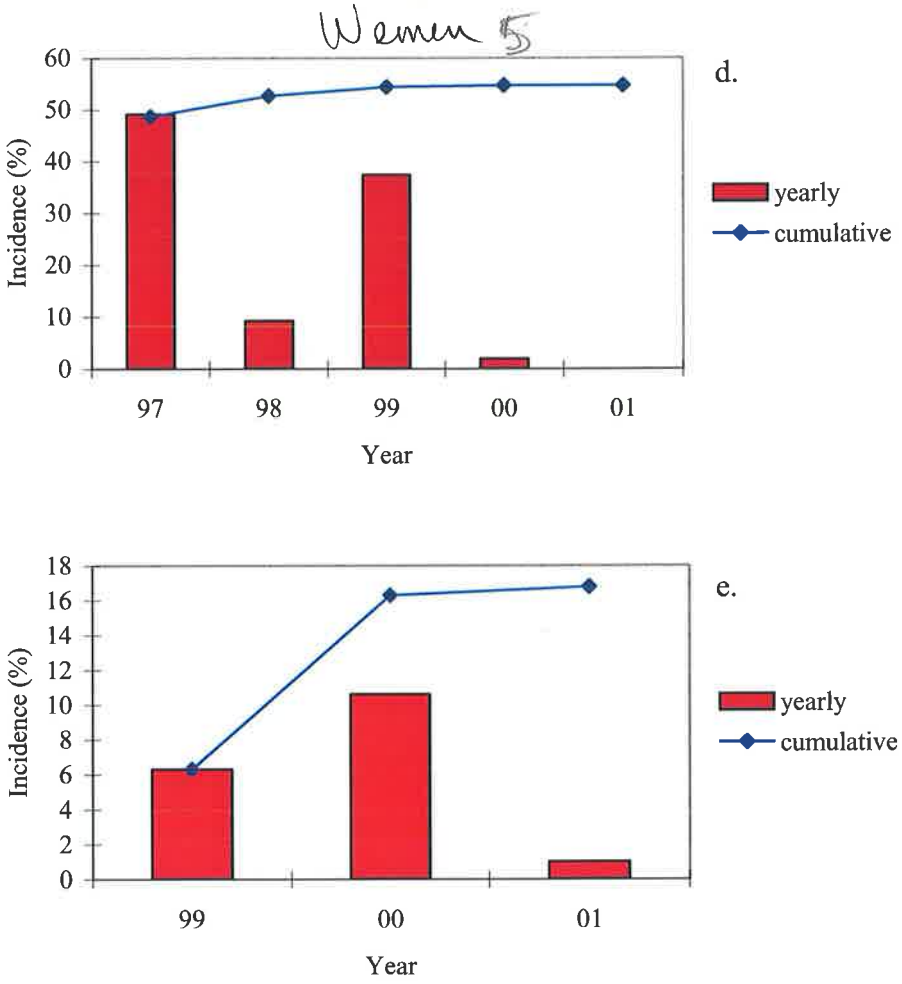



Figure 3.3 continued..

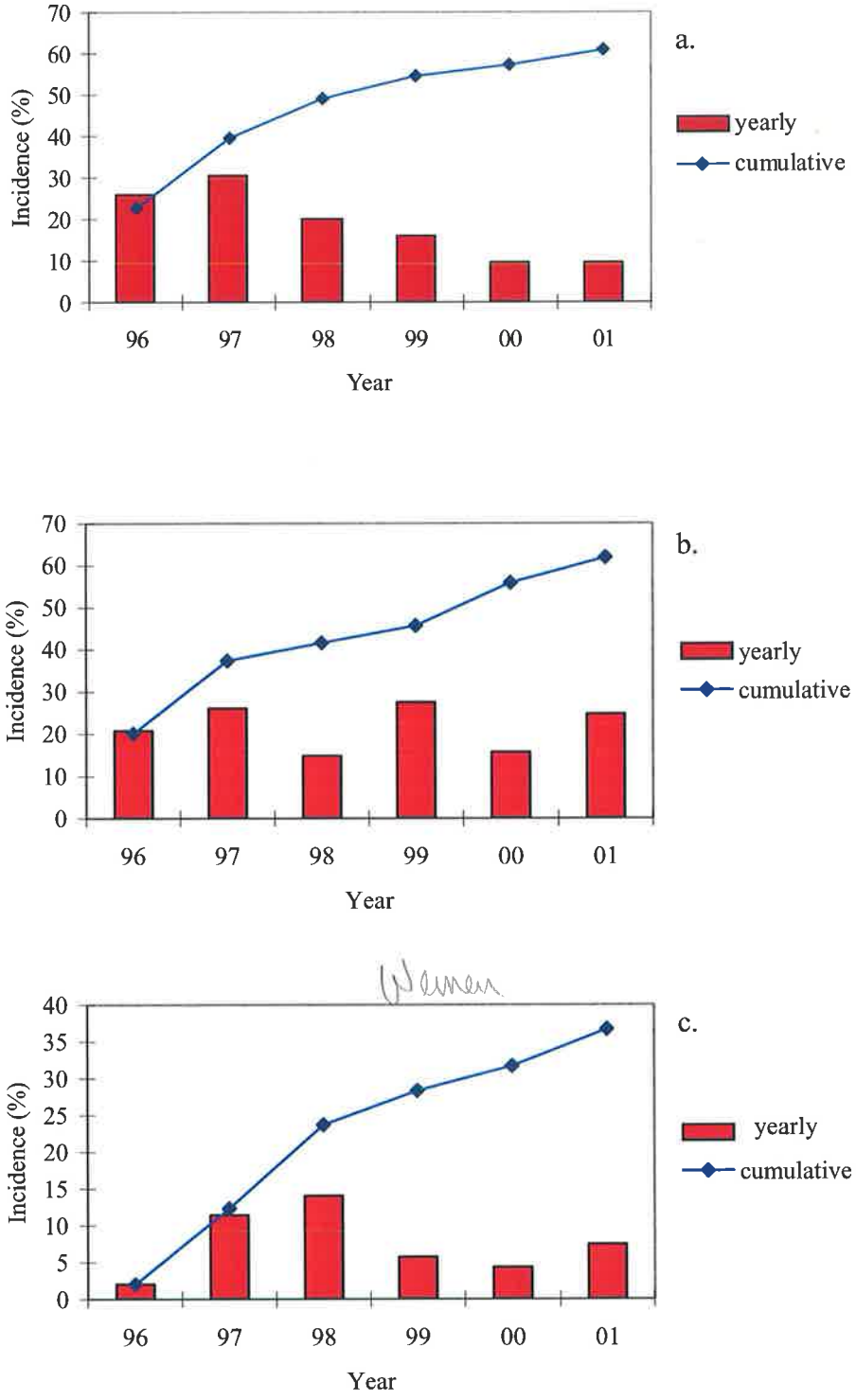



Figure 3.4.The yearly and cumulative incidence (%) of grapevines affected by LSLCd over six seasons at Gol Gol (a), Karadoc (b) and Wemen (c), five years at Wemen (d) and three years at Colignan (e).

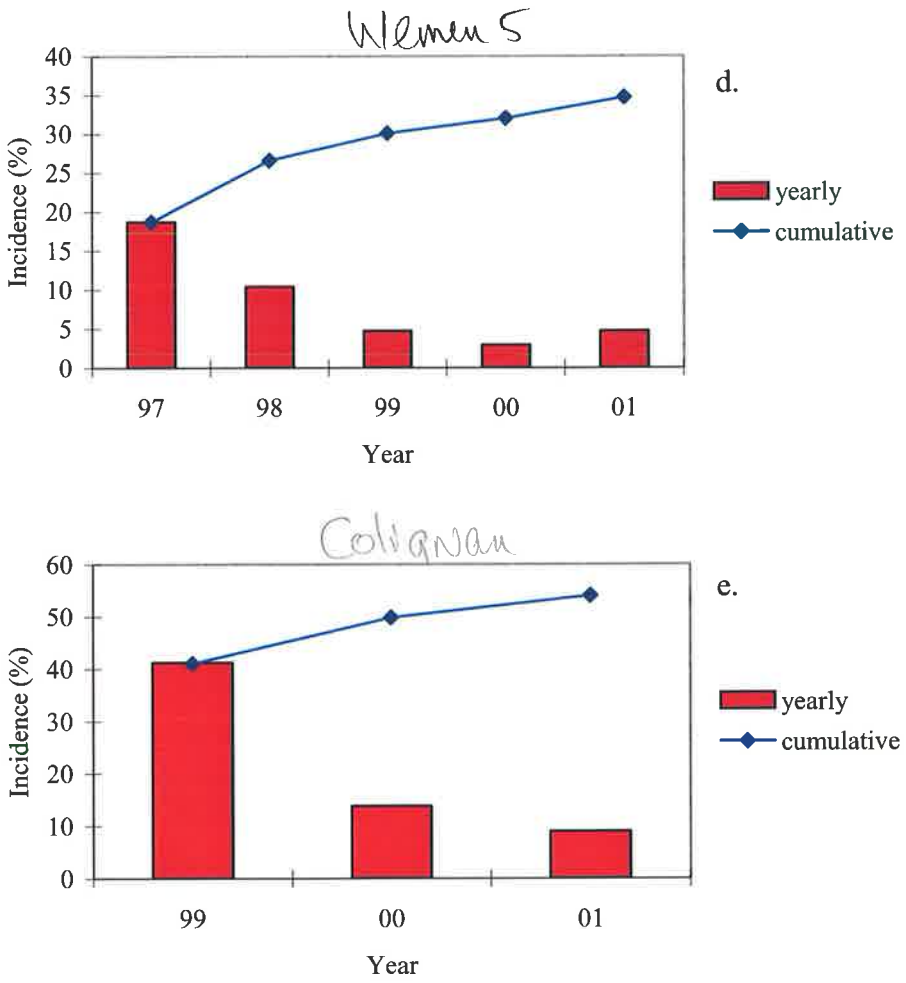



Figure 3.4 continued..

Table 3.2. The percentage of grapevines[a] at Gol Gol, Karadoc, Wemen, Colignan and Paringa that were affected with AGYd, RGd or LSLCd for 0-6 years and the total percentage that had displayed the diseases at sometime during the survey period.

| Years affected | Gol Gol | | | Karadoc | | | Wemen (6 years) | | | Wemen (5 years) | | | Colignan | | | Paringa | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC |
| 6 | 1.8 | 0.3 | 0.1 | 0 | 0.9 | 0.3 | 0 | 0 | 0 | - | - | - | - | - | - | - | - | - |
| 5 | 8.3 | 1.4 | 1.3 | 0.3 | 2.9 | 2.1 | 0.7 | 0 | 0 | 0.1 | 0 | 0 | - | - | - | - | - | - |
| 4 | 19.8 | 6.3 | 2.9 | 3.8 | 8.5 | 5.9 | 1.7 | 1 | 0.3 | 1.9 | 0.4 | 0 | - | - | - | - | - | - |
| 3 | 19.7 | 17.4 | 7.6 | 8.7 | 17.2 | 10.9 | 6.7 | 8.4 | 0.3 | 8 | 1.2 | 0.7 | 4.6 | 0.3 | 0.5 | 0 | 0 | 0 |
| 2 | 19.5 | 23.5 | 18.7 | 24.8 | 21.4 | 15.2 | 17.1 | 22.1 | 6.4 | 15.9 | 5.1 | 5.2 | 38.4 | 0.5 | 9.7 | 0.5 | 0 | 0 |
| 1 | 17.8 | 30.1 | 36 | 38.6 | 25.1 | 26 | 27.9 | 44.6 | 29.2 | 30.5 | 48.7 | 31.8 | 51.9 | 13.4 | 46.2 | 9.1 | 0 | 0 |
| 0 | 13.1 | 21 | 33.3 | 23.7 | 24 | 39.4 | 46 | 23.8 | 63.8 | 43.6 | 44.6 | 62.3 | 5.1 | 85.8 | 43.5 | 90.4 | 100 | 100 |
| Total % affected | 86.9 | 79 | 66.7 | 76.3 | 76 | 60.6 | 54 | 76.2 | 36.2 | 56.4 | 55.4 | 37.7 | 94.9 | 14.2 | 56.5 | 9.6 | 0 | 0 |

[a]The total number of grapevines observed at each site for the entire survey period: Gol Gol = 763 grapevines, Karadoc = 860 grapevines, Wemen (six years) = 298 grapevines, Wemen (five years) = 984 grapevines, Colignan (3 years) = 372 grapevines, Paringa (3 years) = 2170 grapevines

Table 3.3 shows the number of grapevines at each site affected by AGYd, RGd or LSLCd for one year and up to six years and in which years the diseases were observed. The number of grapevines displaying AGYd, RGd or LSLCd for only one year increased or decreased from year to year in a similar pattern to the yearly incidence of each disease at each vineyard. The exception was RGd at Wemen (five years) where the number of grapevines affected for only one year decreased from 1996/97 to 2000/01 but the yearly incidence of RGd decreased from 1996/97 to 1997/98 then increased again to 1998/99 and then decreased to 2000/01. No consistent pattern of AGYd, RGd or LSLCd expression was observed for grapevines that displayed the diseases for two or more years. AGYd, RGd and LSLCd were not always persistently expressed in the same grapevine from season to season.

Table 3.3. The number of grapevines at Gol Gol, Karadoc, Wemen, Colignan and Paringa[a] affected with AGYd, RGd or LSLCd for 1-6 years and in which years they were affected.

| Year[b] | | | | | | Gol Gol | | | Karadoc | | | Wemen (six years) | | | Wemen (five years) | | | Colignan | | | Paringa | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 96 | 97 | 98 | 99 | 00 | 01 | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC |
| ■ | | | | | | 6 | 7 | 72 | 1 | 20 | 33 | 1 | 3 | 2 | | | | | | | | | |
| | ■ | | | | | 53 | 58 | 80 | 5 | 55 | 58 | 23 | 46 | 22 | 102 | 424 | 144 | | | | | | |
| | | ■ | | | | 4 | 81 | 51 | 3 | 67 | 12 | 28 | 32 | 26 | 58 | 37 | 68 | | | | | | |
| | | | ■ | | | 20 | 72 | 33 | 25 | 17 | 62 | 12 | 48 | 13 | 43 | 16 | 31 | 10 | 9 | 125 | 118 | 0 | 0 |
| | | | | ■ | | 29 | 10 | 17 | 118 | 45 | 31 | 6 | 2 | 9 | 32 | 2 | 18 | 140 | 39 | 31 | 79 | 0 | 0 |
| | | | | | ■ | 24 | 2 | 22 | 180 | 12 | 28 | 13 | 2 | 15 | 65 | 0 | 52 | 43 | 2 | 16 | 0 | 0 | 0 |
| **TOTAL – 1 year** | | | | | | **136** | **230** | **275** | **332** | **216** | **224** | **83** | **133** | **87** | **300** | **479** | **313** | **193** | **50** | **172** | **197** | **0** | **0** |
| ■ | ■ | | | | | 9 | 3 | 35 | 0 | 48 | 19 | 1 | 0 | 2 | | | | | | | | | |
| ■ | | ■ | | | | 2 | 3 | 13 | 0 | 21 | 3 | 0 | 3 | 0 | | | | | | | | | |
| ■ | | | ■ | | | 3 | 6 | 7 | 0 | 3 | 5 | 0 | 1 | 1 | | | | | | | | | |
| ■ | | | | ■ | | 2 | 0 | 1 | 5 | 9 | 13 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | | | | ■ | 0 | 1 | 4 | 0 | 5 | 16 | 0 | 0 | 0 | | | | | | | | | |
| | ■ | ■ | | | | 9 | 41 | 28 | 2 | 40 | 9 | 7 | 17 | 6 | 24 | 37 | 21 | | | | | | |
| | ■ | | ■ | | | 21 | 37 | 11 | 1 | 6 | 14 | 4 | 25 | 0 | 20 | 5 | 3 | | | | | | |
| | ■ | | | ■ | | 27 | 3 | 7 | 2 | 6 | 3 | 4 | 3 | 0 | 13 | 5 | 5 | | | | | | |
| | ■ | | | | ■ | 20 | 0 | 3 | 4 | 2 | 7 | 7 | 0 | 2 | 20 | 0 | 8 | | | | | | |
| | | ■ | ■ | | | 3 | 77 | 11 | 6 | 17 | 10 | 12 | 15 | 2 | 16 | 0 | 5 | | | | | | |
| | | ■ | | ■ | | 2 | 4 | 8 | 3 | 10 | 3 | 3 | 0 | 1 | 11 | 2 | 0 | | | | | | |
| | | ■ | | | ■ | 3 | 1 | 3 | 3 | 6 | 4 | 5 | 0 | 3 | 19 | 0 | 4 | | | | | | |
| | | | ■ | ■ | | 12 | 2 | 3 | 21 | 2 | 4 | 2 | 2 | 1 | 5 | 1 | 1 | 13 | 1 | 18 | 11 | 0 | 0 |
| | | | ■ | | ■ | 12 | 1 | 5 | 30 | 2 | 13 | 4 | 0 | 0 | 19 | 0 | 3 | 9 | 0 | 14 | 0 | 0 | 0 |
| | | | | ■ | ■ | 24 | 0 | 4 | 136 | 7 | 8 | 2 | 0 | 1 | 9 | 0 | 1 | 121 | 1 | 4 | 0 | 0 | 0 |
| **TOTAL – 2 years** | | | | | | **149** | **179** | **143** | **213** | **184** | **131** | **51** | **66** | **19** | **156** | **50** | **51** | **143** | **2** | **36** | **11** | **0** | **0** |
| ■ | ■ | ■ | | | | 1 | 1 | 8 | 0 | 38 | 4 | 0 | 0 | 1 | | | | | | | | | |
| ■ | ■ | | ■ | | | 6 | 1 | 8 | 0 | 17 | 6 | 0 | 8 | 0 | | | | | | | | | |

Table 3.3 continued..

| Year[b] | | | | | | Gol Gol | | | Karadoc | | | Wemen (six years) | | | Wemen (five years) | | | Colignan | | | Paringa | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 96 | 97 | 98 | 99 | 00 | 01 | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC | AGY | RG | LSLC |
| ■ | ■ | | | ■ | | 3 | 0 | 4 | 0 | 18 | 3 | 0 | 0 | 0 | | | | | | | | | |
| ■ | ■ | | | | ■ | 3 | 0 | 2 | 1 | 4 | 4 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | ■ | ■ | | | 2 | 5 | 3 | 1 | 6 | 1 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | ■ | | ■ | | 0 | 0 | 3 | 0 | 7 | 1 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | ■ | | | ■ | 0 | 0 | 1 | 0 | 3 | 6 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | | ■ | ■ | | 8 | 0 | 1 | 1 | 1 | 3 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | | ■ | | ■ | 3 | 0 | 0 | 1 | 1 | 12 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | | | ■ | ■ | 2 | 0 | 0 | 4 | 3 | 5 | 0 | 0 | 0 | | | | | | | | | |
| | ■ | ■ | ■ | | | 8 | 92 | 9 | 1 | 18 | 12 | 5 | 13 | 0 | 12 | 8 | 0 | | | | | | |
| | ■ | ■ | | ■ | | 3 | 2 | 2 | 0 | 9 | 5 | 0 | 0 | 0 | 7 | 1 | 1 | | | | | | |
| | ■ | ■ | | | ■ | 2 | 2 | 3 | 1 | 11 | 5 | 3 | 0 | 0 | 10 | 0 | 2 | | | | | | |
| | ■ | | ■ | ■ | | 26 | 5 | 1 | 0 | 2 | 3 | 2 | 1 | 0 | 5 | 2 | 1 | | | | | | |
| | ■ | | ■ | | ■ | 18 | 4 | 2 | 4 | 2 | 14 | 3 | 1 | 0 | 6 | 0 | 1 | | | | | | |
| | ■ | | | ■ | ■ | 23 | 0 | 4 | 3 | 2 | 2 | 0 | 0 | 0 | 3 | 0 | 0 | | | | | | |
| | | ■ | ■ | ■ | | 4 | 12 | 5 | 5 | 4 | 1 | 1 | 2 | 0 | 5 | 1 | 1 | | | | | | |
| | | ■ | ■ | | ■ | 7 | 9 | 1 | 3 | 0 | 4 | 4 | 0 | 0 | 14 | 0 | 0 | | | | | | |
| | | ■ | | ■ | ■ | 6 | 0 | 1 | 3 | 2 | 0 | 2 | 0 | 0 | 9 | 0 | 1 | | | | | | |
| | | | ■ | ■ | ■ | 25 | 0 | 0 | 47 | 0 | 3 | 0 | 0 | 0 | 8 | 0 | 0 | 17 | 1 | 2 | 0 | 0 | 0 |
| **TOTAL – 3 years** | | | | | | **150** | **133** | **58** | **75** | **148** | **94** | **20** | **25** | **1** | **79** | **12** | **7** | **17** | **1** | **2** | **0** | **0** | **0** |
| ■ | ■ | ■ | ■ | | | 3 | 6 | 8 | 0 | 26 | 6 | 0 | 1 | 0 | | | | | | | | | |
| ■ | ■ | ■ | | ■ | | 3 | 0 | 3 | 1 | 14 | 0 | 0 | 0 | 0 | | | | | | | | | |
| ■ | ■ | ■ | | | ■ | 2 | 0 | 1 | 0 | 8 | 1 | 0 | 0 | 0 | | | | | | | | | |
| ■ | ■ | | ■ | ■ | | 9 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | | | | | | | | | |
| ■ | ■ | | ■ | | ■ | 15 | 0 | 3 | 0 | 1 | 9 | 0 | 0 | 0 | | | | | | | | | |
| ■ | ■ | | | ■ | ■ | 3 | 0 | 1 | 0 | 2 | 3 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | ■ | ■ | ■ | | 2 | 1 | 0 | 0 | 0 | 4 | 0 | 1 | 0 | | | | | | | | | |
| ■ | | ■ | ■ | | ■ | 2 | 1 | 0 | 0 | 3 | 2 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | ■ | | ■ | ■ | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | | ■ | ■ | ■ | 8 | 0 | 2 | 1 | 0 | 3 | 0 | 0 | 0 | | | | | | | | | |
| | ■ | ■ | ■ | ■ | | 11 | 15 | 2 | 0 | 6 | 4 | 0 | 0 | 0 | 5 | 4 | 0 | | | | | | |
| | ■ | ■ | ■ | | ■ | 11 | 14 | 0 | 1 | 3 | 7 | 1 | 0 | 0 | 4 | 0 | 0 | | | | | | |
| | ■ | ■ | | ■ | ■ | 6 | 0 | 0 | 0 | 5 | 0 | 1 | 0 | 1 | 3 | 0 | 0 | | | | | | |
| | ■ | | ■ | ■ | ■ | 60 | 4 | 1 | 9 | 1 | 6 | 1 | 0 | 0 | 3 | 0 | 0 | | | | | | |
| | | ■ | ■ | ■ | ■ | 16 | 6 | 1 | 20 | 1 | 5 | 2 | 1 | 0 | 4 | 0 | 0 | | | | | | |
| **TOTAL – 4 years** | | | | | | **151** | **48** | **22** | **33** | **73** | **51** | **5** | **3** | **1** | **19** | **4** | **0** | **0** | **0** | **0** | **0** | **0** | **0** |
| ■ | ■ | ■ | ■ | ■ | | 8 | 3 | 2 | 0 | 7 | 3 | 0 | 0 | 0 | | | | | | | | | |
| ■ | ■ | ■ | ■ | | ■ | 8 | 1 | 4 | 0 | 7 | 5 | 0 | 0 | 0 | | | | | | | | | |
| ■ | ■ | ■ | | ■ | ■ | 1 | 0 | 2 | 0 | 2 | 1 | 0 | 0 | 0 | | | | | | | | | |
| ■ | ■ | | ■ | ■ | ■ | 21 | 0 | 2 | 1 | 2 | 2 | 0 | 0 | 0 | | | | | | | | | |
| ■ | | ■ | ■ | ■ | ■ | 11 | 0 | 0 | 1 | 0 | 3 | 0 | 0 | 0 | | | | | | | | | |
| | ■ | ■ | ■ | ■ | ■ | 14 | 7 | 0 | 1 | 7 | 4 | 2 | 0 | 0 | 1 | 0 | 0 | | | | | | |
| **TOTAL – 5 years** | | | | | | **63** | **11** | **10** | **3** | **25** | **18** | **2** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** |
| ■ | ■ | ■ | ■ | ■ | ■ | 14 | 2 | 1 | 0 | 8 | 3 | 0 | 0 | 0 | | | | | | | | | |
| **OVERALL TOTAL** | | | | | | **663** | **603** | **509** | **656** | **654** | **521** | **161** | **227** | **108** | **555** | **545** | **371** | **353** | **53** | **210** | **208** | **0** | **0** |

[a]The total number of grapevines observed at each site for the entire survey period: Gol Gol = 763 grapevines, Karadoc = 860 grapevines, Wemen (six years) = 298 grapevines, Wemen (five years) = 984 grapevines, Colignan (3 years) = 372 grapevines, Paringa (3 years) = 2170 grapevines

[b]The shaded cells represent the years in which symptoms were observed.

**Spatial analysis of AGYd, RGd and LSLCd**

A fixed grid analysis was used to determine whether AGYd, RGd or LSLCd affected grapevines were clustered. When the number of subunits in the grid laid across the plot was less than 15 the results were not included.

AGYd affected grapevines were significantly clustered in all years at Gol Gol (Table 3.4) and Paringa (Table 3.5) and only in some years at Karadoc (Table 3.6) and Colignan (Table 3.7). The cluster sizes of AGYd affected grapevines were different between most years in each vineyard.

RGd affected grapevines were significantly clustered in all years at Karadoc (Table 3.8), from 1996/97 to 2000/01 at Gol Gol (Table 3.9) and in 1999/00 only at Colignan (Table 3.10). The cluster sizes of RGd affected grapevines were different between most years in each vineyard Clustering of RGd at Gol Gol in 1995/96 or at Colignan in 2000/01 could not be determined due to runtime errors that was encountered while using the computer program, "Patchy".

LSLCd affected grapevines were significantly clustered in all years at Gol Gol (Table 3.11) and Karadoc (Table 3.12). The cluster sizes of LSLCd affected grapevines were different between most years in each vineyard. The distribution of LSLCd affected grapevines at Colignan was random.

Table 3.4. The fixed grid analysis of clustering of AGYd at Gol Gol in each season.

| | 1995/96 | | | | | | | | 1996/97 | | | | | | | | 1997/98 | | | | | | | | 1998/99 | | | | | | | | 1999/00 | | | | | | | | 2000/01 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sub Unit size[a] | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | |
| Row | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 2 | +[b] | + | + | | | | | | | | | | | | | | | | | + | + | | | + | | | + | + | + | | | + | | | | | | | | | | | | | | | | |
| 3 | + | + | + | + | + | | | | | | | | | | | | | + | | + | + | + | + | + | | + | + | + | + | + | + | + | | | | | | | | | | | | | | | | |
| 4 | + | + | | + | + | | | | | | | | | | | | | + | + | + | + | + | + | + | | + | + | + | + | + | + | + | | | | | | | | | | | | | | + | | + | | + | + |
| 5 | + | + | | | | | | | | | | | | | | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | | | | + | | | | | | | | + | + | + | | + | + |
| 6 | + | + | | | | | | | | + | | | | | | | + | + | + | + | + | | | | + | + | + | + | + | | | | | | | | | | | | | | | + | + | + | + | + | + |
| 7 | + | + | | | | | | | | | | | | | | | + | + | + | + | + | | | | + | + | + | | | | | | | | | + | | | | | | | | + | + | + | + | | | |
| 8 | + | + | | | | | | | | + | | | | | | | + | + | + | + | | | | | + | + | + | | | | | | | | | + | + | | | | | | | + | + | + | + | | | |
| 9 | + | + | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | + | | | | | | | + | + | | | | | | | + | + | + | + | + | | | |
| 10 | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | + | | | | | | + | + | + | | | | | | + | + | + | | | | | |
| 11 | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | + | | | | | | | + | + | | | | | |
| 12 | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | | | | | | | | + | + | + | | | | | |
| 13 | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 14 | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 15 | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 16 | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | + | | | | | | |
| 17 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | |
| 18 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | |
| 19 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | |
| 20 | | | | | | | | | | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 21 | | | | | | | | | | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 22 | | | | | | | | | | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 23 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 24 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | |

a. Fixed grid analyses were done using various subunit sizes. Each subunit of the fixed grid analysis consists of a group of adjacent grapevines in rows and columns. b. + = Significant clustering of diseased grapevines ($p<0.05$). When the number of subunits included in the fixed grid analysis is less than 15, the results were not included and this is represented by the shaded cells.

Table 3.5. The fixed grid analysis of clustering of AGYd at in Block 1 at Paringa in each season.

| | Block 1 | | | | | | | | | | | | | | | | | | Block 2 | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1998/99 | | | | | | | | | 1999/00 | | | | | | | | | 1998/99 | | | | | | | | | 1999/00 | | | | | | | |
| | Column | | | | | | | | | Column | | | | | | | | | Column | | | | | | | | | Column | | | | | | | |
| Row | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 3 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | | + | | | + | | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 6 | | | | | + | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 7 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 8 | | | | | | | | | | + | | + | | | | | | | | | | | | | | | | + | | | | | | | | |
| 9 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 10 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 11 | | | | | | | | | | + | | | | | | | | | | | | | + | | | | | + | | | | | | | | |
| 12 | | | | | | | | | | + | | + | | | | | | | | | | | | | | | | + | | | | | | | | |
| 13 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 14 | | | + | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 15 | | + | + | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 16 | | | | | | | | | | + | | + | | | | | | | | | | | | | | | | + | | | | | | | | |
| 17 | | | | | | | | | | + | | + | | | | | | | | | | | | | | | | + | | | | | | | | |
| 18 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 19 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 20 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 21 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 22 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 23 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 24 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 25 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 26 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 27 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |

Table 3.6. The fixed grid analysis of clustering of AGYd at Karadoc in each season.

| | 1995/96 | | | | | | | | 1996/97 | | | | | | | | 1997/98 | | | | | | | | 1998/99 | | | | | | | | 1999/00 | | | | | | | | 2000/01 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sub Unit size | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | |
| Row | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 2 | | | | | | | | | + | + | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | + | + | | | | | | | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | + | | | | | | | | | + | | | + | | | | | | | | | | | | | | | | | + | | | | | | | | | | | | | | | | | |
| 5 | + | + | + | | + | | | | + | + | + | | + | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | | | | | | | | | + | | + | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 7 | | | | | | | | | + | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 8 | + | + | | + | + | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 9 | | + | + | | | | | | + | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 | | | + | | | | | | + | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | + | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 14 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 15 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 16 | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 17 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 18 | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 19 | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 20 | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 21 | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 22 | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 23 | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 24 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Table 3.7. The fixed grid analysis of clustering of AGYd at Colignan in 1999/00.

| | 1999/00 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Column | | | | | | | | |
| Row | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 2 | | | | | | | | | |
| 3 | | | | | | | | | |
| 4 | | | | + | | | | | |
| 5 | | | | + | | | | | |
| 6 | | | | | | | | | |
| 7 | | | | | | | | | |
| 8 | | | | | | | | | |
| 9 | | | | | | | | | |
| 10 | | | | | | | | | |

Table 3.8. The fixed grid analysis of clustering of RGd at Karadoc in each season.

| | 1995/96 | | | | | | | | 1996/97 | | | | | | | | 1997/98 | | | | | | | | 1998/99 | | | | | | | | 1999/00 | | | | | | | | 2000/01 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sub Unit size | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | |
| Row | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 2 | | | + | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | + | | | | | | | | | | | | | | |
| 3 | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | + | | | | + | + | + | | + | | | + | + | + | | | + | + | | |
| 4 | | | | | + | | | | | | | | | | | | | | | | | | | | + | | + | + | + | | | | + | + | + | | + | | | + | | | | | | | | |
| 5 | | | + | + | + | | | | | | | | | | | | | | | | | | | | + | | + | | + | | | | + | + | + | | + | | + | + | | | | | | | | |
| 6 | + | | + | | + | | | | | | | | | | | | | | | | | | | | + | | + | | + | | | | + | + | + | | + | | | | | | | + | + | | | |
| 7 | + | + | + | | | | | | | + | | | | | | | | | | | | | | | + | + | + | | + | | | | | + | + | | + | | | | + | + | + | | + | | | |
| 8 | + | | + | | + | | | | | + | | | | | | | | | | | | | | | + | + | + | | + | | | | + | + | + | | | | | | | | | | | | |
| 9 | + | + | + | | | | | | | + | | | | | | | | | | | | | | | + | + | + | | | | | | + | + | | | | | | | + | | | | | | | |
| 10 | + | + | + | | | | | | | + | | | | | | | | | + | | | | | | + | + | | | | | | | + | + | | | | | | | | | | | | | |
| 11 | + | + | | | | | | | + | + | + | | | | | | + | | + | | | | | | + | + | | | | | | | + | + | | | | | | | + | | | | | | | |
| 12 | + | + | | | | | | | + | + | + | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 13 | + | | | | | | | | | + | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | |
| 14 | + | + | | | | | | | | + | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | | | | | | | | | | | | | |
| 15 | + | + | | | | | | | | + | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | | | | | | | | | | | | | |
| 16 | + | + | | | | | | | | + | | | | | | | + | + | | | | | | | + | + | | | | | | | + | | | | | | | | | | | | | | |
| 17 | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 18 | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 19 | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 20 | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | |
| 21 | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | |
| 22 | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | |
| 23 | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | |
| 24 | | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |

Table 3.9. The fixed grid analysis of clustering of RGd at Gol Gol in each season.

| | 1996/97 | | | | | | | | 1997/98 | | | | | | | | 1998/99 | | | | | | | | 1999/00 | | | | | | | | 2000/01 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sub Unit size | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | |
| Row | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 2 | | | | | | | | | | | | | | | | | | + | | + | + | | | + | + | + | + | + | + | + | | | + | + | + | + | + | | | |
| 3 | | | | | | | | | | | | | | | | | + | + | + | + | + | | + | + | + | + | + | + | + | + | | | + | + | + | + | + | | | |
| 4 | + | | | | | | | | | | + | | + | | | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 5 | + | | | | | | | | + | + | + | + | + | | | | + | + | + | + | + | | | | + | + | + | + | + | + | | + | + | + | + | + | + | + | + | + |
| 6 | + | | | | | | | | | | + | | + | | | | + | + | + | + | + | | | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 7 | + | | | | | | | | + | + | + | | + | | | | + | + | + | | + | | | | + | + | + | + | + | | | | + | + | + | + | + | | | |
| 8 | + | + | | | | | | | | + | + | | | | | | + | + | + | | | | | | + | + | + | + | + | | | | + | + | + | + | + | | | |
| 9 | + | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | + | + | | | | | + | + | + | + | | | | |
| 10 | + | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | + | | | | | | + | + | + | | | | | |
| 11 | + | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | + | | | | | | + | + | + | | | | | |
| 12 | + | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | + | | | | | | + | + | + | | | | | |
| 13 | | | | | | | | | + | + | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 14 | + | | | | | | | | + | + | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 15 | | | | | | | | | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 16 | | | | | | | | | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 17 | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 18 | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 19 | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 20 | | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 21 | | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 22 | | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 23 | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 24 | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |

Table 3.10. The fixed grid analysis of clustering of RGd at Colignan in 1999/00.

| | 1999/00 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Column | | | | | | | | |
| Row | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 2 | | | | | + | | | | |
| 3 | | | + | | + | | | | |
| 4 | | | | | + | | | | |
| 5 | + | + | + | | | | | | |
| 6 | | + | + | | | | | | |
| 7 | | | | | | | | | |
| 8 | | | | | | | | | |
| 9 | | | | | | | | | |
| 10 | + | | | | | | | | |

Table 3.11. The fixed grid analysis of clustering of LSLCd at Gol Gol in each season.

| | 1995/96 | | | | | | | | 1996/97 | | | | | | | | 1997/98 | | | | | | | | 1998/99 | | | | | | | | 1999/00 | | | | | | | | 2000/01 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sub Unit size | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | |
| Row | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 2 | + | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | + | | + | | | | | | | | | | | |
| 3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | + | | | | + | + | + | + | + | + | + | + | | + | + | + |
| 4 | + | + | | | | | | | | | | | | | | | + | | + | + | + | | | | + | | | | | | | | + | + | | | + | + | + | + | + | + | + | | | + | + | + |
| 5 | + | | + | | | | | | | | | | | | | | + | + | + | | + | | | | + | | | | | | | | + | + | + | + | + | + | + | + | + | + | + | + | | + | + | + |
| 6 | + | + | | | | | | | | | | | | | | | + | | + | | | | | | + | + | | | + | | | | + | + | + | + | + | + | + | + | + | + | + | + | | + | + | + |
| 7 | + | + | | | | | | | | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | + | + | + | | | | + | + | + | + | | | | |
| 8 | + | + | | | | | | | | | | | | | | | + | + | | | | | | | + | | | | | | | | + | + | + | + | + | | | | + | + | + | | | | | |
| 9 | + | + | | | | | | | | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | + | + | | | | | + | + | + | + | + | | | |
| 10 | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | + | | | | | | + | + | + | | | | | | + | + | + | | | | | |
| 11 | + | | | | | | | | | | | | | | | | + | + | | | | | | | + | | | | | | | | + | + | + | | | | | | + | + | + | | | | | |
| 12 | + | | | | | | | | | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | + | | | | | | + | + | + | | | | | |
| 13 | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 14 | + | | | | | | | | | | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 15 | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 16 | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | + | | | | | | | + | + | | | | | | |
| 17 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 18 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 19 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 20 | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | |
| 21 | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | |
| 22 | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | + | | | | | | | |
| 23 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |
| 24 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | |

Table 3.12. The fixed grid analysis of clustering of LSLCd at Karadoc in each season.

| | 1995/96 | | | | | | | | 1996/97 | | | | | | | | 1997/98 | | | | | | | | 1998/99 | | | | | | | | 1999/00 | | | | | | | | 2000/01 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sub Unit size | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | | Column | | | | | | | |
| Row | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 2 | | + | + | | + | | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | + | + | + | + | + | | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | + | + | + | + | + | | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | + | + | + | | + | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | + | + | + | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | | | | | | | |
| 7 | + | + | + | | + | | | | | | | | | | | | | | | | + | | | | | | | | | | | | | | + | | + | | | | | | | | | | |
| 8 | + | + | + | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | |
| 9 | + | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 | + | + | + | | | | | | | | | | | | | | | | | | | | | | + | + | | | | | | | | | | | | | | | | | | | | | |
| 11 | + | + | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | | + | | | | | | |
| 12 | + | + | | | | | | | | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | | + | | | | | | |
| 13 | + | + | | | | | | | + | + | | | | | | | | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | |
| 14 | + | + | | | | | | | + | + | | | | | | | | | | | | | | | + | | | | | | | | | + | | | | | | | | | + | | | | | | |
| 15 | + | + | | | | | | | + | + | | | | | | | | | | | | | | | + | | | | | | | | + | + | | | | | | | | | + | | | | | | |
| 16 | + | + | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | | | | | | | | |
| 17 | + | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 18 | + | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 19 | + | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 20 | + | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | | | | | | | | |
| 21 | + | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | | | | | | | | |
| 22 | + | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | | | | | | | | |
| 23 | + | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | + | | | | | | | | | | | | | | | |
| 24 | | | | | | | | | + | | | | | | | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |

Spatial autocorrelation was used to determine whether there was an association between grapevines and their diseased neighbours in the column/row direction and the diagonal direction. If the analysis produced a significant result, the null hypothesis that the spatial distribution of AGYd, RGd or LSLCd is random could be rejected and clustering of disease can be inferred. The results can also suggest a direction of spread if an association between grapevines and diseased neighbours is observed more frequently in one direction. When the total number of joins associated with a significant test result were less than 5% of the total number of grapevines at the test site, the result was disregarded and this is represented by the shaded cells in the table.

*AGYd* At Gol Gol there was a significant association between grapevines and neighbouring AGYd plants in all years (Table 3.13). At Karadoc, Colignan and some areas at Wemen there was a significant association between grapevines and neighbouring AGYd plants in some years only. Clustering of AGYd was not associated with the disease status of the test grapevine because significant associations often occurred between both asymptomatic and AGYd affected grapevines and neighbouring AGYd affected plants in the same years in each vineyard. No directional trends were observed at Gol Gol or Colignan because significant associations between grapevines and neighbouring AGYd affected plants occurred in both the column/row direction and the diagonal direction in the years clustering was observed. At Wemen and Karadoc significant associations occurred more frequently in the column/row direction.

*RGd* At Karadoc there was a significant association between grapevines and neighbouring RGd plants in each year (Table 3.14). At Gol Gol there was a significant association between grapevines and RGd affected neighbours from 1996/97 to 1999/00. At Colignan and some areas at Wemen there was a significant association between grapevines and neighbouring RGd plants in some years only. Clustering of RGd was not associated with the disease status of the test grapevine because significant associations

often occurred between both asymptomatic and RGd affected grapevines and neighbouring RGd affected plants in the same years at Gol Gol, Karadoc and some areas at Wemen. At Colignan and in areas 1 and 2 at Wemen significant associations occurred only between asymptomatic and neighbouring RGd affected plants. No directional trends were observed at Gol Gol or Colignan as significant associations between grapevines and neighbouring RGd affected plants occurred in both the column/row direction and the diagonal direction in the years clustering was observed. At Karadoc and Wemen significant associations occurred more frequently in the column/row direction.

*LSLCd* There was a significant association between grapevines and neighbouring LSLCd was in each year except 1999/00 at Gol Gol and 1997/98 at Karadoc (Table 3.15). At Colignan and some areas at Wemen there was a significant association between grapevines and neighbouring LSLCd plants in some years only. Clustering of LSLCd was not associated with the disease status of the test grapevine because significant associations often occurred between both asymptomatic and LSLCd affected grapevines and neighbouring LSLCd affected plants in the same years in all vineyards. Significant associations occurred more frequently in the column/row direction in all vineyards.

Table 3.13. The association between asymptomatic or AGYd affected grapevines and their AGYd affected neighbours in the row/column direction or the diagonal direction at Gol Gol, Karadoc, the four areas surveyed at Wemen (five years) and Colignan in each year.

| Type of join[a] | Year | Gol Gol | | Karadoc | | Wemen | | | | | | | | Colignan | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Site | | | | | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | | |
| | | H A[b] | AA[c] | H A | AA | H A | | | | AA | | | | H A | AA |
| Rooks | 1995/96 | +[d] | + | + | +[e] | ns[f] | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | 1996/97 | + | + | + | + | | | + | | | | + | | ns | ns |
| | 1997/98 | + | + | | | | | | | | | | + | ns | ns |
| | 1998/99 | + | + | + | | | | | | | | | | | |
| | 1999/00 | + | + | + | + | | | | + | | | | + | + | + |
| | 2000/01 | + | + | | | | + | + | | | + | + | | ns | ns |
| Bishops | 1995/96 | + | + | | | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | 1996/97 | + | + | + | + | | | | | | | | | ns | ns |
| | 1997/98 | + | + | | | | | | | | | | | | |
| | 1998/99 | + | + | | | + | | | | | | | | | |
| | 1999/00 | + | + | | | | | | + | | | | + | + | + |
| | 2000/01 | + | | | + | | | | | | | | | | |

a. Rooks = joins between a grapevines and its neighbours in the row and column direction;
Bishops = joins between a grapevines and its neighbours in the diagonal direction

b. HA = tests between healthy grapevines and AGYd affected neighbours

c. AA = tests between AGYd affected grapevines and AGYd affected neighbours

d. + = significant join count statistic at the 5% level with a z-test statistic ≥ 1.96

e. + in a shaded square = significant join count statistic at the 5% level with a z-test statistic ≥ 1.96
but the number of joins associated with the test result was less than 5% of the total number of vines at the test site

e. ns = vineyard not surveyed

Table 3.14. The association between asymptomatic or RGd affected grapevines and their RGd affected neighbours in the row/column direction or the diagonal direction at Gol Gol, Karadoc, the four areas surveyed at Wemen (five years) and Colignan in each year.

| Type of join | Year | Gol Gol | | Karadoc | | Wemen | | | | | | | | Colignan | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Site | | | | | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | | |
| | | HR[a] | RR[b] | HR | RR | HR | | | | RR | | | | HR | RR |
| Rooks | 1995/96 | | + | + | + | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | 1996/97 | + | + | + | + | + | + | + | | | | + | | ns | ns |
| | 1997/98 | + | + | + | + | | + | | | | + | | | ns | ns |
| | 1998/99 | + | + | + | + | na[c] | + | | | na | + | | + | | |
| | 1999/00 | + | + | + | + | na | + | | | na | + | | | + | |
| | 2000/01 | + | + | + | + | na | na | na | na | na | na | na | na | | + |
| Bishops | 1995/96 | | + | | + | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | 1996/97 | | + | | | + | | | | + | | | | ns | ns |
| | 1997/98 | + | + | | | | | | | | | | | ns | ns |
| | 1998/99 | + | + | | | na | | | | na | + | | | | |
| | 1999/00 | + | + | | | na | | | | na | | | | + | |
| | 2000/01 | | | | | na | | | | na | | | | | + |

a. HR = tests between healthy grapevines and RGd affected neighbours
b. RR = tests between RGd affected grapevines and RGd affected neighbours
c. na = not appropriate to test because the number of affected grapevines were < 2, there fore spatial autocorrelation was not estimated

Table 3.15. The association between asymptomatic or LSLCd affected grapevines and their LSLCd affected neighbours in the row/column direction or the diagonal direction at Gol Gol, Karadoc, the four areas surveyed at Wemen (five years) and Colignan in each year.

| Type of join | Year | Gol Gol | | Karadoc | | Wemen | | | | | | | | Colignan | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Site | | | | | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | | |
| | | HL[a] | LL[b] | HL | LL | HL | | | | LL | | | | HL | LL |
| Rooks | 1995/96 | + | + | + | + | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | 1996/97 | + | + | + | + | + | + | | | + | + | | | ns | ns |
| | 1997/98 | + | + | | | | + | | | | + | | | ns | ns |
| | 1998/99 | + | + | + | + | + | + | | | | | | | + | + |
| | 1999/00 | | + | + | + | | | | | | | | | | |
| | 2000/01 | + | + | + | + | | | | | | | | | | |
| Bishops | 1995/96 | + | + | + | + | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| | 1996/97 | | | | | | | | | | | | | ns | ns |
| | 1997/98 | | + | | | | | | | | | | | ns | ns |
| | 1998/99 | | | | | + | + | | | | | | | | |
| | 1999/00 | | + | | | | | | | | | + | | | |
| | 2000/01 | | + | | | | | + | + | | + | + | | | |

a. HL = tests between healthy grapevines and LSLCd affected neighbours
b. LL = tests between LSLCd affected grapevines and LSLCd affected neighbours

**The association between AGYd and RGd or LSLCd**

*AGYd with RGd and/or LSLCd* There was no pattern of association between AGYd and RGd only, LSLCd only or RGd/LSLCd in the same year in any vineyard (Table 3.16).

Table 3.16. The proportion (%) of AGYd affected grapevines that also had RGd, LSLCd or RGd and LSLCd in each vineyard in each season.

| Vineyard | Disease | 1995/96 | 1996/97 | 1997/98 | 1998/99 | 1999/00 | 2000/01 |
|---|---|---|---|---|---|---|---|
| Gol Gol | RGd | 10.8 | 29.4 | 65.7 | 48.6 | 7.9 | 5.7 |
| | LSLCd | 18.7 | 13.8 | 8.0 | 6.4 | 11.5 | 13.1 |
| | RGd/LSLCd | 1.8 | 16.7 | 16.7 | 10.9 | 1.0 | 1.7 |
| Karadoc | RGd | 11.1 | 27.0 | 35.7 | 20.3 | 27.4 | 8.1 |
| | LSLCd | 11.1 | 21.6 | 12.5 | 18.7 | 12.9 | 23.9 |
| | RGd/LSLCd | 38.9 | 24.3 | 33.9 | 35.7 | 3.1 | 6.4 |
| Wemen (six years) | RGd | 0 | 35.9 | 40.8 | 30.9 | 0 | 0 |
| | LSLCd | 0 | 7.8 | 14.5 | 1.8 | 10.7 | 14.0 |
| | RGd/LSLCd | 0 | 0 | 10.5 | 3.6 | 0 | 0 |
| Wemen (five years) | RGd | | 44.8 | 15.3 | 4.7 | 5.6 | 0 |
| | LSLCd | | 8.4 | 17.8 | 7.0 | 4.0 | 10.6 |
| | RGd/LSLCd | | 11.7 | 2.5 | 0 | 0 | 0 |
| Colignan | RGd | | | | 14.5 | 9.2 | 1.6 |
| | LSLCd | | | | 5.5 | 11.6 | 9.8 |
| | RGd/LSLCd | | | | 3.6 | 1.0 | 0.5 |

The largest proportion of AGYd affected grapevines also affected by RGd only in the same year occurred at Gol Gol (65.7%) in 1997/98. RGd did not affect grapevines with AGYd at Wemen (five years) in 2000/01 or at Wemen (six years) in 1999/00 and 2000/01.

The largest proportion of AGYd affected grapevines also affected by LSLCd only in the same year occurred at Karadoc (23.9%) in 1997/98. LSLCd did not affect grapevines with AGYd at Wemen (six years) in 1995/96.

The largest proportion of AGYd affected grapevines also affected by RGd and LSLCd in the same year occurred at Karadoc (38.9%) in 1997/98. AGYd affected grapevines were not affected by both RGd and LSLCd at Wemen (five years) from 1998/99 to 2000/01 or at Wemen (six years) in 1999/00 and 2000/01.

There was no pattern of association between AGYd and RGd in the following season in each vineyard (Table 3.17). The largest proportion of grapevines with AGYd that displayed RGd in the following season occurred at Karadoc (67.6%) in 1996/97. There were no grapevines with AGYd that displayed RGd in the following year at Colignan in

any year or at Wemen (six years) in 1995/96 and 1999/00 and Wemen (five years) in 1999/00.

Table 3.17. The proportion (%) of grapevines with AGYd that also displayed RGd in the following season at each site for each season of the survey.

| Vineyard | 1995/96 | 1996/97 | 1997/98 | 1998/99 | 1999/00 |
|---|---|---|---|---|---|
| Gol Gol | 48.8 | 53.9 | 64.9 | 5.6 | 5.3 |
| Karadoc | 66.7 | 67.6 | 50.0 | 20.3 | 13.2 |
| Wemen (six years) | 0 | 34.4 | 38.2 | 1.8 | 0 |
| Wemen (five years) | | 15.9 | 4.5 | 2.3 | 0 |
| Colignan | | | | 0 | 0 |

There was no pattern of association between AGYd and LSLCd in the previous season in each vineyard (Table 3.18). The largest proportion of grapevines with AGYd that displayed LSLCd in the previous season occurred at Karadoc (73.2%) in 1997/98. There were no grapevines with AGYd that displayed LSLCd in the previous years at Wemen (six years) in 1996/97 and 2000/01.

Table 3.18. The proportion (%) of grapevines with AGYd that also displayed LSLCd in the previous season at each site for each season of the survey.

| Vineyard | 1996/97 | 1997/98 | 1998/99 | 1999/00 | 2000/01 |
|---|---|---|---|---|---|
| Gol Gol | 25.5 | 33.3 | 24.7 | 20.8 | 14.8 |
| Karadoc | 29.7 | 73.2 | 44.0 | 39.5 | 20.8 |
| Wemen (six years) | 0 | 26.3 | 20.0 | 3.6 | 10.0 |
| Wemen (five years) | | 18.8 | 5.3 | 3.2 | 0 |
| Colignan | | | | 47.3 | 17.6 |

AGYd affected some grapevines in the absence of RGd and LSLCd at each vineyard during the surevy. AGYd alone affected 78 grapevines at Gol Gol, 98 grapevines at Karadoc, 119 grapevines at Wemen (five years), 16 grapevines at Wemen (six years) and 147 grapevines at Colignan.

*RGd with AGYd* There was no pattern of association between RGd and AGYd only or AGYd and LSLCd in the same year in any vineyard (Table 3.19). The largest proportion (58.6%) of grapevines with RGd that also displayed AGYd in the same year occurred at Gol Gol in 198/99. AGYd did not affect grapevines with RGd at Wemen (five years) in 2000/01 or at Wemen (six years) in 1995/96, 1999/00 and 2000/01.

Table 3.19. The proportion (%) of RGd affected grapevines that also had AGYd or AGYd and LSLCd in each vineyard in each season.

| Vineyard | Disease | 1995/96 | 1996/97 | 1997/98 | 1998/99 | 1999/00 | 2000/01 |
|---|---|---|---|---|---|---|---|
| Gol Gol | AGYd | 41.9 | 40.6 | 28.7 | 48.6 | 39.3 | 38.3 |
| | AGYd /LSLCd | 7.0 | 23.2 | 7.2 | 10.9 | 4.8 | 11.7 |
| Karadoc | AGYd | 0.7 | 2.6 | 5.5 | 21.0 | 56.1 | 32.7 |
| | AGYd /LSLCd | 2.4 | 2.3 | 5.2 | 36.9 | 6.3 | 25.7 |
| Wemen (six years) | AGYd | 0 | 19.5 | 35.6 | 14.2 | 0 | 0 |
| | AGYd /LSLCd | 0 | 0 | 9.2 | 1.7 | 0 | 0 |
| Wemen (five years) | AGYd | | 22.0 | 34.1 | 21.6 | 36.8 | 0 |
| | AGYd /LSLCd | | 5.7 | 5.5 | 0 | 0 | 0 |
| Colignan | AGYd | | | | 32.0 | 64.3 | 75.0 |
| | AGYd /LSLCd | | | | 8.0 | 7.1 | 25.0 |

There was no pattern of association between RGd and AGYd in the previous seasons in any vineyard (Table 3.20). A high proportion of RGd affected grapevines that also had AGYd in previous years was observed at Colignan compared to other vineyards, particularly in 2000/01, although the number of grapevines observed with RGd in 2000/01 was only four. At Wemen (six years) in 1995/96 and 200/01, Wemen (five years) in 2000/01 and Colignan in 1999/00 and 2000/01 grapevines with RGd were not affected by RGd in the previous season.

Table 3.20. The proportion (%) of grapevines with RGd that also displayed AGYd in the previous season at each site for each season of the survey.

| Vineyard | 1996/97 | 1997/98 | 1998/99 | 1999/00 | 2000/01 |
|---|---|---|---|---|---|
| Gol Gol | 25.7 | 58.6 | 28.8 | 26.2 | 36.7 |
| Karadoc | 3.1 | 6.9 | 15.9 | 19.6 | 45.1 |
| Wemen (six years) | 0 | 25.3 | 24.2 | 7.7 | 0 |
| Wemen (five years) | | 41.8 | 24.3 | 21.1 | 0 |
| Colignan | | | | 0 | 0 |

RGd affected some grapevines in the absence of AGYd and LSLCd at each vineyard during the surevy. RGd alone affected 22 grapevines at Gol Gol, 61 grapevines at Karadoc, 158 grapevines at Wemen (five years), 80 grapevines at Wemen (six years) and 8 grapevines at Colignan.

*LSLCd with AGYd* There was no pattern of association between LSLCd an AGYd only or AGYd and RGd in affected grapevines in the same year in any vineyard (Table 3.21). The largest proportion (60.2%) of grapevines with LSLCd that also displayed AGYd in the same year occurred at Gol Gol in 2000/01. AGYd did not affect grapevines with LSLCd at Wemen (six years) in 1995/96.

Table 3.21. The proportion (%) of LSLCd affected grapevines that also had AGYd or AGYd and RGd in each vineyard in each season.

| Vineyard | Disease | 1995/96 | 1996/97 | 1997/98 | 1998/99 | 1999/00 | 2000/01 |
|---|---|---|---|---|---|---|---|
| Gol Gol | AGYd | 14.8 | 24.6 | 7.9 | 17.9 | 56.5 | 60.2 |
| | RGd/AGYd | 1.4 | 29.9 | 16.3 | 30.7 | 6.7 | 8.0 |
| Karadoc | AGYd | 1.1 | 3.5 | 5.4 | 14.2 | 37.0 | 50.9 |
| | RGd/AGYd | 3.8 | 3.9 | 14.6 | 27.1 | 8.9 | 13.6 |
| Wemen (six years) | AGYd | 0 | 14.7 | 26.2 | 5.9 | 23.1 | 31.8 |
| | RGd/AGYd | 0 | 0 | 19.0 | 11.8 | 0 | 0 |
| Wemen (five years) | AGYd | | 10.7 | 35.0 | 26.1 | 17.2 | 44.7 |
| | RGd/AGYd | | 15.0 | 4.9 | 0 | 0 | 0 |
| Colignan | AGYd | | | | 1.8 | 7.3 | 52.3 |
| | RGd/AGYd | | | | 1.2 | 5.5 | 2.8 |

There was no pattern of association between LSLCd and AGYd in the following year in any vineyard (Table 3.22). The largest proportion of grapevines with LSLCd that displayed AGYd in the following season occurred at Gol Gol (70.6%) in 1999/00. Grapevines with AGYd did not display RGd in the following year at Colignan in any year, at Wemen (six years) in 1996/97 and 2000/01 and Wemen (five years) in 2000/01.

LSLC d affected some grapevines in the absence of AGYd and RGd at each vineyard during the survey. LSLCd alone affected 29 grapevines at Gol Gol, 15 grapevines at Karadoc, 39 grapevines at Wemen (five years), 4 grapevines at Wemen (six years) and 10 grapevines at Colignan.

Table 3.22. The proportion (%) of grapevines with LSLCd that also displayed AGYd in the following season at each site for each season of the survey.

| Vineyard | 1995/96 | 1996/97 | 1997/98 | 1998/99 | 1999/00 |
|---|---|---|---|---|---|
| Gol Gol | 52.9 | 23.8 | 54.5 | 62.1 | 70.6 |
| Karadoc | 6.1 | 17.8 | 61.5 | 63.4 | 70.4 |
| Wemen (six years) | 0 | 58.8 | 26.2 | 5.9 | 38.5 |
| Wemen (five years) | | 20.3 | 8.7 | 8.7 | 0 |
| Colignan | | | | 84.1 | 61.8 |

**Temporal analysis of AGYd, RGd and LSLCd using Log-linear models**

Log-linear models were used to determine if there were significant interactions between AGYd, RGd and LSLCd and elapsed time at each vineyard. Significant interactions indicated that the expected number of vines for each combination (absence/presence) of the three diseases is significantly different relative to each other and/or significantly different from season to season if year had a significant interaction with the diseases. Graphical representations of the significant interactions that were observed are presented in Appendix

2. B

At Wemen the incidence of RGd changed significantly with time ($p<0.001$).

The incidence of LSLCd affected grapevines changed significantly with time at Gol Gol ($p<0.001$), Karadoc ($p<0.041$) and Wemen ($p<0.001$).

There was a significant interaction between the presence of AGYd and RGd and this interaction changed with time at Gol Gol ($p = 0.035$), Karadoc ($p = 0.033$) and Colignan ($p = 0.02$). That is, the expected number of unaffected grapevines, grapevines affected by AGYd and RGd, AGYd alone and RGd alone were significantly different from each other in some years at each vineyard.

At Wemen there was an interaction between the presence of AGYd and RGd ($p = 0.007$). The graphical representation of the data suggested that the expected number of unaffected grapevines was significantly greater than the number of grapevines affected by

one or both diseases for the entire survey period. The incidence of RGd did not increase with the presence of AGYd.

At Colignan there was an interaction between the presence of AGYd, RGd and LSLCd ($p = 0.052$). The graphical representation of the data suggested that the expected number of grapevines unaffected by RGd was significantly greater than the number of grapevines affected by RGd for the entire survey period, regardless of the presence of AGYd and LSLCd. The incidence of LSLCd did not increase with the presence of AGYd.

There was an interaction between the overall presence of AGYd and LSLCd at Karadoc ($p = 0.004$) and Wemen ($p = 0.011$). For both vineyards the graphical representation of the data suggested that the expected number of unaffected grapevines was significantly greater than the number of grapevines affected by one or both diseases for the entire survey period. The incidence of LSLCd did not increase with the presence of AGYd.

At Colignan there was an interaction between the presence of AGYd and LSLCd and this interaction changed with time ($p = 0.009$). That is, the expected number of unaffected grapevines, grapevines affected by AGYd and LSLCd, AGYd alone and LSLCd alone were significantly different from each other in some years.

There was a significant interaction between the presence of RGd and LSLCd at Gol Gol ($p<0.001$) and Karadoc ($p<0.001$). The graphical representation of the data suggested that the expected number of unaffected grapevines was significantly greater than the number of grapevines affected by one or both diseases for the entire survey period. The incidence of RGd did not increase with the presence of LSLCd.

Log linear modeling was not done for Wemen (six years) due to the small number of grapevines in each block or for Paringa because AGYd was absent in 2000/01.

## 3.4 Discussion

### The expression of AGYd

AGYd in Sunraysia vineyards was characterised by the expression of disease followed by remission in some grapevines and recurrence of disease in others as well as the occurrence of AGYd in previously unaffected grapevines. Some grapevines expressed AGYd in only one growing season while some grapevines expressed AGYd in two or more seasons. However, AGYd expression did not always occur sequentially. Very few grapevines displayed AGYd in every year. In Chapter 2 it was shown that phytoplasmas persistently infect grapevines and that AGYd expression may be related to the number of phytoplasmas present in a grapevine. The inconsistency of AGYd expression between seasons may be a reflection of differences in phytoplasma titre between seasons and new infection events occurring in both diseased and asymptomatic grapevines.

In each season there were new records of AGYd both in established grapevines and in new replacement grapevines. Since AGYd is associated with phytoplasmas this suggests that new phytoplasma infections may occur in each year. However, the expression of disease due to previous phytoplasma infections in existing grapevines prior to the survey and infected planting material cannot be dismissed.

Remission, recurrence and new AGYd incidence would explain the fluctuation of AGYd incidence from year to year that occurred in most vineyards. When comparing surveyed vineyards, none showed the same pattern of AGYd incidence from year to year suggesting that factors local to each vineyard influence the expression of AGYd.

The expression of AGYd in Shiraz at Paringa was different to Chardonnay and Riesling grapevines in other vineyards. In Chardonnay and Riesling AGYd appears from late spring and the incidence of affected grapevines increases until harvest (Magarey, 1986; Magarey and Wachtel, 1983; Magarey and Wachtel 1986a). AGYd appeared in Shiraz grapevines at Paringa from late summer and the incidence of affected grapevines increased until leaf fall.

The symptoms were similar to those described for white varieties except that reddening replaced yellowing (Magarey, 1986; Magarey and Wachtel, 1983; Magarey and Wachtel 1986a). Because the AGYd first appeared in the Shiraz grapevines close to harvest, bunches were not affected.

The incidence of AGYd at Paringa declined from 1999 to 2000 and in 2001 no disease was observed. Of the AGYd affected grapevines that were not removed from the vineyard in the first year of observation, very few grapevines displayed AGYd in the subsequent seasons. Consequently it is unlikely that the removal of grapevines significantly reduced the incidence of disease in the following season. In 1999 another block of Shiraz was planted behind the block that was surveyed. The planting material to establish the block was from a different nursery and hot water treated. AGYd also affected some of the grapevines in the new block of Shiraz in 1999/00. This observation and the observation of new AGYd incidence in the blocks that were surveyed suggests that if phytoplasmas were the cause of AGYd may have been introduced to the vineyard by aerial transmission rather than through infected planting material.

**The spatial distribution of AGYd**

AGYd affected grapevines were often clustered. However, both the fixed grid analysis and spatial autocorrelation showed that clustering did not always occur in each year at each vineyard. The fixed grid analysis indicated that the size of the clusters of AGYd affected grapevines in each vineyard was different from year to year. The difference in the spatial arrangement of AGYd affected grapevines from year to year was likely to have been affected by remission and recurrence of AGYd as well as new AGYd incidence.

Spatial autocorrelation showed that the presence of AGYd in grapevines was not associated with the disease status of immediate neighbours in the same year. This indicates that phytoplasmas may not spread from a diseased grapevine to neighbouring grapevines and cause disease in the same year. Asymptomatic phytoplasma infections can occur (Gibb

*et al* 1999; Chapter 2) and it would be more useful to determine the presence or absence of phytoplasmas in each grapevine in an area by PCR detection and do spatial analyses on such data to see how phytoplasmas can spread within a vineyard.

**The expression of RGd**

RGd was present in every Chardonnay vineyard that was surveyed in Sunraysia and was characterised by remission and recurrence of the disease as well as new observations of RGd. In each season there were new records of RGd both in established grapevines and in new replacement grapevines. However, at Gol Gol and Karadoc a larger number of grapevines were sometimes observed displaying RGd in two or three subsequent years, rather than displaying RGd over several years with remission occurring in between years of expression. The proportion of grapevines displaying RGd symptoms for more that one year decreased with the number of years of observation. Very few grapevines displayed RGd in every year of the survey.

Remission, recurrence and new RGd incidence would explain the fluctuation of RGd incidence from year to year that occurred in most vineyards. In the first three to four years of the survey none of the vineyards that were surveyed showed the same pattern of RGd incidence from year to year, suggesting that local factors can affect the expression of RGd in vineyards over time. However, from 1997/98 or 1998/99 the incidence of RGd declined in most vineyards and this suggests that regional factors may also affect the expression of RGd in vineyards over time. The log linear model indicated that RGd incidence was significantly different with time only at Wemen and the incidence graph (Figure 3.2d) shows that RGd incidence was greatest in 1996/97 compared to other years.

**The spatial distribution of RGd**

Both fixed grid analysis and spatial autocorrelation indicated that RGd affected grapevines were clustered in all years at Karadoc and Gol Gol. Clustering of RGd affected grapevines did not always occur in each year at Colignan. When clustering of RGd was

observed the size of the aggregated groups of grapevines was often different between years in any vineyard. It is likely that the remission and recurrence of RGd as well as new occurrences affect the spatial arrangement of RGd affected grapevines in each year. The presence of RGd in grapevines was not associated with the disease status of immediate neighbours in the same year in most vineyards. This indicates that the cause of RGd may not spread from a diseased grapevine to neighbouring grapevines to cause disease in the same year.

**The association between AGYd and RGd**

The results presented here indicate that AGYd and RGd can occur independently of one another. Consequently, a phytoplasma aetiology cannot be inferred for RGd in all grapevines. The proportion of AGYd affected grapevines that displayed RGd in the same season or the following season is different between vineyards and years. The proportion of grapevines with RGd that displayed AGYd in the same season or the previous season was also variable between vineyards and years. Similarly the proportion of grapevines with AGYd that displayed RGd in the same season or the previous season was also variable between vineyards and years.

The log linear models also indicated that the year to year pattern of incidence of grapevines with both AGYd and RGd was different to the year to year pattern of incidence of AGYd alone and RGd alone indicating that the two diseases are not always associated. At Wemen the log linear model indicated that the overall incidence of RGd during the vineyard survey did not increase when in combination with AGYd indicating that the presence of AGYd and RGd were not associated. The results in Chapter 2 suggested that RGd expression in AGYd affected grapevines might be related to phytoplasmas titre. The inconsistency of RGd expression in AGYd affected grapevines between seasons may be a reflection of differences in phytoplasma titre between seasons.

**The expression of LSLCd**

LSLCd was present in every Chardonnay vineyard that was surveyed in Sunraysia and was also characterised by remission and recurrence of disease as well as new observations of disease. In each season there were new records of LSLCd both in established grapevines and in new replacement grapevines. Some grapevines expressed LSLCd in one growing season only while others expressed LSLCd in more than one season. However, LSLCd does not always occur sequentially. Very few grapevines displayed LSLCd every year.

The log linear model showed that the incidence with LSLCd was significantly different between years at Gol Gol, Karadoc and Wemen. However the years in which the incidence of LSLCd increased and decreased at each vineyard was different. This result indicates that local factors may affect expression of LSLCd. Remission, recurrence and new AGYd incidence would explain the fluctuation of LSLCd incidence from year to year that occurred in most vineyards.

**The spatial distribution of LSLCd**

Both the fixed grid analysis and spatial autocorrelation indicated that clustering of LSLCd affected grapevines occurred at Gol Gol and Karadoc in each year. However the fixed grid analysis indicated that the size of the clusters was different from year to year in both vineyards. It is likely that the remission and recurrence of LSLCd as well as new occurrences affect the spatial arrangement of RGd affected grapevines in each year. At Colignan a random distribution of LSLCd affected grapevines was observed in every year as determined by the fixed grid analysis.

Significant associations between grapevines and neighbouring LSLCd plants occurred more frequently in the column/row direction in all vineyards and it is possible that the disease spreads along rows. Significant associations were observed between both asymptomatic and diseased grapevines and neighbouring LSLCd plants. This result

indicates that the disease status of a grapevine may not influence the disease status of neighbours in that year.

**The association between AGYd and LSLCd**

The results presented here suggest that AGYd and LSLCd can occur independently of one another. The proportion of LSLCd affected grapevines displaying AGYd in the same year or following years was different from year to year and between vineyards. The proportion of AGYd affected grapevines displaying LSLCd in the same years or previous year was also different from year to year and between vineyards. These results support the results of Chapter 2 and indicate that if LSLCd is associated with phytoplasmas ~~the disease~~ LSLCd is not always the first expression of phytoplasma infection. The log linear model indicated that the year to year pattern of incidence of grapevines with both AGYd and LSLCd at Colignan was different to the year to year pattern of incidence of AGYd alone and LSLCd alone indicating that the two diseases are not always associated. The log linear models indicated that the overall incidence of LSLCd during the vineyard surveys did not increase when in combination with AGYd also indicating that the two diseases are not always associated.

In conclusion the results presented here show that AGYd RGd and LSLCd affect many grapevines in Sunraysia vineyards. AGYd, RGd and LSLCd are characterised by remission and recurrence of disease as well as new observations of disease. RGd and LSLCd may be associated with AGYd in some grapevines. However all three diseases can occur independently and phytoplasmas may not be the only cause of RGd and LSLCd.

# CHAPTER 4

# THE CHARACTERISATION OF PHYTOPLASMA CHROMOSOMES ISOLATED DIRECTLY FROM AUSTRALIAN GRAPEVINES

## 4.1 Introduction

Physical and genetic maps can provide information about how phytoplasmas might function at the molecular level and how phytoplasmas might induce disease. The information can also be used to determine molecular variation between species and strains of phytoplasmas. For instance a mild strain of onion yellows phytoplasma (OY-Mp) has a smaller genome size compared to the wild type line (OY-Wp) suggesting that some of the genes associated with pathogenicity are located on the chromosome of OY-Wp but have been deleted from OY-Mp (Oshima *et al* 2001).

This chapter describes the isolation of full length TBBp chromosome and attempted isolation of AGYp chromosome directly from naturally infected grapevines as a step towards the production of physical and genetic maps. The full length and digested TBBp chromosomal DNA from grapevine was compared to full length and digested TBBp chromosomal DNA from two strains maintained in the experimental host periwinkle and naturally infected *Antirrhinum majus* L. (snapdragon) to determine whether there were detectable differences in the chromosomes of different isolates of TBBp.

## 4.2 Methods

### Plant Material

Grapevine material, var. Semillon, infected with TBBp (TBBp-G) was collected from a vineyard in the Barossa Valley, South Australia. Grapevine material, var. Chardonnay and var. Shiraz, infected with AGYp was collected from a vineyard in the Riverland region of South Australia. Grapevine material, var. Semillon, var. Chardonnay and var. Shiraz, in which no phytoplasmas were detected, were used as a control. Dr. Brendan Rodoni of the Institute for

Horticultural Development (IHD), Knoxfield, Victoria provided *Catharanthus roseus* L. (periwinkle) plants that were infected with an isolate of TBBp (TBBp-I) from Victoria. TBBp-I has been maintained by propagation of periwinkle cuttings for more than 12 years and its origin is unknown. Dr. Nuredin Habili provided snapdragon that was naturally infected with TBBp (TBBp-S), from a home garden in Adelaide, South Australia. Dr. Anna Padovan and Dr. Karen Gibb from Northern Territory University provided chromosome preparations of sweet potato little leaf phytoplasma strain V4 (SPLL-V4p) and TBBp from periwinkle (TBBp-N). TBBp-N was isolated from *Lycopersicum esculentum* L. (tomato) with big bud disease collected from Adelaide, South Australia and transferred to and maintained in periwinkle using *Cuscuta australis* R. Br. (dodder; Padovan *et al* 2000).

**Preparation of phytoplasma chromosomes**

The plant tissue used for phytoplasma chromosome preparations from grapevines included vascular scrapings from trunk, cordons and shoots, and leaf petioles and veins. Leaf veins, petioles and stems were used for chromosome preparations from periwinkle. Entire shoots with leaves were used for chromosome preparations from snapdragon. Three methods were used to isolate phytoplasma chromosome from infected plants. For each method all steps were done on ice unless otherwise stated.

Method 1. Phytoplasma chromosomes were prepared from grapevine, periwinkle and snapdragon as previously described by Padovan *et al* (2000), except that the blocks were not pre-electrophoresed prior to pulsed field gel electrophoresis (PFGE). Two g fresh weight of plant material was placed in 12 mL of ice cold isolation buffer (0.1 M $Na_2HPO_4$, 0.03 M $NaH_2PO_4$, 10% (w/v) sucrose, 2% (w/v) PVP-40, 10 mM EDTA, pH 7.6, with 0.15% BSA and 1 mM isoascorbic acid added just prior to use). The tissue was ground with a mortar and pestle. The resulting brei was placed into cold 50 mL centrifuge tubes and centrifuged at 1500 *G* in a Sorvall SS34 rotor for 10 min at 4°C. The supernatant was filtered through a double

layer of cheese cloth and then placed into cold 50 mL centrifuge tubes and centrifuged at 18000 *G* for 30 min in a Sorvall SS34 rotor at 4°C. The green pellets were gently resuspended in 10 mL TSE (20 mM Tris-Cl, 10% Sucrose (w/v), 0.05 M EDTA, pH 8) using a glass rod and homogenised by trituration using a 1 mL plastic transfer pipette. The low and high speed centrifugation steps were repeated. After the high-speed centrifugation step the supernatant was discarded and the tube was inverted and allowed to drain for 1 min. After the final high speed centrifugation step the pellet was resuspended in a minimal volume of TSE. The phytoplasma suspensions were warmed to 37°C for 3-5 min. The suspensions were quickly mixed by gentle trituration using a truncated micropipette tip with an equal volume of molten 2% (w/v) InCert agarose (FMC, Rockland, ME, USA) dissolved in 2 × TES (0.2 M Tris-Cl, 0.2 M NaCl, 20 mM EDTA, pH 8) that had been maintained at 40°C. The phytoplasma/agarose mixture was quickly pipetted into 100 µl rectangular plastic molds (Bio-Rad Laboratories, Hercules, CA, USA) that were taped at the base. The agarose blocks were set on ice for 5-15 min. The tape was removed and the agarose blocks were expelled into lysis buffer (0.5 M EDTA, 1% N-lauroyl-sarcosine (sarcosyl), pH 8.0) in a 25-50 mL plastic tube. One mL of lysis buffer was used per block. Proteinase K (Roche Diagnostic GmbH, Mannheim, Germany) was added to the blocks in lysis buffer from a freshly prepared 20mg/mL stock solution to give a final concentration of 1 mg/mL. The blocks were placed on ice for 30 min before incubating at 50°C in an incubator for 2 days, with 2 changes of lysis buffer and Proteinase K per day.

Method 2. This method was the same as for method 1 except that approximately 100 g fresh weight of plant material was placed in 400 mL of ice cold isolation buffer in the dark at 4°C for 20 min. The tissue was homogenised in a Waring blender for 15 s on the highest setting and then the material was allowed to settle. The homogenisation step was repeated four

times or until the material was well ground. The first low speed centrifuge step was done in cold 250 mL centrifuge bottles and centrifuged at 1500 *G* in a Sorvall GSA rotor for 10 min. The remainder of method 2 was the same as method 1. Approximately 10 –15 agarose blocks were produced using method 2.

Method 3. This method also used 100 g of plant material homogenised in 400 mL of isolation buffer and the differential centrifugation steps were the same as method 2. After the final high-speed centrifugation step the supernatant was discarded and the tube was inverted and allowed to drain for 1 min. The final pellet was resuspended in a total volume of 4 mL of TSE. To further purify phytoplasmas away from plant debris that might interfere with detection of intact and digested phytoplasma chromosome, the resuspended phytoplasma pellet was overlaid on a discontinuous density gradient made from OptiPrep™ (Nycomed Pharma SA, Oslo, Norway). Five volumes of 60% OptiPrep™ stock solution were mixed with one volume of dilution buffer (0.25 M sucrose, 10 mM Tris, pH 8.0) to produce a 50% working solution. The 50% working solution was further diluted with TSE to 18%, 23% and 40%. Eight mL of the 18% solution was underlaid with 10 mL of the 23% solution and 8 mL of the 40% solution in a glass centrifuge tube to form a discontinuous density gradient. Four mL of the phytoplasma suspension was overlaid onto the density gradient and centrifuged in a Sorvall HB4 swing out rotor at 12000 *G* for 1 h at 4°C. The density gradient was sampled from the top layer to the 23:40% interface to determine where the phytoplasmas had banded. A 4 mL fraction was taken from the top of the gradient that represented the 4 mL phytoplasma preparation in TSE. Two 4 mL fractions were taken from the 18% solution of the gradient and the 23% solution was divided into three equal fractions. A 250 µl aliquot was taken from each fraction for PCR detection of phytoplasmas. The remainder of each fraction was placed into 50 mL centrifuge tubes and diluted with 3 volumes of TSE then centrifuged at 20000 g for 30 min at 4°C in a SS34 rotor. The supernatant was discarded and the pellets were resuspended in

a minimal volume of TSE. Agarose blocks were formed and lysed as for method 1. Each fraction yielded one to two agarose blocks.

The 250 µl samples collected for PCR were diluted with 3 volumes of TSE and centrifuged at maximum speed in a microfuge. The supernatant was discarded and the pellets were resuspended in 50 µl of TE. Universal nested PCR detection of phytoplasmas and RFLP analysis was done as described in Chapter 2.

Following lysis the agarose blocks from all three methods were washed 6 times over 2 days with 0.5 M EDTA (pH 8.0) at 50°C to remove the sarcosyl. Unless used immediately the blocks were stored at 4°C in 0.5 M EDTA (pH 8.0) at 1 mL/block.

**Restriction endonuclease digestion**

Prior to pulsed field gel electrophoresis (PFGE) phytoplasma chromosome preparations were incubated with restriction endonucleases to digest the phytoplasma chromosome into smaller fragments that could be more accurately sized. DNA blocks were cut into halves for TBBp-G and into quarters for TBBp-I, TBBp-N and TBBp-S with a glass cover slip. Preceding restriction endonuclease digestion the agarose blocks were washed in 10-20 volumes of ice cold TE. The first wash was for 2 h and the second was overnight. The following four washes were for 15 min each. The agarose blocks were incubated in 500 µl of restriction digestion buffer at 4°C, in the presence of 8 mM spermidine and 2 mg/mL of BSA for 2 h. Restriction endonuclease digestions were carried out overnight in 500 µl of the same buffer with fresh reaction mix and 20 U of endonuclease at the temperature recommended by the manufacturer. The reactions were stopped by replacing the restriction endonuclease mixture with ice cold TE. The plugs were rinsed in 3 changes of ice cold TE prior to running a PFG. The restriction endonucleases were *Apa*I, *Bcl*I, *Bgl*I, *Bam*HI, *Bsm*I, *Bsm*BI, *Bst*BI,

*Bss*HII, *Bste*II, *Eag*I, I-*Ceu*-I, *Kpn*I, *Not*I, *Rsr*II, *Sac*I, *Sal*I, *Sma*I, *Xba*I, *XhoI* (New England Biolabs) and *Not*I, (GeneWorks).

**Pulsed field gel electrophoresis (PFGE)**

PFGE was done by the contour-clamped homogeneous-electric field (CHEF) technique using a CHEF-DRII system (Bio-Rad, Hercules, CA, USA) at 14°C and 200 V. 1% agarose (SeaKem, FMC, Rockland, ME, USA) gels were cast in a 12 × 14 cm tray. DNA blocks were cut with a glass cover slip into halves for TBBp-G and into quarters for TBBp-I, TBBp-N and TBBp-S and whole blocks were used for AGYp. The DNA blocks were then washed in ice cold TE. The first wash was for 2 h and the second was overnight. The following four washes were for 15 min each. The wells of the gel were filled with TE before loading the cut blocks with two flamed spatulas. The TE was then removed from the wells containing the blocks and, to secure the blocks in place, the gaps were filled with molten 1% agarose. Gels were electrophoresed at 6 V/cm in pre-chilled 0.5 × TBE buffer maintained at 14°C. Ramped pulse times varied depending on the size of the DNA being resolved. PFGE of undigested chromosome blocks was for 22 h at a pulse time of 20-80 s. PFGE using restriction endonuclease digested chromosome blocks was for 20 h at a pulse time of 2-40 s. After electrophoresis the PFGs were stained with 1μg/mL ethidium bromide for 0.5 h, then destained in water for 5 h and viewed with a short wavelength UV transilluminator. If the DNA concentration in the blocks was low the gels were not stained with ethidium bromide because this produced a high background on the sensitive film used for autoradiography if the exposure was longer than one week. This background was due to non-specific binding of the radioactivity to excess ethidium bromide. The molecular weight of the chromosome DNA was estimated using a Lambda Ladder PFG marker or a Low Range PFG marker (New England Biolabs).

**Southern transfer of DNA**

Directly after PFGE or after ethidium bromide staining, the DNA contained in the PFG was nicked using 60 mJoules of UV irradiation in the GS gene linker (Bio-Rad). The PFG was shaken in 300 mL of 0.4 M NaOH to denature the DNA. The DNA was transferred overnight from the gels to Hybond $N^+$ membrane (Amersham, Buckinghamshire, England) by Southern transfer (Southern, 1975), using 0.4 M NaOH as the transfer buffer. Prior to prehybridisation the membranes were rinsed in water to remove NaOH.

**Preparation of $^{32}$P-labeled probes**

The probes used to detect TBBp and AGYp chromosome are listed in Table 1.

DNA for the 16S-T and 16S-A probes was amplified by universal nested PCR as described in Chapter 2. DNA for the EF- Tu AGY and EF-Tu AY probes was amplified by single PCR as described in Chapter 2. DNA for the 23S-AY and 23S-PDB probes was amplified using the primer pair P23S5F3 (5'-GTGGATGCCTTGGCACTAAGAGCC-3') and A23S3R3 (5'-ACTTACACACCTGGCCTATCAACC-3') and PCR was also done as described in Chapter 2 except that denaturing was for 1 min at 94°C, annealing was at 55°C for 2 min and extension was at 3.5 min at 72°C (Guo *et al* 2000).

As the concentration of either phytoplasma was likely to be low in grapevine extracts, 20 ng of the DNA used for each probe was labeled by incorporating both $\alpha$-$^{32}$P-dATP and $\alpha$-$^{32}$P-dCTP to increase the sensitivity. The lambda marker ladder was detected using a radioactive labeled probe derived from $\lambda$ DNA/*Hin*dIII markers (GeneWorks).

Table 4.1 Cloned DNA and PCR products used as probes for Southern hybridisation of AGYp and TBBp chromosomal DNA.

| Probe | Defined gene product | Size | Origin of probe | Source |
|---|---|---|---|---|
| E21 | Chromosomal DNA | 1.6 kb | SPPL-V4 phytoplasma (SPPL-V4p) | A. Padovan |
| T11 | EF-Tu | 1.2 kb | TBBp-N | A. Padovan |
| H21 | Chromosomal DNA | 2.1 kb | SPPL V4p | A. Padovan |
| 16S-A | 16S rRNA-tRNA$^{Ile}$-23SRNA | 1.8 kb | AGYp | |
| 16S-T | 16S rRNA-tRNA$^{Ile}$-23SRNA | 1.8 kb | TBBp – grapevine | |
| EF-Tu AGY | Elongation factor (EF) Tu | 1.0 kb | AGYp | |
| EF-Tu AY | EF – Tu | 1.0 kb | Aster Yellows phytoplasma (AYp) | |
| 23S AY | 23S rRNA | 2.7 kb | AYp | |
| 23S PDB | 23S rRNA | 2.7 kb | Papaya dieback phytoplasma (PDBp) | |

Radioactively labeled probes were synthesised by random priming, essentially as described by Feinberg and Vogelstein (1983). Purified DNA (20ng) was combined with 3.0 µl random sequence 9-mer oligonucleotide (0.1 mg/mL), and the mixture was incubated at 95°C for 3 min to denature the DNA. The mixture was cooled on ice for 5 min and combined with 10 µl of probe labelling buffer (0.5 M HEPES, 0.125 M Tris-HCl, 12.5 mM DTT, 12.5 mM $MgCl_2$, 1.0mg/mL BSA), 2.5 µl dNTP mixture (0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP), 1.0 U Klenow fragment, 3.0 µl $\alpha$-$^{32}$P-dATP (10 µCi/µl), 3.0 µl $\alpha$-$^{32}$P-dCTP (10 µCi/µl), and made up to 25 µl with water. The reaction mixture was incubated at room temperature overnight. To make spin columns, 1.0 mL syringes with their plungers removed were plugged at the

bottom with glass wool, filled to the top with Biogel P-10 (Pharmacia, USA) hydrated in TEN solution (0.1 M NaCl, 1.0 mM $Na_2EDTA$, 10 mM Tris-HCl, pH 7.2) and centrifuged at 3000 *G* for 4 min to remove excess TEN. The reaction mixture was combined with 120 µl TEN solution and passed through the column by centrifugation at 3000 *G* for 4 min to remove unincorporated dNTPs.

**Hybridisation and autoradiography**

The prehybridisation and hybridisation procedures used were based on protocols supplied with the Hybond $N^+$ membrane. Each 10 mL of prehybridisation/hybridisation solution was made by combining 3.0mL of 5×HSB solution (3.0 M NaCl, 0.1 M PIPES, 25 mM $Na_2EDTA$, pH 6.8), 2.0 mL of 50 × Denhardt's solution (2.0% ($^W/_V$) BSA, 2.0% ($^W/_V$) Ficoll 400, 2.0% ($^W/_V$) PVP), 3.0 mL 25% ($^W/_V$) dextran sulphate, 1.8 mL water, and 200 µl of 10 mg/mL salmon sperm DNA which was denatured at 95°C for 10 min prior to its addition. Membranes were placed in hybridisation bottles containing 10 mL of prehybridisation/hybridisation solution and prehybridised for 3 to 16 h at 65°C. Following prehybridisation, the probes were denatured by heating for 10 min at 95°C, cooled on ice for 5 min and then added to the hybridisation mixture. Hybridisation was done at 65°C for 12 to 20 h. The membranes were then washed three times for 5 min in 2.0×SSC, 0.1% SDS at 65°C and then three times for 10 min in 0.2×SSC, 0.1% SDS at 65°C. Autoradiography was done for at least 3 h and/or up to 14 days, depending on the signal intensity, at -80°C with Kodak double emulsion BIOMAX MS film and a BIOMAX MS intensifying screen.

## 4.3 Results

### Isolation of intact phytoplasma chromosomes

AGYp and TBBp chromosomes isolated from grapevines by methods 1, 2 or 3 were not detected by ethidium bromide staining (Figures 4.1a, 4.2a, 4.3b, 4.4a, and 4.6a). AGYp and TBBp-G chromosomes isolated from grapevines by method 1 were not detected by Southern hybridisation techniques (results not shown).

Detectable full length TBBp-G chromosome was isolated from infected grapevine material using method 2 (Figure 4.1b, 4.2b and 4.2c), which used larger amounts of grapevine material than method 1. After Southern hybridisation with radioactively labeled 16S-T probe, a signal of approximately 600 kb was detected in the undigested TBBp-G chromosome preparations from infected grapevine but not in preparations from healthy grapevines (Figure 4.1b).

Full length AGYp chromosome preparations using method 2, did not detect a specific signal by Southern hybridisation with the radioactively labeled 16S-A probe (Figure 4.2b). The membrane was exposed to highly sensitive film at –80°C for 24 h and five days. A very faint band that may have represented the AGYp chromosome was occasionally observed in the compression zone of some PFGs after Southern transfer and simultaneous hybridisation to the 16S-A, 23S-PDB, and EF-Tu AGY probes and after autoradiography was done for 7-14 days (results not shown). A similar band was not observed in preparations from healthy grapevine. The faint signal did not appear in every chromosome preparation from AGYp infected grapevines and the background of the autoradiographs was high after the long exposure times, making the band difficult to distinguish.

Full length TBBp-G chromosome was isolated from infected grapevine using method 3 (Figure 4.3), which used the same amount of grapevine material as method 2. PCR results indicated that the phytoplasmas had not settled at the 23:40% interface of the density gradient as expected. Instead, phytoplasmas could be detected throughout the gradient using PCR

(Figure 4.3a). RFLP analysis confirmed that the phytoplasma detected was TBBp (results not shown). Most of the plant material in the chromosome preparations had settled at the 23:40% interface and some plant material was dispersed through the rest of the gradient. No phytoplasma signal was detected by PCR in chromosome preparations from healthy material (Figure 4.3a). After Southern hybridisation with a radioactively labeled 16S-T probe, a phytoplasma signal at a size of approximately 600 kb, was detected in three of the six fractions that were taken from the gradient (Figure 4.3c) in the two 18% fractions and one 23% fraction. No signal was observed in preparations from healthy grapevine after Southern hybridisation with a radioactively labeled 16S-T probe (Figure 4.3c).

Method 2 was used for TBBp-G chromosome mapping because the signal intensities from phytoplasma chromosomes isolated from grapevine using method 2 (Figure 4.1b, 4.1c and 4.2b) were stronger compared to method 3 (figure 4.3b). Method 3 was not pursued due to time constraints.

**Restriction endonuclease digestion of TBBp-G**

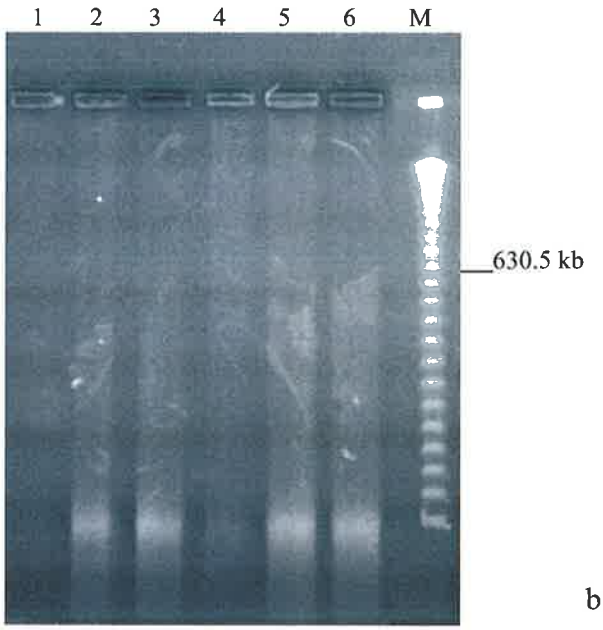
After the TBBp-G chromosome was digested with *Bss*HII and transferred for Southern hybridisation with the radioactively labeled E21, T11, H21, EF-Tu AY, 23S AY and 16S-T probes. Four strong bands, approximately 245 kb, 170 kb, 145 kb and 120 kb (total 680 kb), were detected (Figure 4.4b). The restriction endonuclease I-*Ceu*-I did not digest the TBBp-G chromosome (Figure 4.4b).

Figure 4.1. Isolation of the full length TBBp chromosome from infected grapevine, var. Semillon, by PFGE. DNA was prepared using method 2. DNA preparations from "healthy" (lanes 1-3) and infected grapevine (lanes 4-6). DNA markers (M) are long range lambda concatemer ladders. PFGE parameters were 22 h at a pulse time of 20-80 s.

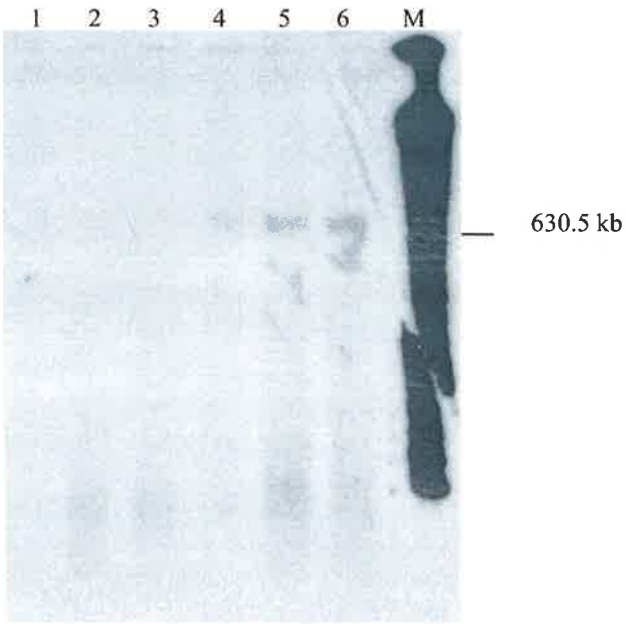
(a) The PFG after ethidium bromide staining

(b) After Southern hybridisation of TBBp chromosomal DNA using a radioactively labeled 16S-T probe the membrane was exposed to sensitive film at -80°C for 5.5 h.

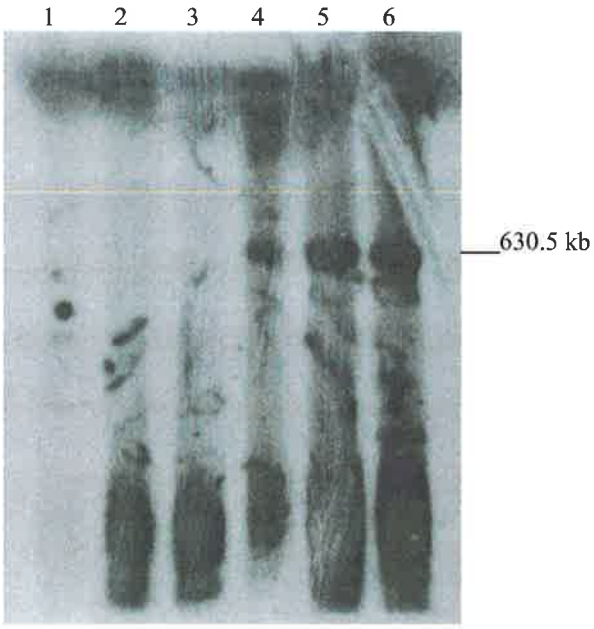
(c) The membrane was also exposed to sensitive film at -80°C for 24 h.




a.




b.

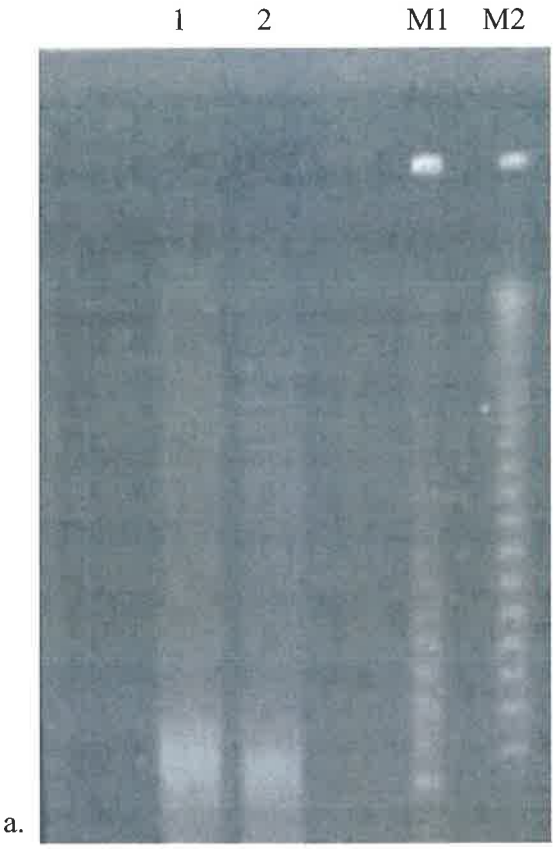



c.

Figure 4.2. Isolation of full length chromosome from AGYp (lane 1) and TBBp (lane 2) from infected grapevine, var. Chardonnay and var. Semillon respectively, by PFGE. DNA was prepared using method 2. DNA markers are low range (M1) and long range (M2) lambda concatemer ladders. PFGE parameters were 22 h at a pulse time of 20-80 s.

(a) The PFG after ethidium bromide staining

(b) Southern hybridisation of AGYp and TBBp chromosomal DNA using radioactively labeled 16S-T and 16S-A probes.




a.


b.

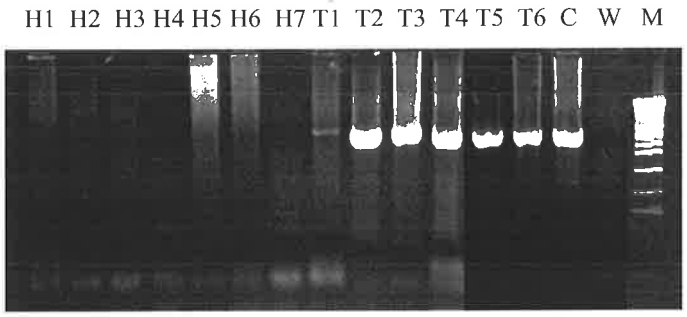
Figure 4.3. Optimising the isolation of the full length TBBp chromosome from infected grapevine, var. Semillon. DNA was prepared from infected and "healthy" grapevines using a density gradient in addition to differential centrifugation.

(a) PCR detection of TBBp after the DNA was prepared using method 3. Lanes H1-H7 are the sequential samples of healthy grapevine from the density gradient. Lanes T1-T6 are the sequential samples of TBBp infected grapevine from the density gradient. A TBBp control (C) and a water control (W) were also included. DNA markers (M) are SPP-1 Phage DNA/*Eco* RI markers

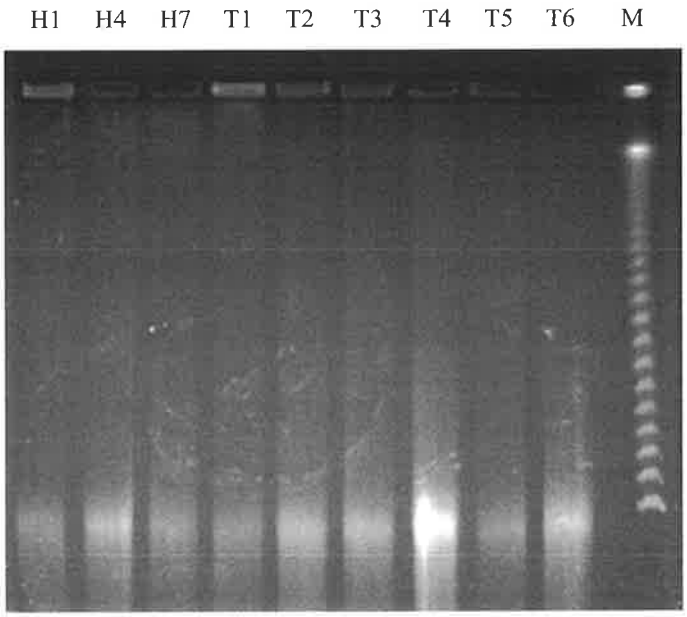
(b) The PFG after ethidium bromide staining. Lanes H1, H4 and H7 are preparations of some of the sequential samples after density centrifugation from '"healthy" grapevine. Lanes T1-T6 are the preparations of the sequential samples after density centrifugation of TBBp infected grapevine. DNA markers (M) are long range lambda concatemer ladders. PFGE parameters were 22 h at a pulse time of 20-80 s.

(c) The same PFG after Southern transfer and hybridisation of TBBp chromosomal DNA using radioactively labeled 16S-T probe. Lanes as for figure 5.3b. The membrane was exposed to highly sensitive film at -80°C for 24 h.

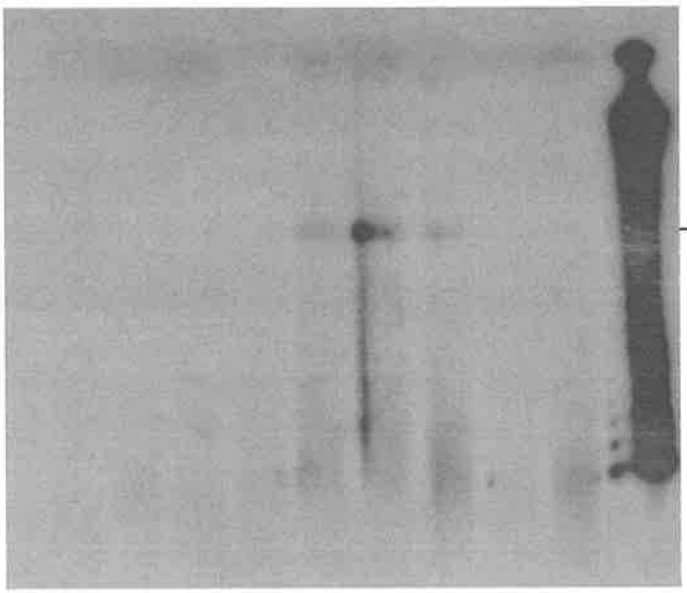
a.


H1 H2 H3 H4 H5 H6 H7 T1 T2 T3 T4 T5 T6 C W M

b.


H1 H4 H7 T1 T2 T3 T4 T5 T6 M

c.


H1 H4 H7 T1 T2 T3 T4 T5 T6 M
630.5 kb

**Comparison of the chromosomes from four isolates of TBBp and SPLL-V4p**

After PFGE and Southern hybridisation with the radioactively labeled 16S-T probe, no significant difference in chromosome size was observed between full length chromosomes from TBBp-G, TBBp-I, TBBp-S, TBBp-N and SPLL-V4 (Figure 4.5).

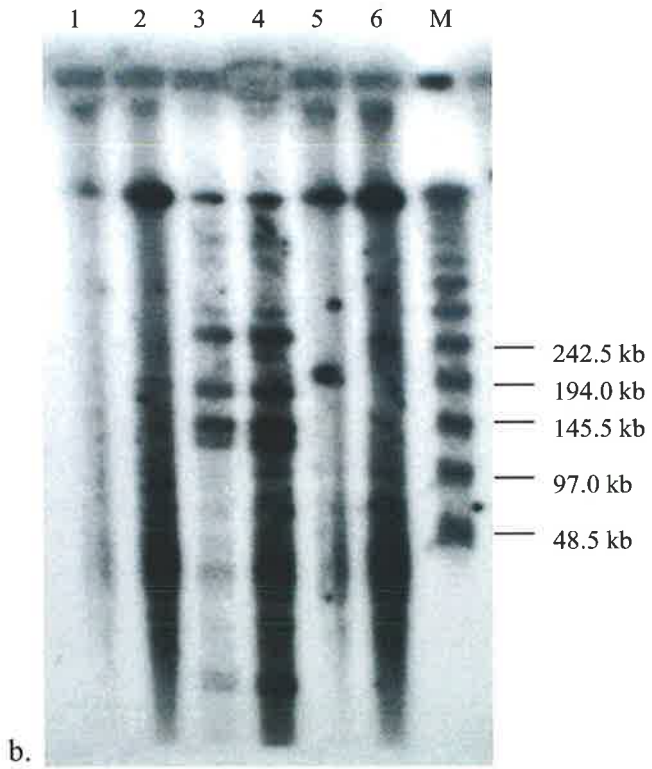
A comparison of the *Bss*HII digested TBBp chromosomes from TBBp-G, TBBp-I, TBBp-S, TBBp-N and SPLL-V4 is shown in Figure 4.6.A after ethidium bromide staining of the PFG signals were clearly observed from digested chromosomal DNA of SPLL-V4, TBBp-N and TBBp-S and no signals were observed from TBBp-G or TBBp-I periwinkle (Figure 4.6a). The higher molecular weight chromosomal DNA TBBp-S gave three signals at approximately 245 kb, 195 kb and 160 kb. These were different in size to the signals from TBBp-N, which were approximately 240 kb, 170 kb, 160 kb and 130 kb. A bright smear that was observed in chromosomal preparations from TBBp-S, TBBp-I and TBB-G may have obscured some bands after ethidium bromide staining of the PFG. After Southern transfer and subsequent, simultaneous, hybridisation to the radioactively labeled E21, T11, H21, EF-Tu AY, 23S AY and 16S-T probes chromosomal DNA was detected in all samples (Figure 4.6b). Some chromosomal DNA bands that were similar in size were difficult to differentiate after hybridisation, most notably the two bands at approximately 170 kb and 160 kb that were present in digested TBBp-N chromosome. Bands were observed in all samples at approximately 194 kb, 48.5 kb and 35 kb. In chromosome preparations from TBBp-G, TBBp-I and TBBp-S a band was observed at 160 kb, while a band was observed 145.5 kb from TBBp-N. A strong band at approximately 242.5 kb was observed from TBB-N that was only faint in TBBp-I, very faint in TBBp-G and not observed from TBBp-S. Low molecular weight bands of a similar size were observed from TBBp-I and TBB-N. Slightly larger low molecular weight bands were observed from TBBp-S. The digestion pattern of TBBp-I, TBBp-G and TBBp-S chromosomes were different from the pattern of the SPLL-V4p chromosome.

Figure 4.4. Digestion of the TBBp chromosome with the restriction enzymes *Bss*HII (lanes 3 and 4) and I-*Ceu*-I (lanes 5 and 6). Lanes 1 and 2 show the undigested TBBp chromosome. DNA markers (M) long range lambda concatemer ladders. PFGE parameters were 20 h at a pulse time of 2-40 s.

(a) The PFG after ethidium bromide staining.

(b) Southern hybridisation of the TBBp chromosomal DNA digested with *Bss*HII to various radioactively labeled phytoplasma gene probes including E21, T11, H21, EF-Tu AY, 23S AY and 16S-T. The membrane was exposed to highly sensitive film at -80°C for 24 h.


a.




b.

Figure 4.5. A comparison of the full length chromosome from TBBp-G (lane1), TBBp-N (lane 2), SPLL-V4p (lane 3), TBBp-I (lane 4) and TBBp-S (lane 5). After PFGE the TBBp chromosome was detected by Southern hybridisation using the radioactively labeled 16S-T probe. The membrane was exposed to highly sensitive film at -80°C for 24 h. DNA markers are long range (M) lambda concatemer ladders. PFGE parameters were 22 h at a pulse time of 20-80 s.

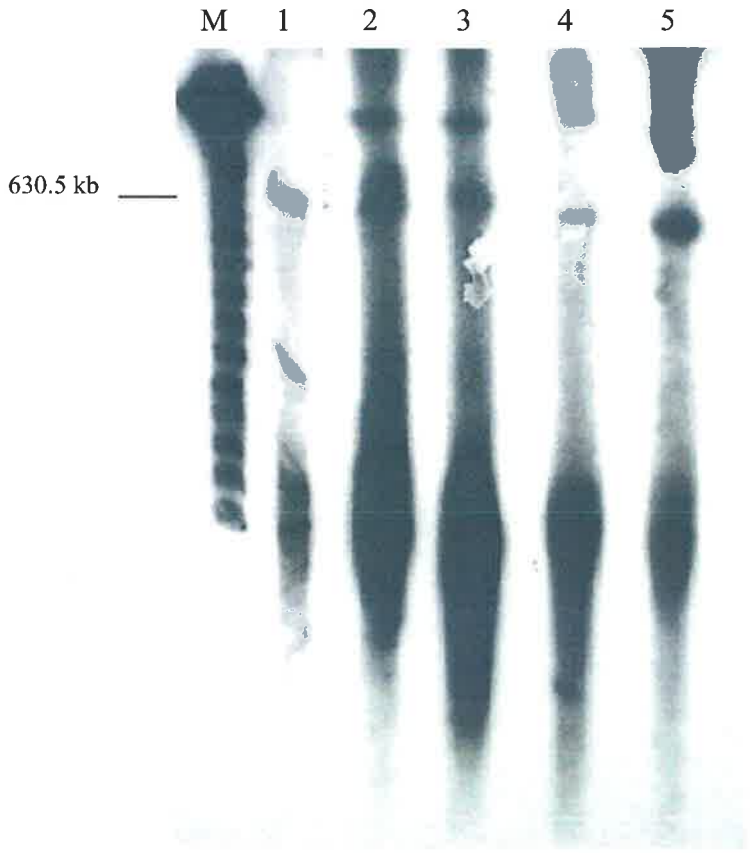
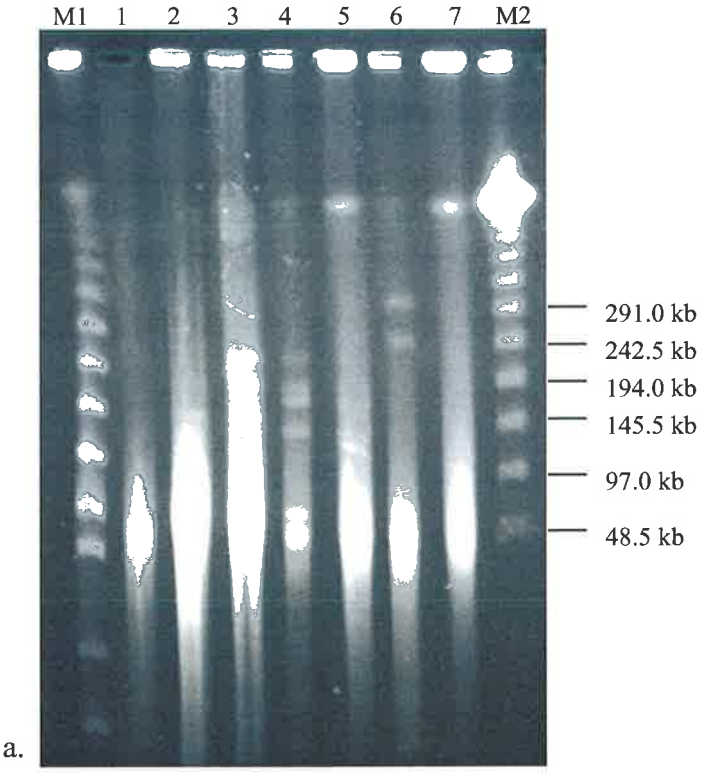

M
1
2
3
4
5
630.5 kb

Figure 4.6. A comparison of the chromosome from TBBp-G (lane1), TBBp-I (lane 2), TBBp-S (lane 3), TBBp-N (lane 4) and SPLL-V4p (lane 6) digested with *Bss*HII. Lane 5 is undigested TBBp-N and lane 7 is undigested SPLL-V4p. After PFGE the fragments of the digested TBBp chromosome from each isolate were detected by Southern hybridisation using combined radioactively labeled E21, T11, H21, EF-Tu AY, 23S AY and 16S-T probes. DNA markers are low range (M1) and long range (M2) lambda concatemer ladders. PFGE parameters were 20 h at a pulse time of 2-40 s.

(a) The PFG after ethidium bromide staining.

(b) Southern hybridisation of the digested TBBp chromosomal DNA to various radioactively labeled phytoplasma gene probes.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242.5 kb
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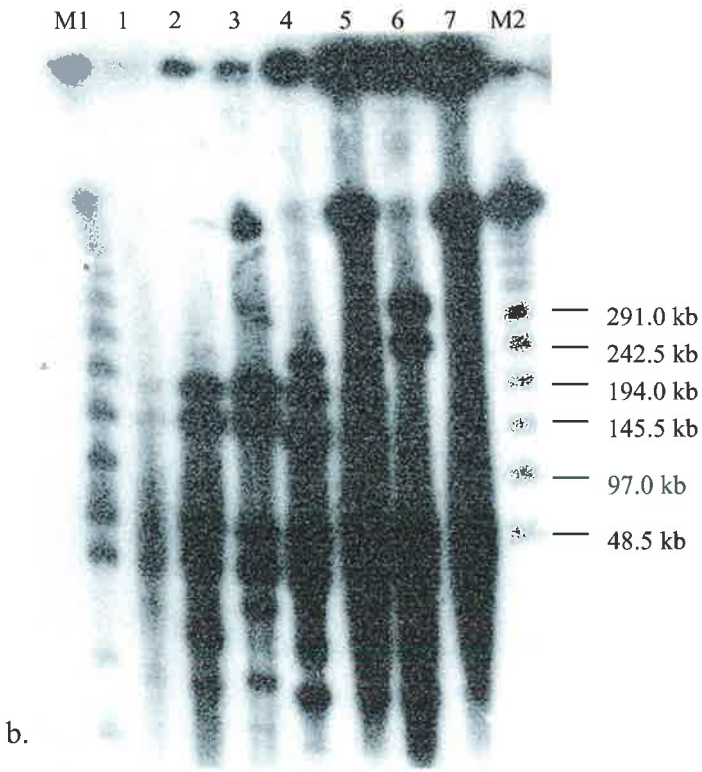
a.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b.

## 4.4 Discussion

Sufficient chromosomal DNA of TBBp was obtained from grapevine to produce a preliminary size estimation of the chromosome, approximately 680 kb, which is similar to that reported by Marcone *et al* (1999, 690 kb) and Padovan *et al* (2000, 662 kb). This is the first report of isolation of an intact phytoplasma chromosome directly from naturally infected grapevine. AGYp chromosomal DNA was not detected.

The results presented here suggest that phytoplasmas are in lower concentrations in grapevine compared to herbaceous hosts such as periwinkle. Also based on the results presented here AGYp is present in lower concentrations than TBBp in grapevine. TBBp and SPLL-V4 chromosomal DNA was detected from periwinkle (Padovan *et al* 2000) and snapdragon by ethidium bromide staining when method 1 was used to prepare phytoplasma chromosomes from 2 g of plant material. Method 1 did not produce sufficient TBBp or AGYp chromosomes for detection in PFGs stained with ethidium bromide or after Southern transfer and hybridisation. AGYp and TBBp chromosomal DNA were not detected in PFGs stained with ethidium bromide when either method 2 or 3 were used to isolate phytoplasma chromosomes from 100 g of grapevine material. TBBp chromosomal DNA from grapevine could only be detected after Southern transfer and probing with radioactively labeled probes using both methods 2 and 3. AGYp chromosomal DNA was not detected after PFGE and Southern hybridisations.

Method 3 was devised to further purify the phytoplasma away from plant material and enrich the phytoplasma preparation using density gradient centrifugation. Similar methods have ~~been~~ also been described for isolation of other phytoplasmas from plants and insect phytoplasmas (Jiang and Chen 1988; Davis *et al* 1988). Observations of the density gradient after centrifugation and the results obtained by PFGE indicated that phytoplasmas were being concentrated well away from the majority of contaminating plant material, which had mostly

settled at the 23:40% interface of the density gradient. While method 3 showed some promise as a useful technique to purify phytoplasmas from grapevine material further optimisation of the technique was not pursued. Method 2 was chosen because more agarose blocks could be prepared from the same amount of starting material as used for method 3. Also after Southern hybridisation the signals from TBBp chromosomal DNA prepared using method 2 were more intense compared to method 3. This result indicated that some phytoplasmas were being lost during the density gradient centrifugation. PCR detection indicated that phytoplasmas were dispersed throughout the 18:23% regions of the density gradient. After PFGE, TBBp chromosomal DNA was detected by Southern hybridisation in preparations from the 18% region of the density gradient and in the top fraction of the 23% region, immediately below the 18:23% interface of the gradient. This also indicated that there was some dispersion of phytoplasmas throughout the density gradient.

None of the endonucleases, which have optimal activity at 37°C, digested TBBp-G. The restriction endonuclease *Bss*HII, which has optimum activity at 60°C, did digest TBBp-G. This suggested that lysis of the phytoplasma membrane was successful and that reduced lysis did not contribute to the inability of other restriction enzymes to digest the phytoplasma chromosome. To test whether high temperature enabled the *Bss*HII endonuclease to digest the TBBp chromosomal DNA several other endonucleases that also had optimal activity at high temperatures were used, including *Bcl*I (50°C), *Bsm*BI (55°C), *Bst*EII (60°C), *Bsm*I (65°C) and *Bst*BI (65°C). These endonucleases did not digest the TBBp chromosome isolated from grapevine.

Full length chromosomes of TBBp from different regions and hosts did not show any significant difference in size. However some variation was observed after restriction endonuclease digestion of chromosomes of different TBBp isolates. After ethidium bromide

staining the fragments from TBBp-S chromosome appeared to be different in size from those of TBBp-N. The radioactively labeled probes that bound to the 242.5 kb fragment of TBBp-N did not bind to the 242.5 kb fragment of TBBp-S. Differences amongst the low molecular weight DNA also occurred. More research needs to be done to confirm these results. Improved isolation of phytoplasmas from the different hosts would provide clearer observations particularly as the presence of a strong smear in some preparations may have obscured the chromosomal DNA fragments from the TBBp isolates after ethidium bromide staining and Southern hybridisation. Some researchers report that subjecting the agarose blocks to electrophoresis at 50V for 1 h prior to PFGE removes much of the low molecular weight material that produces smears (Padovan *et al* 2000). However, when the agarose blocks containing TBBp-G were pre-electrophoresed much of the TBBp-G chromosomal DNA was lost so this step was omitted from the chromosome preparation (results not shown).

TBBp has a very broad host range and is found in many regions of Australia (Davis *et al* 1997; Schneider *et al* 1999) and the results presented here suggest that some genomic diversity exists amongst isolates of TBBp. The methods for isolation of phytoplasma from grapevine presented here may provide a means to study more isolates of TBBp from naturally infected hosts especially if the phytoplasma is in low concentration. By characterising the chromosome of various TBBp isolates of naturally infected hosts and looking for areas of conservation as well as variation, the genes associated with host specificity and perhaps pathogenicity might be determined. Such work could also be pursued for other phytoplasma species.

# CHAPTER 5

# A NEW GRAPEVINE YELLOWS PHYTOPLASMA FROM THE BUCKLAND VALLEY OF VICTORIA

## 5.1 Introduction

An uncharacterised phytoplasma was identified in Chardonnay grapevines with the grapevine yellows disease (GYd) from the Buckland (Ovens) Valley of northern Victoria, Australia (Gibb *et al* 1999). Restriction fragment length polymorphism (RFLP) analysis of the DNA amplified by PCR from the Buckland Valley grapevine yellows phytoplasma (BVGYp) and AGYp indicated that the two phytoplasmas were closely related but distinguishable and that BVGYp may be a variant of AGYp (Gibb *et al* 1999). To date there have been no reports of BVGYp occurring in other regions of Australia or in other vineyards within the Buckland Valley.

The aim of this study was to further characterise BVGYp using polymerase chain reaction (PCR) techniques and DNA sequencing of the 16S rRNA gene and the 16S rRNA/23S rRNA spacer region. Additionally three blocks of Chardonnay were surveyed over four years to determine the distribution of the GYd in the affected vineyards. Multiple samples were taken from each block for PCR detection and RFLP analysis to determine whether other phytoplasmas could also be detected in the affected vineyards. Heteroduplex mobility assay was used to determine whether any variability existed amongst isolates of BVGYp. A regional survey was also conducted to determine whether BVGYp occurred elsewhere in the Buckland Valley and surrounding grape growing regions.

In this chapter GYd is used to refer to grapevine yellows disease in the Buckland Valley to distinguish it from Australian grapevine yellows disease (AGYd) and the associated phytoplasmas AGYp and TBBp.

## 5.2 Methods

### Source of Phytoplasmas

Samples from grapevines with GYd were collected from Chardonnay vineyards in the Buckland Valley of Victoria. Grapevines that tested positive for AGYp or TBBp were collected from the Sunraysia district of northwestern Victoria in 1999. DNA of additional phytoplasmas that have been grouped on the basis of their 16S ribosomal DNA restriction patterns and nucleotide sequences (Schneider *et al* 1993; Seemüller *et al* 1994) were included as reference strains. DNA of Papaya dieback phytoplasma (PDBp) was provided by Dr. K. Gibb (Northern Territory University, Darwin, Australia). DNA of German grapevine yellows phytoplasma (VKp) was provided by Dr. M. Maixner (Institut fuer Pflanzenschutz im Weinbau, Germany).

### Extraction of DNA from grapevine

Leaf veins, petioles and scrapings of vascular tissue from stems were used for DNA extractions from shoots. DNA was isolated as described in Chapter 2.

### Primers for amplification of phytoplasma DNA in grapevine

The primers fP1/rP7 and r16F2n/m23SR were used for universal detection of phytoplasmas as described in Chapter 2. The primer pairs AUSGYF1/AUSGYR2, which are specific to the AGYp, and ftufAY and rtufAY, which amplify the elongation factor Tu (*tuf*) gene and are specific to members of the aster yellows (16Sr I) and stolbur (16Sr XII) phytoplasma groups, were also used for detection of phytoplasmas as described in Chapter 2. The primer pair fstol (5' GCC ATC ATT AAG TTG GGG A 3') and rstol (5' AGA TGT GAC CTA TTT TGG TGG 3'), were used in nested PCR to amplify a region of the 16S rRNA and 16S-23S spacer region, specifically from the stolbur group of phytoplasmas, to which AGYp is closely related (Maixner *et al* 1995).

**Polymerase Chain Reaction (PCR)**

PCR was done as described in Chapter 2. However, for the second round of nested PCR using the primer pair fstol/rstol the annealing temperature was 58°C. Total nucleic acid, extracted from asymptomatic grapevines were subjected to the PCR as a negative control and water controls were included, in which no plant nucleic acid was added to the PCR mix.

**Restriction fragment length polymorphism (RFLP) analysis**

The identity of the phytoplasmas isolated from grapevines in the Buckland Valley was determined using RFLP analysis of the universal nested PCR product with the endonucleases *Alu*I or *Hpa*II as described in Chapter2. To determine whether diversity existed amongst isolates of BVGYp, RFLP analysis of the *tuf* gene of nine isolates of BVGYp was also done using the restriction endonucleases *Alu*I, *Hpa*II and *Csp*I (*Rsa*I) (New England Biolabs) according to the manufacturer's instructions. For routine identification of phytoplasmas, the digested PCR products were run on a 1.3% agarose gel in 0.5×TBE then stained with 1 μg/mL ethidium bromide and viewed with a short wavelength UV transilluminator.

**PCR amplification of DNA for sequencing**

DNA was amplified for sequencing from BVGYp by nested PCR with the first round primer pair fP1/rP7 and the nested primer pair p25f and m23SR as described in Chapter 2. The primer p25f (5' AGG ATT AAC GCT GGC G 3') was designed so that a nested PCR product could be amplified that included more of the 16S rRNA gene at the 5' end of the gene. PCR products gel purified as described in Chapter 2.

**Sequencing directly from PCR products**

PCR products of the 16SrRNA gene from the new phytoplasma were sequenced using the T7 Sequenase PCR Sequencing Kit (Amersham Pharmacia Biotech) according to the

manufacturer's directions. Two isolates of BVGYp were sequenced, and sequences were determined at least twice. The primers used for sequencing are listed in Table 5.1.

Table 5.1. PCR primers used for sequencing the BVGYp 16S rRNA gene and 16S-23S spacer region sequences.

| Primer name (direction) | Primer sequence 5'-3' | Reference |
|---|---|---|
| R16F2n (forward) | GAA ACG ACT GCT AAG ACT GG' | Gundersen *et al* 1996. |
| fU5 (forward) | CGG CAA TGG AGG AAA CT | Lorenz *et al* 1995 |
| ng (forward) | AGG CGG CTT GCT GGG TCT T | Liefting *et al* 1996 |
| fU3 (forward) | TGT TAC AAA GAG TAG CTG AA | Lorenz *et al* 1995 |
| r8 (reverse) | CTC GTT GCG GGA CTT AAC CC | Liefting *et al* 1996 |
| rU3 (reverse) | TTC AGC TAC TCT TTG TAA CA | Lorenz *et al* 1995 |
| rP3 (reverse) – reverse of fP3 | AAG GAG GTG ATC CAT CC | Schneider *et al* 1995b |
| m23SR (reverse) | TAG TGC CAA GGC ATC CAC TGT G | Padovan *et al* 1995 |
| M13f (forward) | GTT TTC CCA GTC ACG ACG TTG TA | |
| M13r (reverse) | TTG TGA GCG GAT AAC AAT TTC | |

**Sequencing cloned PCR products**

When regions of the PCR products could not be sequenced directly, so these regions of DNA were amplified by PCR and gel purified as described in Chapter 2. The primer p593r (5' CCT ACG CAC CCT TTA CGC C 3') was designed and used with the primer p25f to amplify a small region of phytoplasma DNA (568 bp) near the 5' end of the 16S rRNA gene. The primer pair fP3 and m23SR were used to amplify a region of phytoplasma DNA, approximately 350 bp, which incorporated the 16S-23S spacer region. PCR was done as described in Chapter 2 except that the annealing temperature for the p25f/p593r primers was 49°C. The amplified products were cloned using the pGEM®-T Easy Vector System I (Promega, Madison, WI, USA) according to the manufacturers' instructions.

Competent *Escherichia coli* TG1 cells (genotype *supEhsd*Δ5*thi*Δ(*lac-proAB*) F'[*traD*36$^{+}$*proAB*$^{+}$*laqI*$^{q}$*lacZ*ΔM15] ) were prepared and transformed by heat shock (Hanahan 1983). Transformed cells were plated onto Luria-Bertani (LB) agar (1.0% ($^{w}/_{v}$)

bacto-tryptone, 0.5% ($^{W}/_{V}$) Bacto-yeast extract, 0.5% ($^{W}/_{V}$) NaCl, pH 7.0) supplemented with ampicillin (100 µg/mL final concentration) for antibiotic selection and X-gal (20 µl of 50 mg/mL stock per plate) and IPTG (200 µl of 100 mM stock per plate) for colour selection. Recombinants were observed as white colonies after an overnight incubation at 37°C. Individual white colonies were picked using a sterile toothpick and grown overnight at 37°C in 1.5 mL of LB broth supplemented with ampicillin (100 µg/mL final concentration). The culture was transferred to a 1.5 mL tube and centrifuged for 3 min at 20800 *G* (maximum speed) to pellet the cells. The supernatant was discarded and the tube was vortexed briefly to loosen the pellet. Ninety µL of TES (25 mM Tris-Cl, pH 8.0, 10 mM EDTA, 15% w/v sucrose) buffer was added and the tube was vortexed until the pellet was completely resuspended. One hundred and eighty µL of 0.2 M NaOH containing 1% sodium dodecyl sulphate (SDS) was added to the resuspended cells and mixed gently by inversion of the tube. One hundred and thirty µL of 3 M NaOAc (pH 4.6) was added to the suspension and gently mixed again by inversion of the tube. The suspension was then centrifuged for 15 min at 20800 *G*. The supernatant was removed and placed into another 1.5mL centrifuge tube containing 2 µL of 10 mg/mL RNAse A (DNAse free) and incubated at 37°C for 15-30 min. The plasmid DNA was extracted by the addition of 400 µl of phenol equilibrated in 50 mM Tris-HCl pH 8.0 and 400 µL of chloroform. The solution was mixed by vortexing the tube for 10 s and then centrifuged at 20800 *G* for 5 min. The aqueous phase was removed and placed in another 1.5 mL tube and 2.5 volumes of ethanol (stored at –20°C) were added to precipitate the DNA. The tube was incubated at room temperature (RT) for 10 min and then centrifuged at 20800 *G* for 15 min to pellet the DNA. The DNA pellet was washed once with 500 µL of 70% ethanol (stored at –20°C). The pellet was then resuspended in 30 µL of 0.1 mM EDTA pH 7.0.

Three μL of the plasmid DNA, in a 20 μL reaction, was digested for 2 h using the endonuclease *Eco*RI (New England Biolabs) according to the manufacturers' instructions to screen for the presence of the phytoplasma DNA inserts. Ten μL of the reaction was then run on a 1% agarose gel in 0.5×TBE at 100 V for one hour. The gel was stained with ethidium bromide and viewed using a UV transilluminator.

To produce larger quantities of the cloned phytoplasma DNA, 1 μL of the plasmid DNA was placed in a 1.5 mL centrifuge tube, then 100 μL of competent TG1 cells were added and the tube was incubated at RT for 20-30 min. The cells were heat shocked at 42°C for 2 min and then placed on ice. One hundred μL of LB broth was added and the cell suspension was incubated at 37°C for 30 min with gentle shaking. The mixture was plated onto LB agar supplemented with ampicillin (100 μg/mL final concentration) and incubated overnight at 37°C. Individual white colonies were picked using a sterile toothpick and grown overnight at 37°C in 45 mL of LB broth supplemented with ampicillin (100 μg/mL final concentration) with vigourous shaking. The culture was transferred to a 50 mL centrifuge tube and centrifuged at 12000 *G* for 5 min in a Sorvall SS34 rotor. The supernatant was discarded and the tube was vortexed briefly to loosen the cell pellet. Two mL of TES buffer (stored at 4°C) was added and the tube was vortexed until the cell pellet was completely resuspended. Four mL of 0.2 M NaOH containing 1% SDS was added to the resuspended cells and mixed gently by inversion of the tube. Then 2.5 mL of 3 M NaOAc (pH 4.6) was added to the suspension and gently mixed by inversion of the tube. The suspension was centrifuged for 30 min at 27000 *G* in a Sorvall SS34 rotor. The supernatant was removed and placed into another 50 mL centrifuge tube containing 400 μl of 10 mg/mL RNAse A (DNAse free) and incubated at 37°C for 30-120 min. To extract the plasmid DNA, 5 mL of phenol equilibrated in 50mM Tris-HCl pH 8.0 was added to the supernatant and mixed by vortexing. Five mL of chloroform was then added and contents of the tube were mixed again by vortexing. The solution was centrifuged at 12000 *G* for 10

min. The aqueous phase was removed and placed in another 50 mL tube and 2.5 volumes of ethanol (stored at –20°C) was added to precipitate the DNA. The tube was incubated at RT for 20 min and then centrifuged at 27000 *G* for 25 min to pellet the DNA. The DNA pellet was washed once with 5 mL of 70% ethanol (stored at –20°C) and air dried. The DNA pellet was then resuspended in 200 µL of 0.1 mM EDTA pH 7.0. The DNA was purified through a Sepharose Cl-CB (Amersham Pharmacia Biotech), equilibrated in 10 mM Tris-Cl, 0.1 mM EDTA, pH8.0) column made in a 0.5 mL centrifuge tube and centrifuged at 340 *G* for 3 min. The quality and concentration of DNA was determined using a spectrophotometer.

Cloned PCR products of the 16SrRNA gene and the 16S-23S spacer region from the new phytoplasma were sequenced using the T7 Sequenase Sequencing Kit (Amersham Pharmacia Biotech) according to the manufacturers' directions. Two isolates of the new phytoplasma were sequenced in two directions and sequences were determined at least twice. The primers used were those provided with the sequencing kit.

The reaction mixtures from direct sequencing of PCR products or sequencing of cloned DNA were denatured and run on denaturing polyacrylamide gels (6% ($^{W}/_{V}$) acrylamide, 0.3% ($^{W}/_{V}$) bisacrylamide, 7 M urea) in 1×TBE at constant power for 3 to 5 h. Prior to loading the reaction mixtures the gels were pre-electrophoresed at 50 V until the gel temperature was approximately 50°C.

**Sequence analysis of the 16S rRNA gene and the 16S-23S spacer region**

The 16S rRNA gene and 16S-23S spacer region sequences of the new phytoplasma were each compared to several other phytoplasmas using the GCG (Genetics Computer Group, Madison, WI, USA) program Gap (gap weight 5.0 and length weight 0.3), to calculate percentage sequence similarity. The 16S rRNA gene sequence of the new phytoplasma was aligned with other phytoplasma 16S rRNA gene sequences representing the major phylogenetic groups using the EGCG program EclustalW (European

Bioinformatics Institute, EMBL Outstation, Hinxton, UK). The aligned sequences were anlysed by the program Distances which generates a matrix of the pairwise evolutionary distances between aligned sequences using the Jukes-Cantor distance with 0.0 gap weight. The program GrowTree was used to reconstruct a tree from the distance matrix using the neighbour joining method. All programs were accessed through the Australian National Genomic Information Service, Sydney, Australia. The phytoplasmas used for the gap and sequence analyses of the 16S rRNA gene sequence and for the 16S-23S spacer region are listed in Tables 5.2 and 5.3 respectively.

**Heteroduplex mobility assay (HMA)**

HMA of the 16S-23S spacer region and the *tuf* gene was used to determine whether diversity existed amongst isolates of BVGYp. PCR amplification of the 16S-23S spacer region and the *tuf* gene and HMA was done as described in Chapter 2. The standard used for HMA was the BVGYp type isolate sm32, which was also used for sequence analysis. An additional 10 isolates were used as test samples.

**Vineyard surveys for GYd incidence**

Three blocks of the variety Chardonnay from the one vineyard were selected for GYd surveys in 1998. Site 1, consisting of 18 rows each with 50 grapevines, was planted in 1980. Site 2, consisting of 22 rows each with 50 grapevines, was planted in 1991. Site 3, consisting of 20 rows each with 50 grapevines, was planted in 1990.

The study sites were surveyed for incidence of GYd once a year in late February or early March, just prior to harvest. Each grapevine was assessed for the presence of GYd symptoms as described in Chapter 2.

Table 5.2. A list of the phytoplasma species, origins and accession numbers for the 16S rRNA gene.

| Abbreviation | Name | 16Sr group | Origin | Accession number |
|---|---|---|---|---|
| AGY | Australian grapevine yellows | 16Sr XIIB | Australia | X95706 |
| AshY | Ash yellows | 16Sr VIIA | USA | X68339 |
| AT | Apple proliferation | 16Sr XA | Germany | X68375 |
| BB | Tomato big bud | 16Sr IA | USA | L33760 |
| BGWL | Bermuda grass white leaf | 16Sr XIV | Italy | Y16388 |
| CP | Clover proliferation | 16Sr VIA | Canada | L33761 |
| CPh | Clover phyllody | 16Sr IC | Canada | AF222065 |
| EY | Elm yellows | 16Sr VA | USA | L33763 |
| HibWB | Hibiscus witches' broom | 16Sr XV | Brasil | AF147708 |
| LfWB | Loofah witches' broom | 16Sr VIII | Taiwan | L33764 |
| LY | Coconut lethal yellows | 16Sr IVA | USA | U18747 |
| MPV | Mexican periwinkle virescence | 16Sr XIII | Mexico | AF248960 |
| PnWB | Peanut witches broom | 16Sr IIA | Taiwan | L33765 |
| PPWB | Pigeon pea witches' broom | 16Sr IXA | USA | U18763 |
| PYL | Phormium yellow leaf | 16Sr XIIB | New Zealand | U43570 |
| RYD | Rice yellow dwarf | 16Sr XIA | Japan | D12581 |
| SAY | Western aster yellows | 16Sr IB | USA | M86340 |
| Stol | Stolbur | 16Sr XIIA | Serbia | X76427 |
| TBB | Tomato big bud | 16Sr IIB | Australia | Y08173 |
| VK | German grapevine yellows | 16Sr XIIA | Germany | X76428 |
| WX | Western X-disease | 16Sr IIIA | USA | L04682 |

Table 5.3. A list of the phytoplasma species, origins and accession numbers for the 16S-23S spacer region.

| Abbreviation | Name | 16Sr group | Origin | Accession number |
|---|---|---|---|---|
| AGY | Australian grapevine yellows | 16Sr XIIB | Australia | X95706 |
| BB | Tomato big bud | 16Sr IA | Arkansas | AF222064 |
| CPh | Clover phyllody | 16Sr IC | Canada | AF222065 |
| FPWB | Florida periwinkle witches' broom | | USA | AF025426 |
| MPV | Mexican periwinkle virescence | 16Sr XIIIA | Mexico | AF025428 |
| SAY | Western aster yellows | 16Sr IA | USA | M86340 |
| Stol | Stolbur | 16Sr XIIA | France | AF035361 |
| VK | German grapevine yellows | 16Sr XIIA | Germany | AF035362 |

**Regional surveys for GYd incidence**

In February 2000, seven Chardonnay vineyards in the Buckland Valley and two vineyards in the King Valley, Victoria, were surveyed for GYd. The Chardonnay source block in the Barossa Valley, South Australia, from which the planting material was obtained to establish the three study sites, was surveyed for GYd. In 2001 one additional Chardonnay vineyard in the Buckland Valley was surveyed for GYd. Between 400 and 1000 grapevines were surveyed in each vineyard. Any grapevines that had GYd or similar symptoms were sampled and tested for phytoplasmas using PCR methods. Also, blocks of the varieties Pinot Noir, Merlot and Cabernet Sauvignon adjacent to the three study sites were surveyed in each season. In some of the other vineyards, other varieties were also surveyed.

**Analysis of GYd survey data**

A fixed grid analysis and spatial autocorrelation as described in Chapter 3 were used to study the spatial distribution of grapevines with GYd. A Log-linear model, as described in Chapter 3 was used to analyse the temporal incidence of GYd.

## 5.3 Results

### Detection of phytoplasmas by PCR and RFLP

BVGYp was detected by PCR and RFLP analysis in 27 of 29 GYd symptomatic shoot samples collected from the three study sites. Phytoplasmas were not detected in any asymptomatic shoot samples. No variability was observed amongst 18 isolates of BVGYp after RFLP analysis of the nested universal PCR product (Figure 5.1) or amongst the nine isolates for the *tuf* gene (Figure 5.2). No other phytoplasma species was detected.

### Detection of BVGYp using specific PCR primers

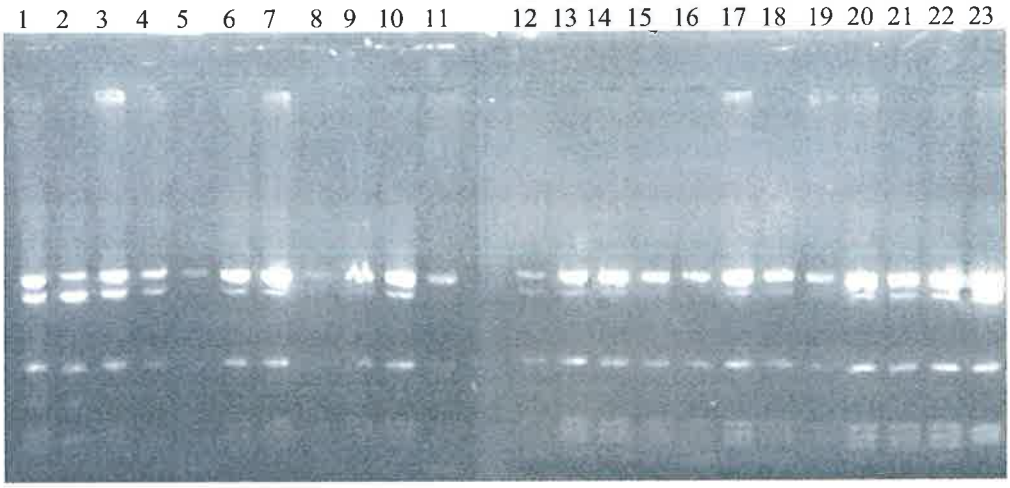
BVGYp was not amplified in PCR using the primer pairs AUSGYF1/AUSGYR1 (Figure 5.3) or fstol/rstol (Figure 5.4). BVGYp was amplified in PCR using the fTufAy/rTufAy primers and the product was of the expected size (1000 bp) (Figure 5.5).

### Sequence analysis of the 16S rRNA gene and Spacer region

The sequence of the 16S rRNA gene and the 16S-23S spacer region of BVGYp is shown in Figure 5.6. The pileup tree guide resulting from the sequence analysis of the 16S rRNA gene showed BVGYp clustering with the aster yellows strain cluster (Figure 5.7). Gap analyses indicated that the greatest sequence similarity was between BVGYp and clover phyllody phytoplasma (97.1%, Table 5.4). Sequence analysis of the $tRNA^{Ile}$ gene indicated that BVGYp had greatest sequence similarity to AGYp (89.0%, Table 5.5).

### Variability amongst isolates of the BVGY phytoplasma as determined by HMA

No heteroduplexes were formed between the standard and the ten test isolates for the 16S-23S spacer region (Figure 5.8) or the *tuf* gene (Figure 5.9).

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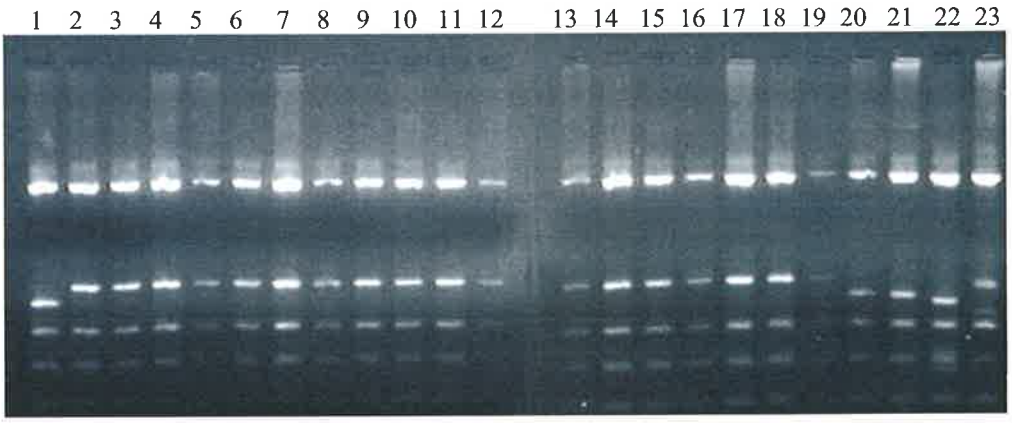


b.

Figure 5.1. RFLP analysis of the PCR products amplified by the primers P1/P7;R16F2n/m23sr using *Alu*1 (a) and *Hpa*II (b). In both gels lane 1 and 20 = AGYp, lanes 2 - 19 = BVGYp isolates, lane 21 = PDBp, lane 22 = VK and lane 23 = AY.

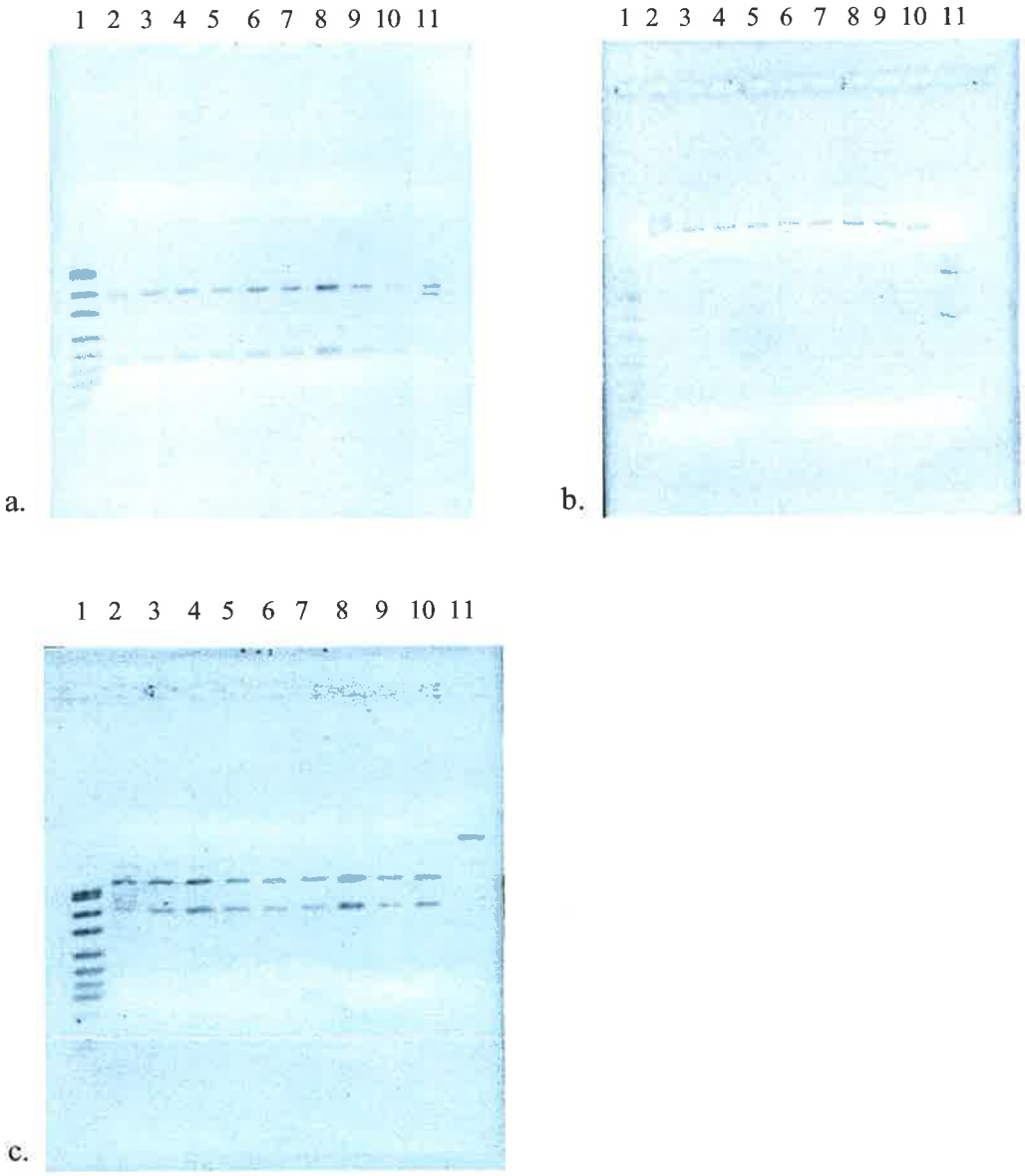


Figure 5.2. Restriction fragment length polymorhism analysis of *tuf* gene from nine isolates of BVGYp using the restriction enzymes *Alu*I (a), *Hpa*II (b) and *Rsa*I (c) Lane 1 = DNA markers are pUC19 DNA/ *Hpa*II, lanes 2-10 = isolates of BVGYp and lane 11 = AGYp.

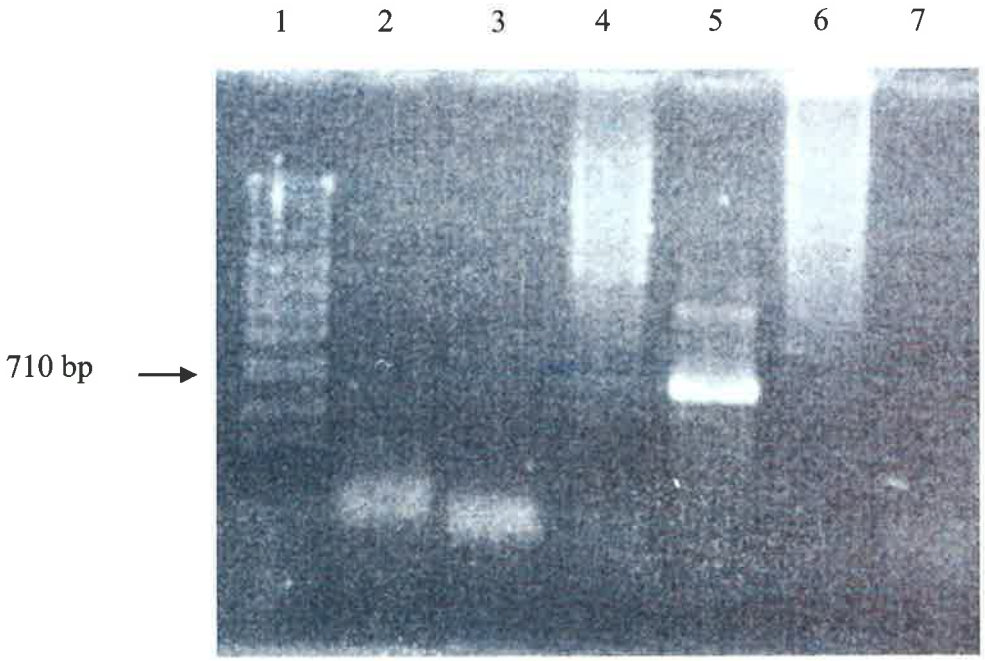


Figure 5.3. PCR amplification of 16S rDNA using the primers AUSGYF1/AUSGYR2. Lane 1 = SPP-1 Phage DNA/*Eco* RI markers (GeneWorks), lanes 2 and 3 = BVGYp, lane 4 = TBBp, lane 5 = AGYp, lane 6 = "healthy" control and lane 7 = water control.

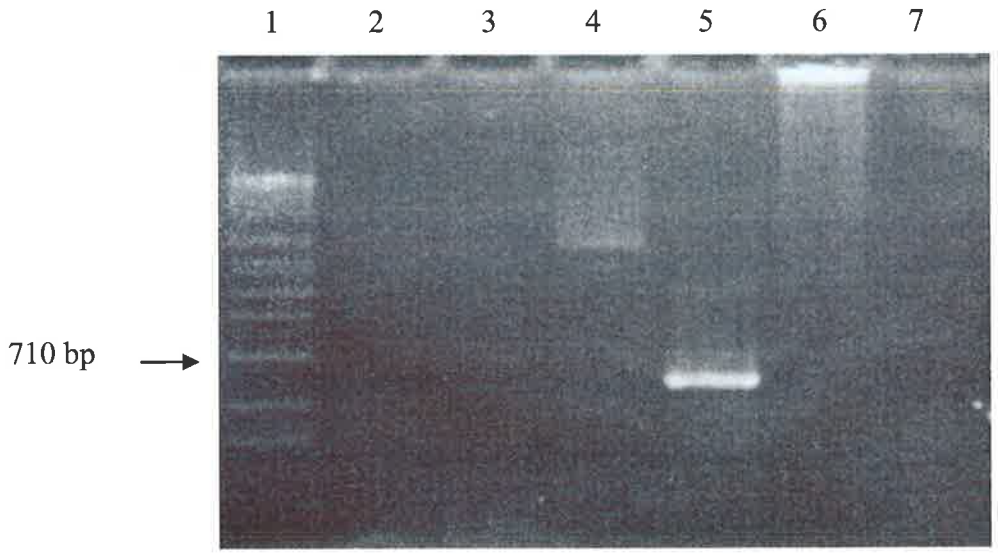


Figure 5.4. PCR amplification of 16S rDNA using the primers fStol/rStol. Lane 1 = SPP-1 Phage DNA/*Eco* RI markers, lanes 2 and 3 = BVGYp, lane 4 = TBBp, lane 5 = AGYp, lane 6 = "healthy" control and lane 6 = water control.

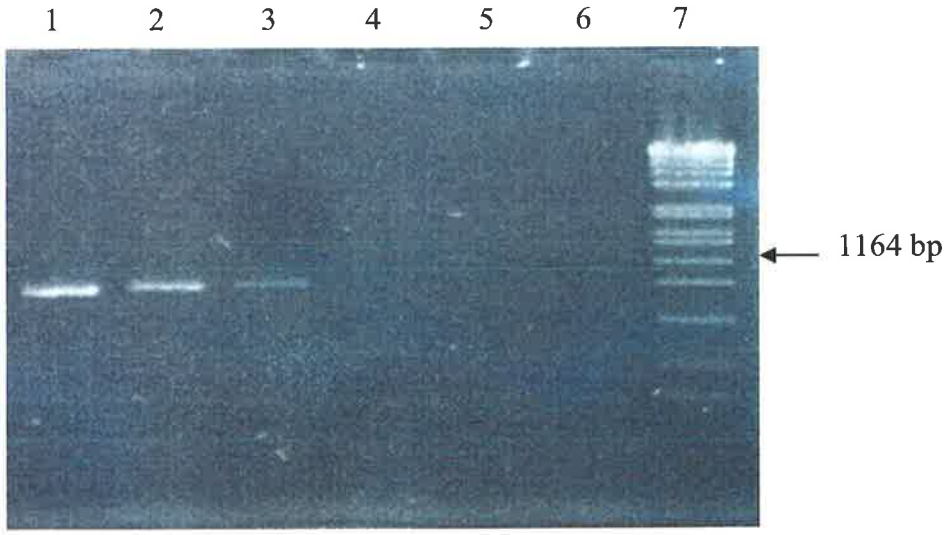


Figure 5.5. PCR amplification of the *tuf* gene using the primers ftufAY/rtufAY. Lanes 1 and 2 = BVGYp, lane 3 = AGYp, lane 4 = TBBp, lane 5 = "healthy" control, lane 6 = water control and lane 7 = SPP-1 Phage DNA/*Eco* RI markers.

```
   1  AGGATTAACG CTGGCGGCGT GCCTAATACA TGCAAGTCGA ACGGAAGTTT
  51  AAGCAATTAA GCTTTAGTGG CGAACGGGTG AGTAACGCGT AACGAATCTG
 101  CCTCTAAGAC GAGGATAACA GTTGGAAACG ACTGCTAAGA CTGGATAGGA
 151  GATAAGAAGG CATCTTCTTA TTTTTAAAAG ACCTAGCAAT AGGTATGCTT
 201  AGAGAGGAGC TTGCGTCACA TTAGTTAGTT GGTAGAGTAA AAGCCTACCA
 251  AGACTATGAT GTGTAGCCGG ACTGAGAGGT TGAACGGCCA CATTGGGACT
 301  GAGACACGGC CCAAACTCCT ACGGGAGGCA GCAGTAGGGA ATTTTCGGCA
 351  ATGGAGGAAA CTCTGACCGA GCAACGCCGC GTGAACGATG AAGTATCTCG
 401  GTACGTAAAG TTCTTTTATT AGGGAAGAAA AAGTGGTGGA AAAACCATTC
 451  TGACGGTACC TAATGAATAA GCCCCGGCTA ACTATGTGCC AGCAGCCGCG
 501  GTAATACATA GGGGGCAAGC GTTATCCGGA ATTATTGGGC GTAAAGGGTG
 551  CGTAGGCGGC TAAGTAAGTT TATGGTCTAA GTGCAATGCT CAACGTTGTG
 601  ATGCTATAAA AACTGCTTTA GCTAGAGTTG GATAGAGGCA AGTGGAATTC
 651  CATGTGTAGT GGTAAAATGC GTAAATATAT GGAGGAACAC CAGGAGCGAA
 701  GGCGGCTTGC TGGGTCTTAA CTGACGCTGA GGCACGAAAG CGTGGGGAGC
 751  AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGAGTACTAA
 801  ACGTTGGGTT AAACCAGTGT TGAAGTTAAC ACATTAAGTA CTCCGCCTGA
 851  GTAGTACGTA CGCAAGTATG AAACTTAAAG GAATTGACGG GACTCCGCAC
 901  AAGCGGTGGA TCATGTTGTT TAATTCGAAG GTACCCGAAA AACCTCACCA
 951  GGTCTTGACA TGCTTCTGCA AAGCTTTAGA AACAAAGTGG AGGTTATCAG
1001  TTGCACAGGT GGTGCATGGT TGTCTTCAGC TCGTGTTGTG AGATGTTGGG
1051  TTTAGTCCCG CAACGAGCGC AACCCTTGTT GTTAATTGCC AGCACGTTAT
1101  GGTGGGGACT TTAACAAGAC TGCCAGTGAT AAATTGGAGG AAGGTGGGGA
1151  TGACGTCAAA TCATCATGCC CCTTATGGCC TGGGCTACAA ACGTGATACA
1201  ATGGCTGTTA CAAAGGGTAG CTGAAACGTA AGTTCTTGGC AAATCTCAAA
1251  AAAACAGTCT CAGTTCGGAT TGAAGTCTGC AACTCGACTT CATGAAGTTG
1301  GAATCGCTAG TAATCGCGGA TCAGCATGTC GCGGTGAATA CGTTCTCGGG
1351  GTTTGTACAC ACCGCCCGTC AAACCACGAA AGTTGGCAAT ACCCAAAGCC
1401  GGTGGCCTAA CTTTGCAAGA AGAGGGAGCC GTCTAAGGTA GGGTTGATGA
1451  TTGGGGTTAA GTCGTAACAA GGTATCCTAC CGGAAGGTGG GGATGGATCA
1501  CCTCCTTTCT AAGGAAAATT ATCATCTTCA GTTTTGAGAG ACTTAAACAA
1551  GTTTTTCATT TTTTTTGTTT TTACCAACAT TTTTCTTAAT TTCTCTTTTT
1601  AAGATCTAAG ATCAAGGGCC TATAGCTCAG CTGGTTAGAG CACACGCCTG
1651  ATAAGCGTGA GGTCGGTGGT TCGAGTCCAT TTAGGCCCAC CACCAATTCC
1701  AAATCTTAAA AAATTAATTG AAAAAGAAGC TCTTTGAAAA GTAGATAAAC
1751  TAAGGTTAAA AAGTAAAGGA AATAAGGGCG CACAGTGGAT CCTTGGCACT
1801  AAATCGAATT CCCGCGGCCG CCATCGGAGG ATGCGACGTC GGCCAATTCG
1851  CCATATGCGT GAGTCGGATT ACA
```

Figure 5.6. 16S rDNA and 16S-23S intergenic spacer region sequence of the BVGYp phytoplasma.

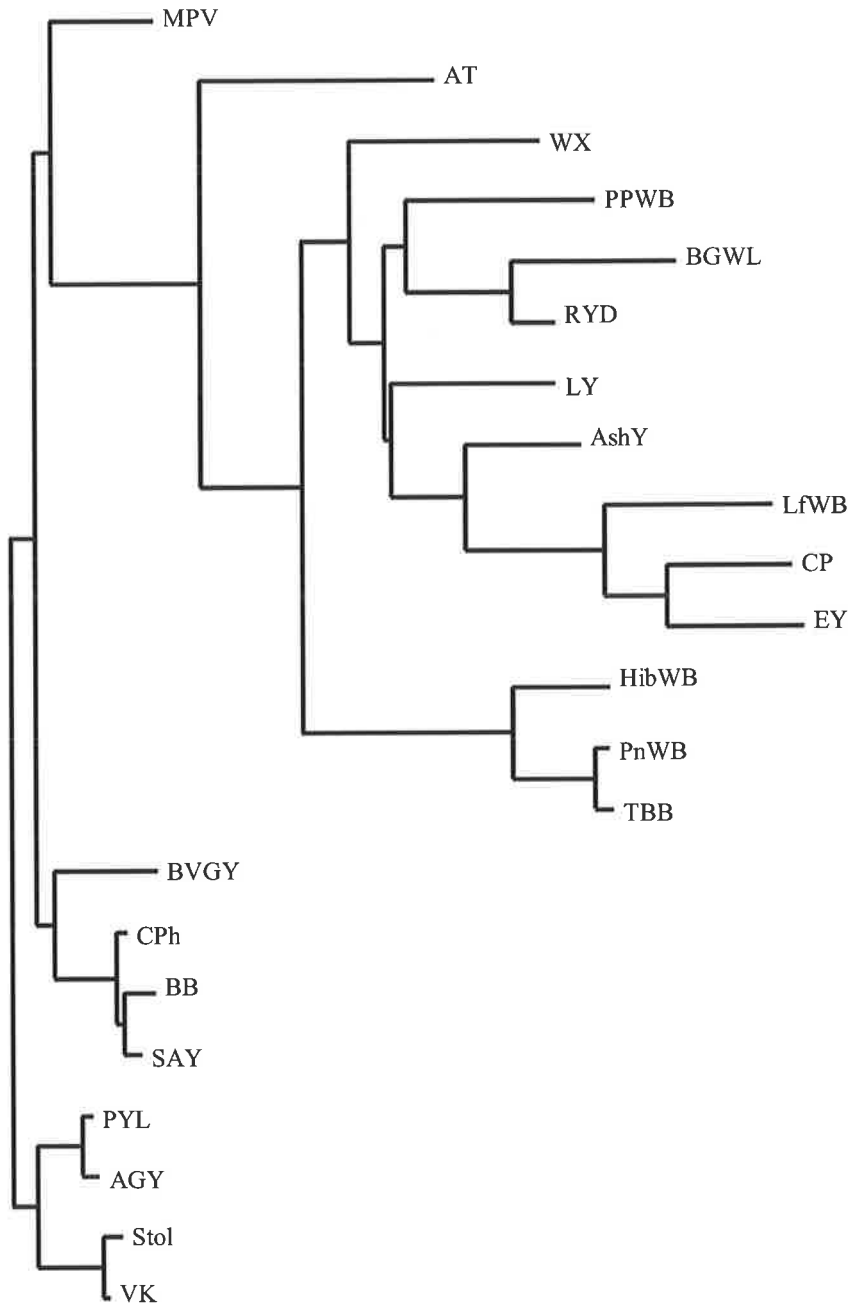


Figure 5.7. A phylogenetic tree constructed from the pairwise analysis of an EClustalW alignment of selected phytoplasma 16S rDNA sequences. Abbreviations are given in Table 5.2.

Table 5.4. Sequence similarity (%) between the 16S rRNA genes of BVGYp and other phytoplasmas.

| | CPh | SAY | BB | AGY | VK | Stol | AAY | MPV |
|---|---|---|---|---|---|---|---|---|
| BVGY | 97.1 | 96.9 | 96.7 | 96.3 | 96.1 | 96.1 | 95.8 | 96.0 |
| CPh | | 99.3 | 99.27 | 96.8 | 96.5 | 96.6 | 98.1 | 96.1 |
| SAY | | | 99.2 | 96.5 | 96.4 | 96.4 | 98.7 | 96.3 |
| BB | | | | 96.5 | 96.1 | 96.2 | 98.2 | 95.9 |
| AGY | | | | | 97.5 | 97.8 | 95.5 | 96.1 |
| VK | | | | | | 99.5 | 95.3 | 96.5 |
| Stol | | | | | | | 95.4 | 96.5 |

Table 5.5. Sequence similarity (%) between the 16S-23S spacer region of BVGYp and other phytoplasmas.

| | AGY | Stol | VK | FPWB | MPV | SAY | BB | CPh |
|---|---|---|---|---|---|---|---|---|
| BVGY | 89.0 | 88.6 | 88.6 | 88.6 | 87.2 | 81.4 | 80.6 | 76.8 |
| AGY | | 94.9 | 94.9 | 94.0 | 93.0 | 92.3 | 92.3 | 91 |
| Stol | | | 99.5 | 95.8 | 93.5 | 92.5 | 92.1 | 88.8 |
| VK | | | | 95.8 | 93.5 | 92.5 | 92.1 | 88.8 |
| FPWB | | | | | 98.2 | 92.5 | 92.1 | 88.8 |
| MPV | | | | | | 90.7 | 90.2 | 86.9 |
| SAY | | | | | | | 97.3 | 95.0 |
| BB | | | | | | | | 93.9 |

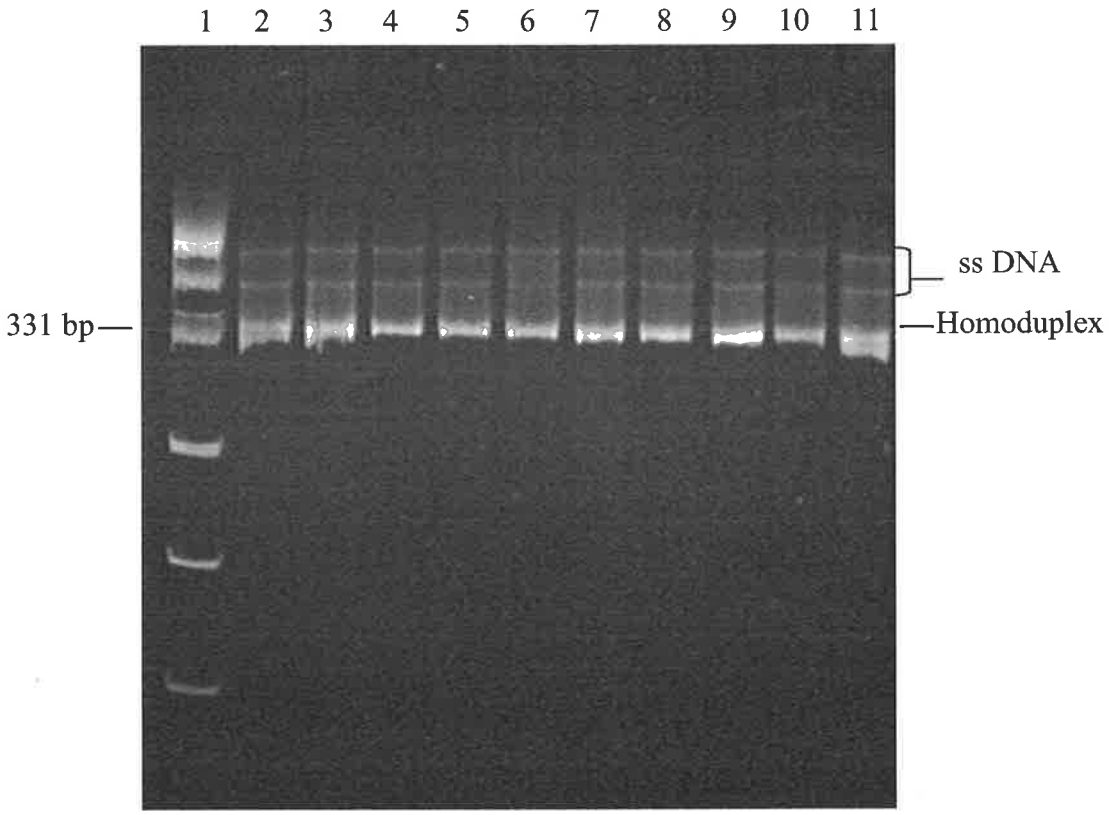


Figure 5.8. Heteroduplex mobility assay of the 16S-23S spacer region sequence amplified from isolates of BVGYp. Lane 1 = pUC19 DNA/*Hpa*II marker, lane 2 = standard to which all other isolates were compared and lanes 3-11 isolates of BVGYp from different grapevines.

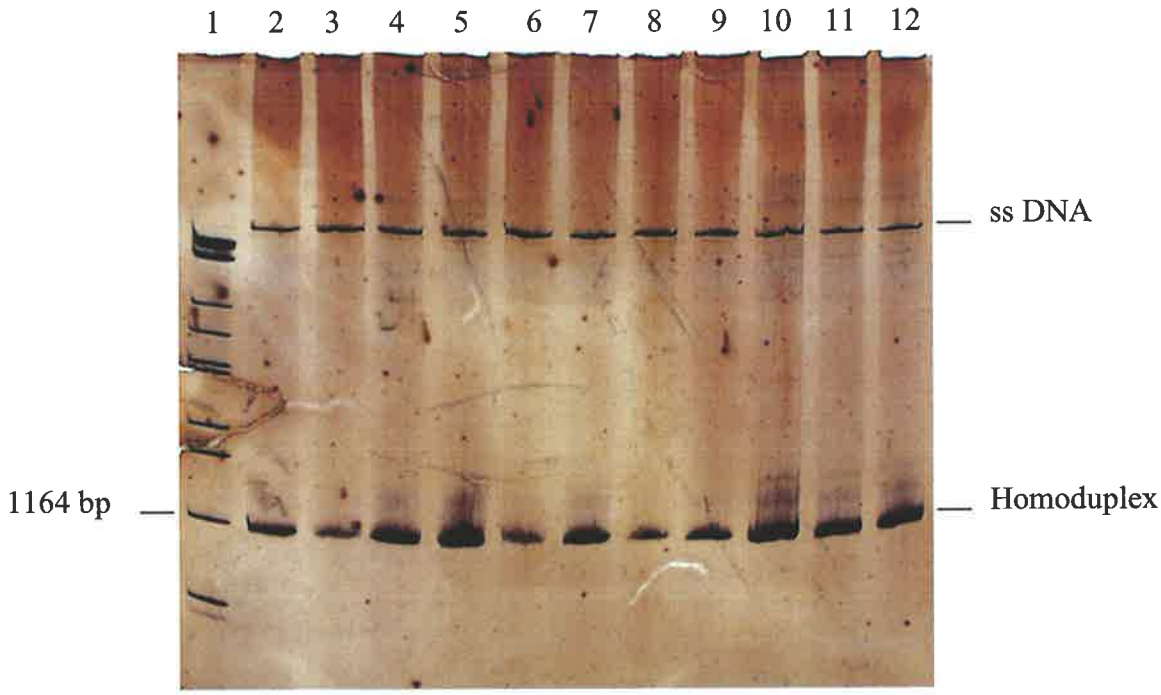


Figure 5.9. Heteroduplex mobility assay (HMA) of the *tuf* gene amplified from isolates of BVGYp. Lane 1 = SPP-1 Phage DNA/Eco RI marker, lane 2 = standard, to which all other samples were compared, lanes 3-12 = isolates of the BVGYp from different grapevines.

**Regional surveys for GYd and BVGYp**

In 2000, shoots with symptoms similar to GYd were collected from two Pinot Noir grapevines adjacent to site 2 at the Buckland Valley study sites. Phytoplasmas were not detected. One grapevine with GYd-like symptoms was observed and sampled from a vineyard at Whorouly, near the Buckland Valley, which was established from planting material collected from the study sites in the Buckland Valley. No phytoplasmas were detected. No symptoms were observed in any other Chardonnay vineyard in the Buckland Valley in 2000, including another vineyard that was also established using planting material from the study sites. GYd symptoms were not observed in any other variety in the Buckland Valley. Two samples from Chardonnay grapevines with GYd-like symptoms were also collected from one vineyard in the King Valley, but no phytoplasmas were detected. Six samples were collected from grapevines in a Chardonnay source block in the Barossa valley, including one with GYd-like symptoms. Again no phytoplasmas were detected. In 2001, two grapevines with GYd symptoms were observed from 1000 grapevines that were surveyed the additional vineyard. BVGYp was detected by universal nested PCR and RFLP analysis in samples from both grapevines.

**Vineyard surveys for disease incidence**

The maps of grapevines affected with GYd in each year are attached in Appendix 1. GYd (Figure 5.10) was indistinguishable from AGYd.

a.

b.

Figure 5.10. a. A Chardonnay grapevine from the Buckland Valley with shoots displaying symptoms of GYd including irregular veinal and interveinal yellowing and downward rolling of leaves, which overlaid one another in a shingled appearance. b. Berry shrivel associated with GYd in the Buckland Valley.

**GYd incidence**

The yearly and cumulative incidence of GYd affected grapevines at sites 1, 2 and 3 are shown in Figures 5.11 a, b and c respectively. The yearly incidence of GYd affected grapevines increased and decreased at sites 1 and 2 in a similar pattern, although the incidence of disease was greater at site 2. At site 3 the incidence of GYd affected grapevines declined from 1998 until 2000 then increased again in 2001. The incidence of GYd affected grapevines was greater at site 3 compared to sites 1 and 2. The cumulative incidence of GYd affected grapevines, which was calculated from the addition of first observations of GYd affected grapevines in one year to the incidence of GYd previous years, increases in a similar pattern at all three sites.

**The expression of GYd over time**

The proportion of grapevines at each site that displayed GYd once was greater than the proportion of grapevines that displayed GYd for two or more years (Table 5.6). The proportion of grapevines displaying GYd for two or more years decreased as the number of years in which GYd was displayed increased (Table 5.6). At site 1 there were no grapevines that expressed GYd in three or four years. At each site the number of grapevines displaying GYd for only one year was variable between years (Table 5.7). The pattern of GYd expression for grapevines that displayed the disease for two years or more was variable (Table 5.7).

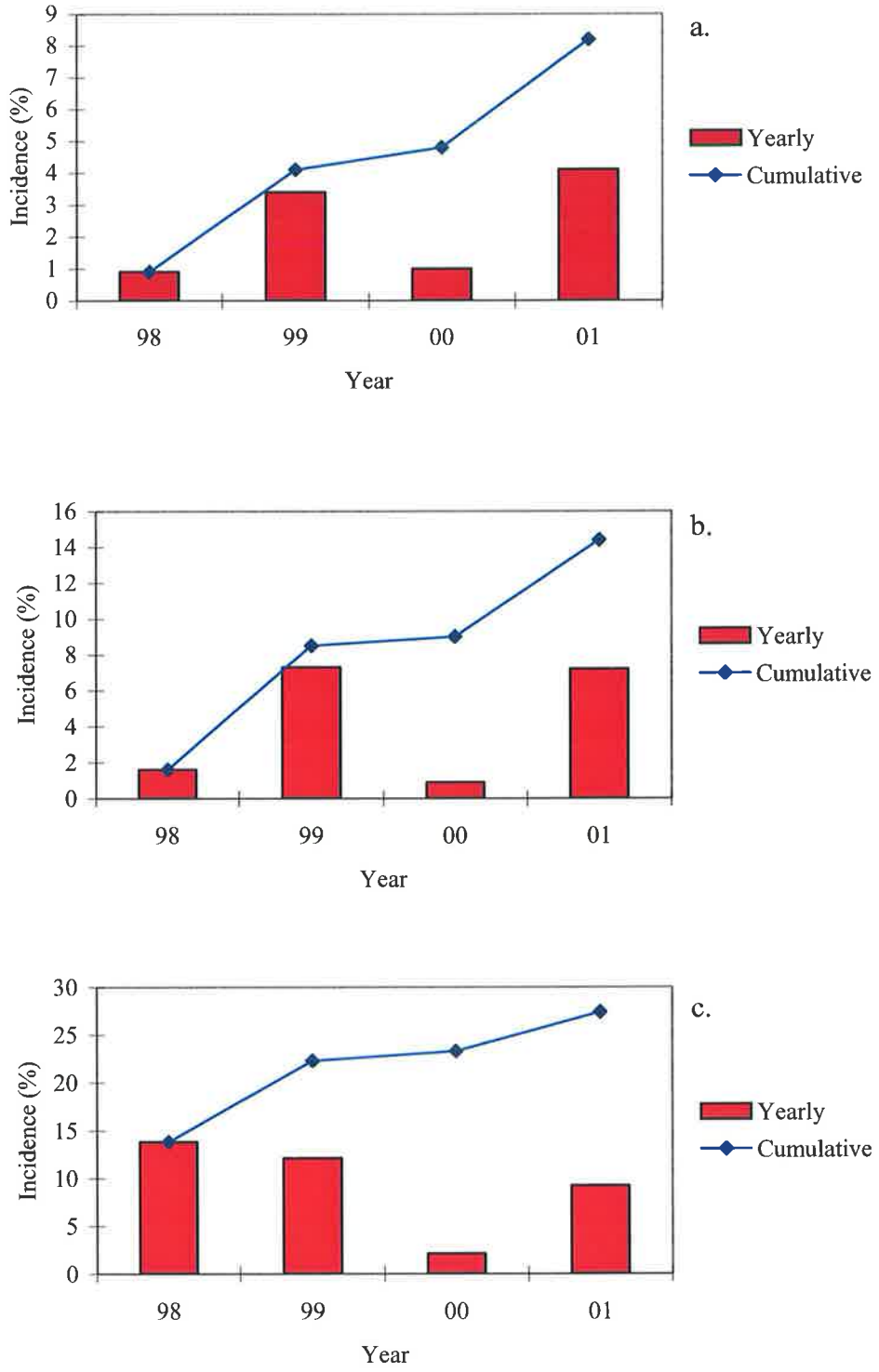


Figure 5.11. The yearly and cumulative incidence (%) of grapevine yellows affected grapevines in three blocks of Chardonnay (a. site1; b. site 2; c. site 3) in the Buckland valley over four years from 1998-2001.

Table 5.6. The number of grapevines that were affected with GYd over four years in the 10 year old, 17 year old and 18 year old blocks of Chardonnay in the Buckland Valley[a] .

| Year[b] | | | | Site 1 | Site 2 | Site 3 |
|---|---|---|---|---|---|---|
| 98 | 99 | 00 | 01 | | | |
| ■ | | | | 4 | 9 | 84 |
| | ■ | | | 27 | 60 | 63 |
| | | ■ | | 2 | 6 | 3 |
| | | | ■ | 30 | 59 | 41 |
| TOTAL - 1 year | | | | 63 | 134 | 191 |
| ■ | ■ | | | 2 | 1 | 28 |
| ■ | | ■ | | 1 | 0 | 3 |
| ■ | | | ■ | 1 | 4 | 13 |
| | ■ | ■ | | 1 | 3 | 2 |
| | ■ | | ■ | 1 | 12 | 17 |
| | | ■ | ■ | 5 | 0 | 7 |
| Total - 2 years | | | | 11 | 20 | 70 |
| ■ | ■ | ■ | | 0 | 0 | 0 |
| ■ | ■ | | ■ | 0 | 3 | 7 |
| ■ | | ■ | ■ | 0 | 0 | 2 |
| | ■ | ■ | ■ | 0 | 0 | 3 |
| Total - 3 years | | | | 0 | 3 | 12 |
| ■ | ■ | ■ | ■ | 0 | 1 | 1 |
| OVERALL TOTAL | | | | 74 | 158 | 274 |

a. The total number of grapevines observed during the entire survey period were: site 1 = 900 grapevines, site 2 = 1100 grapevines, site 3 = 992 grapevines
b.The shaded cells represent the years in which symptoms were observed

Table 5.7. The percentage of grapevines that had been affected with grapevine yellows symptoms for 0-4 years and the percentage that had displayed symptoms at some time during the survey period in three blocks of Chardonnay in the Buckland Valley of Victoria.

| Years affected | Site 1 | Site 2 | Site 3 |
|---|---|---|---|
| 4 | 0 | 0.1 | 0.1 |
| 3 | 0 | 0.3 | 1.2 |
| 2 | 1.2 | 1.8 | 7.1 |
| 1 | 7.00 | 12.2 | 19.3 |
| 0 | 91.8 | 85.6 | 72.4 |
| Total % affected | 8.2 | 14.4 | 27.6 |

**Temporal analysis of GYd using the Log-linear model**

The Log-linear model indicated no significant change ($p=0.064$) in the presence of GYd from season to season or between blocks.

**Spatial analysis of GYd**

The results of the spatial analysis of GYd affected grapevines at site 1, 2 and 3 using fixed grid analyses are given in Table 5.8, 5.9 and 5.10 respectively. If the total number of subunits was less than 15, the results were disregarded, and this is represented by the gray cells in the table. The results indicate significant clustering ($p<0.05$) of subunits of various sizes at each site in most years. No clustering was observed at site 1 in 2001 or at site 3 in 2000.

Spatial autocorrelation indicated a significant association between grapevines and diseased neighbours was variable between years and sites for both the columns/rows direction (Rooks join) and diagonal direction (Bishops join; Table 5.11). A significant association was observed more frequently between "healthy" grapevines and diseased neighbours than between diseased grapevines and diseased neighbours.

Table 5.8. The fixed grid analysis of clustering of GYd at site 1 in the Buckland Valley in each season using various subunit sizes.

| | 1998 | | | | | | | | 1999 | | | | | | | | 2000 | | | | | | | | 2001 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subunit size[a] | Row | | | | | | | | Row | | | | | | | | Row | | | | | | | | Row | | | | | | | |
| Column | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 2 | +[b] | + | | | | + | + | + | | | + | | | | | | + | | | | | | | | | | | | | | | |
| 3 | + | + | | | | + | + | + | | | | | | | | | + | + | + | | | | | | | | | | | | | |
| 4 | + | | | | | + | | | | + | + | + | | | | | + | + | | | | | | | | | | | | | | |
| 5 | | + | | | + | | | + | + | + | + | + | | | | | | | + | | | | | | | | | | | | | |
| 6 | | | | | | | | | + | | + | + | | + | | | + | | + | | | | | | | | | | | | | |
| 7 | | | | | | | | | + | | + | + | | | | | | | | | | | | | | | | | | | | |
| 8 | | | | | | | | | | | + | + | | | | | | + | + | | | | | | | | | | | | | |
| 9 | | | | | | | | | + | + | + | + | + | | | | | + | + | | | | | | | | | | | | | |
| 10 | | + | | | | | | | + | + | + | + | + | | | | | + | + | | | | | | | | | | | | | |
| 11 | | | | | | | | | + | | + | | | | | | | | | | | | | | | | | | | | | |
| 12 | | | | | | | | | + | + | + | | | | | | | | | | | | | | | | | | | | | |
| 13 | | | | | | | | | + | | | | | | | | | + | | | | | | | | | | | | | | |
| 14 | | | | | | | | | + | | | | | | | | | + | | | | | | | | | | | | | | |
| 15 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 16 | + | + | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 17 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 18 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 19 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 20 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 21 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 22 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 23 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 24 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |
| 25 | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | | | | |

a. Fixed grid analyses were done using various subunit sizes. Each subunit of the fixed grid analysis consists of a group of adjacent grapevines in rows and columns. b. + = Significant clustering of subunits with diseased grapevines ($p<0.05$). When the number of subunits included in the fixed grid analysis is less than 15, the results were not included and this is represented by the shaded cells

Table 5.9. The fixed grid analysis of clustering of GYd at site 2 in the Buckland Valley in each season using various subunit sizes.

| | 1998 | | | | | | | | | | 1999 | | | | | | | | | | 2000 | | | | | | | | | | 2001 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subunit size | Row | | | | | | | | | | Row | | | | | | | | | | Row | | | | | | | | | | Row | | | | | | | | | |
| Column | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| 2 | + | | | + | + | + | + | + | + | + | | + | | | | | | | + | | + | + | | | | + | + | + | | | | + | + | | + | + | + | + | + | + | + |
| 3 | + | | | | | | | | + | | | | | | | | + | + | + | + | + | + | | | | + | + | + | | | | + | + | + | + | + | + | + | + | + | + |
| 4 | + | | | + | + | + | | | + | | + | + | | + | | | + | + | + | + | + | | | | | + | | | | | + | + | + | + | + | + | + | + | + | + |
| 5 | + | | | + | + | + | | | | | + | + | + | + | + | + | + | + | + | + | | + | | | + | | | + | | | + | + | + | + | + | | + | + | + | + |
| 6 | + | | | + | | | | | + | | + | + | + | + | + | + | + | + | + | + | | | | | | | | | | | + | + | + | + | + | + | + | + | + | + |
| 7 | + | + | + | + | + | + | | | | | + | + | + | + | + | + | | | | | | | | | | | | | | | + | + | + | + | + | + | | | | |
| 8 | + | + | + | + | + | + | | | | | + | + | + | + | + | + | | | | | | | | | | | | | | | + | + | | + | + | | | | | |
| 9 | + | | | | | | | | | | + | + | + | + | + | + | | | | | | | | | | | | | | | + | + | + | + | + | | | | | |
| 10 | + | + | | + | + | + | | | | | + | + | + | + | + | + | | | | | | | | | | | | | | | + | + | + | + | + | + | | | | |
| 11 | + | | | | | | | | | | + | + | + | + | | | | | | | | | | | | | | | | | + | + | | + | | | | | | |
| 12 | + | | | | | | | | | | + | + | + | + | | | | | | | | | | | | | | | | | + | + | + | + | | | | | | |
| 13 | + | + | | | | | | | | | + | + | + | | | | | | | | | | | | | | | | | | + | + | + | | | | | | | |
| 14 | | + | | | | | | | | | + | + | + | | | | | | | | | | | | | | | | | | + | + | + | | | | | | | |
| 15 | + | + | | | | | | | | | + | + | + | | | | | | | | | | | | | | | | | | + | + | + | | | | | | | |
| 16 | + | + | + | | | | | | | | + | + | + | | | | | | | | | | | | | | | | | | + | + | + | | | | | | | |
| 17 | + | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| 18 | + | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| 19 | + | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| 20 | + | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| 21 | + | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| 22 | + | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| 23 | + | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| 24 | + | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| 25 | + | | | | | | | | | | + | | | | | | | | | | | | | | | | | | | | + | | | | | | | | | |

Table 5.10. The fixed grid analysis of clustering of GYd at site 3 in the Buckland Valley in each season using various subunit sizes.

| | 1998 | | | | | | | | | 1999 | | | | | | | | | 2000 | | | | | | | | | 2001 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subunit size | Row | | | | | | | | | Row | | | | | | | | | Row | | | | | | | | | Row | | | | | | | | |
| Column | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 2 | + | | | + | | | | | | | | | | | | | | | | | | | | | | | | | + | + | + | + | | | | |
| 3 | | | + | + | | | | | | | | | | | | | | | | | | | | | | | | | | | | | + | + | + | + |
| 4 | + | + | + | + | | | | + | + | | | | | | | | | | | | | | | | | | | | | + | + | | + | + | + | + |
| 5 | | + | + | + | + | | + | + | + | | | | | | | | | | | | | | | | | | | | | + | | | + | + | + | |
| 6 | + | + | + | | | | | | + | | | + | | | | | | | | | | | | | | | | + | | + | + | | + | + | + | + |
| 7 | + | + | + | + | + | | | | | | | + | | | | | | | | | | | | | | | | + | + | + | + | + | | | | |
| 8 | + | + | + | + | + | | | | | + | | + | + | | | | | | | | | | | | | | | + | + | + | + | + | | | | |
| 9 | + | + | + | + | | | | | | + | | + | + | | | | | | | | | | | | | | | + | + | + | + | | | | | |
| 10 | + | + | + | + | + | | | | | + | | + | | | | | | | | | | | | | | | | + | + | + | + | | | | | |
| 11 | + | + | + | + | | | | | | + | | + | + | | | | | | | | | | | | | | | + | + | + | + | | | | | |
| 12 | + | + | + | + | | | | | | + | + | + | + | | | | | | | | | | | | | | | + | + | + | + | | | | | |
| 13 | + | | + | | | | | | | + | + | + | | | | | | | | | | | | | | | | + | + | | | | | | | |
| 14 | + | + | + | | | | | | | + | + | + | | | | | | | | | | | | | | | | + | + | | | | | | | |
| 15 | + | + | + | | | | | | | + | + | + | | | | | | | | | | | | | | | | + | + | + | | | | | | |
| 16 | + | + | + | | | | | | | + | + | + | | | | | | | | | | | | | | | | + | + | + | | | | | | |
| 17 | + | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
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| 24 | + | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 25 | + | | | | | | | | | + | | | | | | | | | | | | | | | | | | + | | | | | | | | |

Table 5.11 The association between healthy or GYd affected grapevines and their GYd affected neighbours in the row/column direction or the diagonal direction at each site in each year.

| Type of join | Year | Site 1 | | Site 2 | | Site 3 | |
|---|---|---|---|---|---|---|---|
| | | H A[c] | AA[d] | H A | AA | H A | AA |
| Rooks[a] | 1998 | +[e] | + | + | + | + | + |
| | 1999 | +[f] | | + | + | | |
| | 2000 | | + | | | | |
| | 2001 | | | + | + | + | + |
| Bishops[b] | 1998 | | | | | + | |
| | 1999 | | | + | | + | |
| | 2000 | | | | | | |
| | 2001 | | | + | + | + | |

a. Rooks = joins between a grapevines and its neighbours in the row and column direction
b. Bishops = joins between a grapevines and its neighbours in the diagonal direction
c. HA = tests between healthy grapevines and GYd affected neighbours
d. AA = tests between GYd affected grapevines and GYd affected neighbours
e. + in a shaded square = significant join count statistic at the 5% level with a z-test statistic ≥ 1.96 but the number of joins associated with the test result was less than 5% of the total number of grapevines at the test site
f. + = significant join count statistic at the 5% level with a z-test statistic ≥ 1.96

## 5.4 Discussion

Specific PCR and sequence analysis showed that BVGYp was distinguishable from all other phytoplasmas used for comparison in this study.

BVGYp DNA was not amplified by PCR using the primer pairs AUSGYF1/AUSGYR1 or fstol/rstol, which amplify AGYp (Davis *et al* 1997; Gibb *et al* 1999). This indicated that the new phytoplasma was not a variant of AGYp and was unlikely to be a member of the stolbur group of phytoplasmas. However, PCR amplification with the fTufAy/rTufAy primers, which only amplifies members of the aster yellows phytoplasma group and stolbur phytoplasma group (Schneider *et al* 1997), did amplify a product of the expected size (1000 bp) from grapevine samples infected with BVGYp. This indicated that BVGYp was closely related to members of the aster yellows group.

Sequence analysis of 16SrRNA gene sequences showed that BVGYp was clustering with members of the aster yellows group of phytoplasmas. Gap analyses for sequence similarity of the 16SrRNA gene showed that BVGYp had greatest sequence similarity with clover phyllody phytoplasma (97.1%). Sequence analysis of the 16S-23S spacer region indicated a greater variability in this region compared to other sequences of the same region from different phytoplasmas and the greatest sequence similarity observed was with AGYp (89%).

There was no variation observed amongst isolates of BVGYp as indicated by the results from HMA and RFLP.

BVGYp was the only phytoplasma detected by universal nested PCR techniques and RFLP analysis in GYd samples collected from the Buckland Valley. There have been no reports of BVGYp detected in grapevines from other regions and there have been no reports of BVGYp detected in other Chardonnay vineyards in the same region. Consequently a regional survey of vineyards was conducted in 2000 to assess the distribution of GYd and BVGYp. An additional Chardonnay vineyard was also surveyed for GYd in 2001.

GYd was not observed in any other vineyard in 2000. However several samples that had GYd-like symptoms were collected and tested for phytoplasmas using universal nested PCR and RFLP analyses. The symptoms observed were not identical to GYd and no phytoplasmas were detected in any of the samples that were collected and tested. These results indicate that GYd and, consequently, BVGYp is not widespread in the Buckland Valley.

There is no evidence of transmission of GYd or BVGYp to other vineyards by planting material. The source block, which provided material for the establishment of the original vineyard, was also surveyed in 2000. Five random samples were collected and one sample that had GYd-like symptoms. The symptoms observed were not identical to GYd and no

phytoplasmas were detected in any of the samples. GYd was not observed in the source block. Additionally two vineyards in the Buckland Valley, which had been established using planting material taken from the original GYd affected vineyard, were included in the survey done in 2000 and GYd symptoms were not observed.

Current evidence indicates that BVGYp is unique to the Buckland Valley and has a very localised distribution in the region. In the additional vineyard that was surveyed in 2001, two grapevines with GYd symptoms were observed out of 1000 grapevines surveyed. BVGYp was detected in the samples collected from both grapevines by universal nested PCR and RFLP analysis. This vineyard is close (approximately 4 km) to the vineyard where BVGYp was originally detected, compared to other vineyards included in the survey.

The detection of BVGYp in another vineyard in 2001 provides circumstantial evidence of aerial transmission of this phytoplasma in the Buckland Valley. The planting material used to establish the second vineyard in 1998 was sourced from a different region to that of the original vineyard and because BVGYp has not been reported from any other region it is unlikely to have been introduced to this vineyard through planting material.

Remission and recurrence of GYd in grapevines was observed together with new occurrences of GYd in previously unaffected grapevines. However, grapevines were less likely to display GYd in two or more years or in consecutive years. Only a low percentage of grapevines were affected for two or more years at sites 1 and 2, even when the data were combined, compared to grapevines that were affected for one year only. Similar results were observed for site 3 except that the incidence of grapevines affected by GYd for two or more years was greater. The pattern of disease expression for grapevines affected for two or three years at each site was variable. The cumulative incidence of GYd indicated that there were new records of GYd affected grapevines in each block in each year of the survey. The proportion of new records of GYd in each year was between 81-94% at site 1,

60-94% at site 2 and 45-70% at site 3. Similar remission of grapevine yellows disease in Northern Italy (Osler *et al* 1993) and the Flavescence dorée (FD) disease in France has also been described (Caudwell, 1961; Caudwell, 1964). 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, which is unlike GYd. Incidence and expression of GYd in the Buckland Valley behaves similarly to AGYd except that the incidence of GYd was lower than AGYd in each year and fewer grapevines displayed GYd in subsequent seasons.

Since GYd did not persistently affect the same grapevines, BVGYp may not persistently 'infect' grapevines. A similar study to that carried out in Chapter 2 investigating the seasonal detection and persistence of BVGYp in grapevines would be useful to determine if the remission of GYd in grapevines is due to the "disappearance" or reduction in titre of phytoplasmas in grapevines or if the expression of symptoms is due to the location of phytoplasmas in specific tissues.

The results of the Log-linear model indicated that the presence of GYd in all three blocks did not significantly change from year to year, even though the incidence of GYd fluctuated at each site over the four years of the survey. Thus GYd is endemic and likely to be present in most years during the life of the vineyard.

The fixed grid analysis indicated clustering over various subunit sizes of GYd at each site at some time during the survey. However the clustering was not consistent for subunit size at each site in each year. Clustering was also not consistent between sites in the same year. Because the fixed grid analysis could only be used to analyse clustering amongst groups of diseased grapevines, spatial autocorrelation using the join-count test statistic was also used to determine whether an association between an individual grapevine and GYd affected neighbours existed. If a significant result was obtained in both the column/row direction and the diagonal direction clustering could be inferred. A significant result

obtained in either the column/row direction or diagonal direction would have inferred a direction of the spread of GYd. Like the results from Patchy, evidence for clustering and spread using spatial autocorrelation was inconsistent between sites and years. The inconsistent results from fixed grid analysis and the spatial autocorrelation may have been due to the fluctuation in incidence and variable expression of GYd in each year and between sites.

In conclusion the results presented in this chapter show that BVGYp is a unique phytoplasma that has a localised distribution in the Buckland Valley. BVGYp was associated with GYd. Results from the log-linear model suggested that GYd was endemic to the study sites because there was no significant difference in the incidence of disease between years. However the incidence of disease did fluctuate and many grapevines did not display GYd persistently from year to year. This is reflected in the results generated by the fixed grid analysis and spatial autocorrelation, which showed that clustering and direction spread of GYd was inconsistent between sites and years. These results also suggested that even if GYd is affecting more grapevines with increasing time, the same grapevines are not affected every year. The incidence of GYd was characterised by remission of disease in many affected grapevines and some recurrence in others as well as occurrences in previously unaffected grapevines.

# CHAPTER 6

# DISCUSSION AND CONCLUSIONS

In this thesis several separate investigations have been presented that contribute to the understanding of the biology and epidemiology of Australian grapevine phytoplasmas. The seasonal detection and distribution of phytoplasmas was investigated. The incidence distribution and association between AGYd, RGd and LSLCd in several vineyards was determined. Full length phytoplasma chromosomes were isolated directly from Australian grapevines and characterised. An uncharacterised phytoplasma detected in GYd affected grapevines from the Buckland Valley of Victoria was described. Vineyard surveys were done to determine the incidence of the BVGYp and GYd. A discussion of the results from each study is presented below.

## 6.1 Seasonal detection of phytoplasmas

AGYp and TBBp may persistently infect Australian grapevines throughout the year and between years. However, both phytoplasmas may be unevenly distributed or in uneven titre throughout grapevines. The detection of phytoplasmas in grapevine tissue other than shoots indicates that Australian phytoplasmas spread systemically throughout grapevines.

The results of the seasonal detection and persistence work provide information that will improve PCR detection of phytoplasmas in Australian grapevines throughout the year. There was a seasonal influence on the efficiency of phytoplasma detection by PCR. Like the results from previous studies (Gibb *et al* 1999), AGYd symptomatic shoots were most reliable for detection of phytoplasmas in January compared to asymptomatic shoots. At other times of the year PCR detection of phytoplasmas was more reliable when trunks or cordons were sampled from diseased or asymptomatic grapevines. When the combined results of PCR testing for phytoplasmas in shoot, cordon, and trunk samples were considered the efficiency of the PCR test was greatest in July or October of each year.

The PCR results presented were from individual shoot, cordon, trunk and root samples. For routine and cost effective diagnostic testing for phytoplasmas the separate samples from one grapevine would need to be combined. Further work needs to be done to determine the reliability of PCR detection of phytoplasmas on the bulked shoot, cordon, trunk and root samples.

**6.2 The association of phytoplasmas with AGYd**

Although Koch's postulates have not been fulfilled, phytoplasmas are considered to be the most likely cause of AGYd because of the similarity of AGYd symptoms to those of other grapevine yellows diseases from other countries that are also associated with phytoplasmas. Additionally, electron microscopy (Magarey *et al* 1988), antibiotic treatment (Magarey and Wachtel 1986b) and PCR techniques (Bonfiglioli *et al* 1995; Gibb *et al* 1999; Padovan *et al* 1995) have confirmed an association between phytoplasmas and AGYd.

The results from the seasonal detection studies presented in this thesis suggest that the location phytoplasmas within individual grapevines may influence AGYd expression. Phytoplasmas were most frequently detected by PCR in AGYd affected shoots compared to asymptomatic shoots, even when asymptomatic and AGYd affected shoots were taken from the same grapevine. Other studies, which used shoot material for PCR testing of phytoplasmas, have also shown that phytoplasmas are more frequently detected in AGYd affected grapevines compared to asymptomatic grapevines (Gibb *et al* 1999). It is also possible that AGYd symptoms may not be expressed unless phytoplasmas are present in high enough titre in shoots.

Asymptomatic phytoplasma infections can occur. Phytoplasmas were detected in three of the grapevines examined in the detection and distribution studies, which did not express any disease. In other countries the spread of phytoplasmas through grapevine cuttings has been demonstrated (Caudwell *et al* 1994; Caudwell *et al* 1997; Pavan *et al* 1997). The

spread of phytoplasmas through asymptomatic grapevine cuttings in Australia is not known and needs to be determined. It cannot be assumed that asymptomatic grapevines in Australia are free of phytoplasmas when selecting material for dissemination to growers.

### 6.3 The yearly expression and incidence of AGYd

AGYd affected many grapevines within individual vineyards. Some grapevines showed recurrent disease while others showed remission of disease. When AGYd was recurrent the disease was not always expressed in sequential seasons. In each year of the vineyard surveys, there were new records of AGYd. Consequently, AGYd in Sunraysia vineyards was characterised by the expression of disease followed by remission in some grapevines and recurrence of disease in others as well as the occurrence of AGYd in previously unaffected grapevines.

Recurrence and new observations of AGYd would have contributed to the increases in AGYd incidence that was observed in some years in each vineyard. Remission of AGYd would have contributed to the decreases in AGYd incidence that were observed. The pattern of incidence of AGYd was different between vineyards for the duration of the survey indicating that local factors may have had an important influence on disease expression and incidence. The fluctuation in year to year incidence of AGYd was in contrast to previous research, which showed that the incidence of AGYd may be temporarily high in one year only in some Chardonnay vineyards (Magarey and Wachtel 1986b).

The fixed grid analysis and spatial autocorrelation were useful tools to describe the distribution of AGYd in each year. Both analyses showed that some clustering of AGYd affected grapevines occurred at each vineyard in most years. However, the size of the clusters of AGYd affected grapevines was different in the same vineyard from year to year and between vineyards. Remission, recurrence and new observations of AGYd would also have contributed to the changes in the spatial distribution of AGYd affected grapevines in

each vineyard. Because asymptomatic infections occur, the spatial distribution of AGYd does not reflect the spatial distribution of phytoplasmas. A more reliable way to determine the distribution and spread of phytoplasmas in a vineyard would be to test every grapevine in a vineyard for phytoplasmas rather than using spatial analysis of AGYd, but time and cost of tissue sampling, DNA extractions and PCR analyses makes this approach unrealistic.

The cause of remission and recurrence of AGYd is not known but as suggested earlier AGYd expression could be influenced by phytoplasma location and titre. Recurrence of disease might be influenced by both persistent phytoplasma infections and re-inoculation of phytoplasmas. Remission of disease might be associated with declining phytoplasma titre in shoots. The decline in phytoplasma titre may be due to plant host defence responses or to environmental factors that reduce the replication of the organism.

Many phytoplasmas are transmitted to plants by an insect vector. For example, FDp is transmitted to grapevines by the leafhopper *Scaphoideus titanus,* BNp and VKp are transmitted to grapevines by the planthopper *Hyalesthes obsoletus,* TBBp is transmitted to plants such as tomato by the common brown leafhopper *Orosius argentatus* and PYLp, which is closely related to AGYp, is transmitted to plants in New Zealand by the planthopper *Oliarus atkinsonii.* Additionally netting experiments to exclude insects from papaya plants have shown that PDBp, which is also closely related to AGYp, is excluded as well, suggesting that PDBp is transmitted by an insect (Elder *et al* 2002). The insect vector for AGYp is not known and transmission of TBBp to grapevine by *O. argentatus* has not been demonstrated but it is likely that both phytoplasmas are also transmitted to grapevines by insect vectors. Changes in the numbers of inoculative insects visiting vineyards from year to year could also affect AGYd expression and incidence from year to year.

### 6.4 The association of phytoplasmas with RGd

Phytoplasmas were detected more frequently in shoot, cordon and trunk samples from grapevines with RGd and AGYd compared to samples from grapevines with AGYd alone. These results suggest that location and titre of phytoplasmas within individual grapevines may be associated with RGd expression in AGYd affected grapevines. Other studies have shown a low frequency of detection in shoot samples from grapevines affected by RGd (Bonfiglioli *et al* 1995; Padovan *et al* 1995; Gibb *et al* 1999). Shoot, cordon trunk and root samples from grapevines affected by RGd alone were not tested for phytoplasmas in the seasonal detection and distribution studies and the association of phytoplasmas in grapevines tissues other than shoots of grapevines affected by RGd alone is unknown.

Results from the vineyard surveys showed that RGd does affect grapevines in the absence of AGYd, that is RGd and AGYd are not always associated. This suggests that RGd may not always be associated with phytoplasmas, assuming AGYd is a phytoplasma caused disease. Additionally the proportion of grapevines with AGYd that displayed RGd in the same year or following years was different from year to year in each vineyard. Similarly the proportion of grapevines with RGd that displayed AGYd in the same year or previous years was different from year to year in each vineyard. The log linear models also indicated that AGYd and RGd were not always associated.

### 6.5 The expression and incidence of RGd

RGd affected many grapevines within individual vineyards and was also characterised by remission and recurrence of disease as well as new observations of disease. While the incidence of RGd was different between vineyards in each year, a general decline in the incidence of RGd was observed in most vineyards from 1997/98 or 1998/99. This indicated that local and regional factors may influence the expression of RGd. RGd may have many causes including pathogens such as virus (Bovey and Martelli 1992), pests such as mites (Bernard *et al* 2001) and environmental factors such as winter drought and winter chilling

(Meiering *et al* 1980; Wilson *et al* 1997; Wilson and Hayes 2000). The contribution of these factors may vary from year to year influencing the disease status of grapevines.

RGd affected grapevines were often clustered in each vineyard in each year although the size of the clusters was often different from year to year. Remission and recurrence of RGd together with new observations of RGd are likely to have influenced obsevations of the spatial distribution of RGd. No conclusions can be drawn about the occurrence and spread from the spatial distribution RGd because the cause of RGd is uncertain.

**6.6 The association of phytoplasmas with LSLCd**

The association between phytoplasmas and LSLCd is also uncertain. In one study phytoplasmas were detected in 48/59 LSLCd affected shoot samples (Bonfiglioli *et al* 1995) but in another study phytoplasmas were detected in only 12/126 shoot samples from LSLCd affected grapevines (Gibb *et al* 1999). LSLCd affected 15/20 grapevines examined in the seasonal detection and distribution studies. AGYd also affected the 15 grapevines. Results from the study showed that phytoplasmas were often detected more frequently in the combined shoot, cordon and trunk samples from grapevines with LSLCd and AGYd compared to samples from grapevines with AGYd alone. These results suggest that location and titre of phytoplasmas within individual grapevines may be associated with LSLCd expression in AGYd affected grapevines. Shoot, cordon, trunk and root samples from grapevines affected by LSLCd but never by AGYd, were not tested for phytoplasmas in the seasonal detection and distribution studies so the association of phytoplasmas in grapevines tissues other than shoots of grapevines affected LSLCd alone is unknown.

Results from the vineyard surveys showed that LSLCd affects grapevines in the absence of AGYd, that is LSLCd and AGYd are not always associated. This suggests that phytoplasmas may not always be associated with LSLCd. The proportion of grapevines with AGYd that displayed LSLCd in the same year or following years was different from year to year in each vineyard. Similarly the proportion of grapevines with LSLCd that

displayed AGYd in the same year or previous years was different from year to year in each vineyard. The log linear models also showed that AGYd and LSLCd were not always associated.

### 6.7 The expression and incidence of LSLCd

LSLCd also affected many grapevines in Sunraysia Chardonnay vineyards and remission, recurrence and new observations of disease were characteristic of LSLCd. The incidence of LSLCd was significantly different from year to year within most vineyards but the pattern of incidence from year to year was different between vineyards indicating that local factors influence the expression of this disease in individual grapevines. Clustering of LSLCd was observed in some years and in most vineyards a directional trend along rows and columns was observed using spatial autocorrelation. While spatial autocorrelation does not indicate if the direction trend occurs in either the row direction or column direction, grapevines are in closer proximity along trellises, which is the column direction indicated in the analysis, and it is possible that the cause of this disease in some vineyard spreads in this direction from vine to vine. A statistical analysis that determines gradients of disease in the column and row direction and could have been used to determine a directional trend for LSLCd.

### 6.8 Phytoplasma variation

Schneider *et al* (1997) showed that the *tuf* gene was considerably more variable that the 16S rRNA gene and that it was useful for further differentiating phytoplasmas strains of the aster yellows (16Sr 1) group when RFLP analysis of the 16srRNA gene could not. The *tuf* gene was therefore used in this study to determine if variation existed amongst isolates of AGYp and between AGYp and an isolate of PDBp. Genetic variability amongst isolates of the AGYp was observed using heteroduplex mobility assay (HMA) of the *tuf* gene. HMA using the *tuf* gene also revealed that the PDBp isolate used in this study was different to AGYp. The AGYp *tuf* variants and the PDBp isolate were indistinguishable

when their *tuf* gene PCR products were compared using HMA. Sequence analysis would confirm the similarity between the *tuf* gene of the AGYp *tuf* variants and the PDBp isolate.

The AGYp *tuf* variant occurred with low frequency, as it was not detected in other samples from the same grapevine or other grapevines using HMA. To confirm the low frequency many more AGYp isolates from grapevine would need to be tested. Only one PDBp isolate was used in this study and it would be interesting to determine if the difference between the *tuf* gene of AGYp and PDBp was typical of the two strains of '*Candidatus* Phytoplasma australiense' or if the difference in PDBp was also due to a less frequently occurring variant. HMA could also be used to determine if variation of the *tuf* gene exists in *P. australiense* isolates from other hosts such as strawberry and isolates from other regions.

**6.9 Characterisation of phytoplasma chromosomes isolated directly from grapevines**

Full length chromosomal DNA of TBBp was obtained from grapevine and the chromosome was estimated to be 680 kb and in the range reported for other isolates of TBBp (Marcone *et al* 1999; Padovan *et al* 2000). This is the first report of isolation of an intact phytoplasma chromosome directly from naturally infected grapevine. Detectable amounts AGYp chromosomal DNA was not isolated from grapevine using PFGE and Southern hybridisation.

Large amounts of TBBp infected grapevine material were required for TBBp chromosome isolation compared to herbaceous hosts, such as periwinkle and snapdragon, This suggests that phytoplasmas exist in lower titre in grapevines compared to herbaceous hosts and are much less suitable than herbaceous hosts for the isolation of large amounts of quality phytoplasma chromosomal DNA. Even when herbaceous hosts, such as periwinkle, tomato and various *Nicotiana* sp., are used some researchers report difficulties in isolating enough phytoplasma chromosomes for genomic characterisation using the method of Neimark and Kirkpatrick (1993) because of low phytoplasma titre (Lauer and Seemüller,

2000; Marcone and Seemüller, 2000). Other researchers have modified the same isolation method to produce enough good quality phytoplasma chromosomes from periwinkle for genomic characterisation (Padovan *et al* 2000).

A physical map of the TBBp chromosome was not prepared because most restriction endonucleases did not digest TBBp chromosomal DNA from grapevine. Various grapevine compounds, such as polyphenols and polysaccharides (Lodhi *et al* 1994), were likely to have been co-purified with the TBBp chromosome using method 2 described in Chapter 4. It is possible that the co-purified grapevine compounds inhibited endonuclease activity resulting in undigested TBBp chromosomal DNA. Results indicated a possibility that isolation and physical and genetic mapping of grapevine phytoplasma chromosomes could be achieved if extraction procedures can be improved. In particular the use of density gradients may improve the quality of phytoplasma chromosomal DNA from difficult hosts, such as grapevine, by removing more contaminating plant material than differential centrifugation alone. Similar gradients removed a substantial amount of extraneous protein when maize bushy stunt phytoplasma was isolated from leafhoppers (Davis *et al* 1987). Manipulating the densities of the solutions used for density gradient, centrifugal force and centrifugal times has been shown to affect the amount of phytoplasma recovered from lettuce preparations (Jiang and Chen, 1987). Similar manipulations could be pursued to improve the amount of phytoplasma isolated from grapevines.

Some genomic diversity may exist amongst isolates of TBBp. Continued work in this area would confirm the variability observed amongst TBBp isolates. Physical and genetic mapping could then be used to determine where the differences reside on the chromosome. Identification of genes near those sites could determine whether the differences relate to some biological factor such as host/pathogen specificity.

### 6.10 A new grapevine phytoplasma from the Buckland Valley of Victoria

BVGYp is a unique phytoplasma based on its 16S rRNA gene and 16S-23S spacer region sequences. It is likely that this phytoplasma represents a new subgroup within the aster yellows group (16SrI) of phytoplasmas. The closest sequence similarity observed was 97.1% with Clover phyllody phytoplasma from the USA.

BVGYp was associated with a grapevine yellows disease (GYd). GYd affected many Chardonnay grapevines during the four year survey of one vineyard in the Buckland Valley. The incidence of GYd is characterised by remission of disease in many affected grapevines and some recurrence in others as well as occurrences in previously unaffected grapevines. BVGYp and GYd had a very localised distribution. The two vineyards in which BVGYp and GYd were found are in close proximity to one another, compared to other vineyards in the region. BVGYp has not been reported outside the Buckland Valley. A survey of plants around the two vineyards using PCR detection for phytoplasmas could reveal an alternative host and perhaps an origin for BVGYp.

The detection of GYd and BVGYp in the second vineyard in 2001 provided evidence of aerial transmission of the disease and the phytoplasma in the Buckland Valley. It is unlikely that the infection in 2001 arose from planting material because the material used to establish the second vineyard was obtained from a different source to the other vineyard. To identify candidate vectors for BVGYp, insects could be trapped in and around both vineyards and tested by PCR for phytoplasmas. To confirm vector status phytoplasmas could be localised in insect salivary glands (Webb *et al* 1998). Other techniques such as artificial insect feeding (Tanne *et al* 2001) and feeding trials using BVGYp plant hosts could also be used to confirm phytoplasma transmissibility by candidate vectors.

### 6.11 Conclusions

The results presented in this thesis improve the understanding of how phytoplasmas infect Australian grapevines and how they might cause disease. Phytoplasmas are

associated with AGYd, but AGYd is not necessarily persistently expressed even though phytoplasmas may persistently infect grapevines. More research needs to be done before appropriate management practices can be devised to ameliorate the effect of AGYd and phytoplasmas in vineyards. Controlled experiments looking at various management practices including irrigation, nutrition and pruning methods in individual vineyards might identify means to reduce the effects of phytoplasmas and expression of AGYd. Identifying insect vectors and alternative hosts will assist in understanding the spread and expression of AGYd.

Further molecular research to identify genes will assist in understanding how phytoplasmas function and cause disease. Some research groups have begun to sequence the entire genome of phytoplasmas (Liefting and Kirkpatrick 2000), which will be valuable for identification of genes, particularly those associated with pathogenicity. Others have used various methods to identify genes of interest, such as the design of primers based on conserved regions of genes from closely related organisms (Guo *et al* 2000; Schneider *et al* 1997) and various isolation and cloning techniques (Berg *et al* 1999; Berg and Seemuller 1999; Davis *et al* 1988a, Kirkpatrick and Fraser 1988; Kakizawa *et al* 2001; Kuboyama *et al* 1998; Rekab *et al* 1999). A similar approach could also be applied to the phytoplasmas that infect Australian grapevines to identify genes associated with phytoplasma function and pathogenicity.

The results presented here provide no insight as to the actual cause of RGd or LSLCd. It is possible that phytoplasmas are not the cause of either disease and their association is coincidental.

Transmission of Australian grapevine phytoplasmas to an alternative host such as *Catharanthus roseus* and subsequent transmission back into pathogen free grapevines could be done to further implicate phytoplasmas as a cause for disease. Transmission of both phytoplasmas by dodder and grafting has been attempted but there have been no

reports of successful transmission. Ideally, isolation of phytoplasmas and their inoculation into pathogen free grapevines would answer the question of cause for AGYd, RGd and LSLCd. Phytoplasmas cannot, however, be grown axenically so such an experiment is unlikely, at least in the near future.

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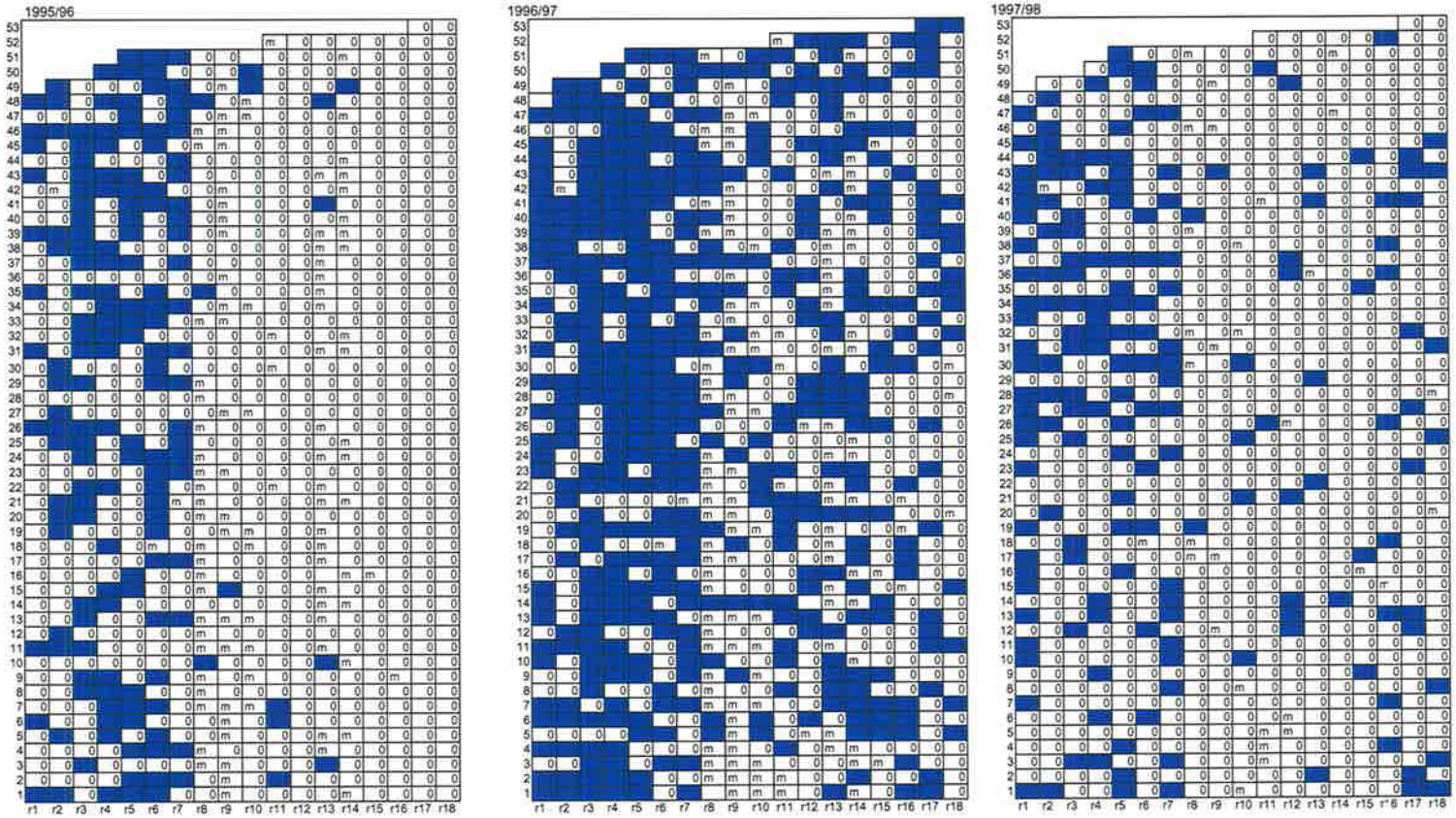
# APPENDIX A:
# DISEASE SURVEY MAPS

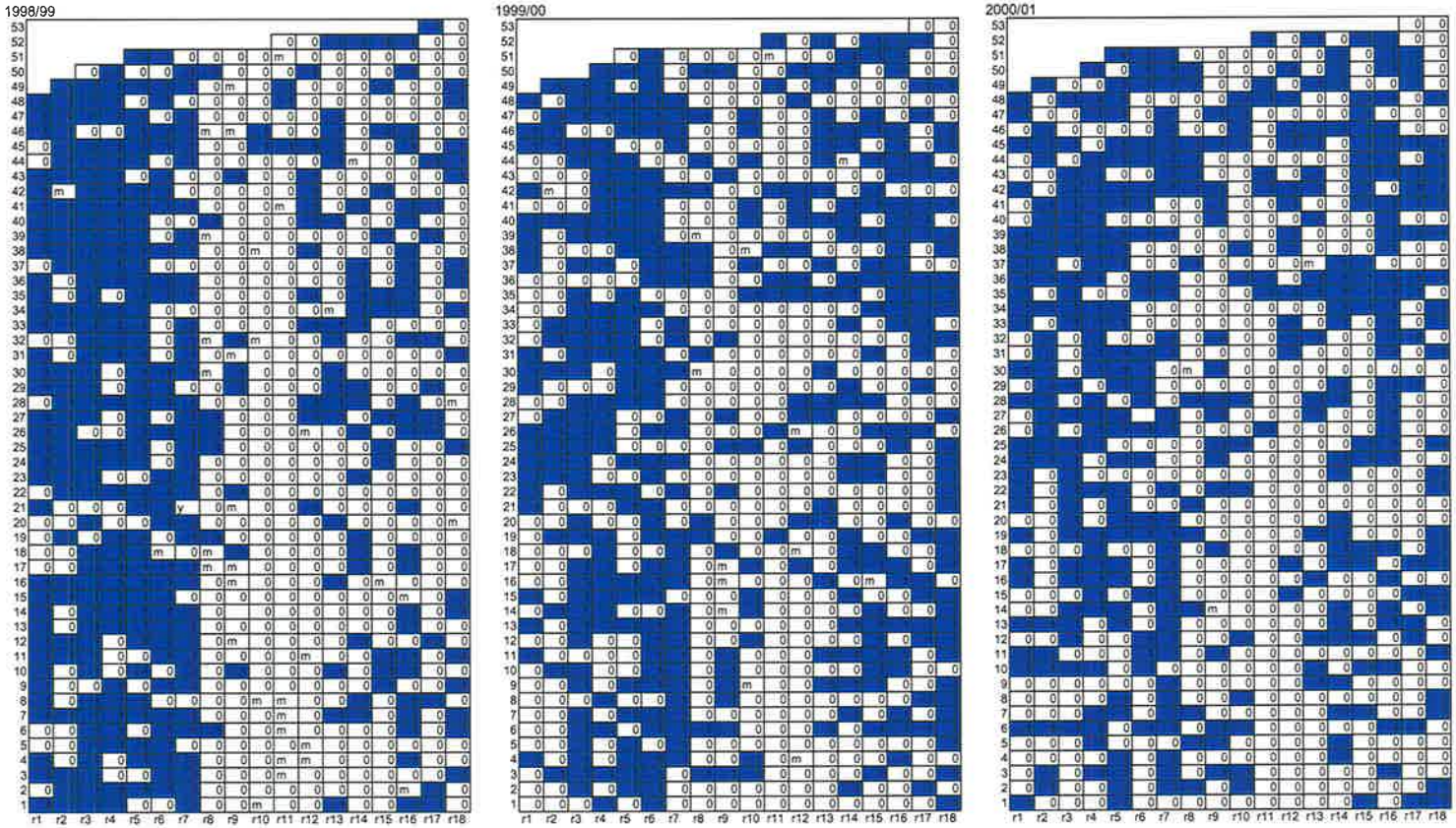
Gol Gol:: Distribution of AGYd affected grapevines

AGYd affected  0 = unaffected  m =missing

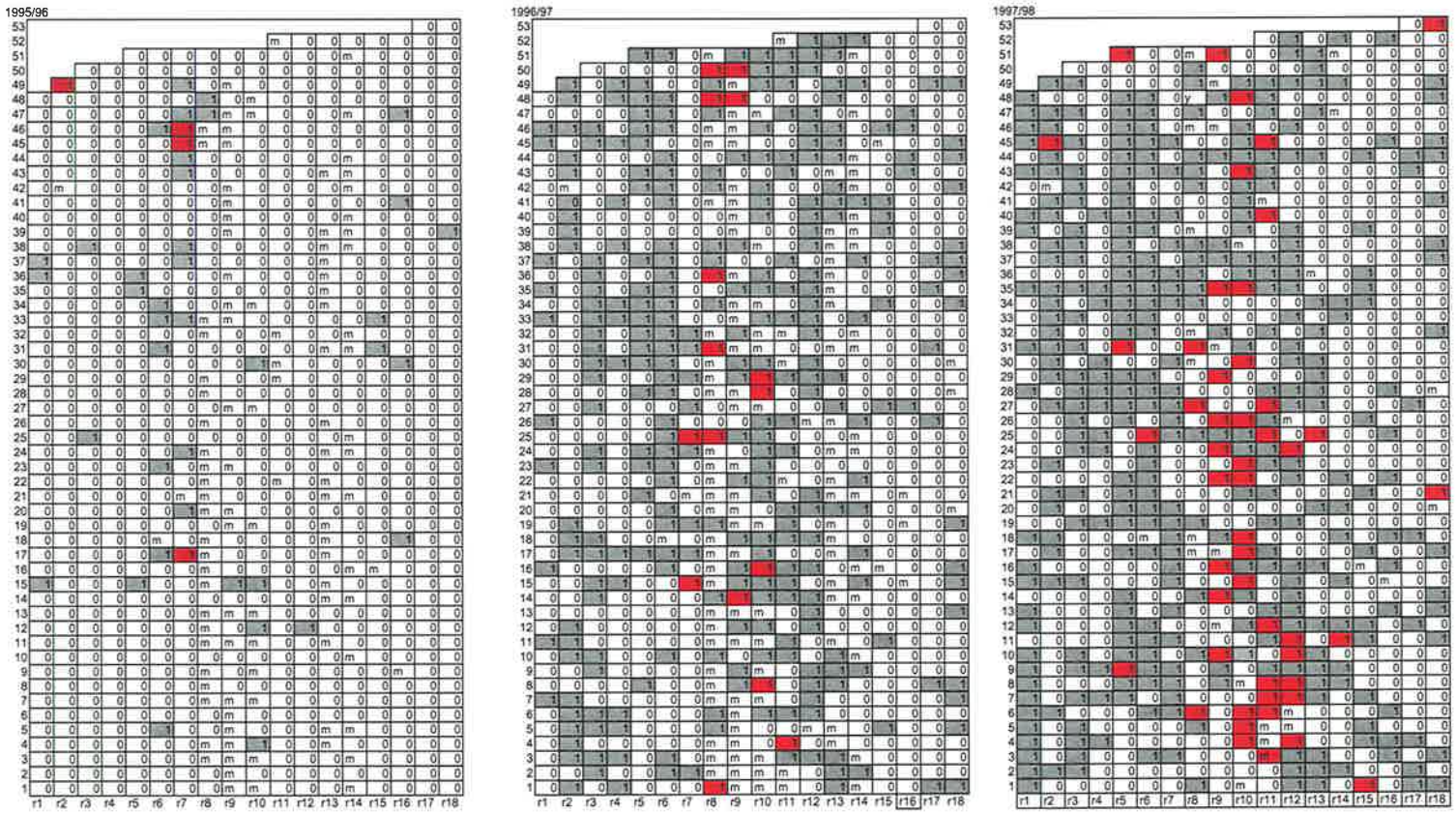


Gol Gol:: Distribution of AGYd affected grapevines

AGYd affected  0 = unaffected  m =missing

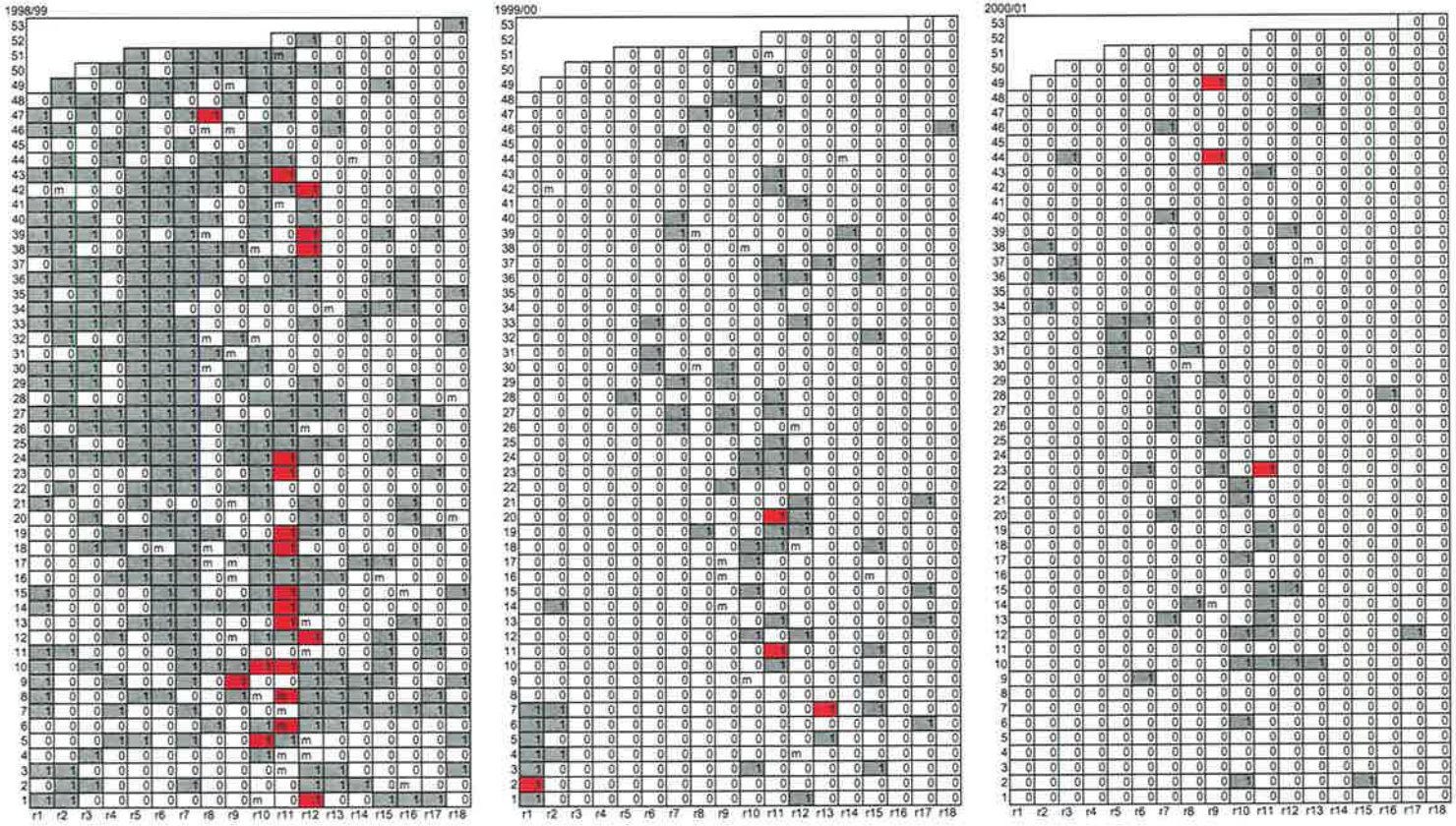


Gol Gol: Distribution of RGd affected grapevines  mild RGd  severe RGd  0 = unaffected  m = missing



Gol Gol: Distribution of RGd affected grapevines

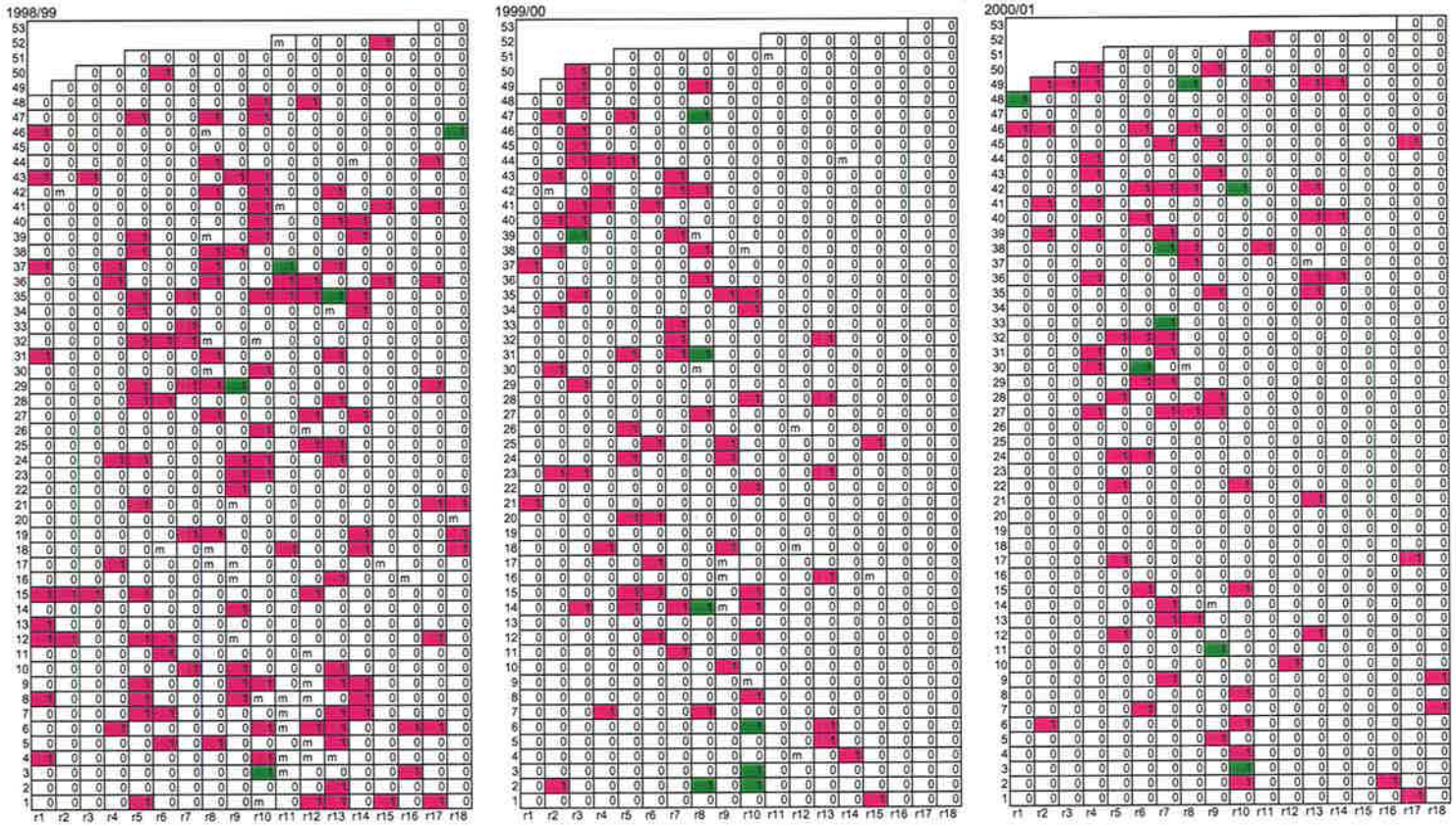
mild RGd severe RGd 0 = unaffected m =missing



Gol Gol: Distribution of LSLCd affected grapevines  mild LSLCd  severe LSLCd  0 = unaffected  m = missing

Gol Gol: Distribution of LSLCd affected grapevines

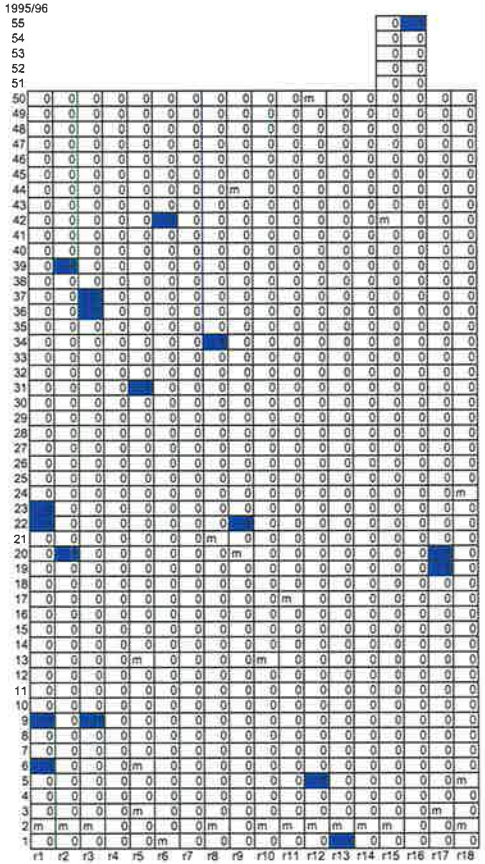
mild LSLCd severe LSLCd 0 = unaffected m =missing



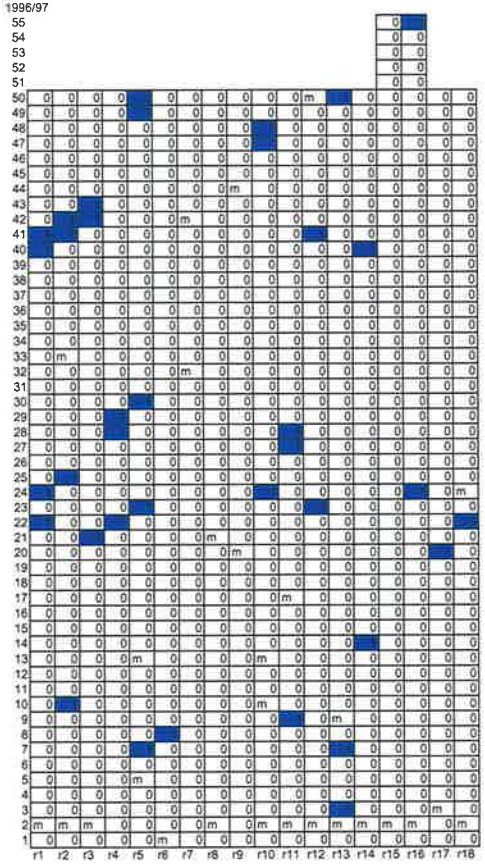
Karadoc: Distribution of AGYd affected grapevines

AGYd affected  0 = unaffected  m =missing

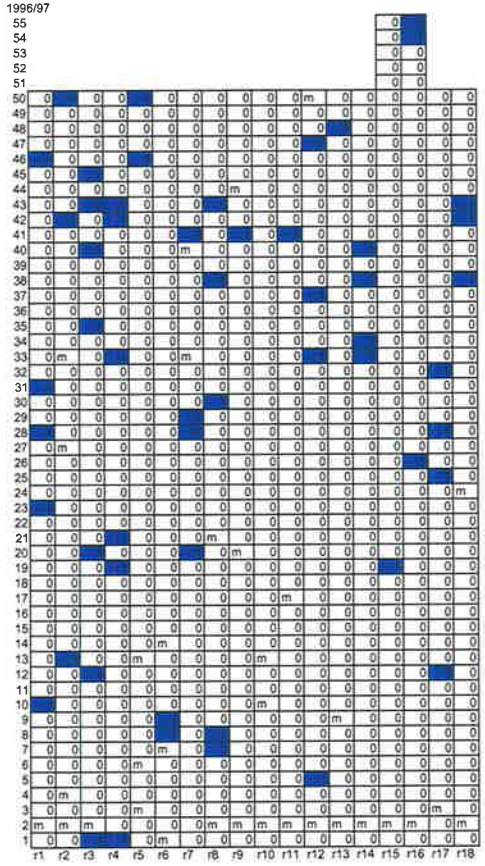
1995/96



1996/97

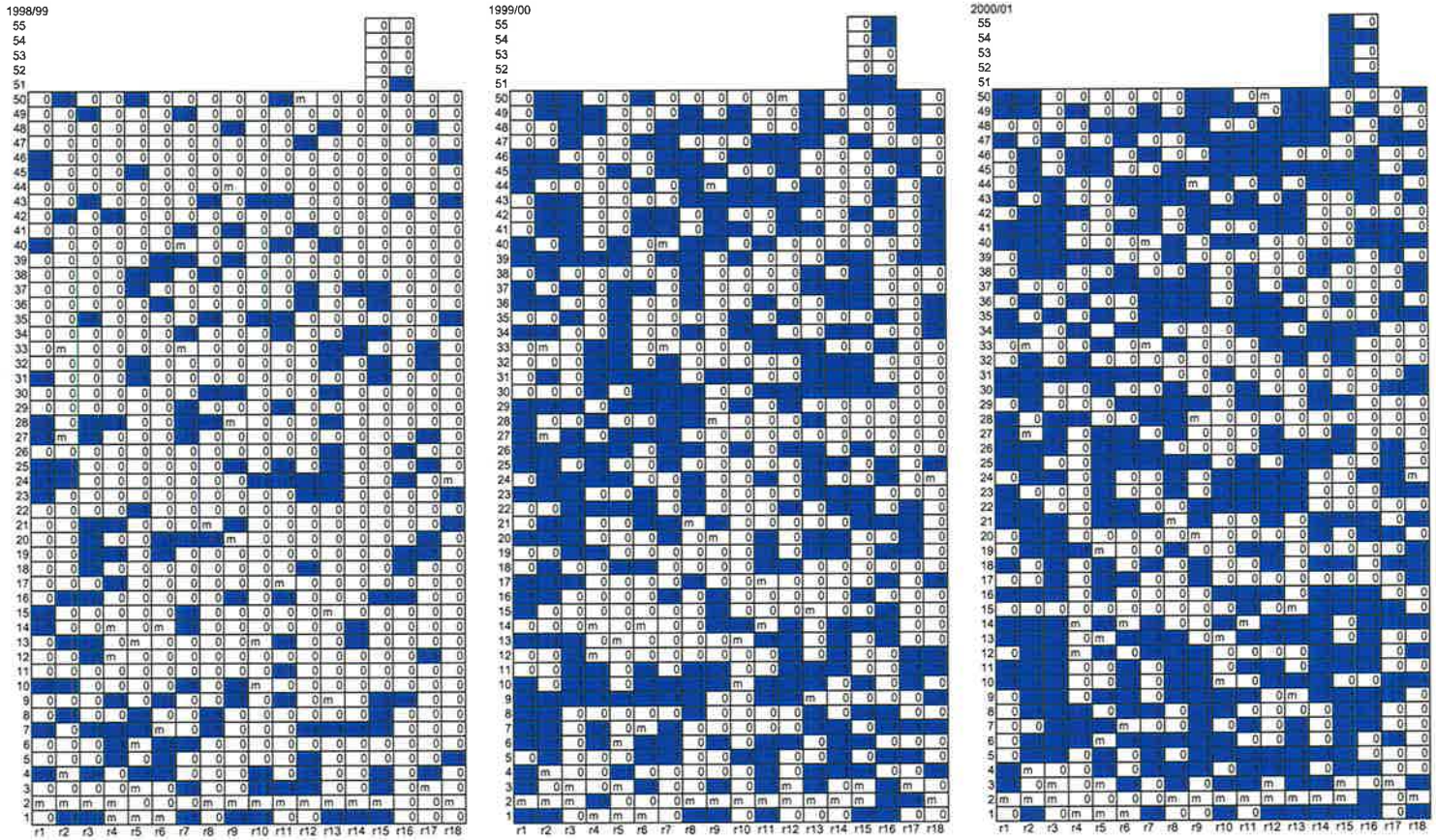


1996/97



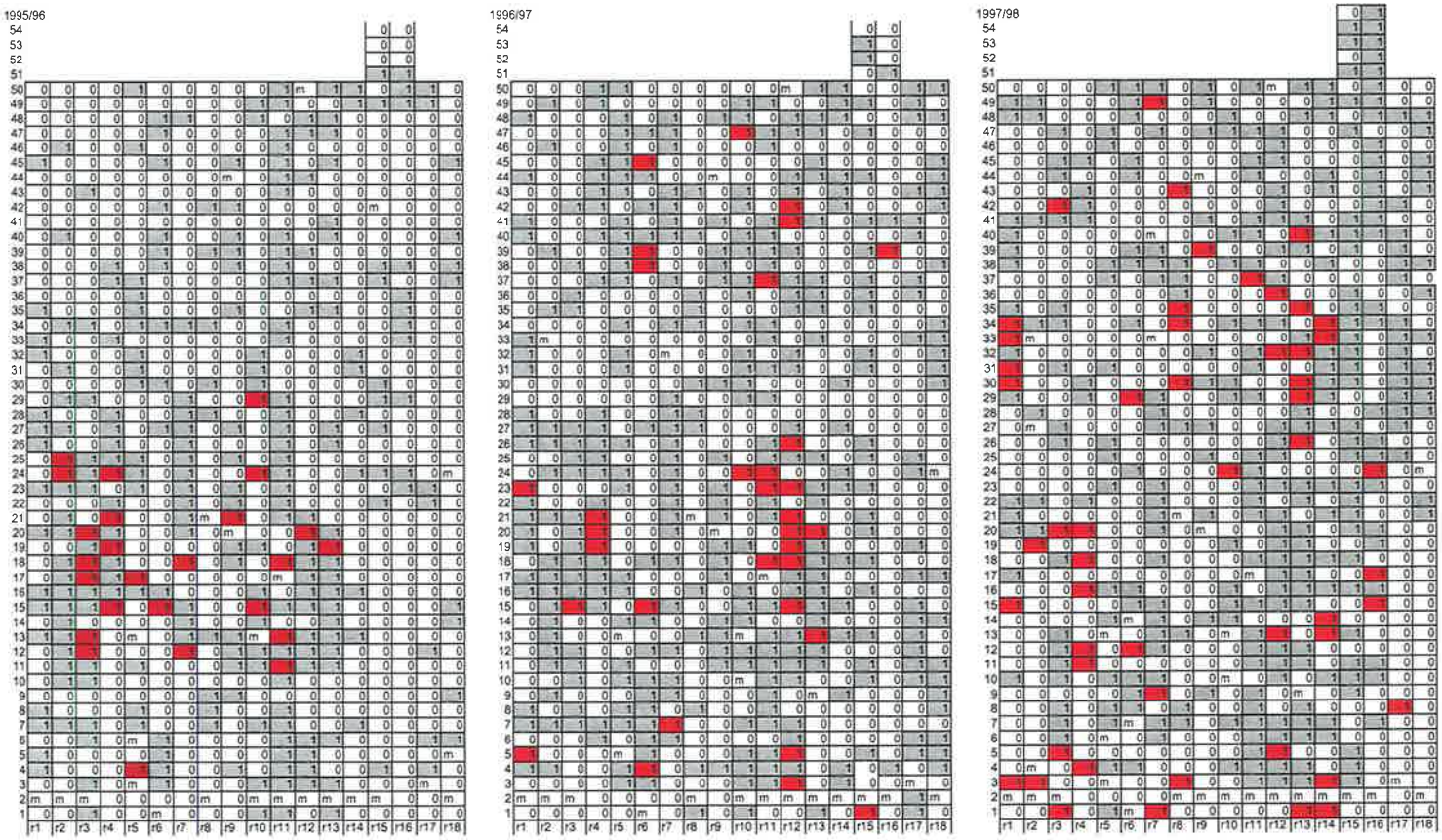
Karadoc: Distribution of AGYd affected grapevines

AGYd affected  0 = unaffected  m =missing



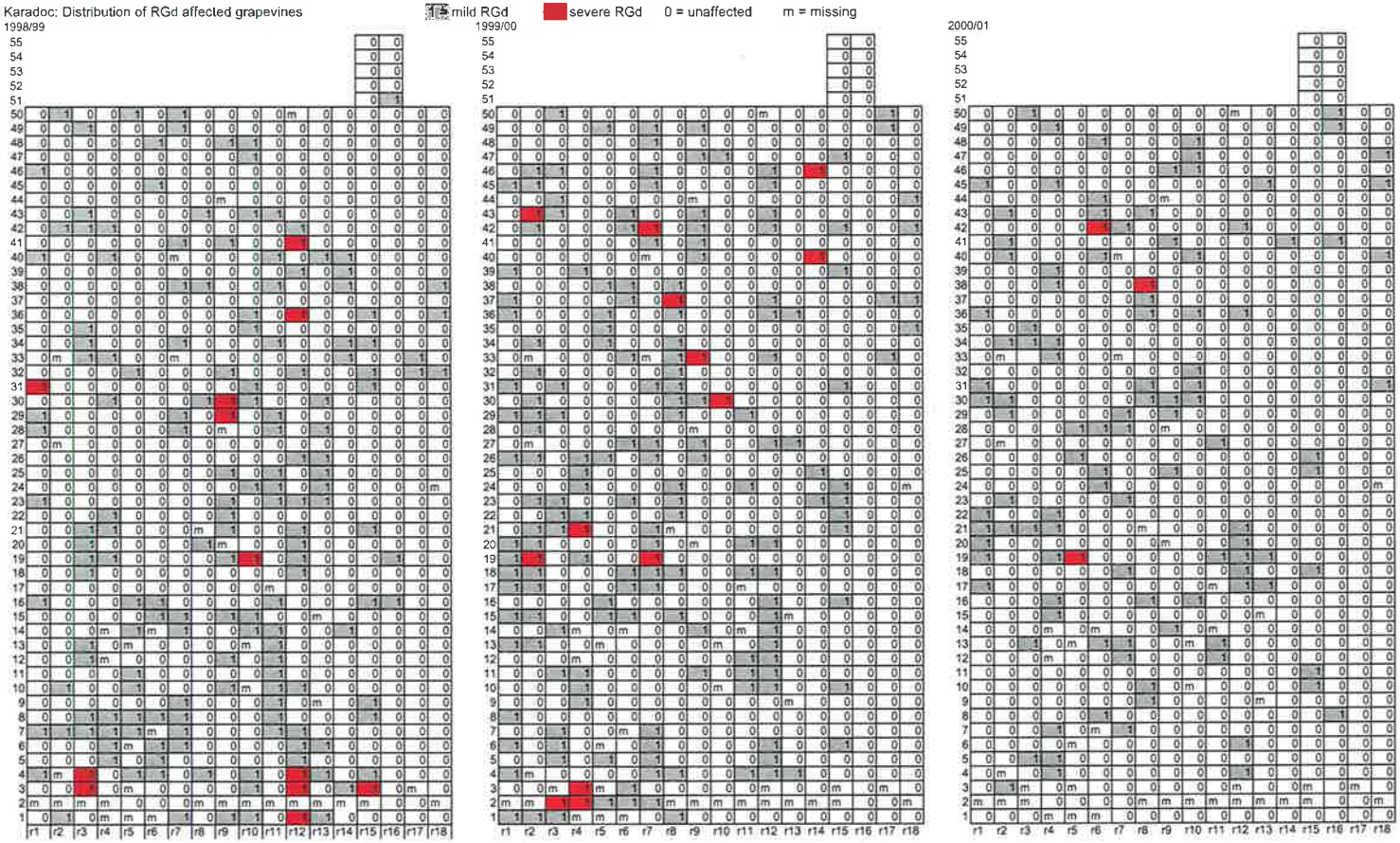
Karadoc: Distribution of RGd affected grapevines

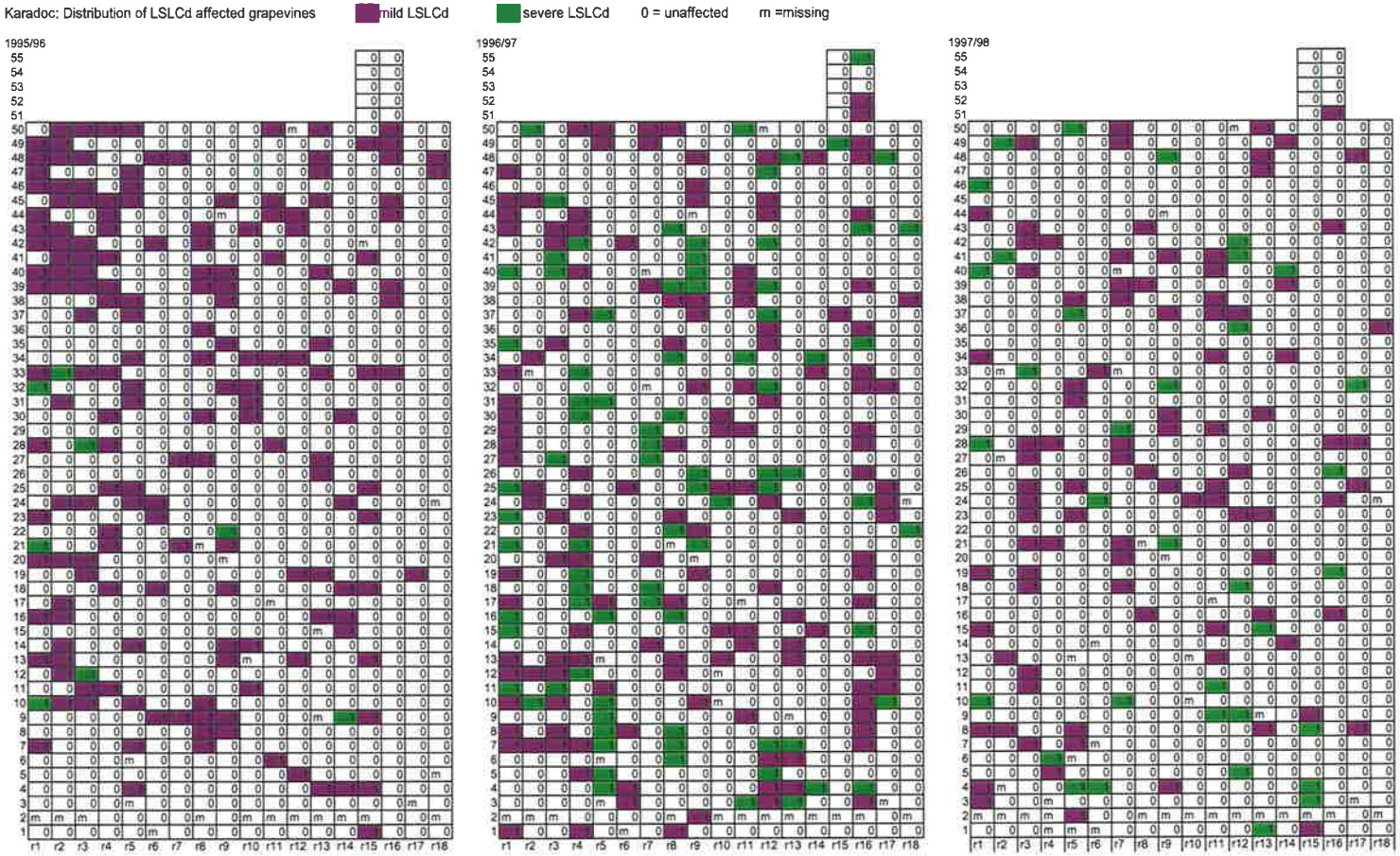
mild RGd severe RGd 0 = unaffected m = missing

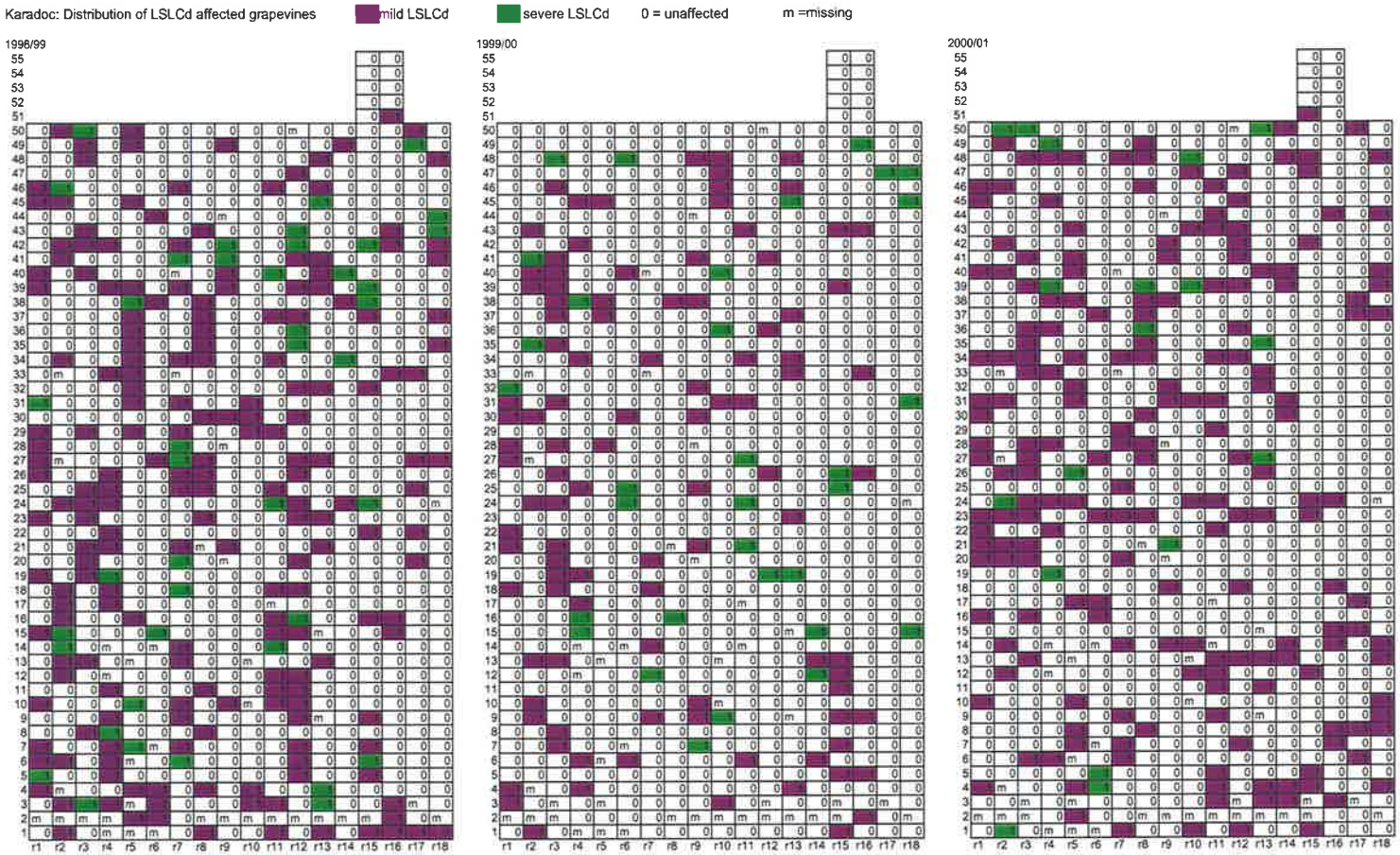


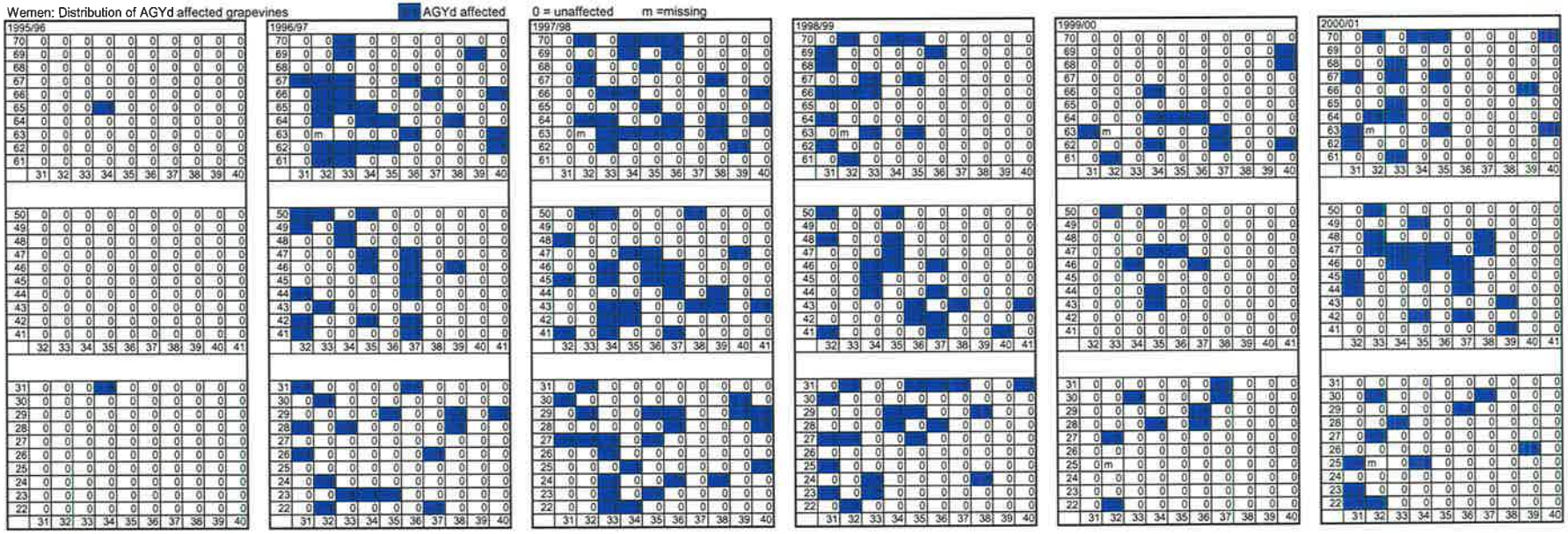
Karadoc: Distribution of RGd affected grapevines

mild RGd  severe RGd  0 = unaffected  m = missing

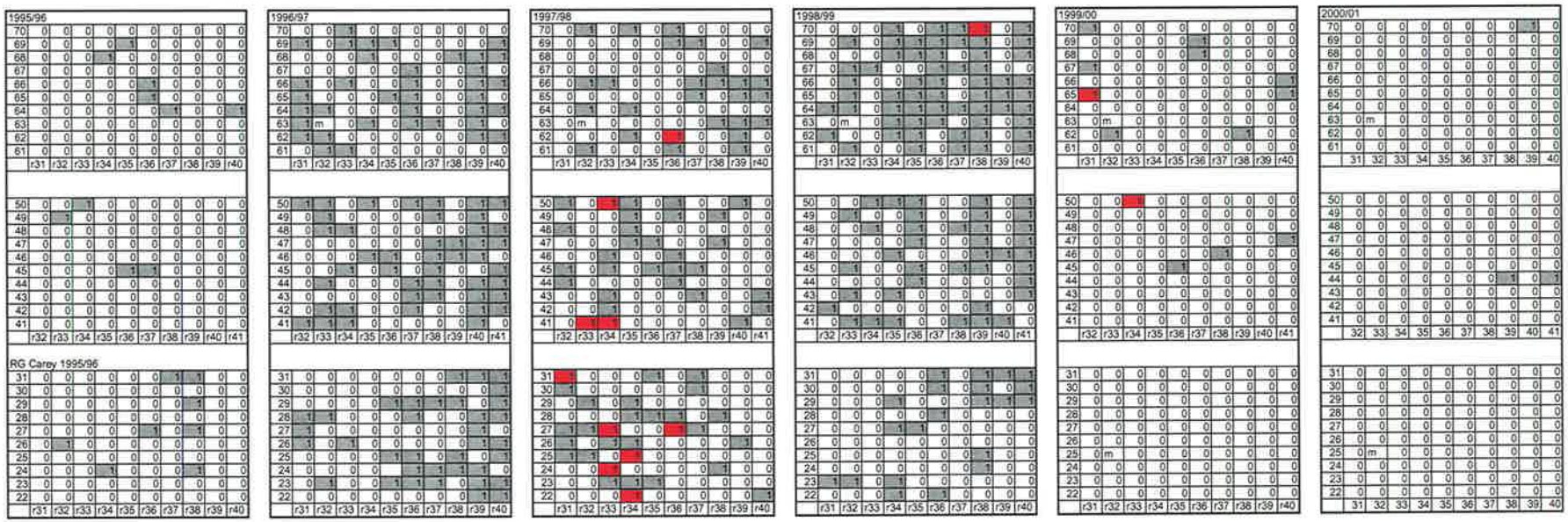



Karadoc: Distribution of LSLCd affected grapevines
mild LSLCd
severe LSLCd
0 = unaffected
m =missing
1995/96
1996/97
1997/98

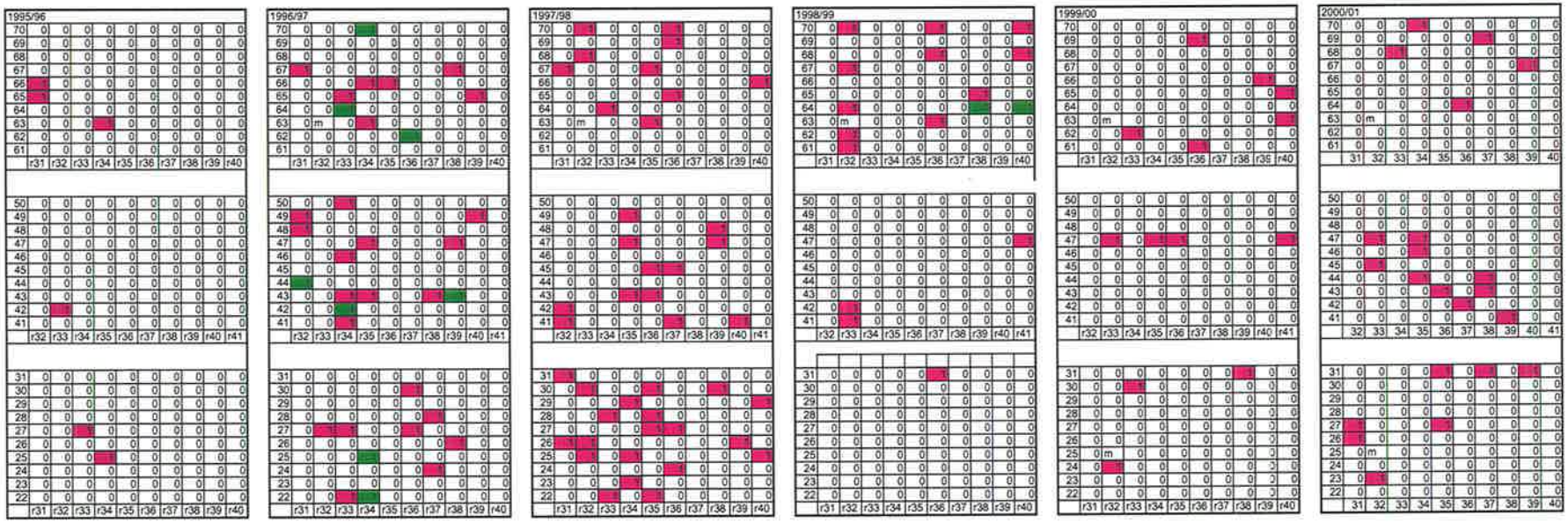

Karadoc: Distribution of LSLCd affected grapevines
mild LSLCd
severe LSLCd
0 = unaffected
m =missing
1998/99
1999/00
2000/01


Wemen: Distribution of AGYd affected grapevines
AGYd affected
0 = unaffected
m =missing
1995/96
1996/97
1997/98
1998/99
1999/00
2000/01

Wemenc: Distribution of RGd affected grapevines  mild RGd  severe RGd  0 = unaffected  m = missing



Wemen: Distribution of LSLCd affected grapevines  mild LSLCd  severe LSLCd  0 = unaffected  m =missing

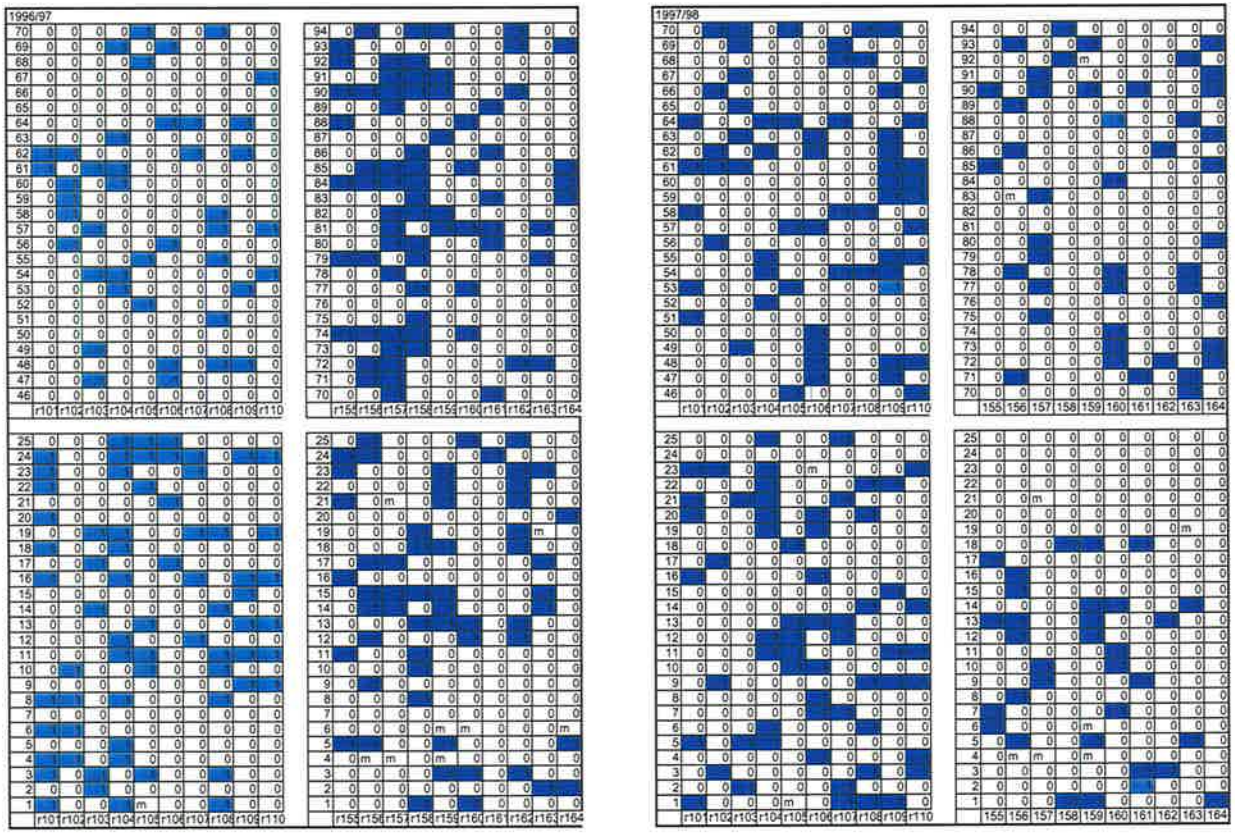


Wernen: Distribution of AGYd affected grapevines

AGYd affected

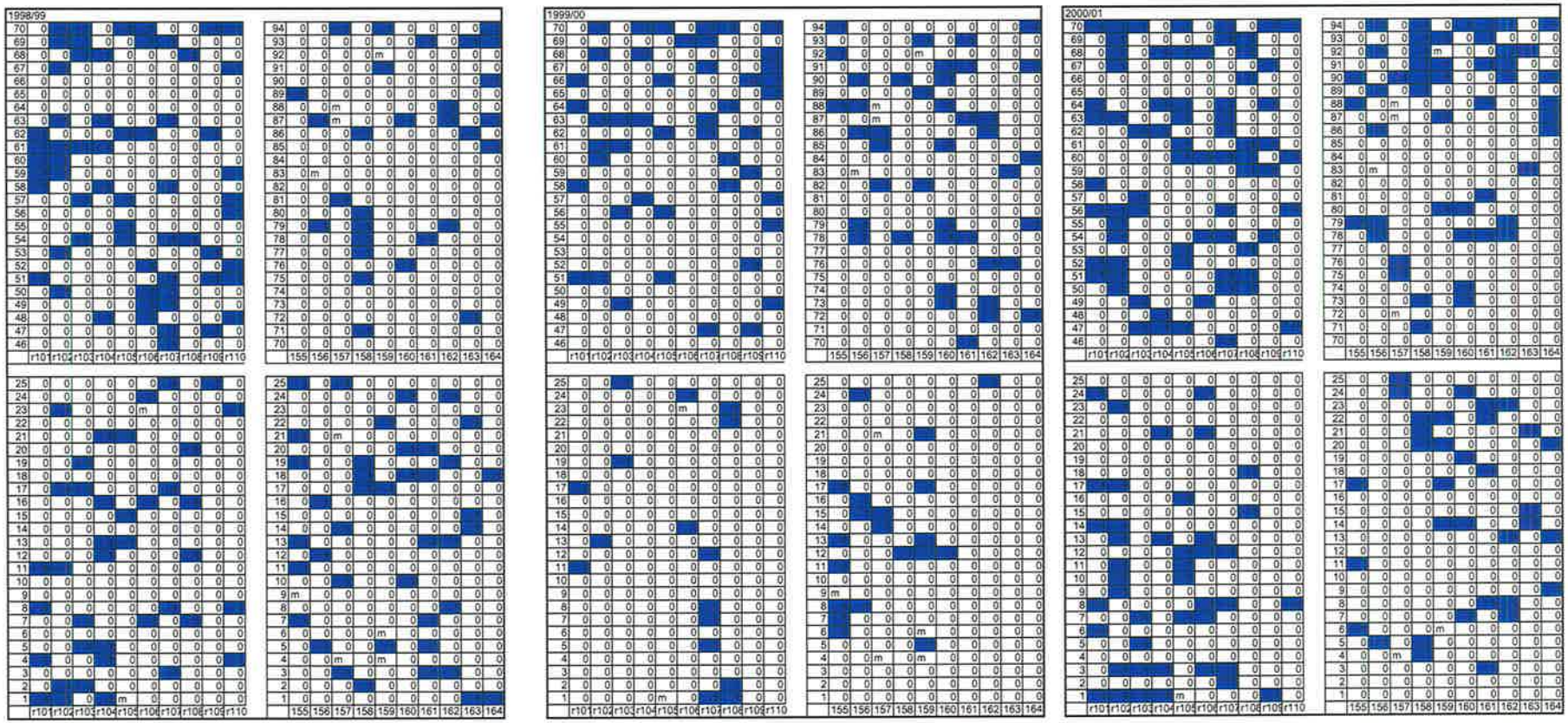
0 = unaffected

m =missing

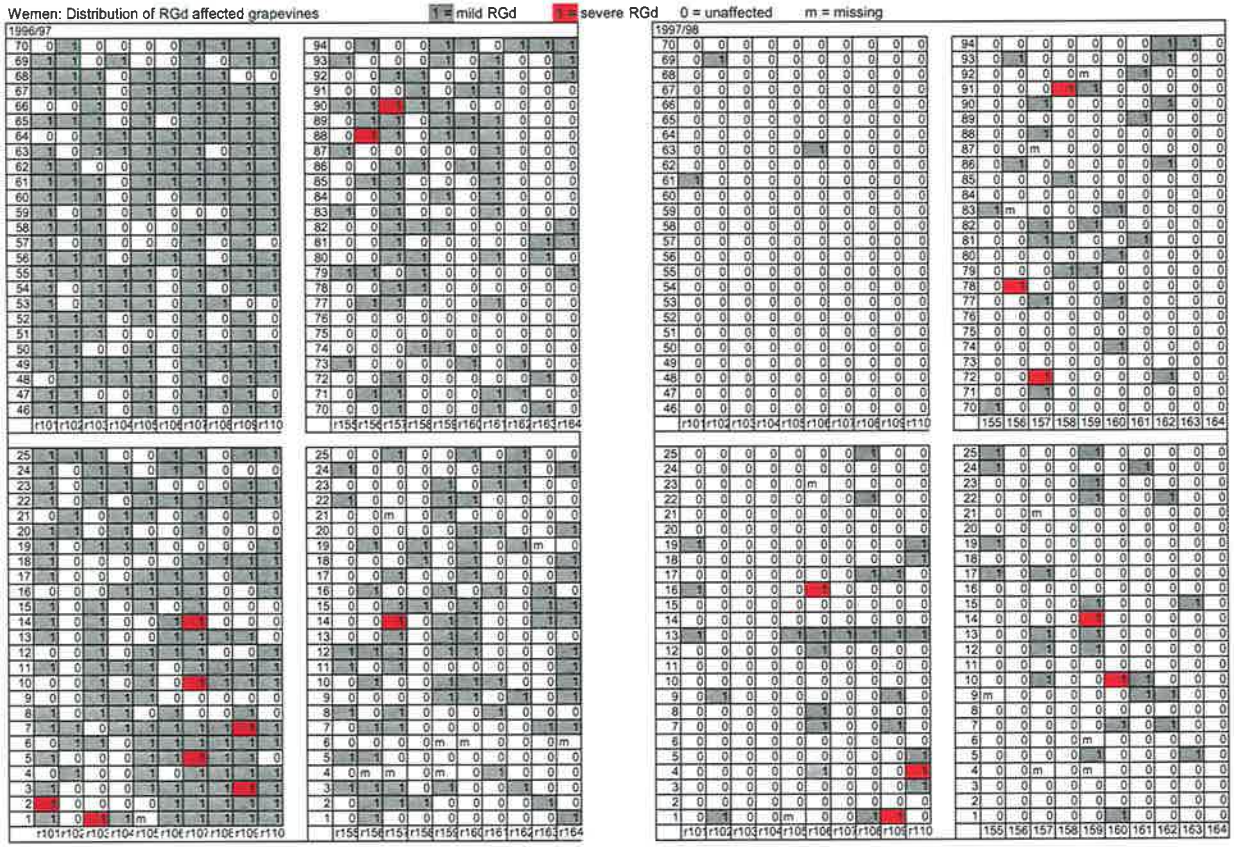


Wemen: Distribution of AGYd affected grapevines

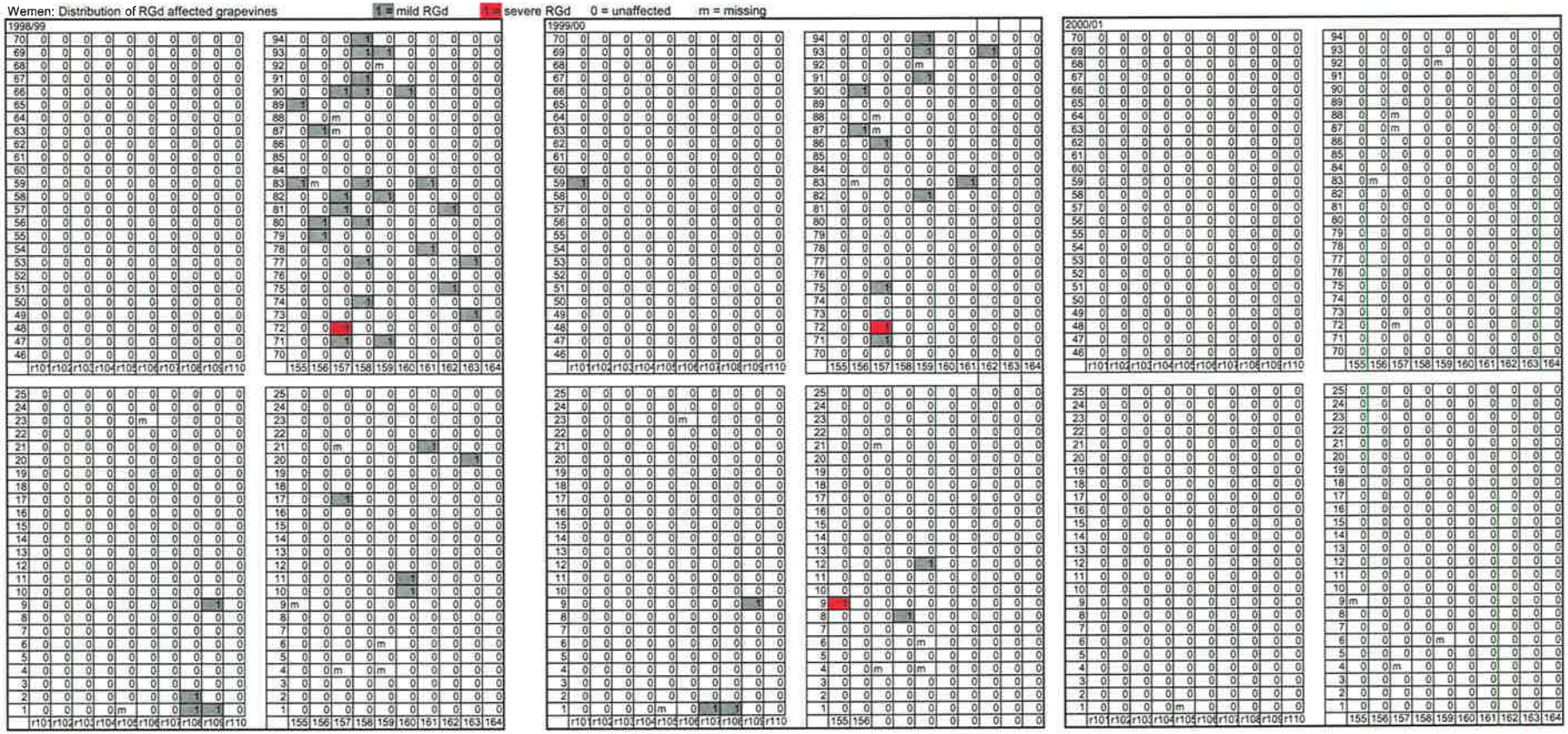
AGYd affected  0 = unaffected  m =missing



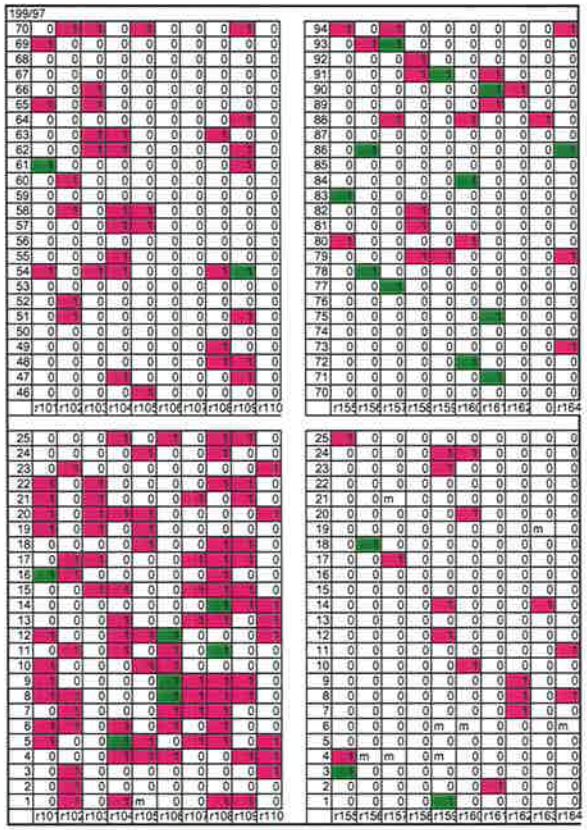
Wemen: Distribution of RGd affected grapevines  1 = mild RGd  1 = severe RGd  0 = unaffected  m = missing

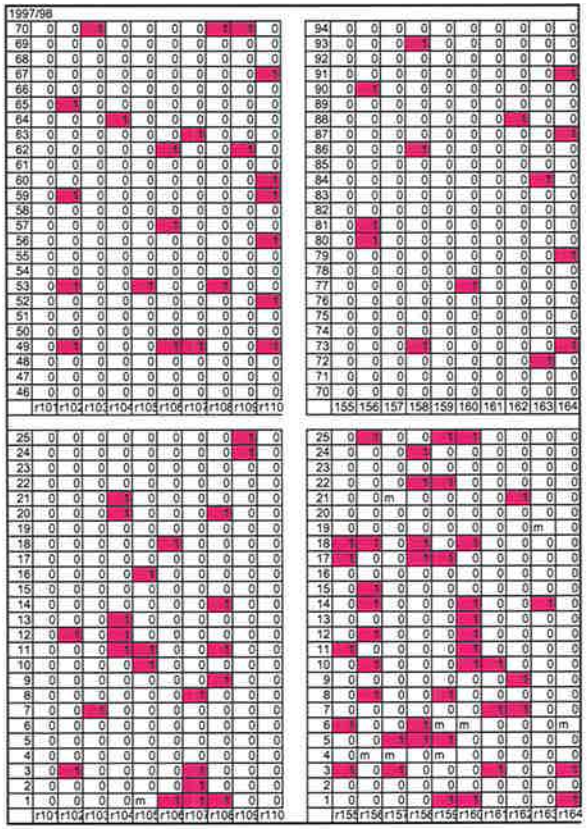


Wemen: Distribution of RGd affected grapevines 1 = mild RGd 1 = severe RGd 0 = unaffected m = missing

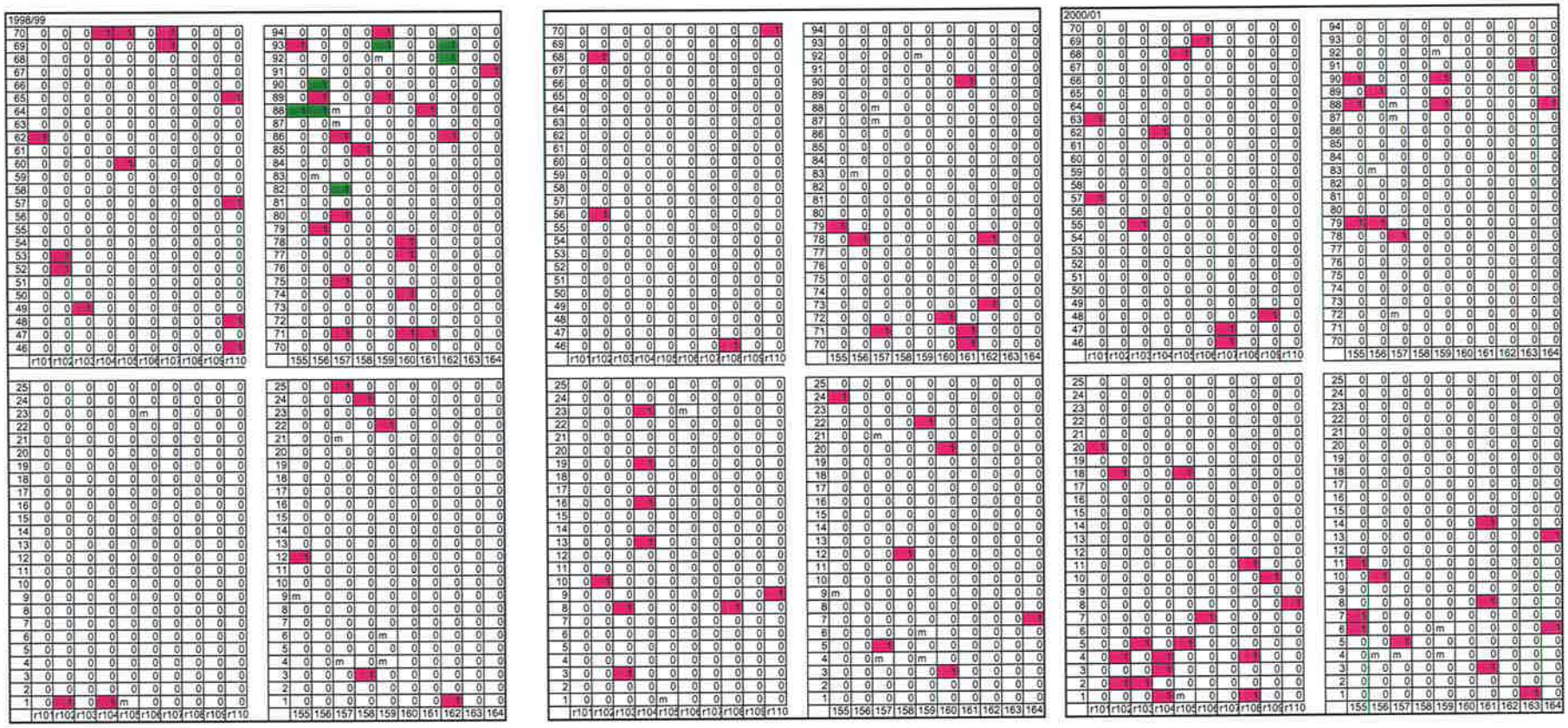


Wemen: Distribution of LSLCd affected grapevines mild LSLCd severe LSLCd 0 = unaffected m =missing

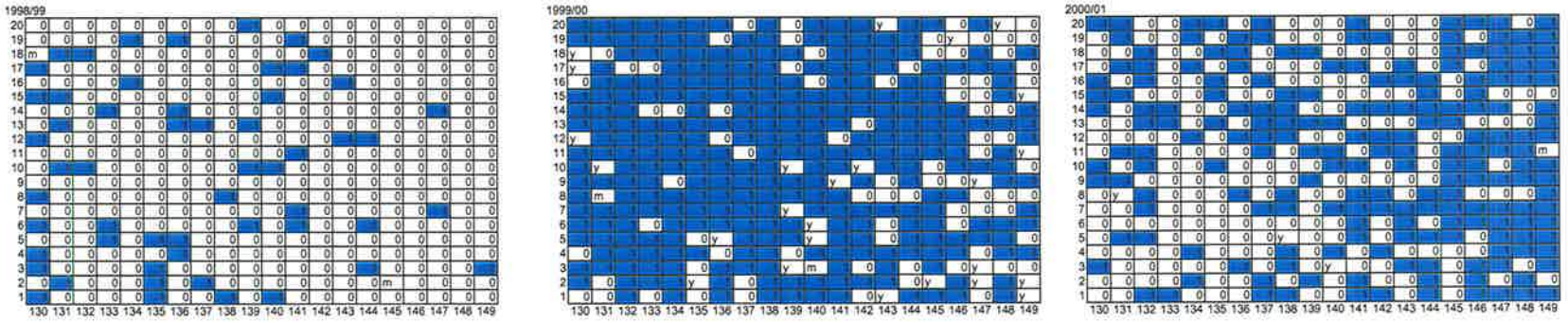


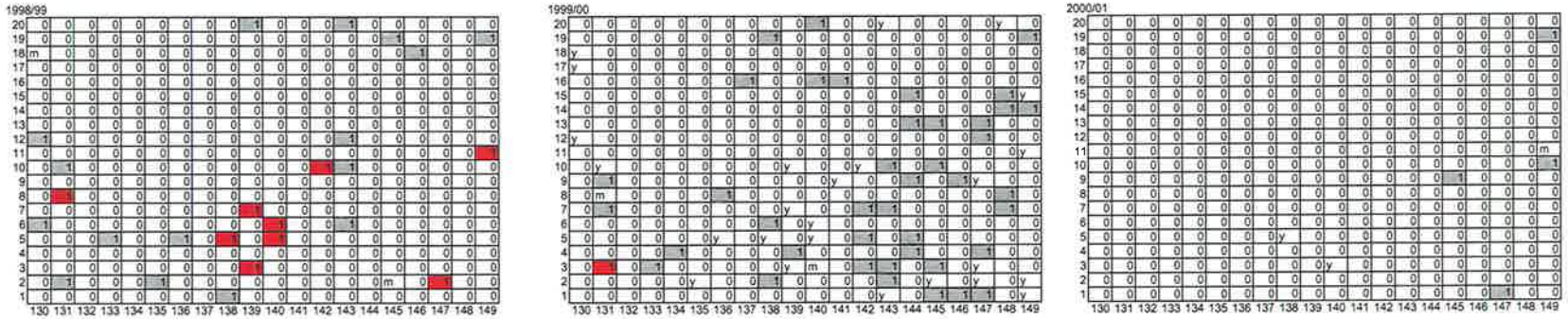
Wemen: Distribution of LSLCd affected grapevines mild LSLCd severe LSLCd 0 = unaffected m =missing



Colignan: Distribution of AGYd affected grapevines AGYd affected 0 = unaffected m =missing

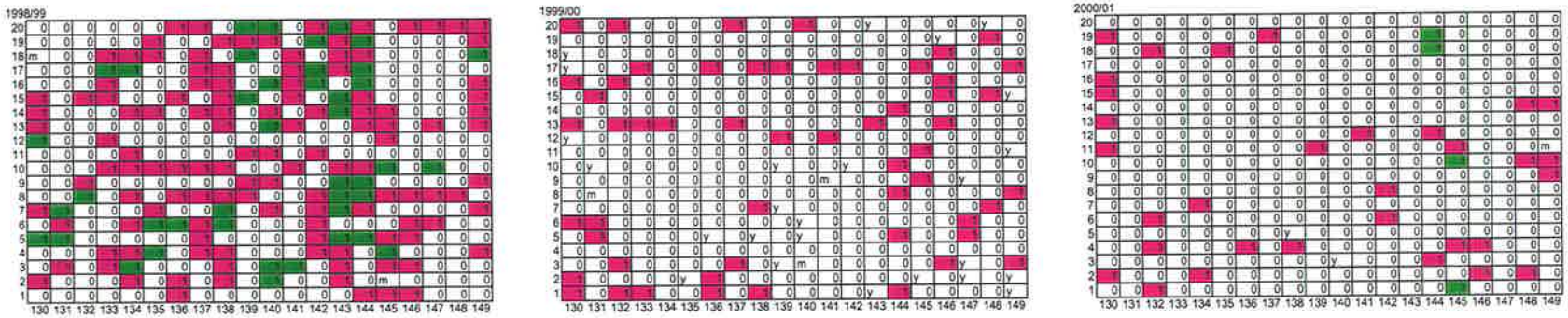



Colignan: Distribution of RGd affected grapevines mild RGd severe RGd 0 = unaffected m = missing y = young



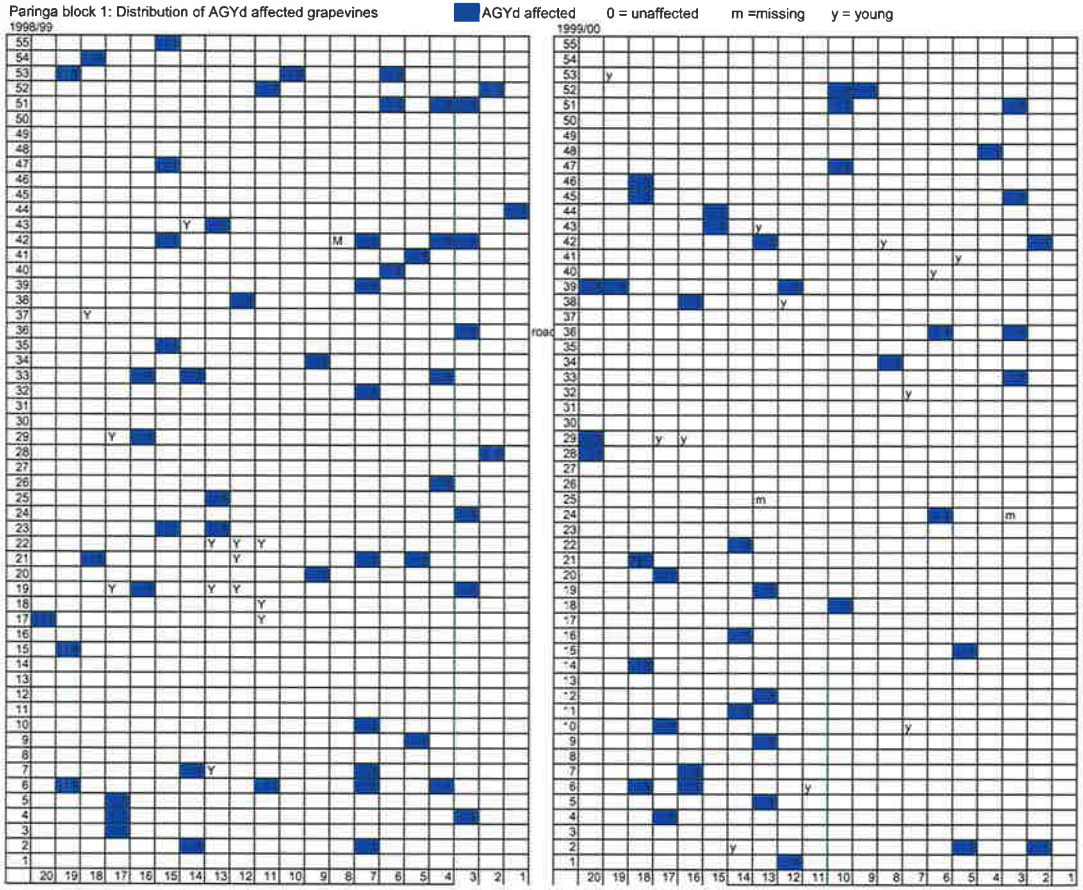
Colignan: Distribution of LSLCd affected grapevines

mild LSLCd  severe LSLCd  0 = unaffected  m = missing  y = young



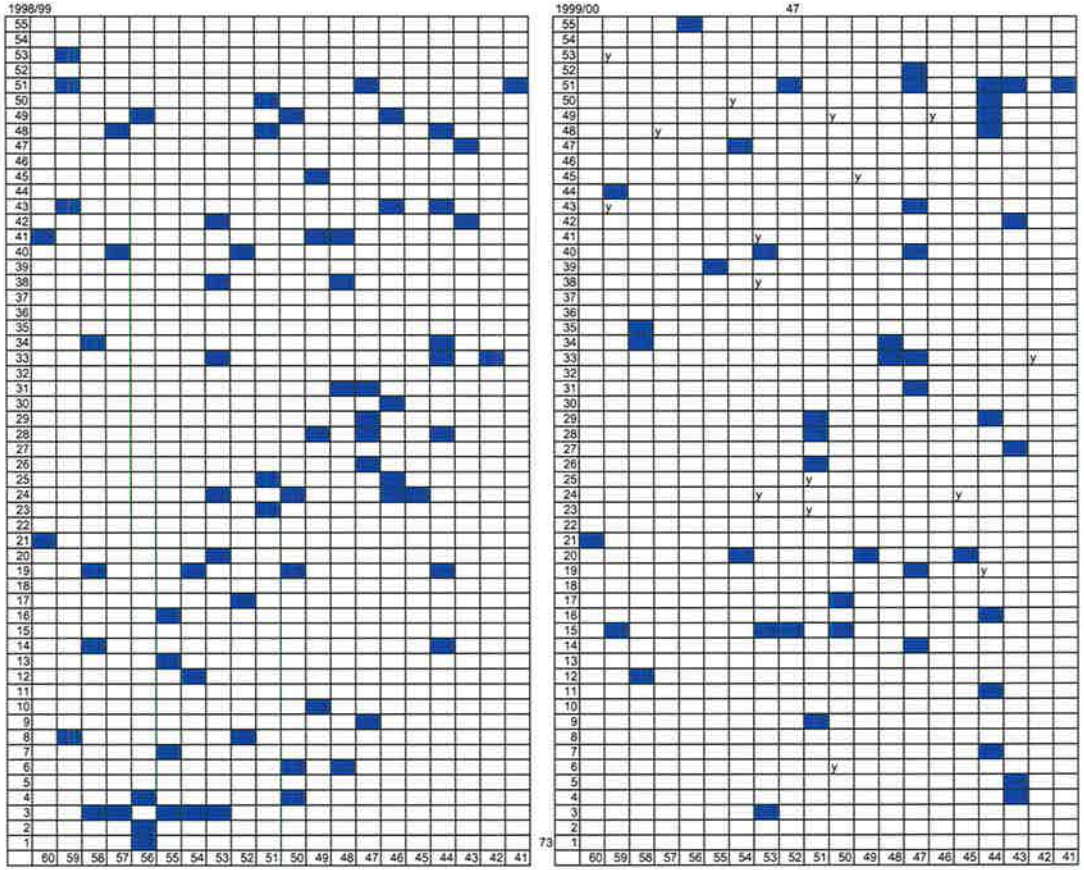
Paringa block 1: Distribution of AGYd affected grapevines

AGYd affected  0 = unaffected  m =missing  y = young



Paringa block 2: Distribution of AGYd affected grapevines

AGYd affected  0 = unaffected  m =missing  y = young



Buckland Valley site 1: Distribution of GYd affected grapevines ■ GYd affected 0 = unaffected m =missing

1997/98

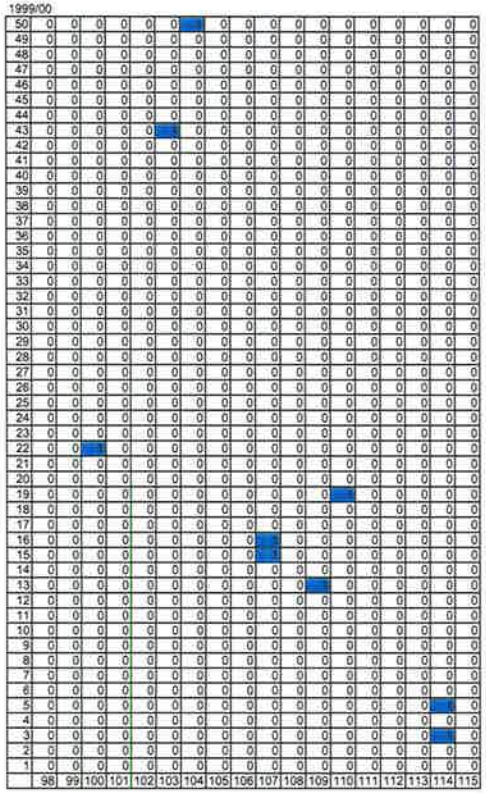
| | 98 | 99 | 100 | 101 | 102 | 103 | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 112 | 113 | 114 | 115 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 49 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 42 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 37 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 34 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 33 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 31 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 29 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 |

1998/99

| | 98 | 99 | 100 | 101 | 102 | 103 | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 112 | 113 | 114 | 115 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 49 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 |
| 44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 |
| 42 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ |
| 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 |
| 37 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 34 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | ■ | 0 | 0 | 0 | 0 | 0 |
| 33 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 31 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | ■ | 0 |
| 29 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 |
| 28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 |
| 24 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | ■ | ■ | 0 | 0 | 0 | ■ | 0 | 0 | ■ | 0 | 0 | ■ |

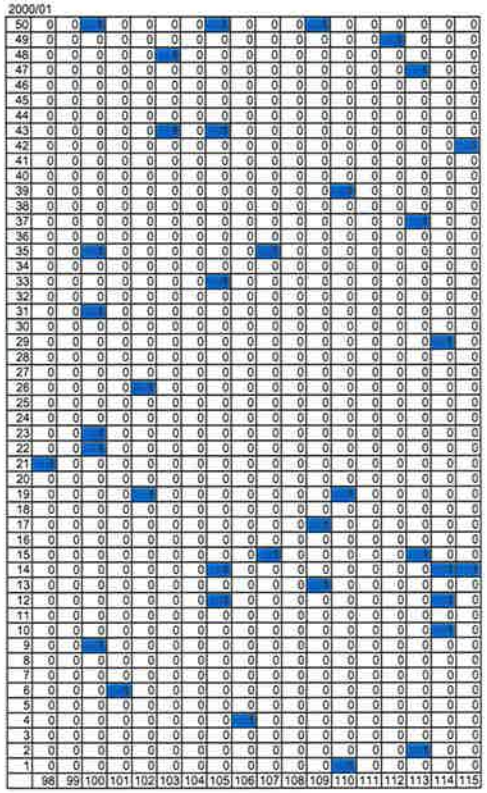
Buckland Valley site 1: Distribution of GYd affected grapevines GYd affected 0 = unaffected m =missing

1999/00



| | 98 | 99 | 100 | 101 | 102 | 103 | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 112 | 113 | 114 | 115 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 49 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 42 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 37 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 34 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 33 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 31 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 29 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

2000/01



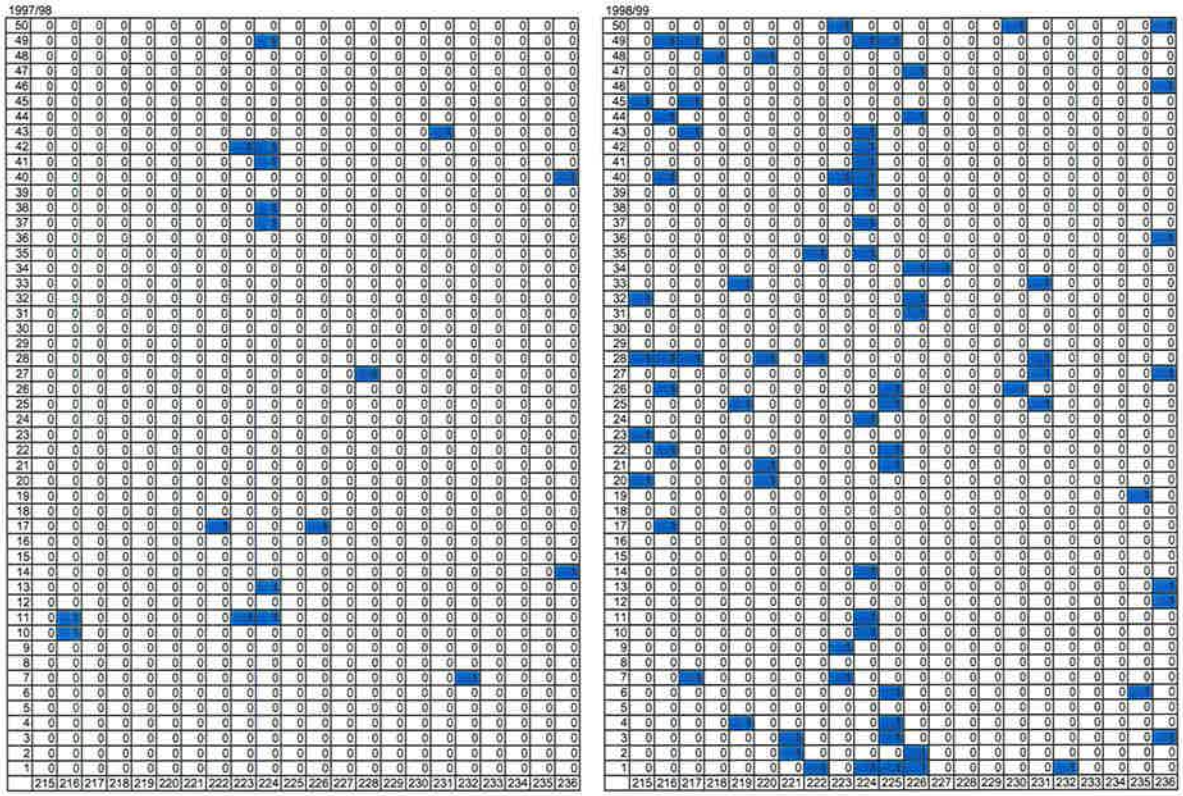
| | 98 | 99 | 100 | 101 | 102 | 103 | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 112 | 113 | 114 | 115 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 0 | 0 | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 |
| 49 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 |
| 48 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 |
| 46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43 | 0 | 0 | 0 | 0 | 0 | | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 42 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 |
| 38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 37 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 |
| 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 34 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 33 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 31 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 29 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 |
| 28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 |
| 9 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 |

Buckland Valley site 2: Distribution of GYd affected grapevines

GYd affected

0 = unaffected

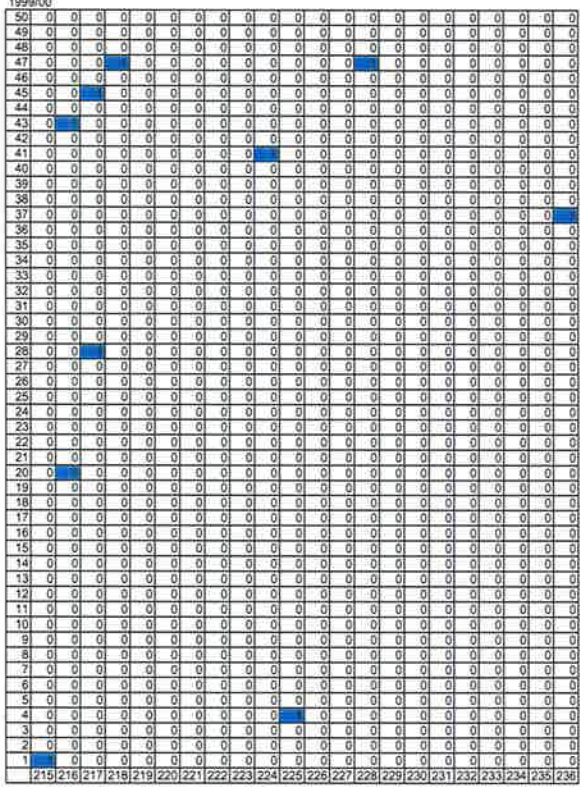
m =missing



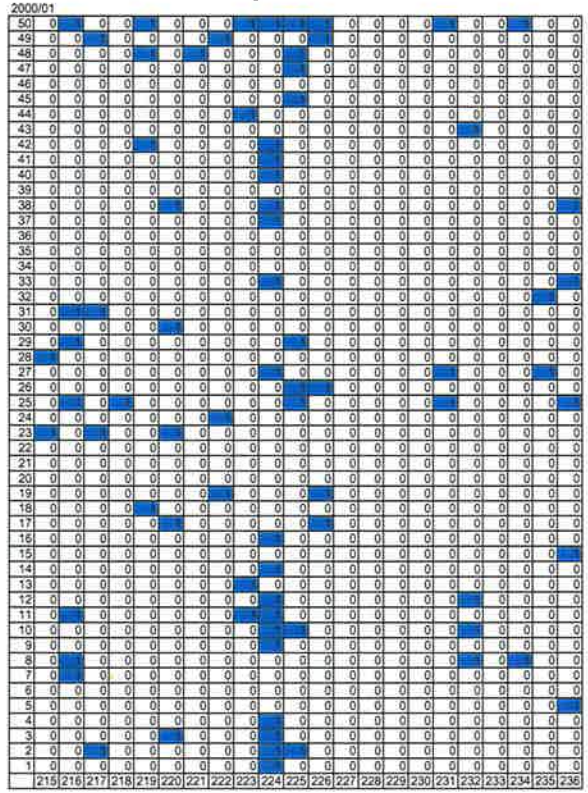
Buckland Valley site 2: Distribution of GYd affected grapevines

GYd affected  0 = unaffected  m =missing

1999/00

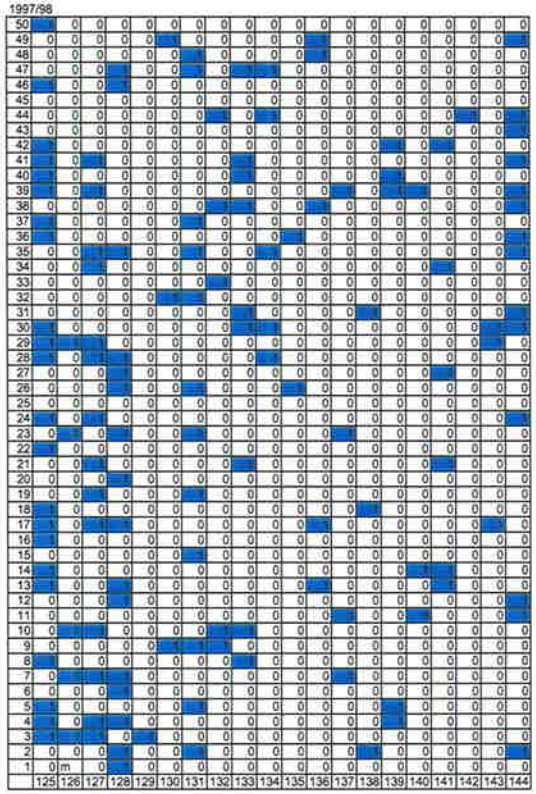


2000/01

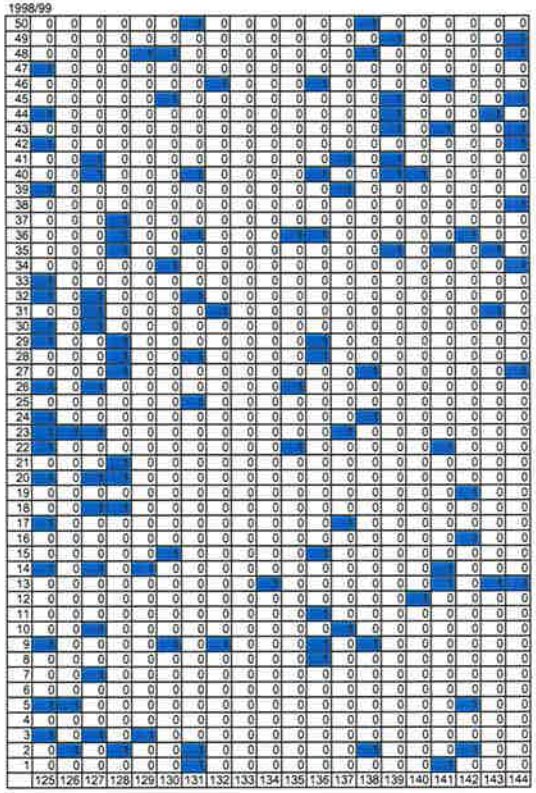


Buckland Valley site 3: Distribution of GYd affected grapevines ■ GYd affected 0 = unaffected m =missing

1997/98

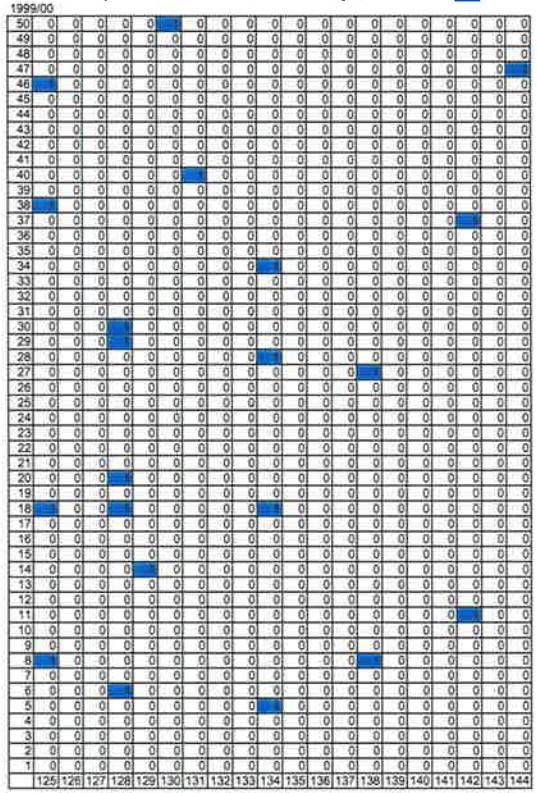


1998/99

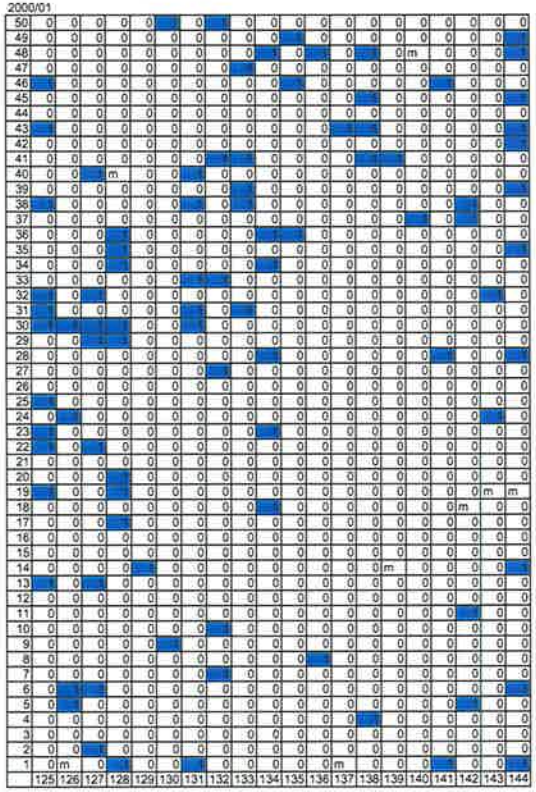


Buckland Valley site 3: Distribution of GYd affected grapevines ■ GYd affected 0 = unaffected m =missing

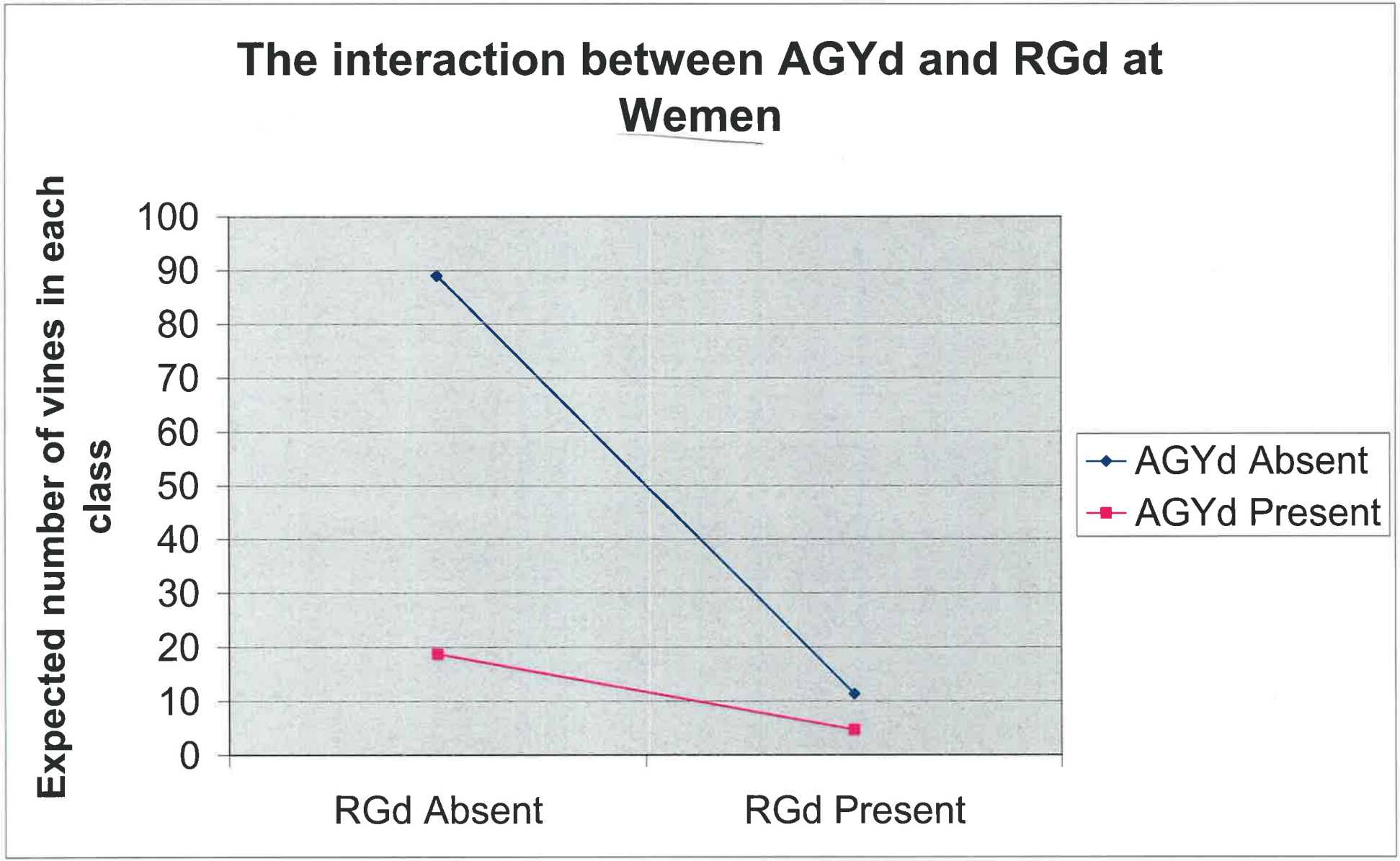
1999/00

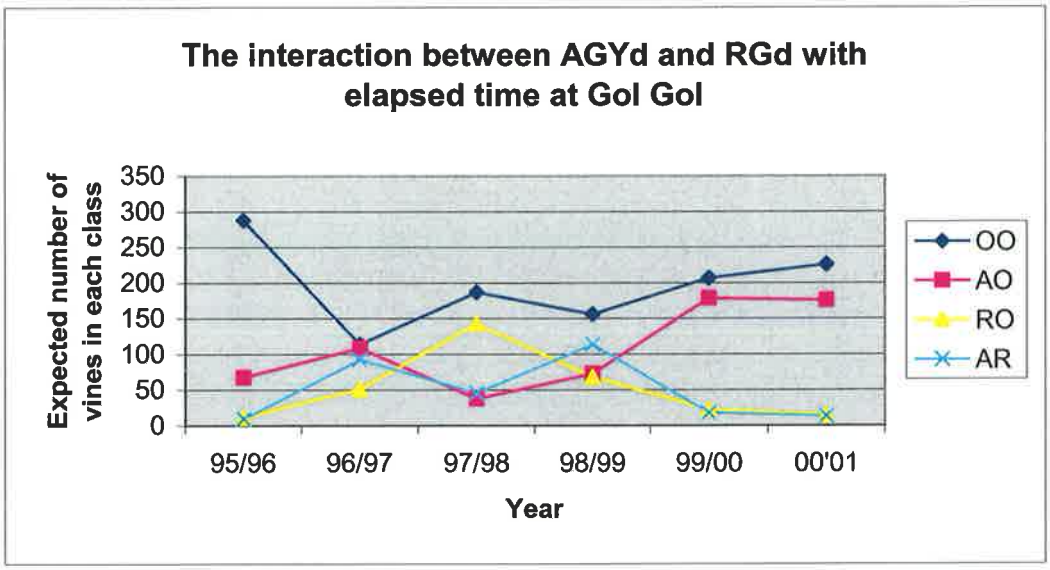


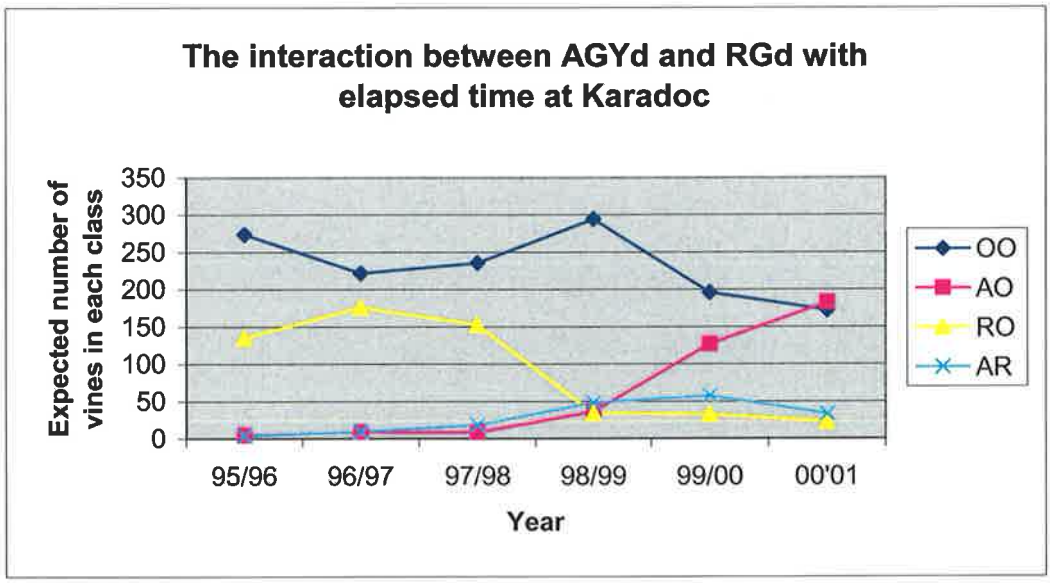
2000/01

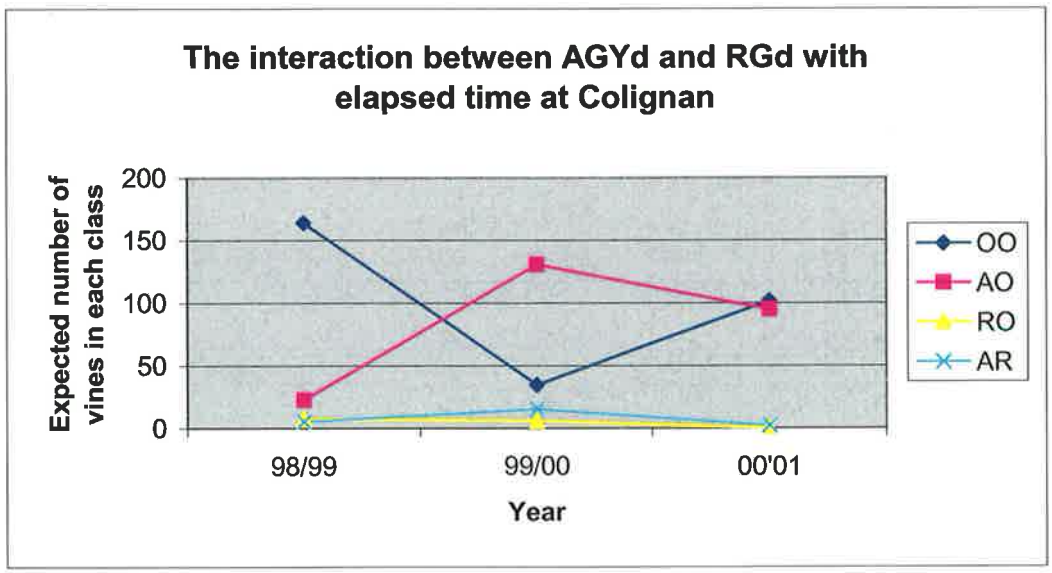


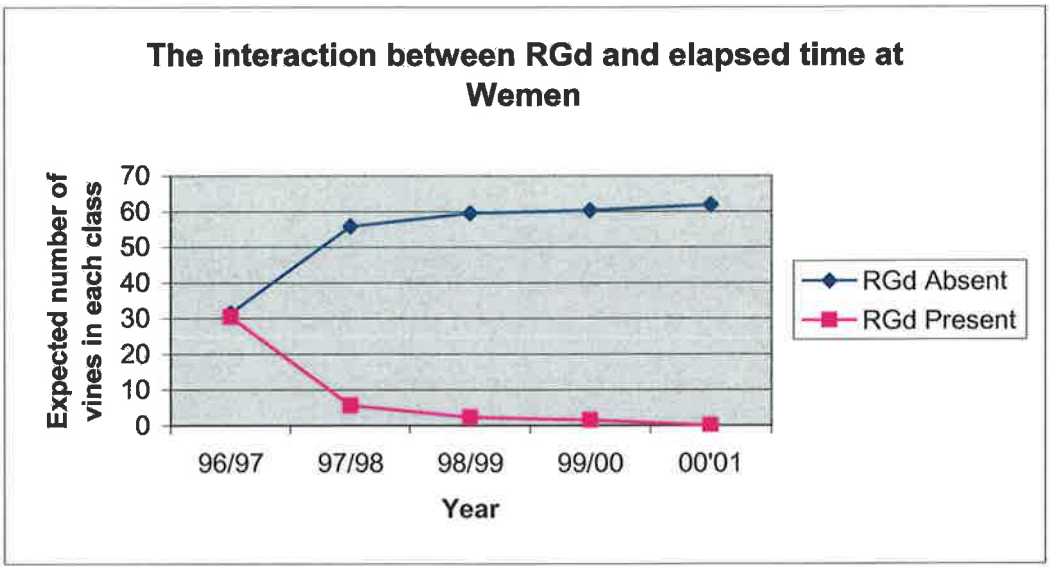
# APPENDIX B:
# LOG LINEAR GRAPHS

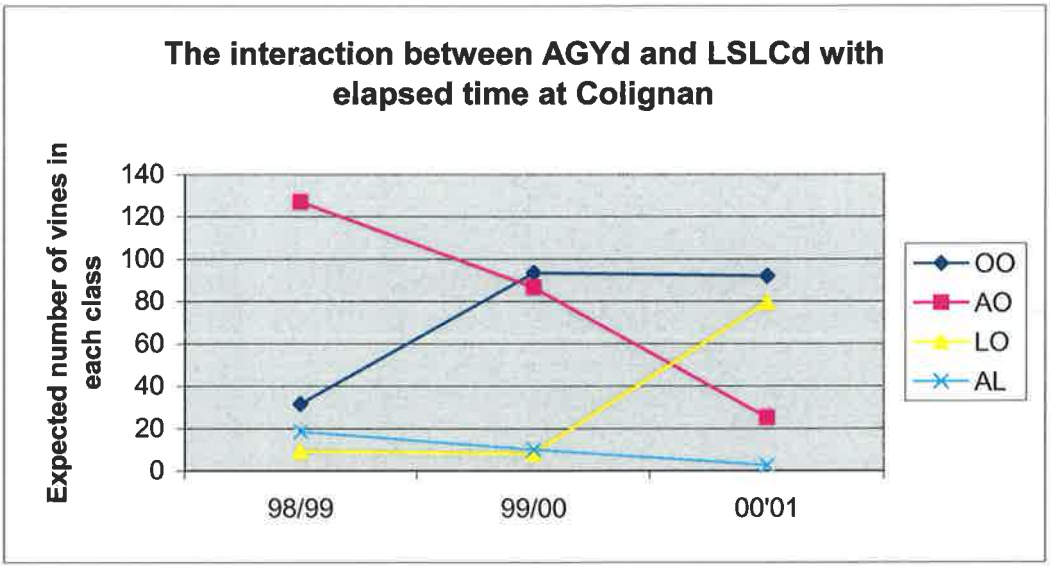

The interaction between AGYd and RGd at Wemen
Expected number of vines in each class
100
90
80
70
60
50
40
30
20
10
0
RGd Absent
RGd Present
AGYd Absent
AGYd Present

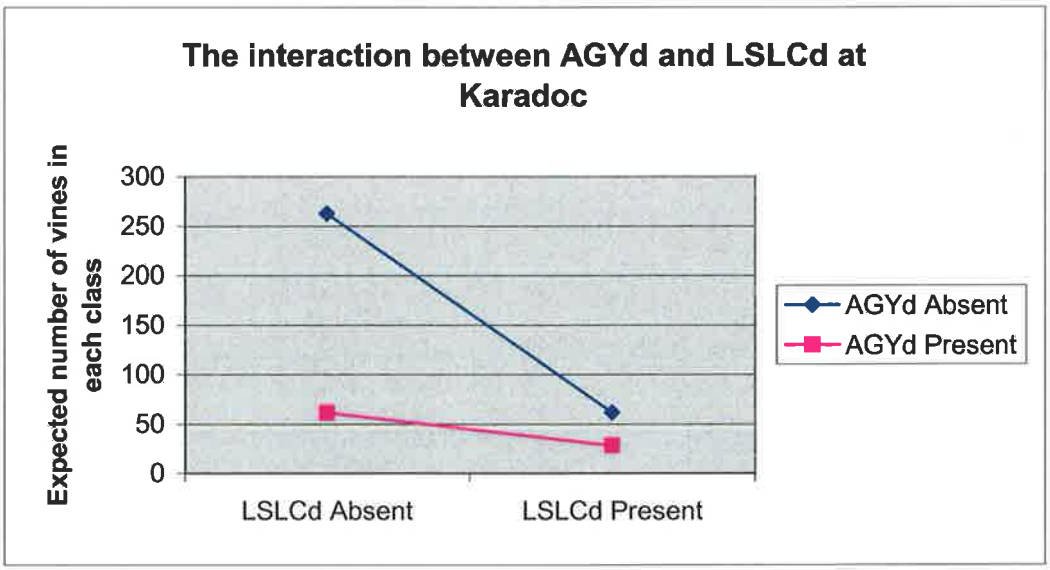

The interaction between AGYd and RGd with elapsed time at Gol Gol
Expected number of vines in each class
350
300
250
200
150
100
50
0
95/96
96/97
97/98
98/99
99/00
00'01
Year
OO
AO
RO
AR

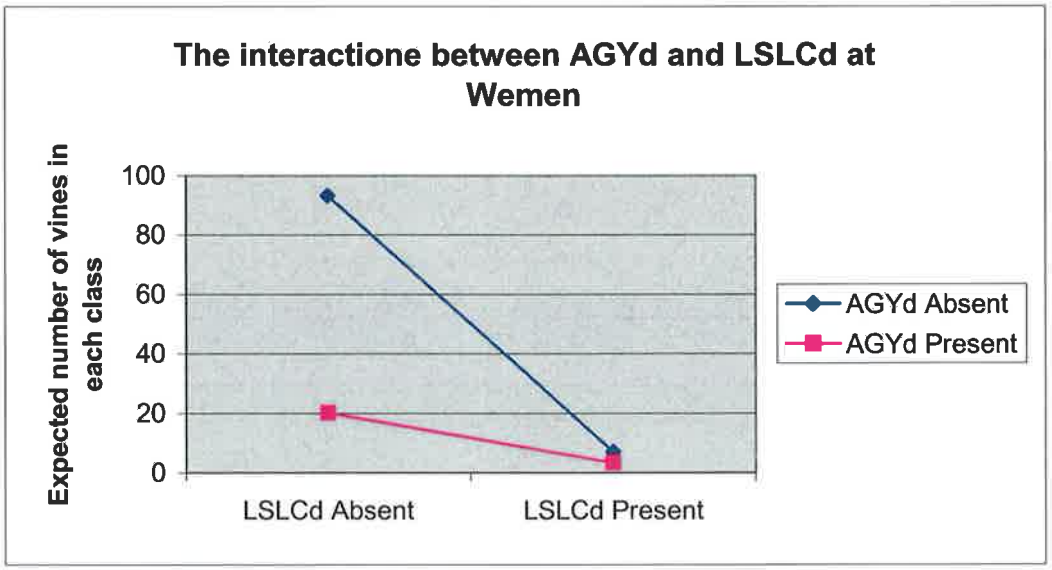

The interaction between AGYd and RGd with elapsed time at Karadoc
Expected number of vines in each class
350
300
250
200
150
100
50
0
95/96
96/97
97/98
98/99
99/00
00'01
Year
OO
AO
RO
AR

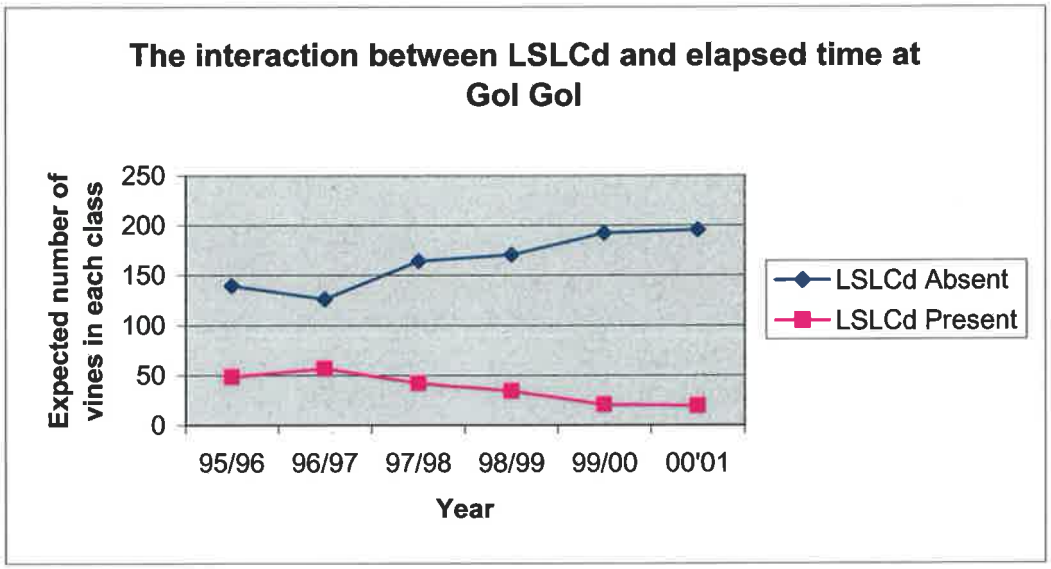

The interaction between AGYd and RGd with elapsed time at Colignan
Expected number of vines in each class
200
150
100
50
0
98/99
99/00
00'01
Year
OO
AO
RO
AR

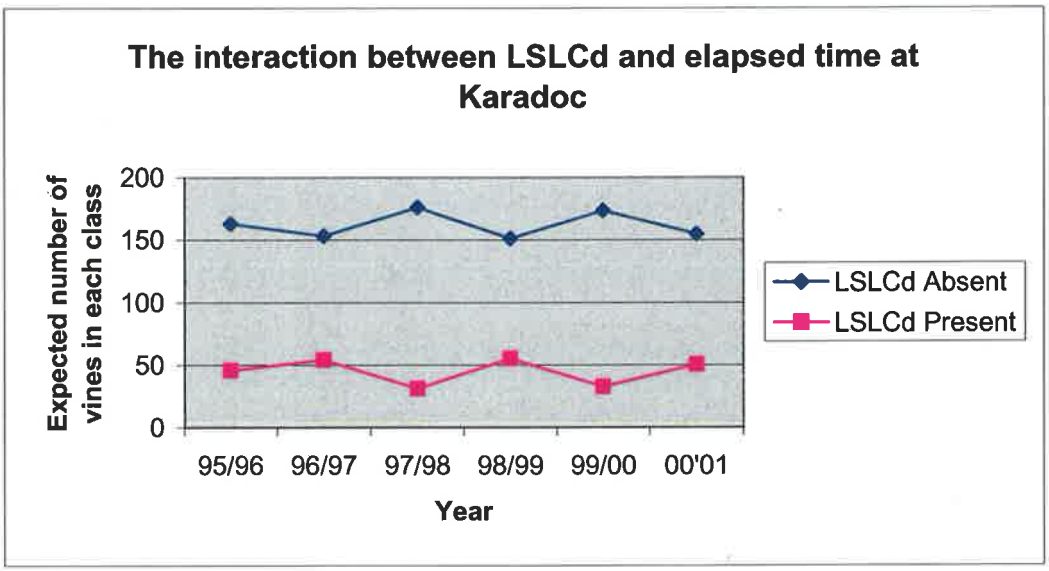

The interaction between RGd and elapsed time at Wemen
Expected number of vines in each class
70
60
50
40
30
20
10
0
96/97
97/98
98/99
99/00
00'01
Year
RGd Absent
RGd Present

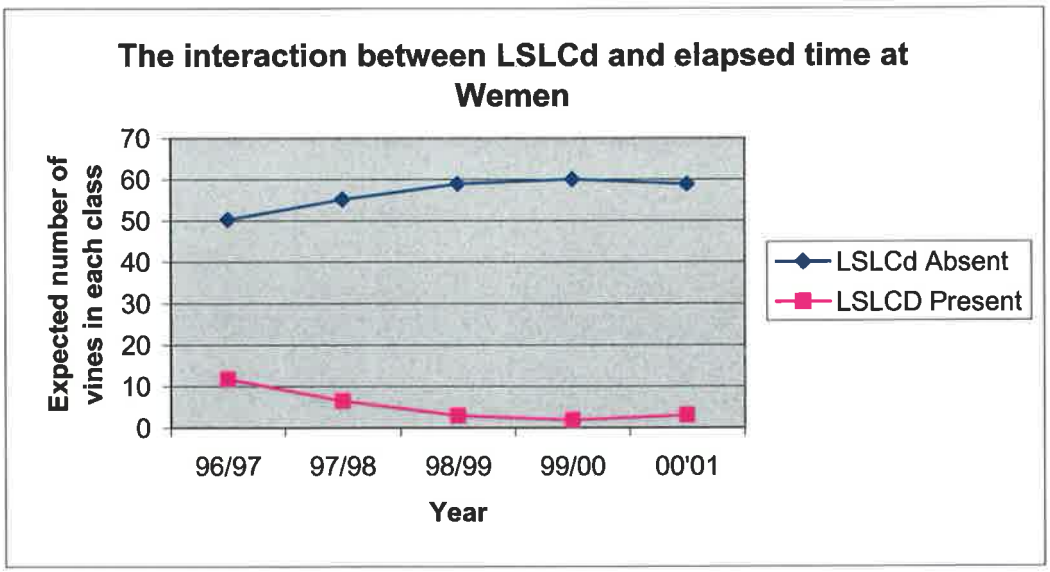

The interaction between AGYd and LSLCd with elapsed time at Colignan
Expected number of vines in each class
140
120
100
80
60
40
20
0
98/99
99/00
00'01
OO
AO
LO
AL

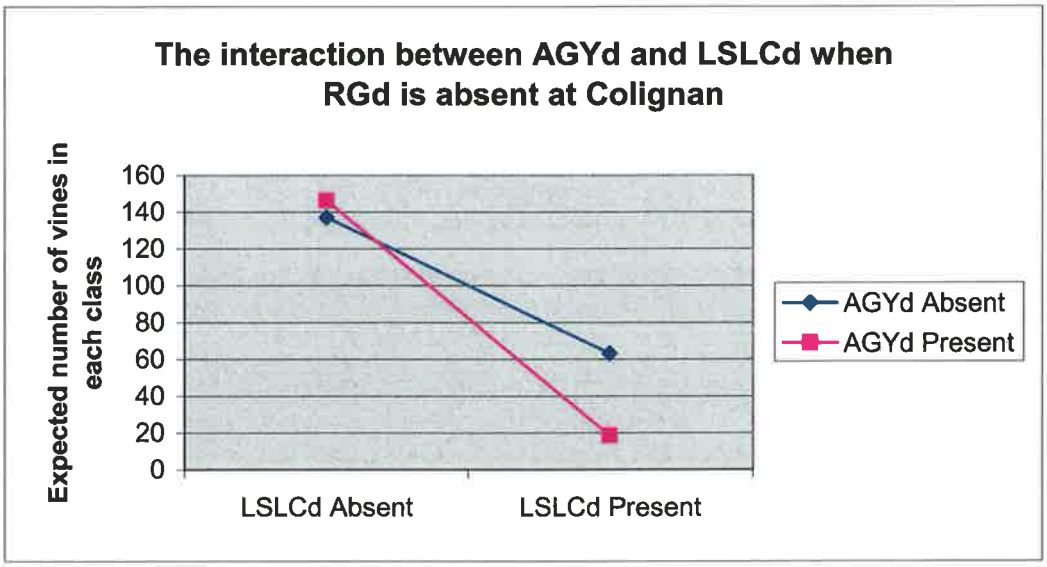

The interaction between AGYd and LSLCd at Karadoc
Expected number of vines in each class
300
250
200
150
100
50
0
LSLCd Absent
LSLCd Present
AGYd Absent
AGYd Present

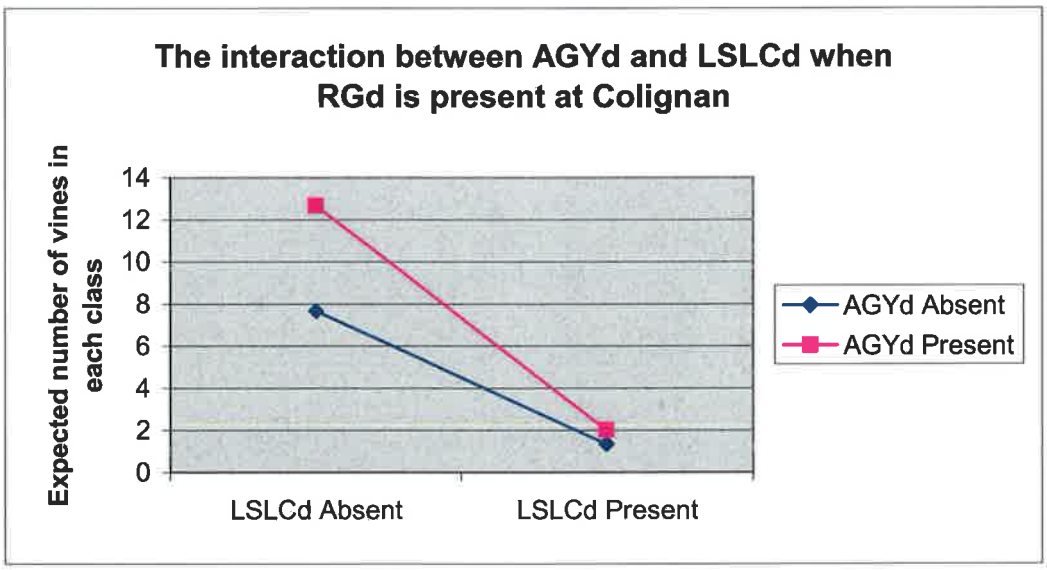

The interactione between AGYd and LSLCd at Wemen
Expected number of vines in each class
100
80
60
40
20
0
LSLCd Absent
LSLCd Present
AGYd Absent
AGYd Present

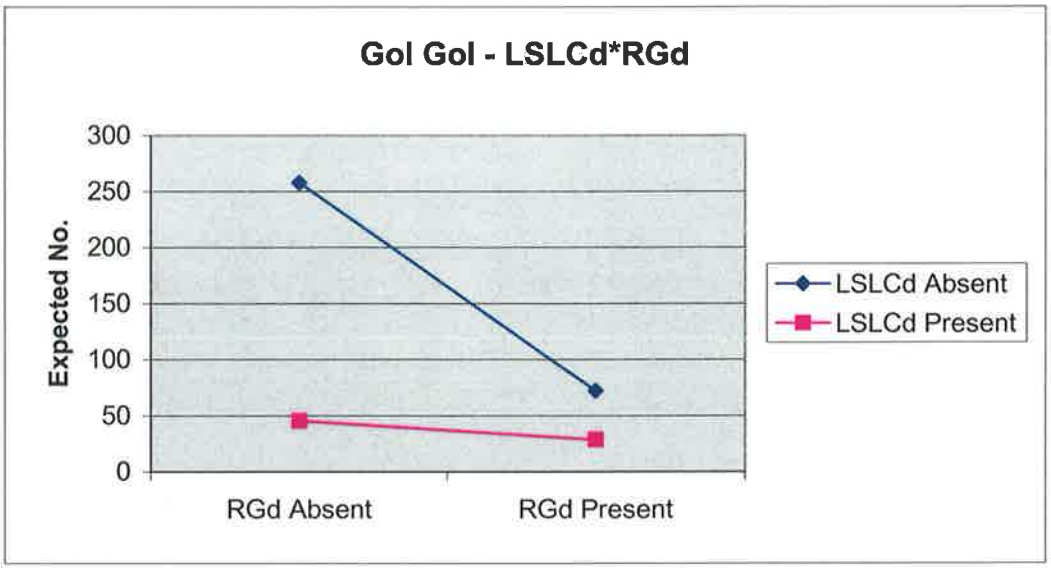

The interaction between LSLCd and elapsed time at Gol Gol
Expected number of vines in each class
250
200
150
100
50
0
95/96
96/97
97/98
98/99
99/00
00'01
Year
LSLCd Absent
LSLCd Present

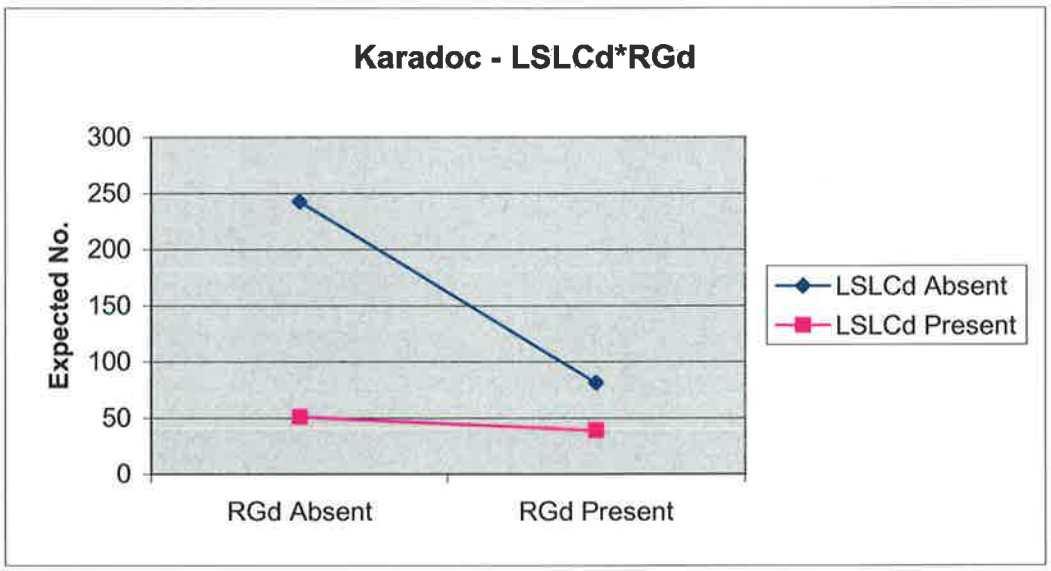

The interaction between LSLCd and elapsed time at Karadoc
Expected number of vines in each class
200
150
100
50
0
95/96
96/97
97/98
98/99
99/00
00'01
Year
LSLCd Absent
LSLCd Present


The interaction between LSLCd and elapsed time at Wemen
Expected number of vines in each class
70
60
50
40
30
20
10
0
96/97
97/98
98/99
99/00
00'01
Year
LSLCd Absent
LSLCD Present


The interaction between AGYd and LSLCd when RGd is absent at Colignan
Expected number of vines in each class
160
140
120
100
80
60
40
20
0
LSLCd Absent
LSLCd Present
AGYd Absent
AGYd Present


The interaction between AGYd and LSLCd when RGd is present at Colignan
Expected number of vines in each class
14
12
10
8
6
4
2
0
LSLCd Absent
LSLCd Present
AGYd Absent
AGYd Present


Gol Gol - LSLCd*RGd
Expected No.
300
250
200
150
100
50
0
RGd Absent
RGd Present
LSLCd Absent
LSLCd Present


Karadoc - LSLCd*RGd
Expected No.
300
250
200
150
100
50
0
RGd Absent
RGd Present
LSLCd Absent
LSLCd Present