

# The incidence and distribution of grapevine yellows disease in South African vineyards

by  
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## ABSTRACT

South Africa is ranked eighth in the world as far as international wine production is concerned and in terms of area under bearing vines South Africa is ranked 12<sup>th</sup>. In 2011 the wine industry contributed R4 204.4 million to the South African economy in state revenue from wine products. The importance of viticulture to the economy of South Africa forces the industry to limit the effect of all disease causing pathogens in order to keep their competitive edge. Aster yellows (AY) phytoplasma 16Srl-B subgroup was reported for the first time in grapevine (*Vitis vinifera* L. (Vitaceae)) in South Africa in 2006. Worldwide phytoplasma diseases of grapevine cause serious damage ranging from lower yields to the death of vines. The lack of knowledge about the epidemiology of AY disease makes it difficult to determine the impact of the disease on the South African wine industry.

The aim of this study was to conduct surveys in disease-affected vineyards in the Vredendal region to determine the incidence and spatial distribution of the disease in a variety of cultivars. The field surveys based on visual symptoms of AY disease were confirmed by polymerase chain reaction (PCR). A survey was also conducted in and around AY-infected vineyards in search of possible alternative host plants of the phytoplasma. Spatial distribution of AY-affected vines were analysed using the PATCHY spatial analysis package.

A rapid decline of AY-affected Chardonnay eventually leading to the death of vines was observed, confirming the sensitivity of Chardonnay towards grapevine yellows infections. Symptomless AY infections occurred and AY could not be detected in all symptomatic vines, which indicate uneven distribution of AY in individual vines. Molecular analyses using PCR-RFLP showed that all vines sampled in the Vredendal vicinity contained AY phytoplasma only. No phytoplasmas were present in any weeds or other possible host plants tested.

Although the mean yearly disease incidences of Chardonnay (29.95%) and Chenin blanc (16.64%) were higher than Pinotage (5.80%) over the four-year survey period, there was

no statistically significant difference between the disease incidences of these three cultivars. The mean yearly disease incidence showed a trend over time and the disease incidence of the first year was significantly lower than that of the other years. Chardonnay showed a cumulative disease incidence of 37.77% at the end of the 4-year study which means that Chardonnay vineyards can be 100% AY infected in ten years' time. Spatial distribution patterns of AY-infected vines were mostly non-random with clustering of disease affected vines along and across vine rows. With the exception of one vineyard, aggregation of AY-affected vines mostly occurred on the edge of vineyards adjacent to infected vineyards.

This epidemiological study gives an indication of the sensitivity of the different cultivars towards AY, the tempo of spreading and the future impact of the disease on the South African wine industry. It also contributes valuable information towards the development of a management strategy for grapevine yellows disease in South African vineyards.

## OPSOMMING

Suid-Afrika is op agtste op die wêreld ranglys wat internasionale produksie van wyn aan betref, en in terme van oppervlakte onder wingerd, is Suid-Afrika 12de. In 2011 het die wynbedryf R4 204.4 miljoen tot die Suid-Afrikaanse ekonomie bygedra in staats inkomste uit wyn produkte. Die belangrikheid van wingerd tot die ekonomie van Suid-Afrika dwing die bedryf om die effek van alle siekteveroorsoekende patogene te beperk, om sodoende hul kompeterende voordeel te behou. Aster vergeling (AY) fitoplasma 16Srl-B subgroep is vir die eerste keer in 2006 in wingerd (*Vitis vinifera* L. (Vitaceae)) in Suid-Afrika waargeneem. Fitoplasma siektes van wingerd veroorsaak wêreldwyd ernstige skade wat wissel van laer opbrengste tot die afsterf van wingerdstokke. Die gebrek aan kennis oor die epidemiologie van astervergeling siekte maak dit moeilik om die impak van die siekte op die Suid-Afrikaanse wynbedryf te bepaal.

Die doel van hierdie studie was om 'n opname te maak in siekte geaffekteerde wingerde in die Vredendal omgewing om sodoende siekte voorkoms en verspreidingspatrone van die siekte in 'n verskeidenheid van kultivars te bepaal. Die veld opnames, gebaseer op visuele simptome van aster vergeling siekte, was bevestig deur polimerase kettingreaksie (PKR). 'n Opname is ook in en om aster vergeling geaffekteerde wingerde uitgevoer, op soek na moontlike alternatiewe gasheer plante van die fitoplasma. Verspreidingspatrone van astervergeling geaffekteerde wingerde is ontleed met behulp van die PATCHY ruimtelike analise pakket.

'n Vinnige agteruitgang van AY geaffekteerde Chardonnay, wat uiteindelik gelei het tot die afsterf van wingerde, is waargeneem, wat die sensitiwiteit van Chardonnay teenoor wingerdvergeling infeksie bevestig. Simptoomlose astervergeling fitoplasma infeksies kom voor en astervergeling fitoplasma kon nie opgespoor word in alle simptomatiese wingerdstokke nie, wat op oneweredige verspreiding van AY fitoplasma in individuele wingerdstokke dui. Molekulêre ontledings met behulp van PKR-RFLP het getoon dat alle wingerdstokke, wat in die Vredendal omgewing getoets is, slegs astervergeling fitoplasma

bevat. Geen fitoplasmas was teenwoordig in enige onkruid of ander moontlike gasheer plante.

Hoewel die gemiddelde jaarlikse siekte voorkoms van Chardonnay (29,95%) en Chenin Blanc (16,64%) oor die vier-jaar opname periode hoër was as dié van Pinotage (5,80%), was daar geen statisties beduidende verskil tussen die siekte voorkoms van hierdie drie kultivars nie. Die gemiddelde jaarlikse siekte voorkoms het 'n tendens oor tyd getoon, en die siekte voorkoms van die eerste jaar was betekenisvol laer as dié van die ander jare. Chardonnay het 'n kumulatiewe siekte voorkoms van 37.77% aan die einde van die 4-jaar studie getoon, wat beteken dat Chardonnay wingerde binne 10 jaar 100% besmet kan wees met AY. Verspreidingspatrone van AY geaffekteerde wingerdstokke was meestal nie-ewekansig met bondeling van geaffekteerde wingerdstokke in en oor wingerd rye. Bondeling van AY geaffekteerde wingerdstokke het, met die uitsondering van een wingerd, meestal op die kant van wingerde aanliggend aan besmette wingerde, voorgekom.

Die epidemiologiese studie gee 'n aanduiding van die sensitiwiteit van die verskillende kultivars ten opsigte van AY, die tempo van die verspreiding en die toekomstige impak van die siekte op die Suid-Afrikaanse wynbedryf. Dit dra ook waardevolle inligting by tot die ontwikkeling van 'n strategie vir die bestuur van wingerdvergelings siekte in Suid-Afrikaanse wingerde.

## ABBREVIATIONS

AAP	acquisition access period
AGY	Australian grapevine yellows
APIS	Agricultural Product Inspection Services
AY	aster yellows
AY-WB	aster yellows phytoplasma strain witches' broom
BN	Bois noir
bp	base pair
BVGY	Buckland Valley grapevine yellows
Ca	<i>Candidatus</i>
CFIA	Canadian Food Inspection Agency
CTAB	cetyl-trimethyl-ammonium bromide
cv	cultivar
CYP	chrysanthemum yellows phytoplasma
DAPI	4', 6-diamidino-2-phenylindole
ELISA	enzyme-linked immunosorbent assay
EY	elm yellows
FD	Flavescence dorée
GLM	General Linear Models
GNA	<i>Galanthus nivalis</i> agglutinin
GY	grapevine yellows
GDP	gross domestic product
HWT	hot water treatment
ICSB	International Committee of Systematic Bacteriology
ISEM	immunosorbent electron microscopy
LAMP	loop-mediated isothermal amplification
LP	latent period
MAbs	monoclonal antibodies
MLOs	mycoplasma-like organisms
NAGY	North American grapevine yellows

NCBI	National Center for Biotechnology Information
nPCR	nested PCR
OY-M	onion yellows phytoplasma strain M
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
RFLP	restriction fragment length polymorphisms
rRNA	ribosomal RNA
SAWIS	SA Wine Industry Information & Systems
SLY	strawberry lethal yellows
TE	Tris EDTA
THRIP	Technology and Human Resources for Industry Programme
V/M	variance to mean ratio
WB	witches' broom
WL	white leaf
Y	yellows

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***“Chemical industry and plant breeders have forged fine technical weapons, but only epidemiology sets the strategy”***

Vanderplank (1963)



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# 1 INTRODUCTION

## 1.1 General introduction

The South African wine industry makes a significant contribution to the South African economy. This agricultural industry went from strength to strength with exports growing by 253% between 2000 and 2011. The most recent statistics by the SA Wine Industry Information & Systems (SAWIS) showed that R4 204.4 million was generated in state revenue in 2011 from wine products (SA Wine Industry Statistics nr 36, 2012). In 2008 some 275 600 people were employed in the wine industry according to a study by SAWIS. The wine industry contributed approximately R26.2 billion in gross domestic product (GDP) to the regional economy, of which R4.3 billion was generated through wine-tourism in the Western Cape wine area. Currently 3 527 farmers cultivate 100 568 hectares of land under vines. However, from 2006 the total area under vines showed a steady decrease. This could be because vines are no longer profitable due to age, disease or that a cultivar is no longer in demand for winemaking. It is clear that wine farmers are forced to increase yields on the decreasing vineyard area to survive financially, which is detrimental to fruit quality and the lifespan of the vines. Fruit quality is a prerequisite for international market access and competitiveness. As far as international wine production is concerned, South Africa is ranked eighth with 3.6% (966 500 000 liter) of the total and in terms of area under bearing vines South Africa is ranked 12<sup>th</sup> in the world. The majority of wine grapes in South Africa are planted in the Western Cape Province. In the Northern Cape wine grapes are also planted in the Orange River area and near Douglas (Figure 1).



**Figure 1.** Wine Regions of South Africa (<http://www.wineanorak.com/safricamap.htm>)

Grapevine yellows (GY) is a disease induced by phytoplasmas that cause severe symptoms on a variety of *Vitis vinifera* cultivars worldwide. It occurs in the viticulture areas of Europe, North and South America and Australia. All yellows diseases are almost identical in symptom expression but differ with respect to the susceptibility of cultivars and epidemiology (Martelli et al., 2006). Chardonnay is very susceptible to all phytoplasma diseases. Infected vines show severe symptoms and ultimately die. The most important phytoplasma diseases of grapevine are Flavescence dorée (FD), Bois noir (BN), Australian grapevine yellows (AGY) and Aster Yellows (AY). FD is transmitted from vine to vine by insect vectors and spreads epidemically. An increase in incidence from 5% to 30% was recorded over 4 years in Italy (Angelini, 2008). BN on the other hand is transmitted from weeds to vine by vectors and not from vine to vine. This disease has an endemic behaviour and the sanitary situation in a vineyard is usually stable year after year. Bois noir is distributed more or less randomly in a vineyard and the spreading is very slow (Magarey, 1986). AGY, for which a vector has not yet been identified, showed an increase in the number of symptomatic vines from 1.6% in the first year to 16.8% in the second year (Bonfiglioli et al., 1997). With AGY, remission of the disease was observed in

some vines, while the disease recurred in other previously unaffected Chardonnay vines (Constable et al., 2004). Disease incidence fluctuated in vineyards from year to year due to remission of the disease and new infections that were recorded. Beanland et al. (2006) found in an eight year study on two northern Virginia vineyards that vines infected with AY and X-disease were non-randomly distributed and clusters occurred near vineyard edges bordering woodlands. Disease incidences in these 2 vineyards varied between 1.5% and 6.3% and the disease incidence of Chardonnay seemed to be higher than that of Riesling and Sauvignon blanc, although it could not be evaluated statistically.

Worldwide phytoplasmas are transmitted by phloem-feeding insect vectors particularly leafhoppers, planthoppers and psyllids (Maixner et al., 1993; Maixner et al., 2006). Literature showed that the distribution of phytoplasma-infected plants can give more information on the source of disease inoculum, the source of insect vectors as well as the identity and behaviour of phytoplasma transmitting insects (Weintraub & Beanland, 2006). These distribution patterns can also give more information on the mode of transmission that took place. For example, a patchy or clustered distribution indicates vine-to-vine transmission (Wolf, 2000), while a gradient from one side to the other indicates transmission from weeds next to the vineyard or from an adjacent infected vineyard. A random distribution may indicate transmission of the disease by planting material. Several programmes like two-dimensional distance class (2DCLASS) (Nelson et al., 1992; Uyemoto et al., 1998) and PATCHY (Maixner, 1993) are available to analyse spatial distribution and determine if diseased vines are clustered or randomly distributed.

A mixed infection of phytoplasmas (16SrXII-A and 16SrII-B) was reported to be present in South African grapevines (Botti et al., 2006a). Aster yellows phytoplasma (AY, 16SrI-B subgroup) was however officially recorded for the first time in grapevine (*Vitis vinifera* L. (Vitaceae)) in South Africa in 2006 (Engelbrecht et al., 2010). Initially the disease occurred on grapevines in two wine producing areas only, Vredendal (Olifants River) and Wabooms River area (Breedekloof) (Burger, 2008), but recently it was also found at three other localities namely Robertson, Trawal (Olifants River) and Montagu (Klein Karoo) in the Western Cape Province. An insect vector, *Mgenia fuscovaria* (Stal), was identified (Douglas-Smit et al., 2010). Visual symptom observations (Jeff Joubert, Vinpro Consultant

Vredendal, personal communication) indicated that the disease has spread and that it covers an area of approximately 107ha in the Vredendal area. In the other areas only a few vineyards are affected by the disease.

Worldwide phytoplasma diseases of grapevine cause serious damage ranging from lower yields (20-30% yield loss, but sometimes as high as 80%) to the death of vines (Magarey, 1986). In Australia, yield loss due to AGY was estimated to be 40% (Tanne, 1996). AY disease, like all other phytoplasma diseases affects the yield negatively because bunches dry out before ripening. There is however no data available in literature on the effect of AY on yield of grapevine. Observations in two Chardonnay vineyards in Vredendal (Petrie Burger, producer Vredendal, personal communication) indicated yield losses of 29% and 30%, respectively.

The epidemiology of FD, BN, AGY and North American grapevine yellows (NAGY) have been studied extensively (Constable, 2010), but little epidemiological knowledge for AY of grapevine existed up to now.

## **1.2 Project Proposal**

The aim of this study was to conduct surveys in disease-affected vineyards in the Vredendal area to determine the incidence and distribution of the disease in a variety of cultivars. The field surveys of AY disease incidence, based on visual symptoms will be confirmed by polymerase chain reaction (PCR). Alternative hosts will be sampled and tested for phytoplasma presence by PCR-RFLP. This epidemiological study will give an indication of the tempo of spreading and the future impact of the disease on the South African wine industry. It will also contribute valuable information towards the development of a management strategy for grapevine yellows disease in South African vineyards.

## 2 LITERATURE REVIEW

### 2.1 Introduction

Phytoplasma associated plant diseases affect several hundred plant species, including economically important crops such as vegetables, fruit, ornamental plants and trees. These bacterial plant pathogens can cause disease with devastating yield losses in a wide variety of high and low value crops worldwide (Hoy et al., 1992; Lee et al., 2000; Bertaccini, 2007). In one very rare instance phytoplasma infections are used to the benefit of growers. Infected poinsettias have a bushy dwarfed growth, which is a desirable trait for producing showy multi-flowered potted poinsettia plants (Lee et al., 1997). Grapevine yellows has been an economically important disease in many countries. The best known of these phytoplasma-associated diseases are FD and BN (Boudon-Padieu et al., 1998). Bois noir, also called Legno nero, Vergilbungskrankheit or Schwarzholkkrankheit occurs widely in Europe. Other important phytoplasma diseases that affect grapevine are Australian grapevine yellows, Buckland Valley grapevine yellows (BVGY) and North American grapevine yellows (NAGY) (Boudon-Padieu, 2005). Yield losses of 20 - 74% (FD) and 11 - 15% (BN) were mentioned in literature (Magarey, 1986). Estimated crop losses of 13% (Chardonnay) and 3 - 8% (Riesling) due to AGY was reported (Magarey et al., 1983) and in 1996 Tanne estimated the crop losses in Australia to be as high as 40%. Grapevine yellows is caused by a variety of phytoplasmas which is transmitted by different insect vectors (Table 1) (Boudon-Padieu, 2005). The first occurrence of a mixed phytoplasma infection on grapevine in South Africa was reported in 2006 (Botti et al., 2006a) and shortly afterwards AY had been reported to occur on grapevine in several cultivars in the Vredendal grape producing area of South Africa (Burger, 2008; Engelbrecht et al., 2010). The vine and wine industry immediately identified research projects, which could elucidate the phytoplasma problem through their research coordinating and funding body, Winetech. State revenue of R4204.4 million was generated in 2011 from South African wine products (SA Wine Industry Statistics nr 36, 2012). The importance of viticulture and ultimately wine products to the economy of South Africa forces the industry to limit the effect of all disease causing pathogens in order to keep their competitive edge. Background information on phytoplasmas and the

associated diseases will be given in this chapter as reviewed by several authors (Magarey, 1986; Lee et al., 1992; Krake et al., 1999; Lee et al., 2000; Christensen et al., 2005; Weintraub et al., 2006; Hogenhout et al., 2008; Hogenhout, 2009; Bertaccini et al., 2009; Olivier et al., 2009; Gasparich, 2010).

**Table 1.** Current status of molecular characterization, biology and vectors of phytoplasmas associated with grapevine yellows diseases (Boudon-Padieu, 2005).

Grapevine yellows disease	Phytoplasma name	Ribosomal group (subgroup)	Known insect vector to grapevine	Preferred host plants of vector	Alternative hosts of the phytoplasma	Occurrence
Flavescence dorée	Flavescence dorée (FD; 'Candidatus Phytoplasma vitis'*)	16SrV (-C, -D) or elm yellows (EY)	Scaphoideus <i>titanus</i> Ball	<i>Vitis sp.</i>	<i>Clematis alba</i>	France, Italy, Spain, Serbia,
Palatinate grapevine yellows	Palatinate grapevine yellows (PGY)	16SrV or EY	<i>Oncopsis alni</i> Schrank	<i>Alnus glutinosa</i>		Germany
Bois noir, Legno nero, Vergilbungskrankheit, Schwarzholzkrankheit	Stolbur (STOL, 'Candidatus Phytoplasma solani'*)	16SrXII-A or stolbur	<i>Hyalesthes obsoletus</i> Signoret	<i>Convolvulus arvensis</i> . <i>Urtica dioica</i> , <i>Ranunculus spp.</i> , <i>Solanum spp.</i> , <i>Lavandula spp.</i>	<i>C. arvensis</i> , <i>U. dioica</i> , <i>Ranunculus spp.</i> , <i>Lavandula spp.</i>	Europe, Israel, Lebanon
Australian grapevine yellows	'Candidatus Phytoplasma australiense'	16SrXII-B	ND <sup>†</sup>	ND	<i>Maireana brevifolia</i>	Australia
Australian grapevine yellows	Tomato big bud (TBB)	16SrII-D	ND	ND		Australia
Buckland Valley grapevine yellows	Buckland Valley grapevine yellows (BVGY)	16SrI-related or AY-	ND	ND		Australia
Grapevine yellows	Aster yellows	16SrI (-B, -C) or AY	ND	ND		Italy, Chile
North American grapevine yellows (NAGY)	Virginia grapevine yellows 1 (NAGY1)	16SrI-A or AY	ND	ND	<i>Vitis spp.</i> , Various herbaceous hosts	Virginia (USA)
	Western X Virginia grapevine yellows III (NAGYIII)	16SrIII-I or WX	ND	ND	<i>Vitis spp.</i> , <i>Prunus spp.</i>	New York (USA), Virginia (USA)
Grapevine yellows	'Candidatus Phytoplasma fraxini'	16SrVII	ND	ND	ND	Chile
Grapevine yellows	X-disease	16SrIII	ND	ND	ND	Italy, Israel

\*Suggested *Candidatus* phytoplasma names: however, the species are still to be described

†ND = not determined.

## 2.2 Phytoplasmas

### 2.2.1 History of phytoplasma diseases

Many phytoplasma diseases showing yellows, dwarfing and witches' broom symptoms occur throughout the world. During the Tokugawa Period (1603 – 1868) mulberry dwarf disease was first observed in Japan. This phytoplasma disease spread widely and caused severe damage to mulberry plants (Okudu, 1972). Paulownia witches' broom disease, rice yellow dwarf disease and other yellows diseases have been reported since the early 1900s (Kunkel, 1926; Lee et al., 2000; Okudu, 1972). At first these yellows diseases of plants were thought to be caused by viruses because they were transmitted by insect vectors and through grafting and the symptoms were similar to those of viral diseases (Lee et al., 2000). However, no virus particles could be found in disease-affected plants. In 1967, Doi et al. discovered that wall-less prokaryotes were associated with these yellows diseases and they were called mycoplasma-like organisms (MLOs) because of their similarity with mycoplasmas. However, these MLOs could not be cultured *in vitro* (Lee et al., 1986) like other mycoplasmas. In 1993 the International Committee of Systematic Bacteriology (ICSB) replaced the name of MLO with phytoplasma (ICSB, 1993; Martelli et al., 2006).

### 2.2.2 Classification of phytoplasmas

Traditionally phytoplasmas were identified and classified according to biological properties such as the symptoms induced in plants, the range of plants that act as hosts and the specific insect vectors which transmit the phytoplasma. The establishment of PCR and modern sequencing technologies allowed partial genome sequences of many new phytoplasma strains to be determined. Phylogenetic analyses of especially the 16S ribosomal RNA (rRNA) gene, revolutionised the taxonomical classification of phytoplasmas. All phytoplasmas now comprise a single clade within the class Mollicutes (Gasparich, 2010). They are cell wall-less microbes of very small size, approximately 1-2 $\mu$ m in diameter and small genome size of 530 Kb-2220 Kb. Phytoplasmas are classified in different phylogenetic groups (I, II, III...) and subgroups (A, B, C etc.) on the basis of the sequence of their ribosomal DNA and other conserved genes. Later a new taxon, '*Candidatus* (Ca.) Phytoplasma' was proposed for these organisms (IRPCM, 2004). In this system, phytoplasmas are classified based on the nucleotide sequence of the 16S rRNA



gene (Firrao et al., 2005). A strain can be described as a novel 'Ca. Phytoplasma' species if its 16S rRNA gene sequence has less than 97.5% similarity to that of any 'Ca. Phytoplasma' species that was described previously (Hogenhout et al., 2008). Thus far, 30 Ca. species have been described and 5 more have been informally proposed as 'Ca. Phytoplasma' species (Table 2).

([http://plantpathology.ba.ars.usda.gov/pclass/pclass\\_phytoplasmaclassification\\_system2.html](http://plantpathology.ba.ars.usda.gov/pclass/pclass_phytoplasmaclassification_system2.html)).

**Table 2.** 16S rDNA RFLP group-subgroup classification and '*Candidatus* Phytoplasma' species (Dr RE Davis, United States Department of Agriculture, Phytoplasma Resource Centre).

Phytoplasma/ Disease common name <sup>1</sup>	16S rDNA group- subgroup <sup>2</sup>	GenBank no. <sup>3</sup>	Named ' <i>Candidatus</i> Phytoplasma' species	Informally proposed ' <i>Candidatus</i> Phytoplasma' species <sup>4</sup>
Aster yellows (AY)	16SrI	M30790	' <i>Candidatus</i> Phytoplasma asteris'	
WB disease of lime	16SrII-B	U15442	' <i>Ca.</i> Phytoplasma aurantifolia'	
Papaya yellow crinkle	16SrII-D	Y10097	' <i>Ca.</i> Phytoplasma australasiae'	
Western X-disease	16SrIII-A	L04682	' <i>Ca.</i> Phytoplasma pruni'	
Palm lethal yellowing	16SrIV-A	U18747		' <i>Ca.</i> Phytoplasma palmae'
Elm yellows	16SrV-A	AY197655	' <i>Ca.</i> Phytoplasma ulmi'	
Jujube WB	16SrV-B	AB052876	' <i>Ca.</i> Phytoplasma ziziphi'	
Flavescence dorée	16SrV-C	AF176319		' <i>Ca.</i> Phytoplasma vitis'
Clover proliferation	16SrVI-A	AY390261	' <i>Ca.</i> Phytoplasma trifolii'	
Ash yellows	16SrVII-A	AF092209	' <i>Ca.</i> Phytoplasma fraxini'	
Loofah WB	16SrVIII-A	AF086621		' <i>Ca.</i> Phytoplasma luffae'
Almond lethal disease	16SrIX-D	AF515636	' <i>Ca.</i> Phytoplasma phoenicium'	
Apple proliferation	16SrX-A	AJ542541	' <i>Ca.</i> Phytoplasma mali'	
Pear decline	16SrX-C	AJ542543	' <i>Ca.</i> Phytoplasma pyri'	
Spartium WB	16SrX-D	X92869	' <i>Ca.</i> Phytoplasma spartii'	
European stone fruit Y	16SrX-F	AJ542544	' <i>Ca.</i> Phytoplasma prunorum'	
Rice yellow dwarf	16SrXI-A	AB052873	' <i>Ca.</i> Phytoplasma oryzae'	
Stolbur phytoplasma	16SrXII-A	AF248959	' <i>Ca.</i> Phytoplasma solani'	
Australian GY	16SrXII-B	Y10097	' <i>Ca.</i> Phytoplasma australiense'	
Hydrangea phyllody	16SrXII-D	AB010425	' <i>Ca.</i> Phytoplasma japonicum'	
Strawberry yellows	16SrXII-E	DQ086423	' <i>Ca.</i> Phytoplasma fragariae'	
Mexican periwinkle Vir	16SrXIII-A	AF248960		No ' <i>Candidatus</i> ' name proposed <sup>5</sup>
Bermuda grass WL	16SrXIV	AJ550984	' <i>Ca.</i> Phytoplasma cynodontis'	
Hibiscus WB	16SrXV	AF147708	' <i>Ca.</i> Phytoplasma brasiliense'	
Sugarcane yellow leaf	16SrXVI	AY725228	' <i>Ca.</i> Phytoplasma graminis'	
Papaya bunchy top	16SrXVII	AY725234	' <i>Ca.</i> Phytoplasma caricae'	
Potato purple top wilt	16SrXVIII	DQ174122	' <i>Ca.</i> Phytoplasma americanum'	
Chestnut WB	16SrXIX	AB054986	' <i>Ca.</i> Phytoplasma castaneae'	
Buckthorn WB	16SrXX	X76431	' <i>Ca.</i> Phytoplasma rhamnii'	
Pine shoot proliferation	16Sr XXI	AJ632155	' <i>Ca.</i> Phytoplasma pini'	
Nigerian Awka disease	16Sr XXII-A	Y14175		' <i>Ca.</i> Phytoplasma cocosnigeriae'
Buckland Valley GY	16SrXXIII-A	AY083605		No ' <i>Candidatus</i> ' name proposed <sup>5</sup>
Sorghum bunchy shoot	16SrXXIV-A	AF509322		No ' <i>Candidatus</i> ' name proposed <sup>5</sup>
Weeping tea WB	16SrXXV-A	AF521672		No ' <i>Candidatus</i> ' name proposed <sup>5</sup>
Sugarcane yellows phytoplasma D3T1	16SrXXVI-A	AJ539179		No ' <i>Candidatus</i> ' name proposed <sup>5</sup>
Sugarcane yellows phytoplasma D3T2	16SrXXVII-A	AJ539180		No ' <i>Candidatus</i> ' name proposed <sup>5</sup>
Derbid phytoplasma	16SrXXVIII-A	AY744945		No ' <i>Candidatus</i> ' name proposed <sup>5</sup>
Cassia italica WB	16SrXXIX	EF666051	' <i>Ca.</i> Phytoplasma omanense'	
Salt cedar WB	16SrXXX	FJ432664	' <i>Ca.</i> Phytoplasma tamaricis'	
Allocauarina yellows	Undetermined	AY135523	' <i>Ca.</i> Phytoplasma allocasuarinae'	
Parsley leaf of tomato	"	EF199549	' <i>Ca.</i> Phytoplasma lycopersici'	
Tanzanian lethal disease	"	X80117		' <i>Ca.</i> Phytoplasma cocostanzaniae'
Chinaberry yellows	"	AF495882		No ' <i>Candidatus</i> ' name proposed <sup>5</sup>

<sup>1</sup>Abbreviations are as follows: AY, aster yellows; WB, witches'-broom; Y, yellows; GY, grapevine yellows; Vir, virescence; WL, white leaf.<sup>2</sup>Group and subgroup are determined on the basis of RFLP patterns of 1.2 kbp segments of 16S rDNA that are delimited by the annealing sites of PCR primers R16F2n and R16R2. '*Candidatus* Phytoplasma' species are distinguished on the basis of 16S rDNA nucleotide sequence homology/identity.<sup>3</sup>GenBank accession number of 16S rRNA gene sequence documenting description of the '*Candidatus* Phytoplasma' species or strain.<sup>4</sup>Potentially distinct '*Candidatus* Phytoplasma' species names noted by the IRPCM Phytoplasma/Spiroplasma Working Team - Phytoplasma Taxonomy Working Group (2004. International Journal of Systematic and Evolutionary Microbiology 54:1243-1255.). These names have not been formally published.<sup>5</sup>Recognized in Wei et al. 2007 (International Journal of Systematic and Evolutionary Microbiology 57:1855-1867.) as potential representatives of new '*Candidatus* Phytoplasma' species.

### **2.2.3. Phytoplasma genome sequences**

Scientists published the first full phytoplasma genomic sequence in 2004, namely that of 'Ca. Phytoplasma asteris' OY-M (onion yellows phytoplasma strain M) (Oshima et al., 2004) and since then four additional genome sequences had been completed, namely aster yellows phytoplasma strain witches' broom (AY-WB) (Bai et al., 2006); 'Ca. Phytoplasma mali' (Kube et al., 2008); 'Ca. Phytoplasma australiense' subgroup tuf-Australia I; rp-A (Tran-Nguyen et al., 2008) and strawberry lethal yellows (SLY) isolate of 'Ca. Phytoplasma australiense' (Andersen et al., 2013). The availability of complete genomic sequences of individual phytoplasma species will enable researchers to develop new analytical techniques and detection methods in order to determine the source of new outbreaks.

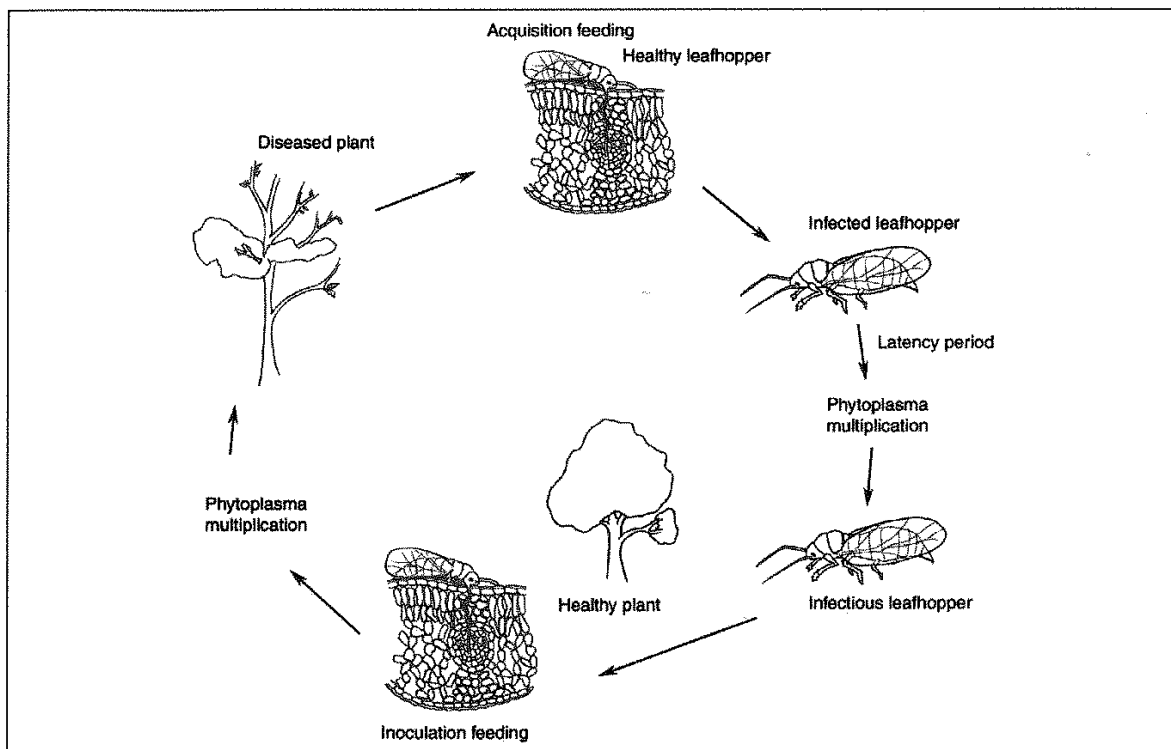
### **2.2.4 Host range of phytoplasmas**

Phytoplasmas are plant pathogens that are limited to the phloem and can mostly be found in the sieve elements of infected plants. The number of insect vectoring species that can transmit a phytoplasma and the feeding behaviour of these vectors will determine the plant host range for a specific phytoplasma. Phytoplasmas in general have broad plant host ranges (Lee et al., 2000) and can occur in lime, plum, cherry, apples, papaya, parsley, lettuce, cabbage, spinach, wheat, oats, triticale, pear, peach and flower plants (Alhudaib et al., 2009; Landi et al., 2007; De Salvador et al., 2007; Siddique et al., 1998; Olivier et al., 2009). Phytoplasmas can also be found in wild plants in hedges around vineyards (Filippin et al., 2008) and in weeds (Arzone et al., 1995; Batlle et al., 2000; Weaver, 2001; Radonjić et al., 2009; Olivier et al., 2009). Aster yellows phytoplasma can infect host plants such as barley, lettuce, carrots, celery, rhadiola, asparagus, oilseed rape, tomato, potato and grapevine (Urbanaviciene et al., 2005; Hollingsworth et al., 2008; Zhou et al., 2002; Duduk et al., 2009; Lee et al., 2003; Hwang et al., 2009; Fránová et al., 2010; Mori et al., 2010; Holguín-Peña et al., 2007; Cheng et al., 2011; Ember et al., 2011; Seruga et al., 2003; Avramov et al., 2008; Engelbrecht et al., 2010). *Ca* Phytoplasma asteris (16Srl subgroup) can be transmitted by approximately 30 polyphagous insect species to between 200 and 300 diverse plant species (Hogenhout et al., 2008). Plant species can be infected by more than one type of phytoplasma simultaneously. For research purposes a plant

such as periwinkle is often used as a source plant to maintain phytoplasmas in culture because it is able to harbour the majority of known phytoplasmas.

### 2.2.5 Dual life cycle

Phytoplasmas are unique in the sense that they require plant and insect hosts for their survival and spread (Figure 2). Phytoplasmas can be found in the phloem of most organs of infected plants. The highest concentration of phytoplasmas can be found in mature sieve tubes of plants (Christensen et al., 2004). Insects feed on the phloem of infected plants where they acquire the phytoplasma and then transmit them to healthy plants. In insects, phytoplasmas move from gut cells to various tissues of the insect, where they replicate, and move to the salivary glands in order to be introduced into new host plants via the saliva during feeding.



**Figure 2.** Host cycle of phytoplasmas (Christensen et al., 2005).

## **2.2.6 Insect vectors and transmission**

### **2.2.6.1 Vectors and specificity**

Phytoplasmas are vectored by insects from the Order Hemiptera, mainly leafhoppers, planthoppers or psyllids belonging to the families Cicadellidae, Cixidae, Psyllidae, Derbidae and Delphacidae (Weintraub et al., 2006). Some phytoplasmas have low insect vector specificity such as California aster yellows phytoplasma (16SrI-B), which is transmitted by 24 different species of leafhoppers. American elm yellows phytoplasma (16SrV-A) and pear decline phytoplasma (16SrX-C) are examples of phytoplasmas with a high vector specificity and are transmitted by only one or a few vector species (Tsai, 1979). Phytoplasmas persist in their vectors but vertical transmission from infected insects to their progeny was believed not to be possible. However, evidence of low transmission rates to plant embryos and insect progeny has since been reported (Botti et al., 2006b; Cordova et al., 2003; Khan et al., 2002; Nipah et al., 2007; Alma et al., 1997).

### **2.2.6.2 Factors affecting acquisition, latency and transmission**

Phytoplasmas are acquired during insect feeding on an infected plant. The phytoplasma is then multiplied in specific organs of the vectors (McCoy et al., 1989) before it can be transmitted to another plant. The acquisition access period (AAP) is the length of time a vector needs to feed on an infected plant in order to acquire enough phytoplasma to become infective and able to transmit the disease to other plants. Once the phytoplasmas are acquired, the vector remains infective for life. The AAP can range from a few minutes to several days and depends on the titre of the phytoplasma and where the phytoplasma is located in the host plant (Siddique et al., 1998), the vector species (Pedrazzoli et al., 2007), the age and gender of the vector ((Murrall et al., 1996; Beanland et al., 2000), the phytoplasma strain (D'Amelio et al., 2007) and the species or variety of the host plant (Bressan et al., 2005). The latent period (LP) or sometimes called the incubation period, is the time from acquisition of the phytoplasma until the vector is able to transmit it to other plants. This LP depends on temperature (Murrall et al., 1996), leafhopper species and phytoplasma strain (Oshima et al., 2001; D'Amelio et al., 2007). Phytoplasmas are transmitted to healthy plants during feeding via the saliva of insect vectors. The rate of phytoplasma transmission depends on several factors, such as feeding behaviour and

gender of vectors (Beanland et al., 1999), host plant species and the phytoplasma strain involved (Mori et al., 2002) as well as season (Tanne et al., 2001).

### **2.2.6.3 Other means of transmission of phytoplasma diseases**

Phytoplasmas persisting in perennial plants can serve as a reservoir of phytoplasmas for spreading to plants in the next spring. A wide variety of weeds such as *Convolvulus arvensis*, *Solanum nigrum*, *Chenopodium album*, *Urtica* species and *Conyza canadensis* are known to act as hosts for phytoplasmas or insects (Batlle et al., 2000; Weaver, 2001; Radonjić et al., 2009). Phytoplasmas can be transmitted by parasitic dodder (*Cuscuta* sp.) from phytoplasma infected plants to healthy plants (Maixner et al., 1994) or by means of vegetative propagation through cuttings, tubers or bulbs (Lee et al., 1992). Grapevine phytoplasmas have been reported to be graft transmitted, although at a low transmission rate (10 - 40%) (Caudwell, 1957). However, there is no proof of mechanical transmission of grapevine phytoplasmas through infected sap during pruning (Angelini, personal communication). Transmission of FD and BN through infected grapevine rootstocks had been demonstrated (Zorloni et al., 2011).

## **2.2.7 Symptoms**

### **2.2.7.1 General symptoms induced by phytoplasmas**

Phytoplasmas induce symptoms that resemble imbalances of growth regulators and interference with plant development, like virescence (green coloration of non-green flower parts); phyllody (development of green leaf-like structures instead of flowers), sterility of flowers, proliferation of axillary buds which gives a “witches’ broom” effect, shortened internodes; reddening of leaves and stems; generalised yellowing and general stunting of plants (Bertaccini, 2007). Factors such as the phytoplasma and host plant involved, development stage of the disease, age of plant at the time of infection and environmental conditions can influence the symptoms that are displayed (McCoy, 1979; McCoy et al., 1989; Lee et al., 2000; Seemüller et al., 2002).

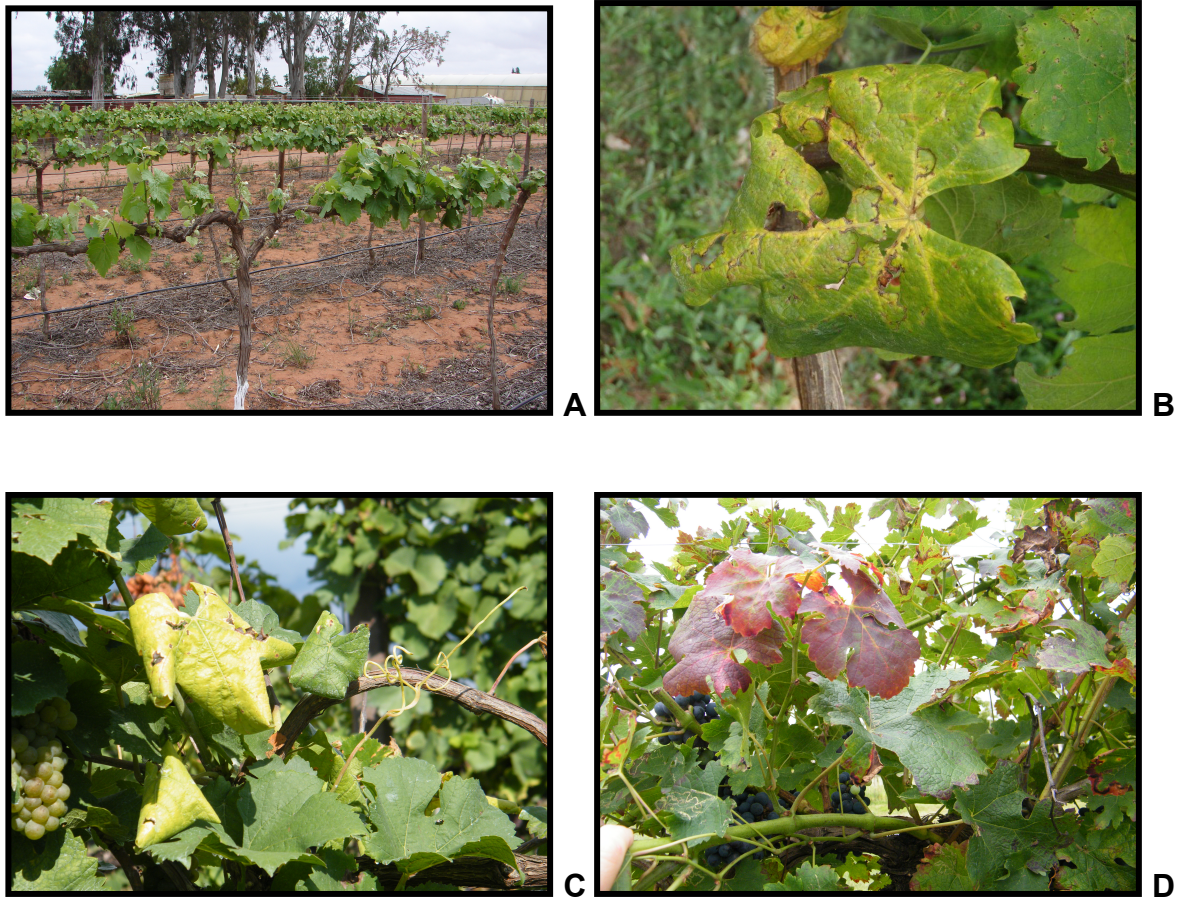
### **2.2.7.2 Grapevine yellows symptoms**

Grapevine yellows diseases all produce similar symptoms (Martelli et al., 2006) irrespective of the specific phytoplasma/s associated with the disease (Boudon-Padieu et

al., 1998). Many plant parts are affected including shoots, growth tips, leaves and bunches. Symptoms appear during summer, but infected vines can be identified from spring onwards by their delayed bud break and reduced growth (Figure 3A). Usually a combination of symptoms will develop on plants (Osler et al., 1993; M'hirsi et al., 2004; Carstens, 2008; Radonjić et al., 2009). Some symptoms however, appear to be variety specific (Wolf, 2000). Usually symptoms appear one year after inoculation in adult vines and 3 to 6 months after inoculation in grafted vines (Angelini, 2008).

Leaves of infected vines become yellow (white varieties) or red (red varieties). Yellowing can start as spots and then enlarge to form bands along the veins (Figure 3B). Colour of the spots and bands varies with the cultivar in that white-fruited cultivars will show creamy yellow spots and red-fruited cultivars will show reddish spots. This yellowing (Figure 3C) or reddening (Figure 3D) will gradually cover the whole leaf. Leaves roll downwards and leaf blades become thick and brittle later in summer. Some red varieties show a sectorial discoloration of leaves (Angelini, 2008). Leaf rolling can result in a typical triangular shape (Ćurković Perica et al., 2001). Some phytoplasma diseases show symptoms of downward rolling leaves overlaying one another in a shingled appearance (Constable et al., 2003a; Constable et al., 2004). Vines showing GY symptoms often display light green foliage (Bonfiglioli et al., 1997). Leaves of infected vines fall later than that of healthy vines, however premature leaf fall has been reported in cases of vines infected by AGY (Habibi et al., 2001; Constable et al., 2003a). Shoots lignify partially or not at all, which gives the vines a drooping appearance. Small black pustules may appear on the base of shoots of some cultivars. Bunches can dry out early in the season or berries can shrivel later in summer, which will cause a decrease in yield.





**Figure 3.** Grapevine yellows symptoms: (A) Affected vine showing delayed bud break (Photo: Jeff Joubert); (B) Yellow spots on infected leaves enlarge to form bands along the veins; (C) Yellowing of leaves indicative of grapevine yellows infection on a white variety and (D) Red varieties infected with grapevine yellows show leaf reddening.



## **2.2.8 Detection of phytoplasmas**

### **2.2.8.1 Biological detection**

Phytoplasmas have been poorly characterised because of low and variable titre in plants and the fact that it cannot be cultured *in vitro*. Biological properties such as the symptoms induced in infected plants, plant host range, the relationship with specific insect vectors or microscopic observations of ultra-thin sections of phloem tissue (Lee et al., 1992) were therefore primarily used for the identification and classification of phytoplasmas. The use of DNA-binding fluorescent dyes such as 4', 6-diamidino-2-phenylindole (DAPI) became a standard procedure for the detection of phytoplasmas in many laboratories (Seemüller et al., 1996). The methods used to determine these biological properties were time-consuming and labour intensive and very often researchers ended up with inconsistent results. For some phytoplasma diseases, the vector had not been determined (Kelly et al., 1998), which complicated the identification based on biological properties. Other factors that also complicated this method of identification were the fact that different phytoplasmas can cause similar symptoms in the same plant species and the same phytoplasma can cause different symptoms and diseases in different plant species. Indexing was regularly applied for identification purposes. This was done by transmission of phytoplasma from a suspected positive plant to an indicator plant such as periwinkle by using parasitic dodder (*Cuscuta* sp.) (Maixner et al., 1994).

### **2.2.8.2 Serological detection**

In the 1980s, phytoplasma disease diagnostics developed quite rapidly and serological methods such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy where monoclonal antibodies (MAbs) were used, became one of the most reliable means of detection and identification of phytoplasma diseases (Lin et al., 1986). Polyclonal antisera however, could not always differentiate between phytoplasma diseases. Immunosorbent electron microscopy (ISEM) has also been used to detect phytoplasma diseases especially in individual insect vectors.

### **2.2.8.3 Nucleic acid-based detection**

Molecular detection and diagnosis of phytoplasmas based on genomic DNA was achieved using methods such as dot and Southern blot hybridization and PCR technology (Padovan

et al., 1995; Guo et al., 2003). After the first cloning of phytoplasma DNA (Kirkpatrick et al., 1987) nucleic acid-based probes were used to study inter-relationships among phytoplasmas and to identify phytoplasma groups and subgroups. Primers based on cloned DNA fragments specific to a given phytoplasma provided a very specific and sensitive detection of phytoplasmas, but broad-spectrum primers based on conserved sequences, for example the 16S rRNA, provided detection of a wide array of phytoplasmas. Additional information can be obtained by Southern blot hybridization as well as restriction fragment length polymorphisms (RFLP) analysis following PCR. RFLP analysis can differentiate between strains that are indistinguishable on the basis of dot hybridization. Quantitative (real-time) PCR (qPCR) was developed in mid 1990s and rapidly gained popularity (Walker, 2002). Diagnostic procedures based on qPCR are very sensitive and specific and allow for high throughput and rapid sample processing (Angelini et al., 2007; Hren et al., 2007; Herath et al., 2010). qPCR and bio-imaging techniques to quantify phytoplasma levels in plants and insects have been developed. This technique can also be used to determine the distribution of phytoplasma in the plant (Christensen et al., 2004). Rapid and reliable field detection of phytoplasmas can also be done by performing DNA extraction and loop-mediated isothermal amplification (LAMP) assays (Tomlinson et al., 2010).

Phytoplasmas can be detected in infected grapevine tissues at different phenological stages, but the highest concentration was found in leaf tissue extracts at the stage of berry ripening (Del Serrone et al., 1996). Sometimes vines can show up phytoplasma negative even if samples are taken from symptomatic areas of the vine (Gibb et al., 1999). Uneven distribution of phytoplasmas in the vine (Terlizzi et al., 2007) often complicates detection.

Phytoplasma vectors are cold-sensitive and prefer higher temperatures. Temperature increase caused by global warming could therefore lead to an increase of phytoplasma-related diseases. Currently there is an increase in organic agriculture with less pesticides being used and this can definitely contribute to an increase in phytoplasma outbreaks. Accurate and sensitive detection techniques to determine the presence of phytoplasmas will therefore become even more important in future.

### **2.2.9 Control of Phytoplasma Diseases**

Control of phytoplasmas, which are obligate parasites, is usually directed towards prevention rather than cure. It is very difficult to control phytoplasma diseases because of their dual host life cycle.

#### **2.2.9.1 Vector control**

The application of insecticides to control phytoplasma diseases is common practise. The choice of insecticide and timing of application is important to consider as this can influence the effectiveness of control (De Klerk CA, personal communication). Timing of application will depend on factors such as numbers of vector insects present and the mobility of the insects (Saracco et al., 2008). Limited success with the use of biological control agents have been reported for the control of aster leafhopper and *Scaphoideus titanus* Ball (Oraze et al., 1989; Nusillard et al., 2003). Managing of weeds and wild plants that can act as hosts for insect vectors is a non-chemical method of control that had been used previously (McClure, 1980; Nesten et al., 1995). Nymphs of *Hyalesthes obsoletus*, the vector of BN, live underground on plant roots and controlling of weeds can decrease the survival of eggs and nymphs (Sforza et al., 1998). Mulching can also be used to repel insect vectors (Howard et al., 1998). Although some parasitoids of leafhoppers were identified (George, 1959), they may not provide effective control because of the migrant nature of the vector. In Italy, research showed that foundation vineyards, which is the source of clonal material used to establish commercial mother blocks, can be kept clean of phytoplasma infection by covering it with insect proof netting (Mannini, 2007).

#### **2.2.9.2 Reducing and preventing disease inoculum**

Roguing of infected trees (Uyemoto et al., 1998) and other host plants (Lukens et al., 1971) has been used successfully in controlling phytoplasma diseases. Different pruning methods and pollarding (cutting the trunk 10 – 15cm above the graft union) was used successfully to reduce Bois noir disease inoculum, resulting in yearly recovery rates of 66 – 84% in the following season. Rootstock had an effect on recovery rates as well as the severity of the infection and the sensitivity of the cultivar (Riedle-Bauer et al., 2010). Proper quarantine practices to prevent importation of plant material containing phytoplasmas are essential for avoiding new phytoplasma diseases. Meristem tip culture

can be used to eliminate phytoplasmas from infected plant material such as sweet potato (Green et al., 1989). In the case of grapevine, disease-free propagation material can also be obtained through hot water treatment (HWT) of dormant grapevine material and it is recommended for establishment of new plantings. Caudwell et al. (1997) found that by dipping plant material infected with FD into water at 30°C for 72h, the phytoplasma could be eliminated from 80% of the infected plant material. After more experiments with different regimes for water temperatures and immersion times they found that a hot water treatment regime of 50°C for 45 min was not only effective for phytoplasma (FD) elimination, but it also effectively eliminated live leafhopper eggs present on the wood (Caudwell et al., 1997). Quarantine services of several countries adopted HWT as control measure against a variety of pathogens. The Canadian Food Inspection Agency (CFIA) implemented mandatory hot water treatment of all vines imported from Europe in 2007 (CFIA directive D-95-08) to make sure that phytoplasmas do not enter the country through infected plant material. HWT should be applied carefully according to protocol otherwise it could affect the survival rate of the treated plant material (Boudon-Padieu et al., 2002).

<http://web.pppmb.cals.cornell.edu/fuchs/icvg/data/icvghotw.pdf>

### **2.2.9.3 Resistant plant varieties**

The development of disease resistance in plants to control phytoplasma diseases is the most promising control method and has been used with limited success (Thomas et al., 1998). Breeding varieties resistant to vector feeding could also be used to control phytoplasma disease. Genetically modified host plants were developed to provide resistance to planthoppers and leafhoppers and an example is transgenic rice expressing snowdrop lectin [*Galanthus nivalis* agglutinin (GNA)] in the phloem tissue (Nagadhara et al., 2003).

### **2.2.9.4 Curing infected plants**

In the 1960s, tetracycline was applied by spraying onto or injection into infected plants to treat phytoplasma infections. This gave limited control and the infection recurred when treatment was discontinued (McCoy, 1982). A single application of oxytetracycline hydrochloride injected into grapevine showed to reduce the incidence of AGY by 97% and it remained effective for about 5 years (Magarey et al., 1986). This treatment was not

used commercially as it is very expensive, the chance is there that resistance may develop and the possibility that antibiotics may enter into the human food supply makes it an unacceptable practice.

#### **2.2.10 *Candidatus* Phytoplasma asteris**

'Ca Phytoplasma asteris' (16Srl subgroup) represents the most diverse and widespread phytoplasma group and has been reported to infect between 200 and 300 diverse plant species worldwide (Hogenhout et al., 2008). The name AY derived from the fact that China aster, *Callistephus chinensis* L. (Nees) (Asteraceae), was the first crop that was affected by this phytoplasma disease in the United States of America (Kunkel, 1926). Based on 16S rRNA gene sequences, strains from this group are most closely related to the stolbur phytoplasma subclade (Lee et al., 2004). Subgroup 16Srl-B, to which aster yellows belongs, represents the largest and most diverse strain cluster in the group.

#### **2.2.11 Epidemiology of aster yellows disease**

Epidemiology is described as the study of epidemics or the development of disease in space and over time. The environment and human interferences can have an influence on the development of disease. In order to improve understanding of plant disease epidemics a wide range of mathematical models has been developed and reviewed by epidemiologists (Madden et al., 2006). Some of these disease models had been incorporated into computer software programmes. Disease progress data can provide new insights into epidemic development and play a key role in management decisions (Jeger, 1999; Jeger, 2004). The epidemiology of four of the grapevine yellows diseases, including FD, BN, AGY have been studied extensively (Constable, 2010), but only one survey was performed on NAGY, caused by aster yellows and X-disease phytoplasma.

##### **2.2.11.1 Disease incidence**

Disease incidence is one of the first parameters determined after the outbreak of a new disease. It can give an indication of the severity of disease and also determine the impact of the disease on the crop and ultimately on the economy of the country. Disease incidence data can be collected by different sampling methods such as sparse sampling or intensive mapping. When sparse sampling is performed, disease status of a restricted

number of samples is recorded without the spatial location of a sample. Different sampling methods can be used, for example an X- or W-shaped path covering the whole field or a smaller demarcated area or a diagonal path covering the whole field (Lin et al., 1979). Intensive mapping allows the recording of both disease status and spatial location of a large number of sampling units, for example inspection of all vines in a vineyard. Assessing disease incidence is quite straightforward and can be done by visually assessing the plant and recording it as healthy or diseased. This categorization can also be based on molecular diagnostic assays.

Numerous studies to determine the disease incidence of phytoplasma diseases on grapevine had been performed and the main conclusions are described below. The number of Chardonnay vines showing typical GY symptoms in the Sunraysia region in Australia increased from 1.6% in the first year to 16.8% in the second year of study (Bonfiglioli et al., 1997). Boselli (1999) showed that the Italian cultivar Vermentino is more severely affected by GY than a cultivar such as Albarola. Vermentino had a higher mean disease index and higher percentage of dead plants compared to Albarola. This study also showed that plants were more susceptible to disease when they were highly productive and planted in shallow soils with specific characteristics. In Virginia, disease incidence of GY, caused by AY and X-disease phytoplasmas, was found to exceed 4% per year, causing vineyards to be marginally profitable after 10 years (Wolf, 2000). When the main viticulture areas in Spain were monitored for GY diseases, the incidence of the disease in a BN-infected vineyard increased from 3.4% in 1994 to 18.4% in 1997 (Batlle et al., 2000). Symptoms of BN infection were found to vary according to cultivar and total incidence of symptomatic vines of 14% in Vermentino, 83% in Vernaccia di Oristano, 61% in Cannonao and 17% in Chardonnay was found (Garau et al., 2004). After the first observations of GY symptoms in Montenegro a survey was conducted and phytoplasma infection was confirmed in nine vineyards. The disease incidence in these vineyards varied between a single vine and 20% of the total plants in the vineyard (Radonjić et al., 2009).

From the abovementioned studies one could conclude that the variation in disease incidence depends on factors such as sensitivity of the cultivar, disease causing

phytoplasma, inoculum source of phytoplasma available in vicinity, soil type and vector efficiency, to name a few.

#### **2.2.11.2 Spatial distribution of disease**

A spatial pattern of disease is described as the dispersion in space of pathogens. Diverse analytical procedures exist to characterise spatial distribution. Statistical analysis of spatial distribution of a disease usually begins with a test for spatial randomness (Diggle, 2003), which can be determined by ordinary runs analysis or doublets (Madden et al., 1982; Vanderplank, 1946). Spatial patterns can be either random or non-random. A non-random pattern is also referred to as clustered, clumped or aggregated. Spatial distribution can also be characterised by using semivariograms, such as used in a survey of Pierce's disease (Park et al., 2011). Gray et al. (1986) developed a two-dimensional distance class analysis for characterising spatial relationships of virus-infected plants in row crops, but was designed for a mainframe computer. Nelson et al. (1992) adapted Gray's program into two-dimensional distance class analysis software for a personal computer (2DCLASS). This computer programme was used to evaluate the spatial distribution of X-disease, a phytoplasma disease in sweet cherry orchards in California (Uyemoto et al., 1998). Another spatial analysis computer software program, PATCHY, which is often used for the incidence and spatial distribution of phytoplasma (Constable et al., 2003b) and virus diseases (Pietersen, 2006) of grapevine, was developed by Maixner (1993). 2DCLASS analysis is useful for the detection of non-random spatial patterns as well as edge effects, but was inappropriate when the number of infected plants was either very small or very large, in relation to the total number of plants in a lattice (Nelson et al., 1992). Because of this disadvantage the programme could not be used for the AY survey. The spatial distribution of disease in an area provides clues to the nature of the disease. Spatial patterns of phytoplasma-associated diseases can indicate the location of alternative hosts and vectors and also shed light on the mode of transmission (Orenstein et al., 2003). Spatial pattern maps can be used to identify potential sources of primary infection and evaluate secondary spread of disease.

In California, spatial distribution of sweet cherry trees infected with X-disease indicated random distribution and secondary spread concentrated around infected trees (Uyemoto

et al., 1998). The trees therefore served as sources from which the leafhopper vectors could acquire the phytoplasmas. Management measures such as removal of infected trees; general tree sanitation and an insecticide spray prior to the removal of the trees provided enough protection to ensure a low incidence of new infections, while maintaining a productive orchard.

The epidemiology of several grapevine phytoplasma diseases has been studied worldwide. Spatial distribution studies of North American grapevine affected by yellows (aster yellows and X-disease) indicated a non-random distribution of diseased vines with significant clustering (Beanland et al., 2006) and the authors thus reasoned that transmission of the phytoplasmas was through insect vectors and not by infected nursery material. It was found that clustering of infected vines often occurred on the sides of vineyards near wooded areas which could indicate that vectors came from the trees into the vineyard to feed. In this study, although it could not be statistically proven, the incidence of yellows in Chardonnay was higher than in Sauvignon blanc and Riesling. Yellows-infected vines in Virginia usually die within the first three years after initial symptom expression (Wolf, 2000), which is different from what happens with phytoplasma-infected vines in Europe and Australia, where symptoms are expressed in one year after which recovery of some vines may be experienced.

The spatial distribution of BN-infected vines in the majority of north Italian vineyards that were surveyed showed a randomized pattern. In contrast, clustering of infected vines occurred in seven vineyards where nettle bordered the vineyard edge (Mori et al., 2008). This indicated the importance of surrounding vegetation as possible source of phytoplasma inoculum or as host plant for insect vectors. Insecticide treatment of vineyard canopies was not effective in controlling spread of BN in these vineyards, unlike FD-infected vineyards where insecticide treatments of vineyard canopies gave effective control of the vectors and limited the spread of FD. In the case of BN no specific insecticide treatments of vineyards is therefore recommended.



### 3 MATERIALS AND METHODS

#### 3.1 Identification of experiment vineyards

Thirteen vineyards (sites 1-13) comprising of seven different cultivars (Chenin blanc, Shiraz, Chardonnay, Cabernet franc, Sauvignon blanc, Pinotage and Colombar) with low to medium AY disease incidence were identified in 2009 (Table 3). In 2010 a new vineyard cv Chenin blanc (site 14) was planted next to site 5 and was also included in the study. Ages of vineyards at the time of the first disease incidence survey ranged from as young as 6 months to 18 years. All these vineyards were situated west of Vredendal along the road to Lutzville and in the centre of the area most infected by yellows disease as mapped by the Agricultural Product Inspection Services (APIS) of the Department of Agriculture, forestry and Fisheries.

**Table 3.** Detail of experiment vineyards.

Site	Cultivar	GPS coordinates (South and East)	Vineyard planting date	Amount of vines surveyed	Duration of survey
1	Chenin blanc	31.66786 18.47053	2009	7611	2010
2	Shiraz	31.66842 18.47206	1999	4635	2010 - 2011
3	Chardonnay	31.66200 18.48228	2004	2216	2009 - 2013
4	Chardonnay	31.66306 18.48347	2005	2905	2010 - 2013
5	Chenin blanc	31.66008 18.47772	2008	4301	2010 - 2013
6	Pinotage	31.29666 18.46255	2009	2412	2010 - 2013
7	Sauvignon blanc	31.66483 18.46986	1992	984	2010 - 2011
8	Sauvignon blanc	31.66483 18.46919	1992	2015	2010 - 2011
9	Colombar	31.66717 18.46786	2001	2024	2010 - 2011
10	Cabernet franc	31.66758 18.46825	1996	1280	2010 - 2011
11	Chenin blanc	31.65942 18 47717	2009	4048	2010 - 2013
12	Pinotage	31.66561 18.48264	1998	3530	2010 - 2013
13	Pinotage	31.66908 18.48617	1997	5970	2010 - 2013
14	Chenin blanc	31.66008 18.47772	2010	3761	2011 - 2013

### 3.2 Vineyard surveys for GY incidence

GY disease incidence surveys were conducted annually from 2009 to 2013. Intensive mapping, where both the disease status and spatial location of all vines in a vineyard are recorded, were used for the surveys. Disease incidence assessment was conducted during late summer (late January or early February) just before harvest, when symptoms were most apparent. Visual assessment of disease incidence was performed and each vine was characterised as healthy, GY affected and missing/dead. Vines were considered GY-affected if any one of the following visual symptoms of the disease were present: (1) aborted bunches, (2) downward rolling and yellowing/reddening of leaves, (3) green, immature canes and/or (4) die back of shoot tips and shoots. The yearly incidence (%) was determined for each vineyard (= number of vines showing disease symptoms in the current year), as well as the cumulative incidence (%) (= sum of all new records of grapevines showing disease symptoms in the current year and all records of diseased grapevines in previous years). In the field this data was mapped on Excel sheets and transferred to the PATCHY computer programme (Maixner, 1993) for disease incidence and spatial analysis.

Vine-to-vine visual analysis was performed in site 1 during the 2010 season. Before the 2011 season the vines were cut back 30 cm above the ground. This site was surveyed until 2013. Site 2, 7, 8, 9 and 10 were surveyed in 2010 and 2011 only. The Shiraz vines (site 2) were infected with a combination of leafroll and grapevine yellows, which made the visual survey extremely difficult. During the 2011 survey no phytoplasma symptoms could be found and the survey was terminated. In November 2010 shortly before the second survey, yellows-affected shoots were removed at sites 7, 8, 9 and 10. The vineyards at these four sites all belong to one producer and GY symptoms were removed every year since November 2010.

Site 3 was surveyed for 5 years from 2009-2013 and sites 4-6 and 11-14 were surveyed for 4 years from 2010-2013. Before the second survey in 2011 some of the vines that showed disease symptoms at site 4 and site 5 were pollarded by the producer (cut back about 60 cm above the ground). During the 2011 survey the pollarded vines at these two sites were assumed to be AY affected.

After the identification of the insect vector, *Mgenia fuscovaria* (Stal) in 2010 producers treated vines with the systemic neonicotinoid insecticide, imidacloprid. All vineyards in the survey were treated with imidacloprid in the spring of 2010 and after harvest in March 2012.

Statistical analyses were performed on the disease incidence data of 7 vineyards (sites 3-6 and 11-13), which were all surveyed for 4 years. Contingency tables were set up for years against yearly disease incidence and new infections for each site. Chi-squared tests were conducted to determine if disease incidence is independent of year. Analysis of variance (Anova) was performed on yearly and cumulative disease incidences as well as new infections, using GLM (General Linear Models) Procedure of SAS software (Version 9.2; SAS Institute Inc., Cary, USA). Observations over years were combined in a split plot Anova considering sites as random replicates for cultivars, with cultivar as main plot factor and years as subplot factor (Little, 1972). Shapiro-Wilk test was performed to test for normality (Shapiro, 1965). Percentages were subjected to arc-sine transformation to improve normality (Snedecor, 1980). Student's t-least significant difference was calculated at the 5% level to compare treatment means (Ott, 1998). A probability level of 5% was considered significant for all significance tests.

### **3.3 PCR detection of grapevine phytoplasmas**

Every season a maximum of 5 symptomatic and 5 asymptomatic vines were randomly sampled per vineyard and subjected to PCR and RFLP analysis to confirm disease status of the vines in order to correlate disease status with visual evaluation and to confirm the phytoplasma involved (Engelbrecht et al., 2010). Some of the vineyards had fewer than 5 infected vines and in those cases the available samples were analysed.

#### **3.3.1 DNA isolation**

Total nucleic acid was extracted according to Angelini et al. (2001): Extraction was done from leaf veins using the cetyl-trimethyl-ammonium bromide (CTAB) method. Leaf vein tissue (1 g) was ground in 7 ml of buffer (3% CTAB, 100 mM Tris-HCl, pH8, 10 mM EDTA, 1.4 M NaCl, 0.1% 2-mercaptoethanol). The suspension (1 ml) was transferred to an Eppendorf tube and incubated for 20 min at 65°C. Extraction was done with an equal

volume of chloroform and the aqueous phase was recovered. Nucleic acids were precipitated with an equal volume of isopropanol and collected by centrifugation. DNA pellet was washed with 70% ethanol, dried and dissolved in 100  $\mu$ l of TE buffer (10 mM Tris, 1 mM EDTA, pH7.6).

### **3.3.2 PCR assays and phytoplasma detection**

The PCR assay was first optimised with samples from infected and healthy vines collected in Vredendal vineyards in December 2009. To detect phytoplasmas in extracted DNA samples, 20  $\mu$ l reaction mixtures contained 0.5  $\mu$ l DNA, 0.2  $\mu$ M of each primer pair and 10  $\mu$ l of GoTaq<sup>®</sup> Green Master Mix (Promega, WI, USA). Final extracted DNA (0.5  $\mu$ l) was standard use for all assays. PCR controls included a healthy vine (negative control), sterile water (negative control) and total DNA from a verified AY infected grapevine (positive control). Nested PCR (nPCR) was performed using two sets of universal primers (P1+P7, followed by R16R2+R16F2n) (Table 4) that amplifies the 16S-23S ribosomal rRNA genes of all phytoplasmas. Half a microlitre of a 1/10 dilution of extracted total nucleic acid was used as template for a first round of PCR in a MJ Research Engine (Biorad, South Africa) with primers P1+P7, and 0.5 $\mu$ l of the first PCR product was used as template for PCR with the nested primers R16R2+R16F2n. PCR parameters used were as follows: 94°C for 4 min followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, with a final extension step at 72°C for 7.5 min. Nested PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide, visualised and photographed using the Ingenious Gel documentation system (Syngene, Vacutec, South Africa).

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

Following nPCR, 4  $\mu$ l of each PCR product was individually digested with Thermo Scientific Fastdigest restriction enzymes *Rsa*I, *Hha*I, *Alu*I and *Hpa*II (Inqaba Biotechnology, South Africa) according to the manufacturer's instructions. Additional restriction enzymes, namely *Kpn*I, *Taq*I and *Tru*1I were included from the second season onwards. The digested products were analysed by electrophoresis in a 2% agarose gel. The resultant restriction fragments of the samples were compared with the restriction

patterns of the positive control, which had previously been sequenced to confirm its identity as AY phytoplasma.

**Table 4.** Primers used for phytoplasma detection.

Primer name	Sequence (5'-3')	Position	Description	Reference
P1	AAGAGTTTGATCCTGGCTCAGGATT	16S rDNA	<b>Universal phytoplasma primer P1.</b> Amplification with primers P1 and P7 yields a 1792 base pair (bp) product.	Deng et al., 1991
P7	CGTCCTTCATCGGCTCTT	23S rDNA	<b>Universal phytoplasma primer P7.</b>	Schneider et al., 1995
R16F2n	GAAACGACTGCTAAGACTGG	16S rDNA	<b>Universal phytoplasma primer R16F2n.</b> Amplification with primers R16F2n and R16R2 yields a 1244 bp product.	Gundersen et al., 1996
R16R2	TGACGGGCGGTGTGTACAAACCCCG	16S rDNA	<b>Universal phytoplasma primer R16R2.</b>	Lee et al., 1993
R16(V)F1	TTAAAAGACCTTCTTCGC	16S rDNA	Fwd primer R16(V)F1, group V-specific primer for elm yellows and related phytoplasmas	Lee et al., 1994
R16(V)R1	TTCAATCCGTAAGACTACC	16S rDNA	Rev primer R16(V)R1, group V-specific primer	Lee et al., 1994
R16(III)F2	AAGAGTGGA AAAACTCCC	16S rDNA	Fwd primer R16(III)F2, group III-specific primer, which includes peach X disease	Lee et al., 1994
R16(III)R1	TCCGAACTGAGATTGA	16S rDNA	Rev primer R16(III)R1, group specific primer for group III	Lee et al., 1994
R16(I)F1	TAAAAGACCTAGCAATAGG	16S rDNA	Fwd primer R16(I)F1, group specific primer for group I, which includes aster yellows	Lee et al., 1994
R16(I)R1	CAATCCGAACTGAGACTGT	16S rDNA	Rev primer R16(I)R1, group specific primer for group I	Lee et al., 1994
R16(X)F1	GACCCGCAAGTATGCTGAGAGATG	16S rDNA	Fwd primer R16(X)F1, group X-specific primer for apple proliferation and related phytoplasmas	Lee et al., 1995
R16(X)R1	CAATCCGAACTGAGACTGT	16S rDNA	Rev primer R16(X)F1, group X-specific primer for apple proliferation and related phytoplasmas	Lee et al., 1995

### **3.4 Spatial analysis of GY diseased vines**

Data collected during the disease surveys were analysed using the PATCHY spatial analysis package (Maixner, 1993). PATCHY uses both fixed grid analysis and ordinary runs analysis to determine disease patterns. The fixed grid analysis lays a grid of subunits over the plot, which was used for calculations. A tolerance level for missing plants was set and all subunits exceeding this tolerance level for missing plants was not used for calculations. The fixed grid analysis calculates the mean number of affected plants in each subunit of the grid and it also determines the variance amongst subunits across the plot. The variance to mean ratio (V/M) is then calculated using this information (Madden, 1989). A random disease pattern will be inferred if V/M equals or is not significantly different from 1. If V/M is significantly greater than 1 the disease shows a clustering pattern. Fixed grid analysis only analyse clustering between groups of diseased plants, whereas ordinary runs analysis can be used to analyse clustering between individual plants (Madden et al., 1982).

For ordinary runs analysis the disease status of plants was recorded by using a symbol of 0 representing disease-free plants and 1 representing infected plants. If there is a succession of one or more identical symbols, which are followed or preceded by a different symbol or no symbol at all, it is defined as a run. There will be few runs if a pathogen spreads or is transmitted from plant to plant causing a clustering or aggregation of infected plants and of healthy plants. The Z-statistic will be a large negative number if there is clustering of diseased plants (Madden et al., 1982; Uyemoto et al., 1998). A random mixing of healthy and diseased plants and a resulting large number of runs will be the case if the disease is not transmitted from plant to plant. The PATCHY spatial analysis package (Maixner, 1993) was used to test for randomness or clustering by ordinary runs analysis. Spatial distribution maps were generated for each vineyard in each year using the fixed grid analysis with 2 x 2 grid size.

### **3.5 Alternative host plants**

Weeds and other possible host plants were collected in and around vineyards infected with AY. Weeds were collected from 13 different sites at the end of March 2010, 2011 and 2012. Identification of the weeds was performed by Dr. Johan Fourie, weeds expert at

ARC Infruitec-Nietvoorbij, Stellenbosch and Edwina Marinus of the South African National Biodiversity Institute, Compton Herbarium, Kirstenbosch Research Institute, Cape Town. Total nucleic acid extraction and PCR analysis was performed as described for grapevine. DNA quality was assessed by agarose gel electrophoresis prior to PCR. To test for the presence of phytoplasmas, nPCR was performed as described for the grapevine samples using two sets of universal primers (P1/P7, followed by R16F2n/R16R2). One microlitre of a 1/10 dilution of the extracted total nucleic acid was used as template for a first round of PCR with primers P1/P7, and 1  $\mu$ l of the first PCR product was used as template for PCR with the nested primers R16F2n/R16R2. PCR controls included a “no template” (negative) control, total DNA from AY-affected grapevine (positive control) as well as 1  $\mu$ l of the 1/10 dilution of the weed samples’ total nucleic acid spiked with the positive control DNA. The latter control was included to rule out false negative results due to the presence of PCR inhibitors in the diagnostic sample. PCR parameters used were as described for grapevine.

Following nested amplification with the universal primers, PCR-positive samples were further analysed using phytoplasma group specific primers (Table 4). One microlitre of the P1/P7 PCR product was used as template for group-specific PCR with the following PCR parameters: 94°C for 2 min, 35 cycles of 94°C for 1 min, 50°C for 2 min, 72°C for 3 min, and a final extension step at 72°C for 10 min. The products of nPCR were resolved on 1% agarose gels. After amplification with R16R2+R16F2n, 4  $\mu$ l of the nPCR products were digested with Thermo Scientific Fastdigest restriction enzymes, *AluI*, *HhaI*, *RsaI* and *TruI* and the DNA fragments resolved on 2% agarose gels. The resultant PCR-RFLP profiles were compared to those in literature as well as the AY phytoplasma included as a positive control in the analysis.

Amplicons from the nPCR were excised from 1% agarose gels (1200 bp products, see Figure 19) and purified using the QIAEX II DNA purification kit (Qiagen, Whitehead Scientific, South Africa) according to the manufacturers’ instructions. The purified DNA fragments were ligated into the vector pJET1.2/Blunt using the ClonJet PCR Cloning kit (Thermo Scientific) according to the accompanying manual. The inserts were sequenced at the University of Stellenbosch Central Analytical Facility, using the pJET1.2 forward and



reverse sequencing primers. Sequences were analysed using the BLASTn program of the National Center for Biotechnology Information (NCBI; Altschul et al., 1997).

## 4 RESULTS

### 4.1 Disease assessment

Every vine of every vineyard was visually assessed for the presence of disease symptoms. A vine was characterised as grapevine yellows infected when any one of the following symptoms were observed on the vine:

- Symptoms of AY infection can be localised on a few shoots or one cordon of the vine. In other cases the entire vine can be affected. These affected shoots are thin and rubbery (Figure 4A), giving the vine a drooping appearance (Figure 4B).
- Affected shoots display tip death followed by dieback of the shoots, node-by-node (Figure 4C).
- Shoots can have shortened internodes with a zigzag growth pattern (Figure 4D) and later show partial or total lack of lignification.
- The stems of affected shoots sometimes develop a blue-grey waxy appearance and remain rubbery (Figure 4E), a symptom which was regularly recorded on cultivars such as Chardonnay and Chenin blanc.
- Suckers that are visually unaffected develop on the trunk and arms of affected vines, especially young vines.
- Early in the season, affected leaves have a wrinkled appearance (Figure 4F) and later they become thicker than normal leaves, are crisp and roll downwards. Some leaves show a general yellowing/reddening and turn to a golden yellow on white cultivars (Figure 4G) or a red colour on red cultivars (Figure 4H). Pinotage sometimes shows a sectorial discoloration of leaves (Figure 4I). Leaf rolling can result in a typical triangular shape as recorded on Chardonnay (Figure 4E).
- Fruit set is reduced on grapevines as some bunches dry out and fall off early in the season (Figure 4J). Later in the season, fully developed bunches become dry and shrivelled before fruit can ripen (Figure 4K).



A



B



C

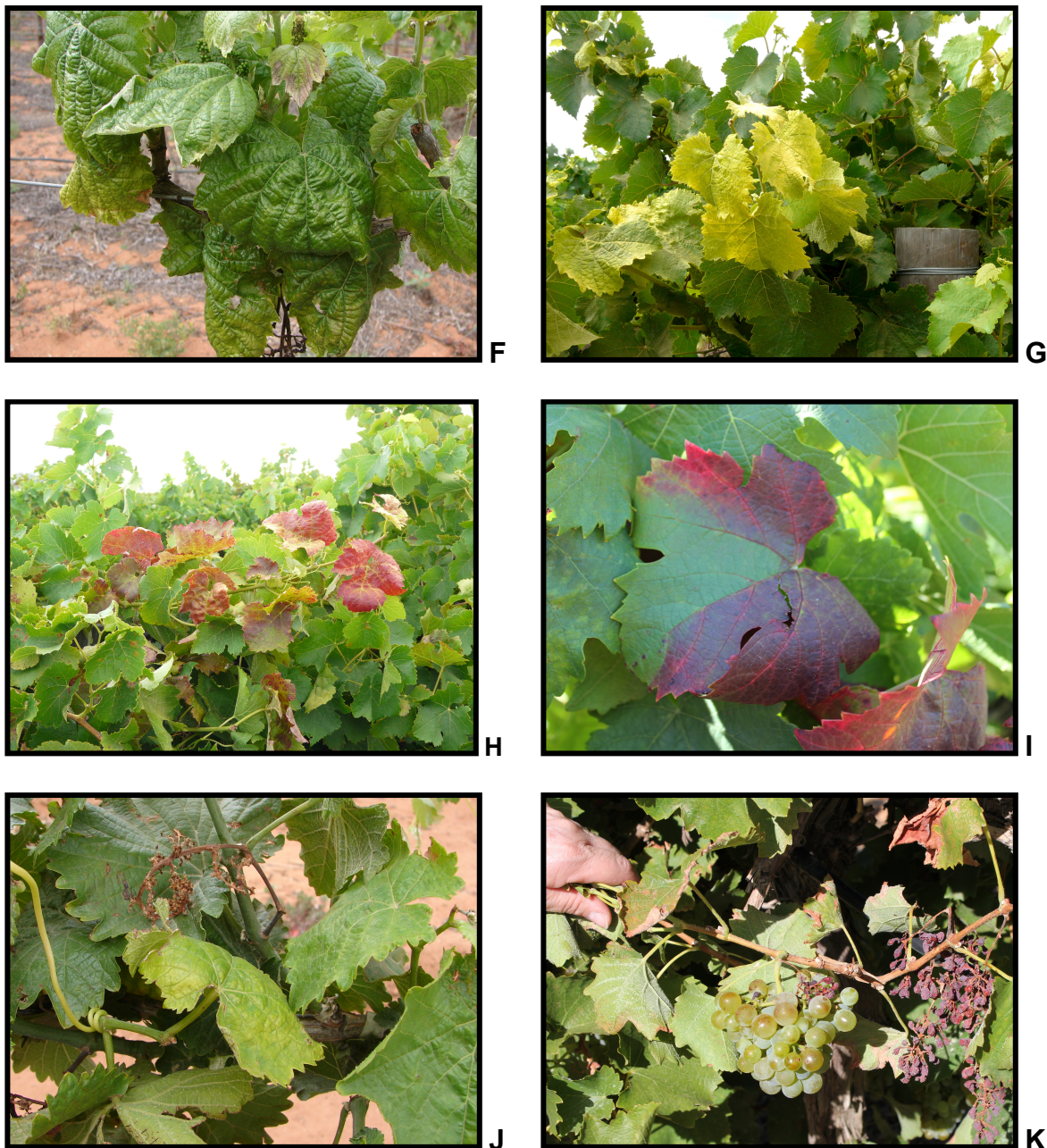


D



E





**Figure 4.** Aster yellows symptoms: (A) Thin stunted shoots that do not lignify. (B) Rubbery shoots giving the vine a weeping or drooping appearance. (C) Growth tip death followed by die back of shoot node-by-node. (D) Zigzag growth of non-lignified shoot and total reddening of leaves on infected Pinotage. (E) Chardonnay shoots show a typical grey waxy substance and have a triangular shape. (F) Leaves of infected vines have a wrinkled appearance early in the season. (G) Leaves of infected white cultivars turn to bronze yellow and roll downwards. (H) Infected vines of a red cultivar show red discoloration of leaves. (I) Sectorial reddening of leaves is a typical symptom of phytoplasma infection on some red cultivars. (J) Bunch dries out and aborts early. Yellow leaf with wrinkled appearance in foreground (Photo: Jeff Joubert). (K) Infected bunches shrivel and dry out before ripening.

## 4.2 Disease incidence

All vines in the 14 selected vineyards in the Vredendal wine growing region were visually assessed for AY symptoms late summer (late January/early February) for a varying number of seasons from 2009-2013. A total of 144 514 vines were visually assessed over the survey period. Varied disease incidences were recorded for the different vineyards. During 2010, producers were made aware of the fact that GY infections occurred in their vineyards and as a result of this some producers cut back vines or removed shoots with AY symptoms as soon as it became apparent, to try and reduce the available sources of phytoplasma. Due to these practices followed by producers some vineyards (sites 1, 2, 7, 8, 9 and 10) could not be surveyed for the full survey period. The yearly disease incidences of these vineyards are shown in Table 5. The systemic insecticide, imidacloprid, was applied to all AY affected vineyards surveyed in this study. The applications were made in the spring of 2010 and after harvest in 2012.

The Chenin blanc vineyard (site 1) was planted in September 2009 and the first GY symptoms were observed in November of the same year. It was surveyed in February 2010 and showed a disease incidence of 7.4%. The vineyard was pollarded in the same season by cutting vines 30 cm above the ground and allowed to grow out again. No AY symptoms could however be observed on any of these vines during the following season and the survey was terminated.

The Shiraz vines (site 2) were surveyed in 2010 and 2011 and showed yearly disease incidences of 10.6% and 3.7%, respectively. This vineyard also showed leafroll virus symptoms. In 2011 leafroll symptoms were observed, but much less AY symptoms. In 2012 only leafroll symptoms could be observed and no AY symptoms could be found. The vineyard was therefore not surveyed again the following season.

The vineyards at sites 7, 8, 9 and 10 all belong to the same producer and were surveyed late January/early February 2010. Yearly disease incidences of 2.6%, 4%, 8.2% and 0.1%, respectively were recorded. During November 2010 when the first symptoms became visible, shoots with AY symptoms were removed at all these sites. Disease incidences of 4.5%, 3.1%, 11.3% and 0.1%, respectively, were recorded in 2011. The

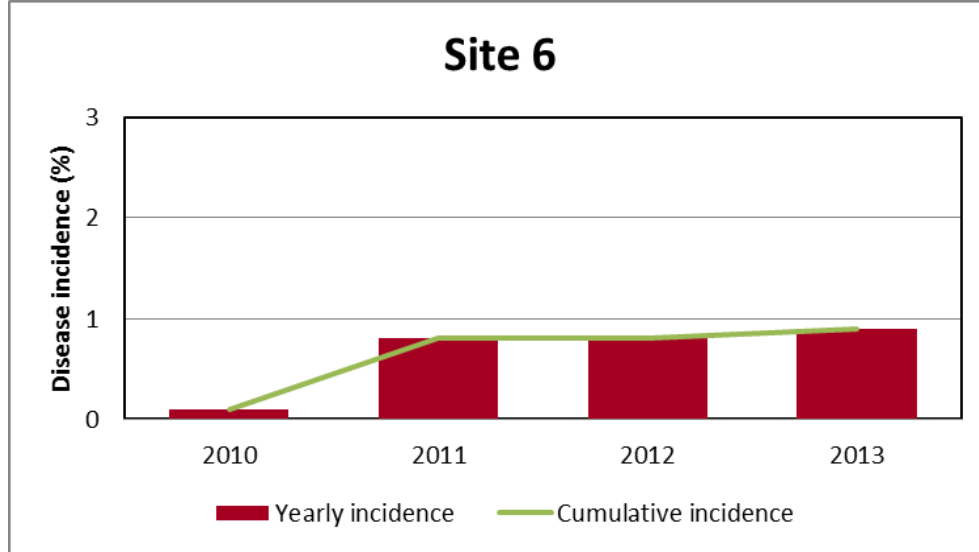
producer proceeded to remove AY symptoms every year and therefore these vineyards were not surveyed after 2011.

**Table 5.** AY incidence of vineyards that could not be surveyed for the full period from 2010 - 2013.

Site	Cultivar	Yearly disease incidence (%)		
		2010	2011	2012
1	Chenin blanc	7.4	0	-
2	Shiraz	10.6	3.7	0
7	Sauvignon blanc	2.6	4.5	-
8	Sauvignon blanc	4.0	3.1	-
9	Colombar	8.2	11.3	-
10	Cabernet franc	0.1	0.1	-

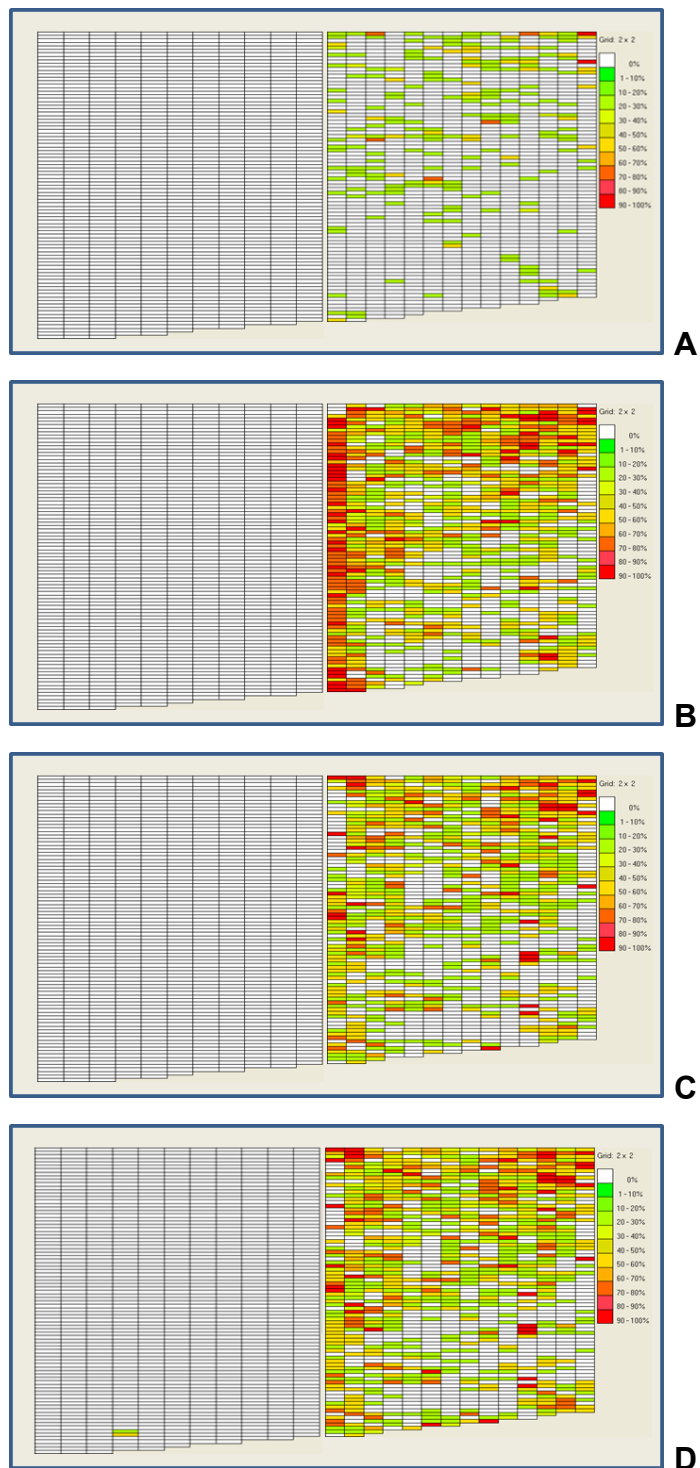
- = not surveyed.

The Pinotage vineyard (site 6) was planted in September 2009 and the first AY symptoms were observed in November of the same year. Fifteen AY diseased vines out of 2412 vines (0.6%) were identified and immediately rogued and replanted. From February 2010 to 2013 visual disease assessments were performed annually and yearly disease incidences (= number of vines showing disease symptoms in the current year) of 0.1%, 0.8%, 0.8% and 0.9% and cumulative disease incidences (= sum of all new records of grapevines showing disease symptoms in the current year and all records of diseased grapevines in previous years) of 0.1%, 0.8%, 0.8% and 0.9%, respectively, was recorded (Figure 5).



**Figure 5.** Yearly and cumulative disease incidence (%) of AY-affected grapevines in a Pinotage vineyard (site 6).

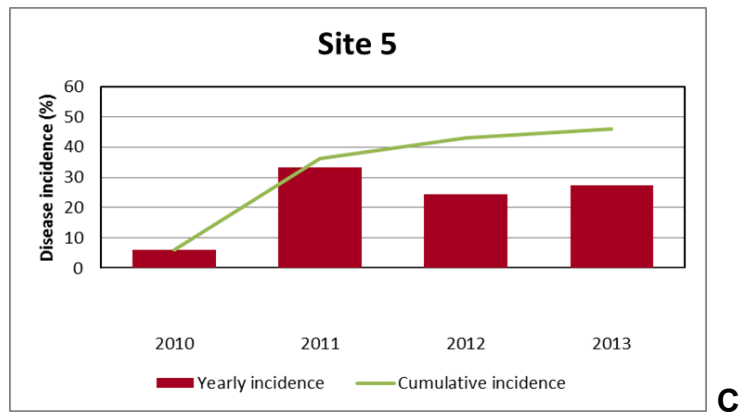
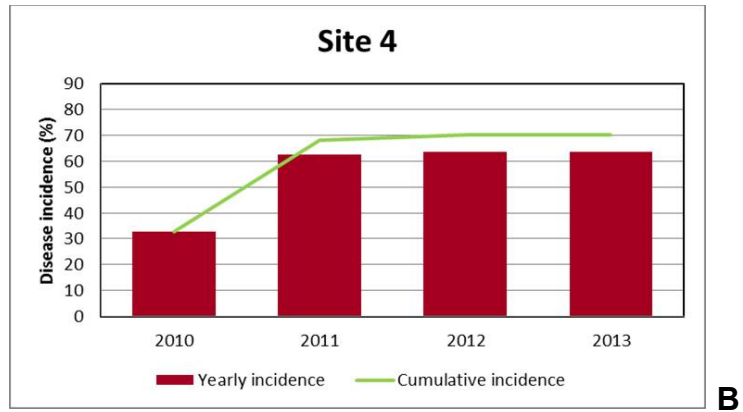
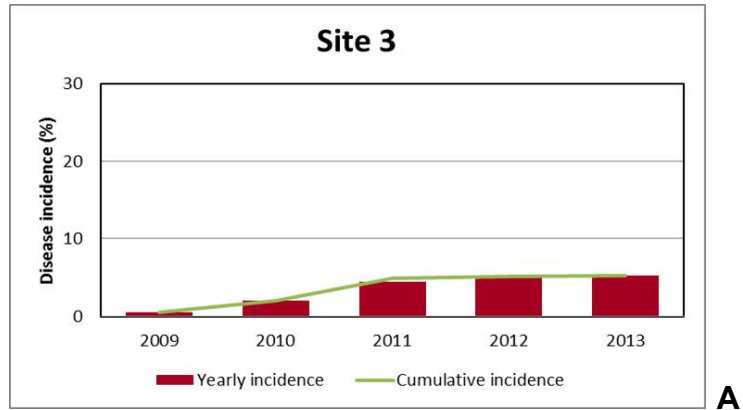
A new Chenin blanc vineyard (site 14) (Figure 6 – left) was planted in August 2010 next to a two year-old Chenin blanc vineyard (site 5) (Figure 6 – right) showing 6% disease infection in 2010. Site 5 was surveyed from 2010 – 2013 and site 14 from 2011 - 2013. Site 14 was treated with imidacloprid shortly after planting and again after harvest in 2012. The two year-old Chenin blanc vineyard (site 5) showed yearly disease incidences of 6%, 33.3%, 24.4% and 27.4%. Cumulative disease incidences of 6%, 36.1%, 43% and 45.9% were recorded (Figure 7C). The new Chenin blanc vineyard (site 14) showed yearly and cumulative disease incidences of 0%, 0% and 0.1% (data not shown). In 2013, three years after planting the new Chenin blanc vineyard (site 14) AY symptoms could be observed for the first time and three adjacent vines out of 3761 vines tested positive for AY (Figure 6d, left).

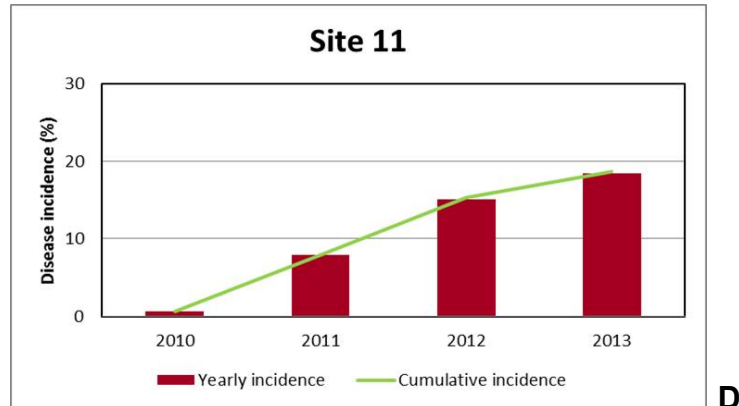


**Figure 6.** Spread of AY from an infected Chenin blanc vineyard (right – site 5) to an adjacent new Chenin blanc vineyard (left – site 14) planted in 2010 (A) and surveyed during (B) 2011, (C) 2012 and (D) 2013 as displayed in PATCHY using a 2 x 2 grid size.

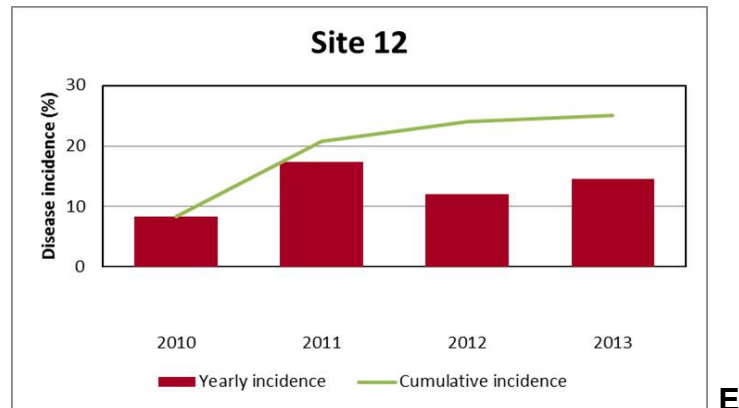


Vineyards of the following cultivars: Chardonnay (site 3, 4); Chenin blanc (site 5, 11) and Pinotage (site 12, 13) were surveyed from 2009/2010 until 2013 and varied disease incidences were recorded as shown in Figure 7A, B, C, D, E and F, respectively. The yearly and cumulative incidences of AY-affected grapevines at sites 3 (Figure 7A), 4 (Figure 7B), 11 (Figure 7D) and 13 (Figure 7F) followed similar patterns, although the incidence of disease was higher at site 4 with cumulative disease incidence reaching 70%. The yearly and cumulative incidences of disease at site 5 (Figure 7C) and 12 (Figure 7E) showed similar patterns with a higher disease incidence at site 5. The cumulative incidence at site 5 was 45.9%. Year-by-year progression of disease incidence is illustrated in Figure 12-18.

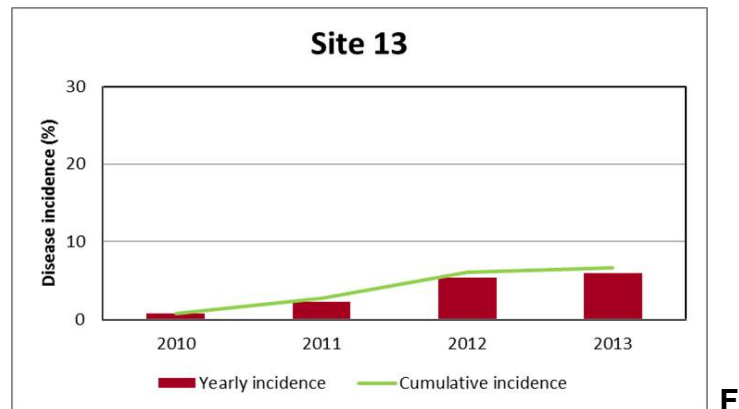




D



E



F

**Figure 7.** Yearly and cumulative disease incidence (%) of grapevines in two Chardonnay vineyards [(A) site 3, (B) site 4]; two Chenin blanc vineyards [(C) site 5, (D) site 11] and two Pinotage vineyards [(E) site 12, (F) site 13] surveyed from 2009 to 2013 in the Vredendal wine producing area.

Mean yearly disease incidences of the cultivars studied varied between 5.8% and 29.95%. Although the mean yearly disease incidences of Chardonnay and Chenin blanc were higher than that of Pinotage, there were no statistical significant difference between these three cultivars (Table 6). The mean yearly disease incidence did however show a significant trend over time and the disease incidence of the 2010 season was significantly lower than that of 2011, 2012 and 2013 (Table 7).

**Table 6.** Mean yearly disease incidence of the different cultivars.

<b>Cultivar</b>	<b>Mean</b>	<b>N</b>	<b>T Grouping*</b>
Chardonnay	29.95	8	A
Chenin blanc	16.64	8	A
Pinotage	5.80	12	A
LSD ( $p \leq 0.05$ )	50.396		

\*Values followed by the same letter do not differ significantly.

**Table 7.** Mean yearly disease incidence over the survey period.

<b>Year</b>	<b>Mean</b>	<b>N</b>	<b>T Grouping*</b>
2013	19.470	7	A
2012	18.072	7	A
2011	18.399	7	A
2010	7.240	7	B
LSD ( $p \leq 0.05$ )	7.0609		

\*Values followed by the same letter do not differ significantly.

The average percentage new infections for Chardonnay, Chenin blanc and Pinotage was 9.2%, 8% and 2.7%, respectively. New AY infections did not differ significantly between the three cultivars (Table 8). The new infections that occurred in 2011 (12.9%) was significantly higher than that of 2012 (3.1%) and 2013 (1.2%), but it did not differ significantly from 2010 (7.2%). The percentage new infections that occurred in 2010 did not differ significantly from that of 2012 and 2013 (Table 9).

**Table 8.** Average percentage new AY infections in the different cultivars.

<b>Cultivar</b>	<b>Mean</b>	<b>N</b>	<b>T Grouping*</b>
Chardonnay	9.2	8	A
Chenin blanc	8.0	8	A
Pinotage	2.7	12	A
LSD ( $p \leq 0.05$ )	17.092		

\*Values followed by the same letter do not differ significantly.

**Table 9.** Average percentage new AY infections over the survey period.

<b>Year</b>	<b>Mean</b>	<b>N</b>	<b>T Grouping*</b>
2011	12.9	7	A
2010	7.2	7	A B
2012	3.1	7	B
2013	1.2	7	B
LSD ( $p \leq 0.05$ )	9.1326		

\*Values followed by the same letter do not differ significantly.

The cumulative incidence of AY-affected vines, which was calculated by adding the new records of diseased grapevines in each year to all records of diseased grapevines in previous years, increased from year to year over the survey period at all the sites, except for Pinotage (site 6) where no new infections occurred in 2012 and the cumulative incidence of 2011 and 2012 were therefore the same. By the end of the survey (2013) the cumulative incidence of AY at each site was: Chardonnay (site 3) 5.3%, Chardonnay (site 4) 70.3%, Chenin blanc (site 5) 45.9%, Chenin blanc (site 11) 18.7%, Pinotage (site 6) 0.9%, Pinotage (site 12) 25.1% and Pinotage (site 13) 6.6%. The mean cumulative disease incidence of Chardonnay, Chenin blanc and Pinotage was 37.77%, 32.31% and 10.87%, respectively, with no significant difference between the cultivars (Table 10).

**Table 10.** Mean cumulative disease incidence of the different cultivars at the end of the survey period (2013).

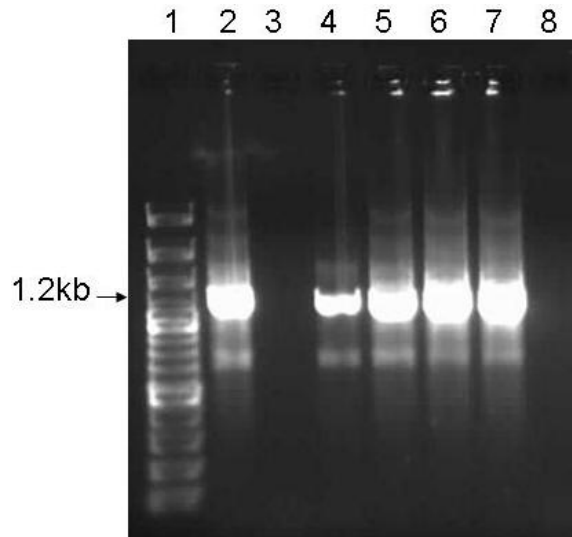
<b>Cultivar</b>	<b>Mean</b>	<b>N</b>	<b>T Grouping*</b>
Chardonnay	37.77	2	A
Chenin blanc	32.31	2	A
Pinotage	10.87	3	A
LSD ( $p \leq 0.05$ )	69.271		

\*Values followed by the same letter do not differ significantly.

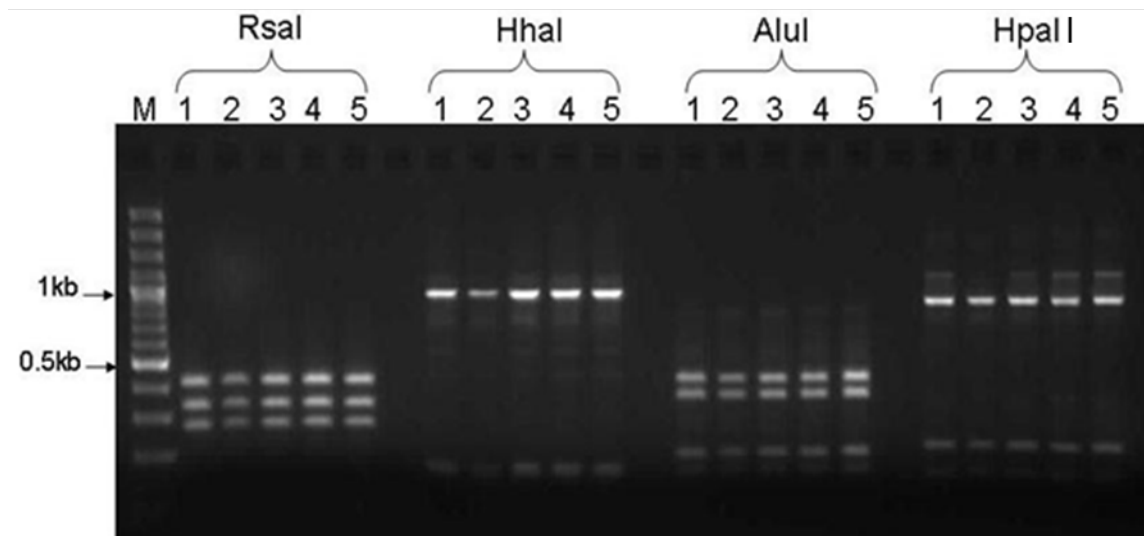
### 4.3 Phytoplasma detection by PCR and RFLP analysis

The PCR process was optimised with samples from infected and healthy vines collected in Vredendal vineyards in December 2009. The four samples analysed (Hanepoot, Datal, Cabernet franc, Chenin blanc) tested positive for the presence of a phytoplasma, while the Red Globe sample tested negative (Figure 8). RFLP analysis of samples 1-4 showed that they produced the same RFLP pattern (Figure 9) as the AY positive control, indicating infection of the four plant samples with only AY phytoplasma.

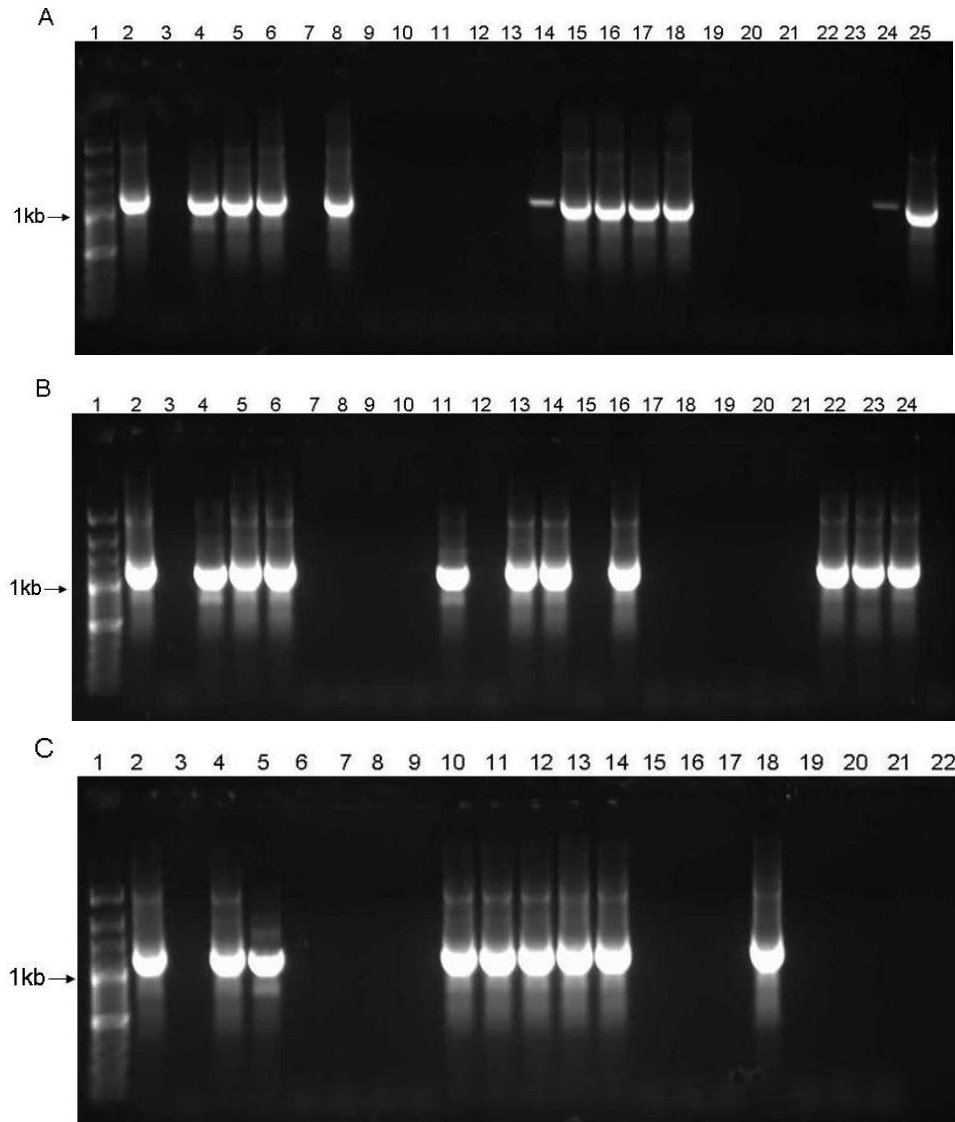
Nested PCR detection of phytoplasma from grapevine samples using universal primers P1/P7 followed by R16F2n/R16R2 confirmed phytoplasma presence in some of the samples analysed every year (2010 to 2013) from all vineyards. Figure 10 shows the results for 2012, which are representative of results obtained from 2010 to 2013. RFLP analysis of PCR products digested with *AluI*, *HhaI*, *HpaII*, *KpnI*, *RsaI*, *TaqI* and *TruI* confirmed the presence of only AY phytoplasma in the samples analysed from all vineyards during this survey from 2010 to 2013 (Figure 11).



**Figure 8.** Agarose gel analysis of amplicons following nPCR with universal phytoplasma primers. Lane (1) 100 bp plus DNA marker (Thermo Scientific), (2) AY positive control, (3) negative control, (4) Hanepoot, (5) Datal, (6) Cabernet franc, (7) Chenin blanc, (8) Red Globe.

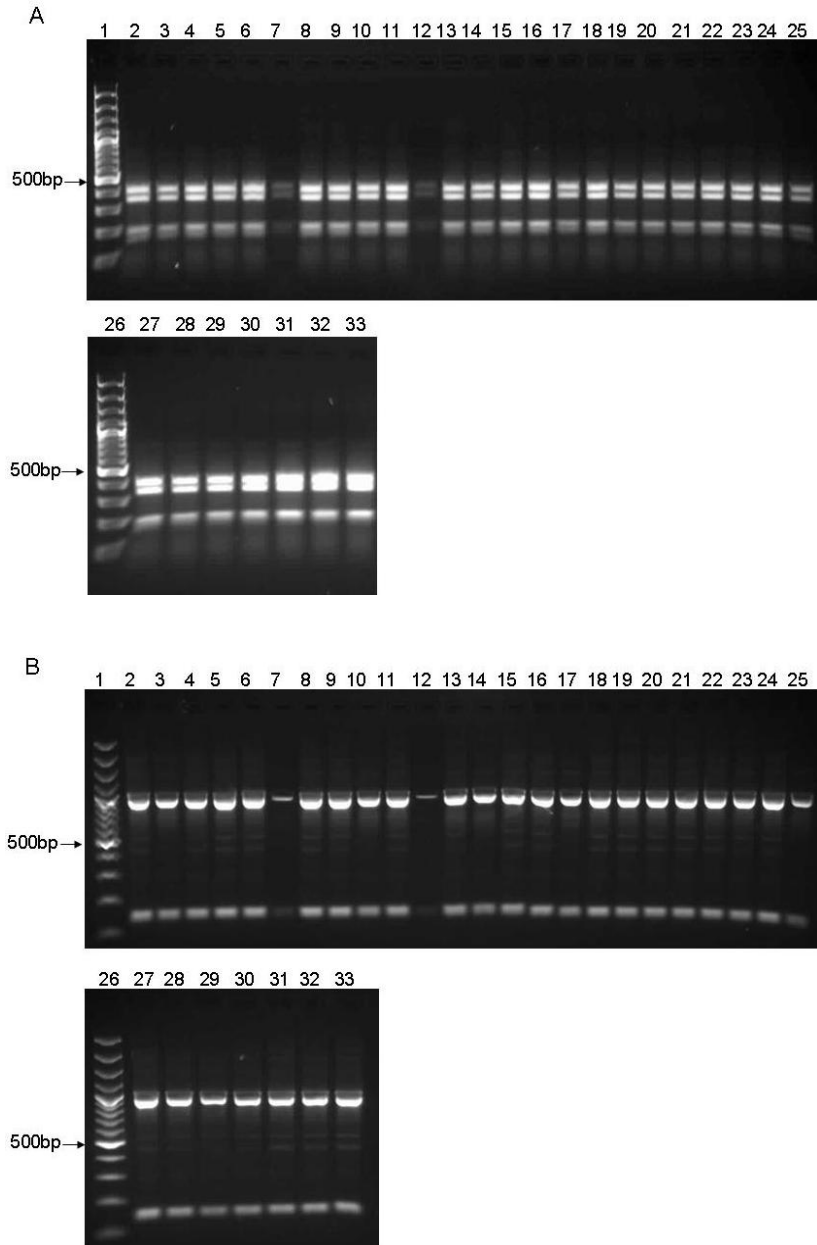


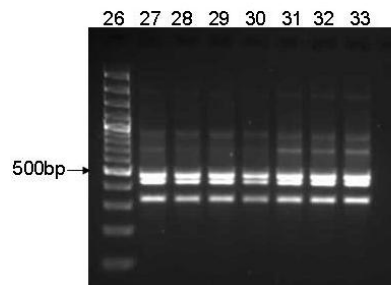
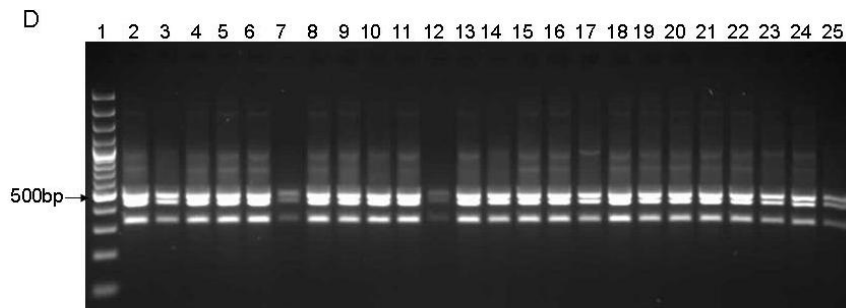
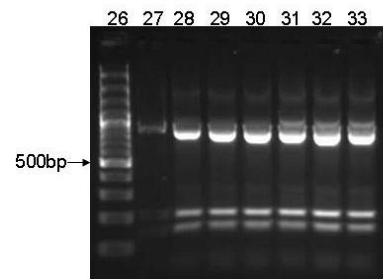
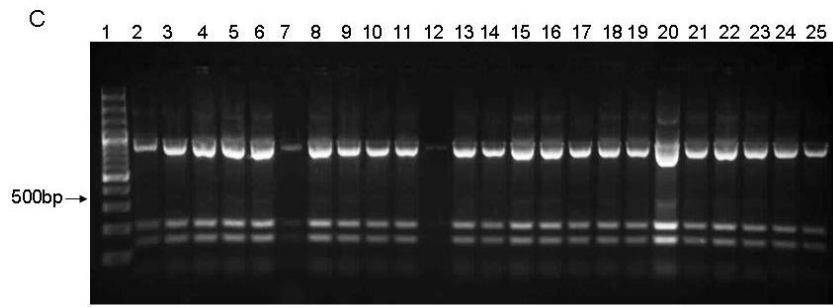
**Figure 9.** RFLP analysis of nPCR amplicons. Lane (1) AY positive control; (2) Hanepoot; (3) Datal; (4) Cabernet franc; (5) Chenin blanc; (M) 100 bp plus DNA marker (Thermo Scientific).

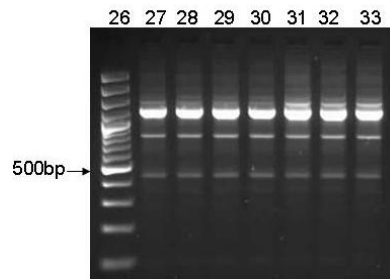
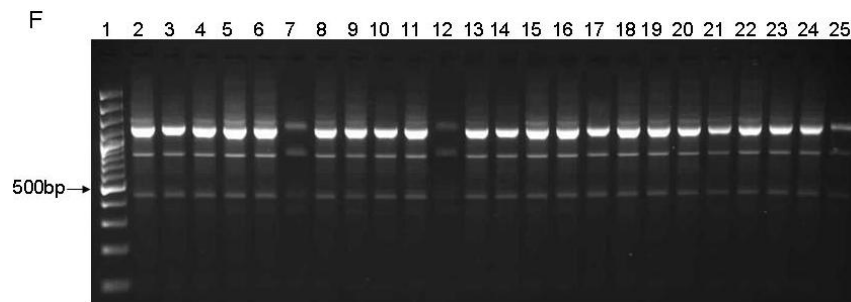
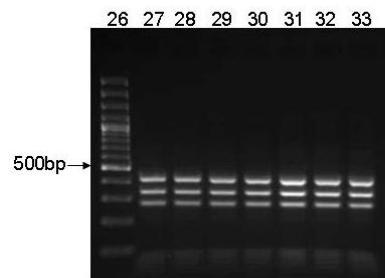
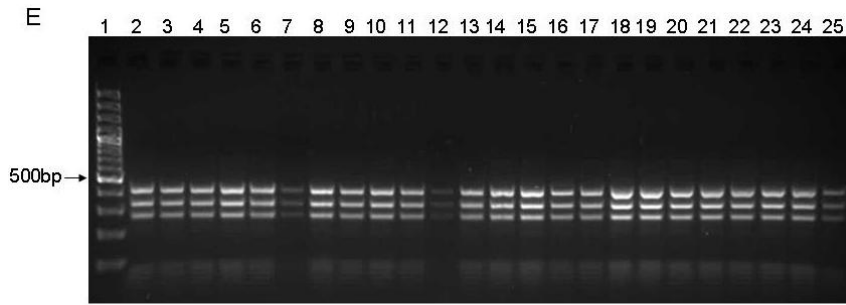


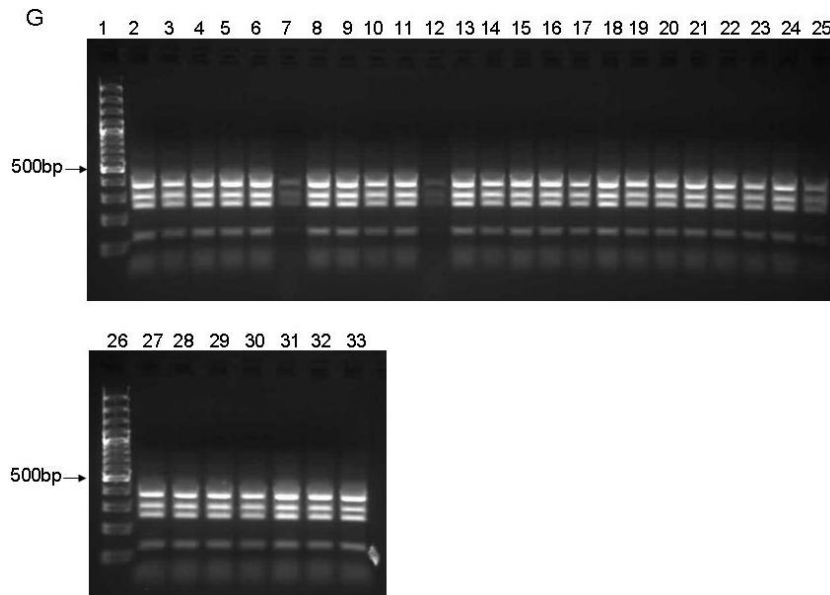
**Figure 10.** Nested PCR detection of phytoplasma of grapevine samples collected during the 2012 season, using universal primers P1/P7 followed by R16F2n/R16R2. PCR products were electrophoresed through a 1% agarose gel. Lane 1) 100bp Plus molecular weight marker (Thermo Scientific), (2) AY-positive control, (3) No template-negative control. A: Lanes (4-25) grapevine samples MP1-10, PBC1-10, RC1-2. B: Lanes (4-24) RC3-9, LP1 RC10, LP2-10, RW1-4. C: Lanes (4-22) grapevine samples RW5-10, TB1-10, KJS1, KO.











**Figure 11.** RFLP analysis of 16S rDNA amplified from grapevine samples collected during the 2012 season, via nested PCR with the universal primers P1/P7 followed by R16F2n/R16R2. PCR products were digested with (A) *AluI* (B) *HhaI* (C) *HpaII* (D) *KpnI* (E) *RsaI* (F) *TaqI* and (G) *TruI*. Lane (1) 100bp Plus molecular weight marker (Thermo Scientific), (2) AY phytoplasma, (3) MP1, (4) MP2, (5) MP3, (6) MP5, (7) PBC1, (8) PBC2, (9) PBC3, (10) PBC4, (11) PBC5, (12) RC1, (13) RC2, (14) RC3, (15) RC5, (16) LP1, (17) LP2, (18) LP3, (19) LP5, (20) RW1, (21) RW2, (22) RW3, (23) RW4, (24) RW5, (25) RW6, (26) 100bp Plus molecular weight marker (Thermo Scientific), (27) AY phytoplasma, (28) TB1, (29) TB2, (30) TB3, (31) TB4, (32) TB5, (33) TB9.

PCR and RFLP analysis to confirm disease status of the vines was performed every year from 2010-2013 for the available samples. The results were used to correlate disease status with visual symptom evaluation. Correlation between disease status tested by PCR-RFLP and visual evaluation was 78%, 83%, 95% and 90% in the respective years as described in Table 11. During the survey period AY had been detected in 8 out of 178 grapevines (4.5%), which did not express any GY symptoms. AY had been detected in 138 out of 186 (74.2%) symptomatic grapevines.

**Table 11.** Correlation between disease status tested by PCR-RFLP and visual assessments over the survey period.

<b>Year</b>	<b>Number of symptomatic plants nPCR positive out of number tested</b>	<b>Number of asymptomatic plants nPCR positive out of number tested</b>	<b>Correlation between PCR and visual symptom analysis (%)</b>
2010	41/67	2/62	78
2011	44/59	4/56	83
2012	29/30	2/30	95
2013	24/30	0/30	90
<b>TOTAL</b>	<b>138/186</b>	<b>8/178</b>	<b>86</b>

#### 4.4 Spatial analysis of AY

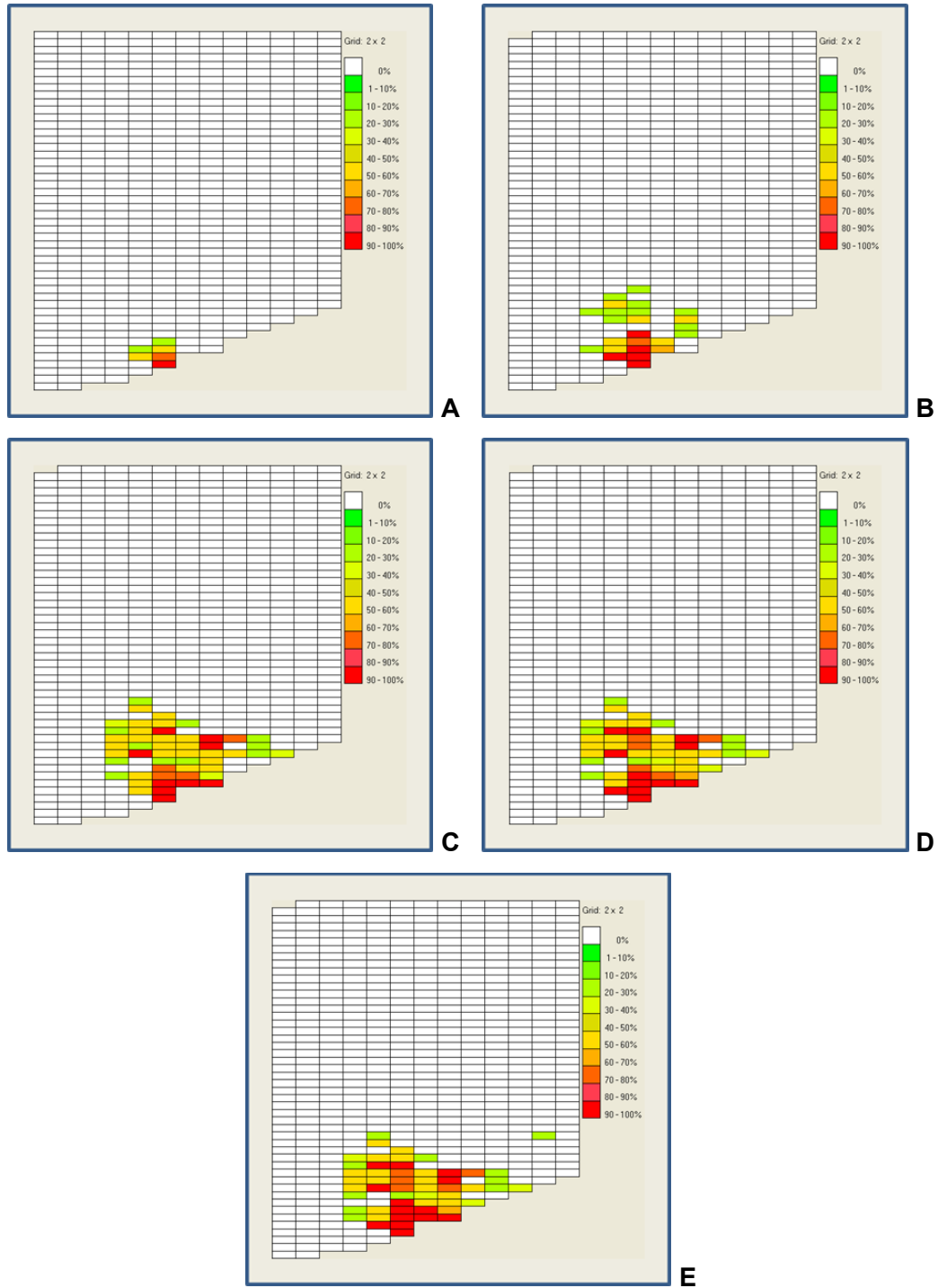
The PATCHY spatial analysis package (Maixner, 1993) was used to test for randomness or clustering of AY disease affected vines by ordinary runs analysis along and across rows. Spatial analysis results are indicated in Table 12 and year-by-year progression of disease incidence, which gives an indication of clustering, is illustrated in Figure 12-18. The Pinotage vineyard planted in 2009 (site 6; Figure 15) showed random spread along rows in the first year of the survey (2010) but from 2011 to 2013 non-random spread or clustering occurred along rows. The disease spread of this specific vineyard remained random across rows throughout the survey. The Chenin blanc vineyard planted in 2010 (site 14) next to an infected vineyard showed no disease until 2013 and then clustering along the row occurred. Across row disease distribution was random in 2013 (Figure 6 left). All other vineyards at site 3 (Figure 12), 4 (Figure 13), 5 (Figure 14), 11 (Figure 16), 12 (Figure 17) and 13 (Figure 18) showed non-random or clustered patterns along and across rows during all the years of the survey. Edge effects with a gradient of infection from one side of a vineyard were observed in some of the vineyards at site 4, 5, 11, 12 and 13 (Figure 13, 14, 16, 17, 18). All of these instances could be correlated to adjacent vineyards infected with grapevine yellows. The Chardonnay vineyard at site 3 (Figure 12)

showed spread from a single point on one side of the vineyard that is not adjacent to another vineyard.

Table 12. Ordinary runs analysis performed on vineyards.

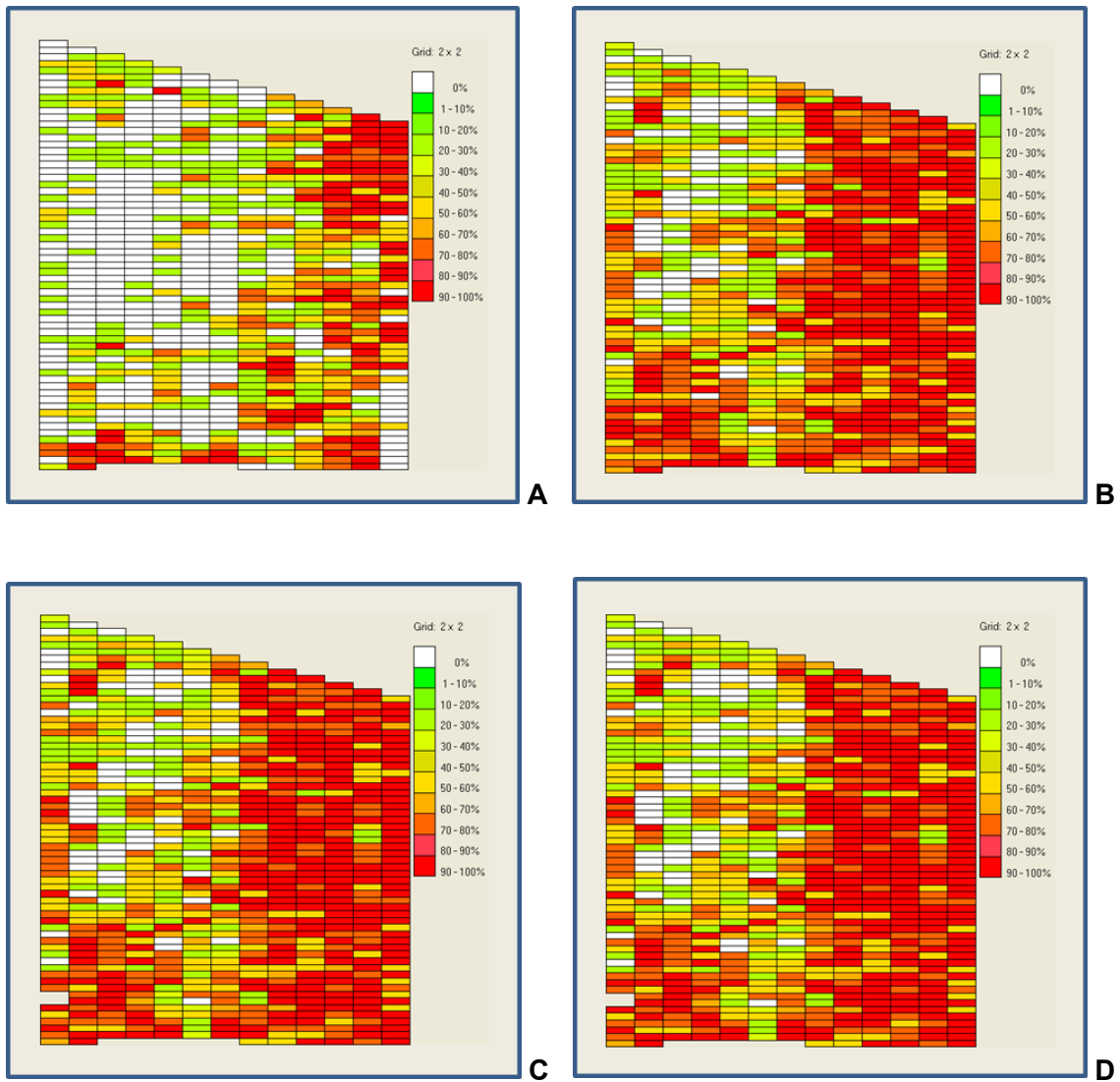
		Site 3	Site 4	Site 5	Site 6	Site 11	Site 12	Site 13	Site 14
2009	Z-statistic	-37.3757	■	■	■	■	■	■	■
	Random / Non-random	N	■	■	■	■	■	■	■
	Z-statistic	-13.1183	■	■	■	■	■	■	■
	Random / Non-random	N	■	■	■	■	■	■	■
2010	Z-statistic	-28.7428	-25.4142	-7.4106	7.0922	-1.2813	-17.5701	-28.7079	■
	Random / Non-random	N	N	N	R	N	N	N	■
	Z-statistic	-22.3103	-15.5042	-5.5403	7.0922	-1.2813	-18.4029	-16.1785	■
	Random / Non-random	N	N	N	R	N	N	N	■
2011	Z-statistic	-33.1345	-19.8604	-18.8656	-3.9719	-5.5952	-19.7985	-24.7638	*
	Random / Non-random	N	N	N	N	N	N	N	*
	Z-statistic	-19.8181	-13.2047	-13.4765	1.0396	-6.5728	-13.0579	-17.9332	*
	Random / Non-random	N	N	N	R	N	N	N	*
2012	Z-statistic	-33.7915	-20.9819	-12.6907	-3.9719	-11.9193	-13.3500	-39.3268	*
	Random / Non-random	N	N	N	N	N	N	N	*
	Z-statistic	-23.9048	-13.3751	-7.4808	1.0396	-8.9066	-7.6596	-25.7224	*
	Random / Non-random	N	N	N	R	N	N	N	*
2013	Z-statistic	-33.1582	-20.9311	-13.6996	-5.8073	-13.0584	-16.4267	-42.8378	-39.2377
	Random / Non-random	N	N	N	N	N	N	N	N
	Z-statistic	-25.4629	-13.3150	-8.2538	-1.2520	-10.5908	-10.3012	-27.6626	5.5991
	Random / Non-random	N	N	N	R	N	N	N	R

■ = not surveyed; \* = No statistics (no infection); N= Non-random; R= Random

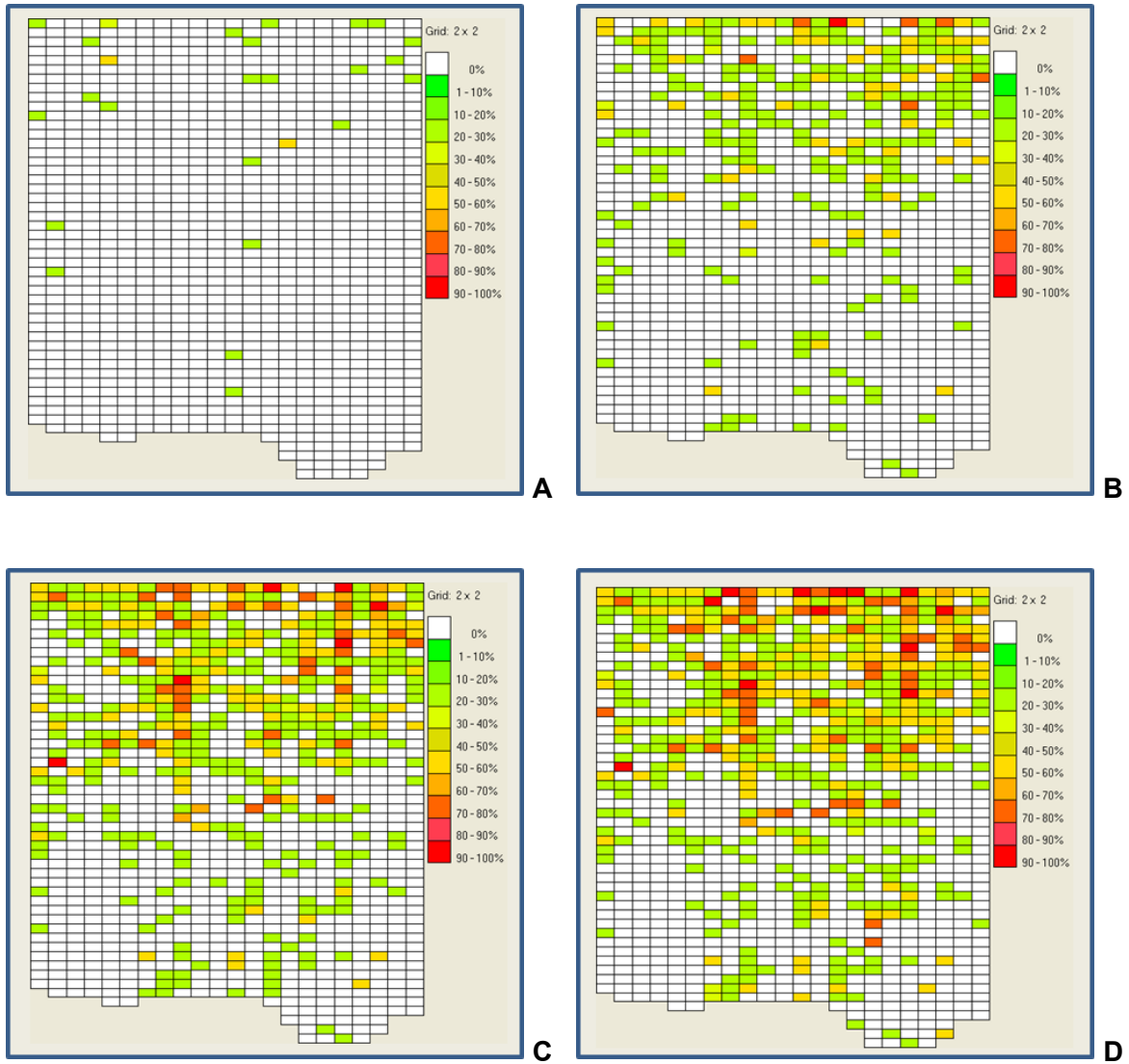


**Figure 12.** Year-by-year progression of AY incidence in a Chardonnay vineyard (site 3) surveyed in (A) 2009, (B) 2010, (C) 2011, (D) 2012 and (E) 2013 as displayed in PATCHY using a 2 x 2 grid size.

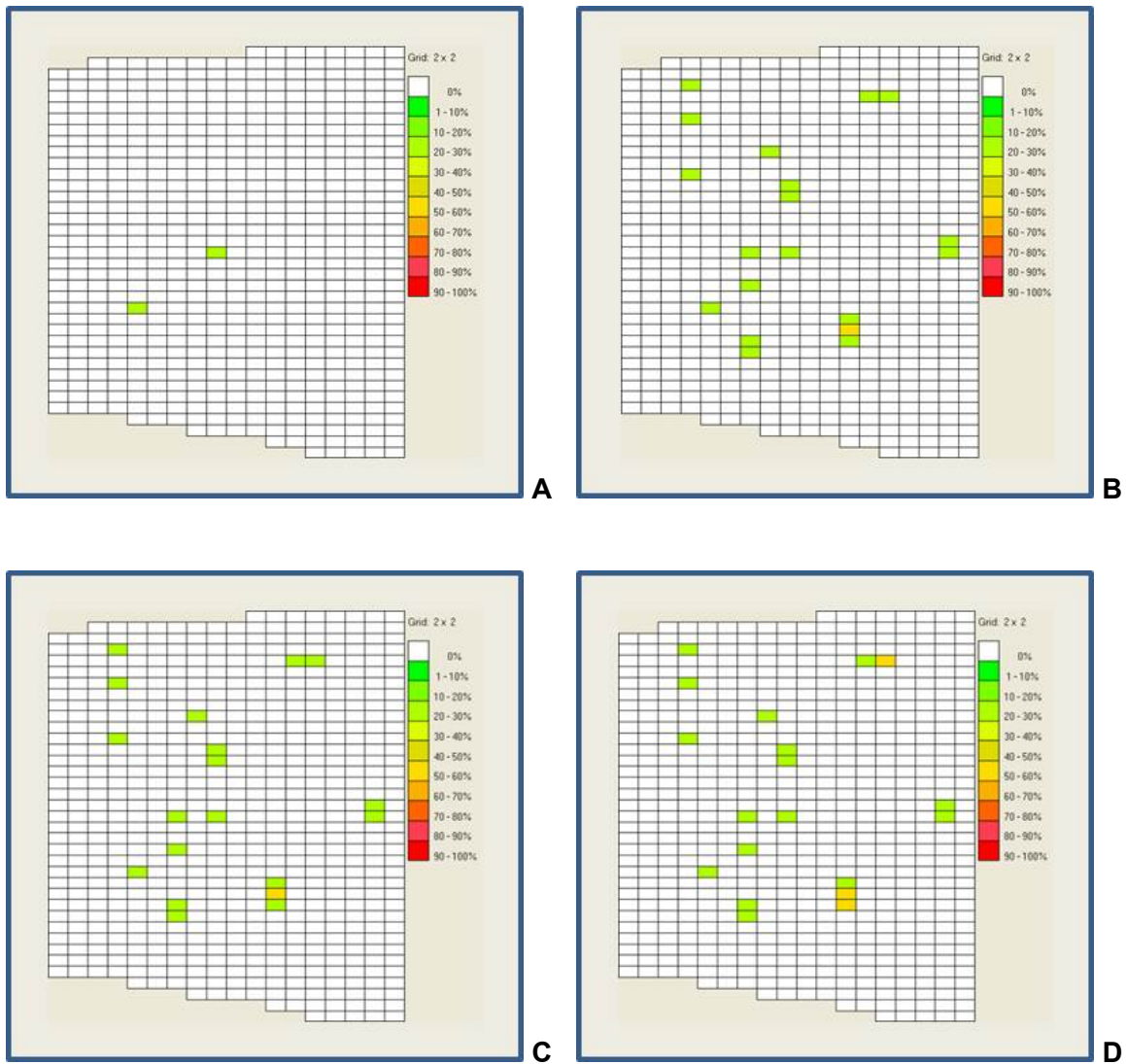




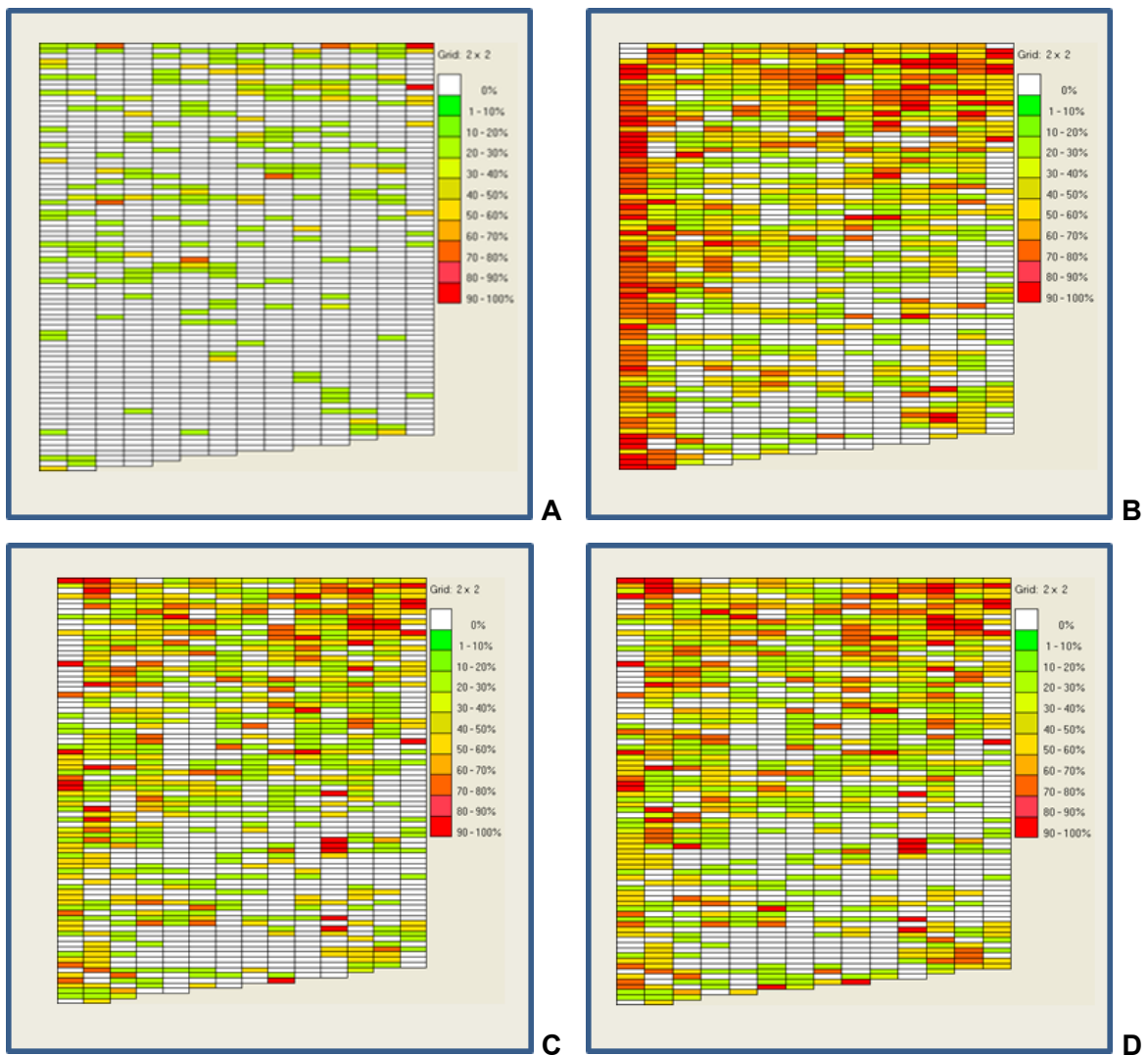
**Figure 13.** Year-by-year progression of AY incidence in a Chardonnay vineyard (site 4) surveyed in (A) 2010, (B) 2011, (C) 2012 and (D) 2013 as displayed in PATCHY using a 2 x 2 grid size.



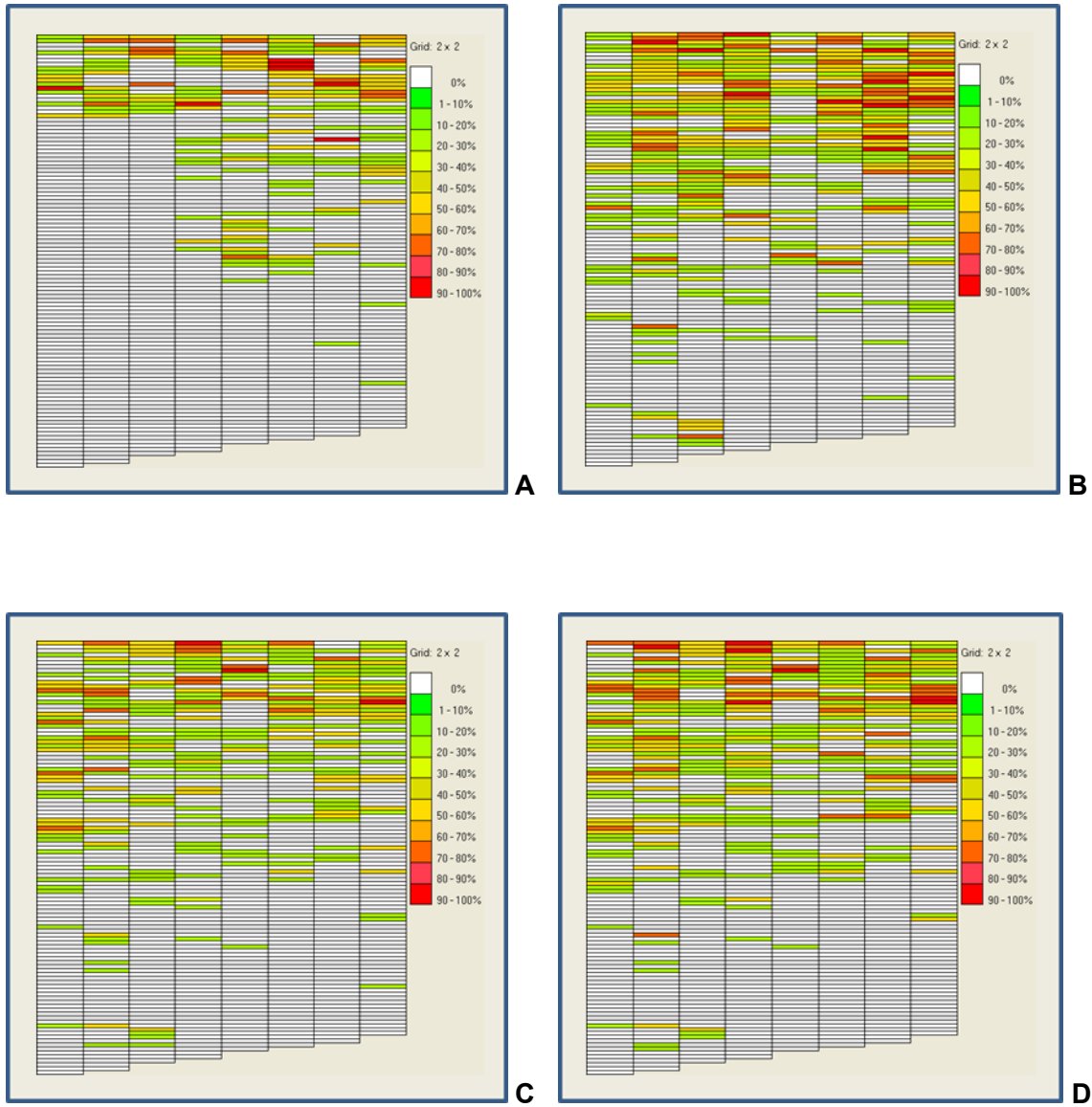
**Figure 14.** Year-by-year progression of AY incidence in a Chenin blanc vineyard (site 5) surveyed in (A) 2010, (B) 2011, (C) 2012 and (D) 2013 as displayed in PATCHY using a 2 x 2 grid size.



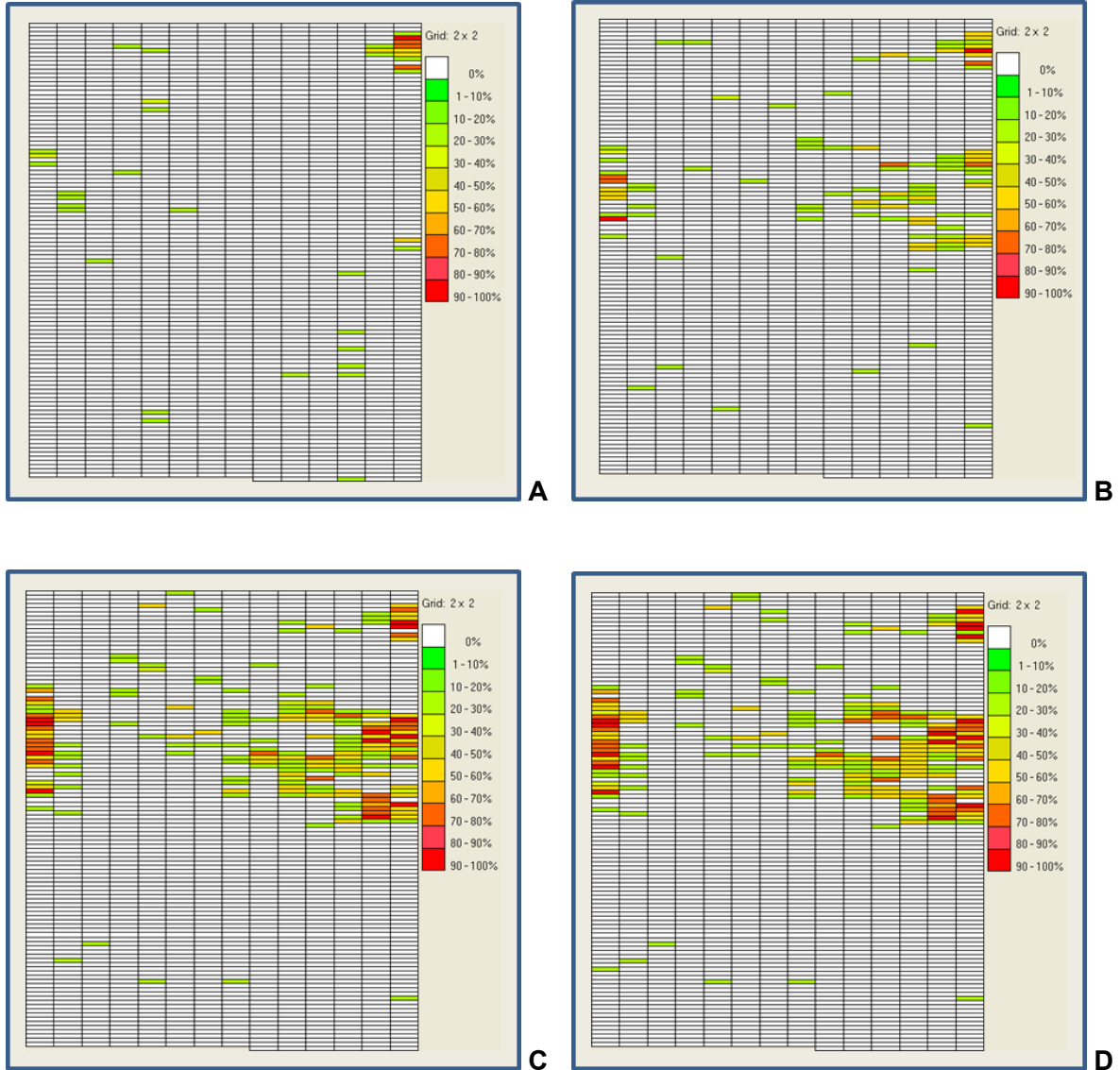
**Figure 15.** Year-by-year progression of AY incidence in a Pinotage vineyard (site 6) surveyed in (A) 2010, (B) 2011, (C) 2012 and (D) 2013 as displayed in PATCHY using a 2 x 2 grid size.



**Figure 16.** Year-by-year progression of AY incidence in a Chenin blanc vineyard (site 11) surveyed in (A) 2010, (B) 2011, (C) 2012 and (D) 2013 as displayed in PATCHY using a 2 x 2 grid size.



**Figure 17.** Year-by-year progression of AY incidence in a Pinotage vineyard (site 12) surveyed in (A) 2010, (B) 2011, (C) 2012 and (D) 2013 as displayed in PATCHY using a 2 x 2 grid size.



**Figure 18.** Year-by-year progression of AY incidence in a Pinotage vineyard (site 13) surveyed in (A) 2010, (B) 2011, (C) 2012 and (D) 2013 as displayed in PATCHY using a 2 x 2 grid size.

#### 4.5 Alternative host plants

Weeds and other possible host plants (55 samples comprising of 31 species, Table 13) were collected from 13 different sites in and around vineyards infected with AY in March 2010, 2011 and 2012 and tested for the presence of phytoplasma using the universal primers (Table 4).

The following species collected in 2010 produced a band of the correct size (1200 bp), similar to that of AY, following nested PCR with universal primers: *Chenopodium album* (white goosefoot), *Urtica urens* (small stinging nettle), *Setaria verticillata* (sticky bristle grass), *Cyperus esculentus* (yellow nut sedge) (Figure 19; lane 5, 7, 9 and 15). Of the weeds tested in 2011 *Atriplex lindleyi* subsp *inflata* (salt bush), *Datura stramonium* (common thorn apple) and *Eleusine indica* (ox grass) consistently yielded an approximately 1200 bp band following nPCR and gel electrophoresis, indicating possible phytoplasma infection (Figure 20B; lane 16, 17 and 18).

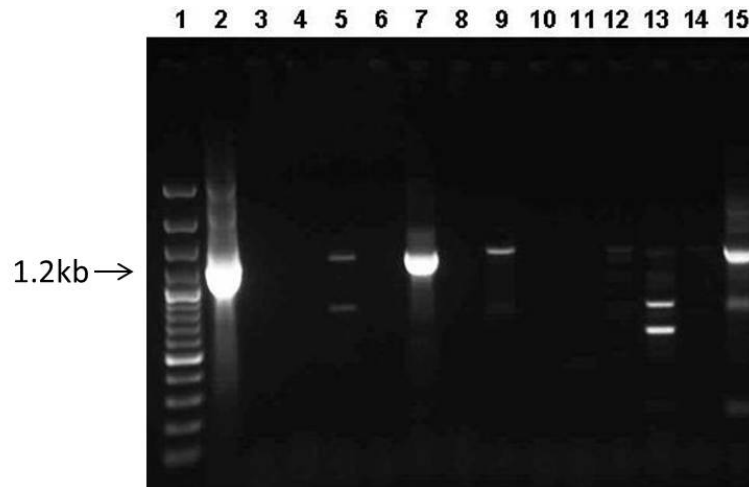
Group-specific PCR and PCR-RFLP of samples showed that the phytoplasmas in the weed samples were not AY, nor did they belong in Phytoplasma Groups I, III, V or X. Results for *Atriplex lindleyi* subsp *inflata* (salt bush), *Datura stramonium* (common thorn apple) and *Eleusine indica* (ox grass) are shown in Figure 21 (2010 RFLP results not shown). Hence the PCR products for the nine weed samples, comprising of seven different species, were cloned and sequenced to determine the identity of the amplicons. BLAST analysis showed the ~1200 bp DNA fragment to have a high sequence similarity to the 16S rRNA gene of two Gram-positive bacteria belonging to the genera *Bacillus* and *Friedmaniella*.

**Table 13.** Possible host plants collected in and around infected vineyards and analysed with PCR RFLP for presence of phytoplasmas.

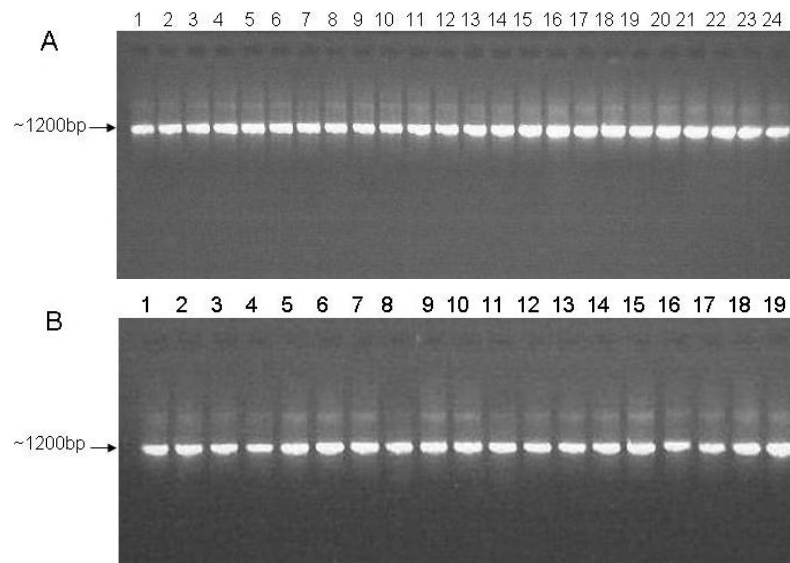
Family	Latin name	Common name	Number of plants nPCR positive out of number tested
Chenopodiaceae	<i>Salsola kali</i>	Russian tumbleweed	0/2
Chenopodiaceae	<i>Chenopodium album</i>	White goosefoot	1/3
Chenopodiaceae	<i>Atriplex lindleyi</i> subsp <i>inflata</i>	Salt bush	1/1
Chenopodiaceae	<i>Atriplex semibaccata</i>	Australian Salt bush	0/1
Amaranthaceae	<i>Amaranthus viridis</i>	Slender amaranth	0/1
Amaranthaceae	<i>Amaranthus spinosus</i>	Thorny pigweed	0/1
Poaceae	<i>Setaria verticillata</i>	Sticky bristle grass	2/3
Poaceae	*	Eragrostis sp.	0/1
Urticaceae	<i>Urtica urens</i>	Small stinging nettle	1/1
Cyperaceae	<i>Cyperus esculentus</i>	Yellow Nut sedge	1/2
Asteraceae	<i>Bidens bipinata</i>	Spanish Blackjack	0/2
Asteraceae	<i>Sonchus oleraceus</i>	Thistle	0/3
Asteraceae	<i>Conyza sumatrensis</i>	Tall fleabane	0/1
Asteraceae	<i>Eleusine indica</i>	Ox grass	1/1
Asteraceae	<i>Flaveria bidentis</i>	Smelter's bush	0/1
Convolvulaceae	<i>Ipoemea purpurea</i>	Morning Glory	0/1
Commelinaceae	<i>Commelina benghalensis</i>	Wandering Jew	0/1
Rosaceae	*	Rose	0/1
Solanaceae	<i>Datura stramonium</i>	Common thorn-apple	2/2
Solanaceae	<i>Solanum nigrum</i>	Black nightshade	0/2
Solanaceae	<i>Lycopersicon esculentum</i>	Tomato	0/8
Malvaceae	*	Aromatic malva	0/1
Malvaceae	<i>Malva parviflora</i>	Small mallow	0/1
Fabaceae	<i>Medicago sativa</i>	Lucerne	0/2
Fabaceae	<i>Phaseolus</i>	Bean	0/5
Fabaceae	<i>Melilotus indica</i>	Annual yellow sweet clover	0/2
Euphorbiaceae	<i>Euphorbia hypericifolia</i>	Milk purslane	0/1
Euphorbiaceae	<i>Chamaesyce hirta</i>	Red milkweed	0/1
Zygophyllaceae	<i>Tribulus terrestris</i>	Devil's thorn	0/1
Brassicaceae	<i>Raphanus raphanistrum</i>	Wild radish	0/1
Portulacaceae	<i>Portulaca oleracea</i>	Purslane or pigweed	0/1
<b>TOTAL</b>			<b>9/55</b>

\*Could not be identified on species level.

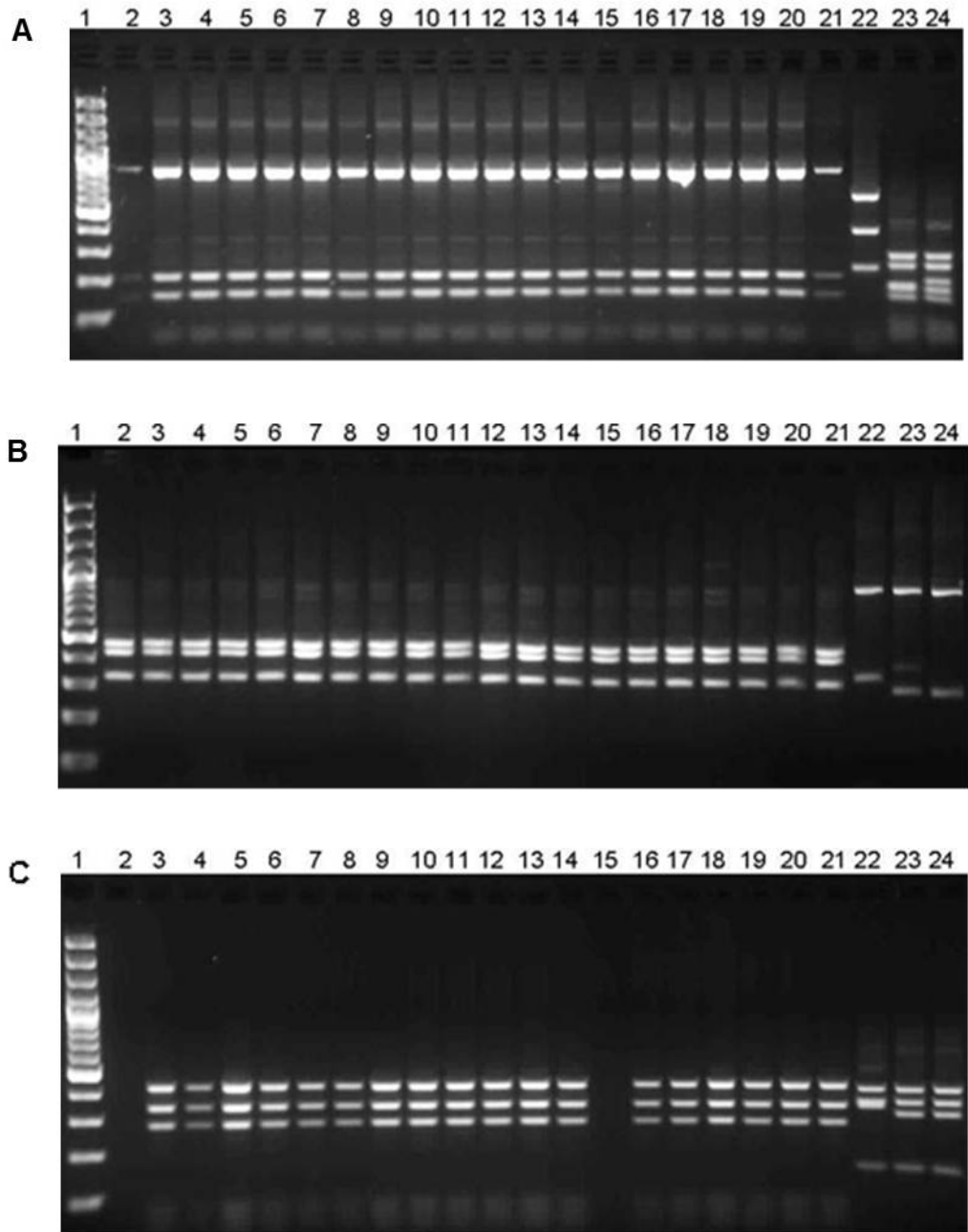




**Figure 19.** Nested PCR detection of phytoplasma in vineyard weed samples (year 2010) using universal primers P1/P7 followed by R16F2n/R16R2. Lane 1) 100bp Plus molecular weight marker (Thermo Scientific), 2) AY positive control, 3) water negative control, 4) *Ipoemea purpurea*, 5) *Chenopodium album*, 6) *Salsola kali*, 7) *Urtica urens*, 8) *Cyperus esculentus*, 9) *Cyperus esculentus*, 10) *Chenopodium album*, 11) *Sonchus oleraceus*, 12) *Datura stramonium*, 13) *Amaranthus viridis*, 14) *Setaria verticillata*, 15) *Setaria verticillata*.



**Figure 20.** Nested PCR detection of a number of grapevine and weed samples (year 2011), which tested positive for the presence of a phytoplasma using universal primers P1/P7 followed by R16F2n/R16R2. PCR products were electrophoresed through a 1% agarose gel. A: lane 1) JNO1, 2) PDP1 3) PDP3 4) PDP4 5) CN1 6) CN2 7) CN8 8) TB1 9) TB2 10) TB3 11) TB4 12) TB5 13) TB9 14) SVZ3 15) SVZ4 16) PBS10 17) RC1 18) RC2 19) RC3 20) RC4 21) RC5 22) RC12 23) JNB1 24) JNB2  
 B: lanes 1) JNB3 2) JNB4 3) LP2 4) PDP3 5) PDP5 6) CN3 7) CN4 8) CN5 9) TB8 10) RC14 11) RC11 12) JNO2 13) LP3 14) LP4 15) LP5 16) *Atriplex lindleyi* subsp *inflata* 17) *Datura stramonium* 18) *Eleusine indica* 19) SVZ5.



**Figure 21.** RFLP analysis of 16S rDNA amplified from grapevine and three weed samples via nested PCR with the universal primers P1/P7 and R16F2n/R16R2. PCR products were digested with (A) *HpaI* (B) *KpnI* and (C) *RsaI*. Lane 1) 100bp Plus molecular weight maker (Thermo Scientific), 2) AY phytoplasma, 3) SVZ4, 4) SVZ5, 5) PBS10, 6) RC1, 7) RC2, 8) RC3, 9) RC4, 10) RC5, 11) RC11, 12) RC12, 13) RC14, 14) LP2, 15) LP3, 16) LP4, 17) LP5, 18) JNB1, 19) JNB2, 20) JNB3, 21) JNB4, 22) *Atriplex lindleyi* subsp *inflata* 23) *Datura stramonium* 24) *Eleusine indica*.

## 5 DISCUSSION

The epidemiology of four of the grapevine yellows diseases FD, BN, AGY and NAGY have been studied extensively (Constable, 2010), but worldwide little epidemiological knowledge for AY disease of grapevine existed up to now. This study determined the incidence and distribution of grapevine yellows disease in a variety of cultivars in the Vredendal wine producing region in order to understand the impact of the disease on the crop; and represents the first report on disease incidence and spatial distribution of AY disease on grapevine in South Africa. Visual assessments of AY disease incidence were performed annually on a variety of cultivars in the Vredendal region and disease status was confirmed with PCR. Disease incidence and spatial distribution of AY-affected vines were analysed. Weeds and other alternative host plants were surveyed in and around disease-affected vineyards.

In the Wabooms River region only a couple of vineyards were infected with grapevine yellows and because the area forms part of a bigger wine producing area where mother blocks of the Plant Improvement Organizations are situated, immediate actions were taken to control further spread of the disease by removing some of the infected vineyards and applying imidacloprid to infected vineyards. Although it was planned to perform the survey in more than one area, the abovementioned removal of infected vineyards limited the survey to the Vredendal area only, where more infected vineyards of a larger variety of cultivars were available. The survey started with seven different cultivars, but because of reasons mentioned (see Results) surveys of Shiraz, Sauvignon blanc, Colombar and Cabernet franc were terminated and statistical analysis could be performed for seven vineyards, consisting of three cultivars, namely Chardonnay, Chenin blanc and Pinotage. Grapevine yellows symptoms were observed in the Vredendal area on both wine and table grape varieties, but this study was limited to wine grape varieties.

Phytoplasma diseases worldwide generally exhibit similar symptoms. However, specific symptoms that were rarely reported in literature to be associated with grapevine yellows disease were observed during this study. A blue grey waxy appearance that can be

rubbed off was regularly found on AY-affected Chardonnay shoots. Dead shoot tips and dieback of shoots were observed on disease-affected vines of all cultivars surveyed in this study. Both these symptoms were reported to appear on vines affected with grapevine yellows disease in Australia (Constable et al., 2003a), but was not regularly observed on GY-affected vines in Italy, Germany or Hungary (personal observations). Grapevine cultivars differ in the severity of expression of GY disease symptoms. Worldwide, Chardonnay is severely affected by GY and it was confirmed to be similar in AY-affected Chardonnay in South Africa. During this study a rapid decline, which eventually leads to death of phytoplasma-infected vines, was observed in Chardonnay. Chardonnay covers 8.1% of the total area under wine grapes in South Africa. In the Olifants River region, where the first symptoms of AY disease were observed, 5% of the total Chardonnay in the country is planted. From 2006 to 2011 a total of 178 hectares of Chardonnay has been uprooted in the Olifants River region according to SAWIS, which could to some extent probably be ascribed to phytoplasma disease. Other cultivars such as Colombar are not affected so severely and only show a few affected bunches and yellow leaves without much effect on the growth of vines and the yield. This severe effect that the disease has on Chardonnay might force producers in the Vredendal wine producing region to plant less Chardonnay and concentrate on cultivars that are less affected by the disease. Results of this study indicate that some AY-affected vines do not show symptoms every year. This phenomenon could be ascribed to seasonal variation in phytoplasma titre; environmental factors (Lee et al., 2000) and remission or recovery from disease as reported for BN (Riedle-Bauer et al., 2010), FD (Caudwell, 1961, 1964) and AGY (Constable et al., 2003b). During the 2011/12 season symptoms were only fully visible later in the season (end January), compared to previous seasons when symptoms could already be observed in November and December. This late expression of symptoms could possibly be ascribed to climatic differences between the seasons. GY symptoms were observed in a Pinotage (site 6) and Chenin blanc (site 1) vineyard in the Vredendal region three months after planting, which is different from GY in North America, where GY infection was never found in the first year of newly-established vineyards (Wolf, 2000). In Italy symptoms usually appear one year after inoculation in adult vines and 3 to 6 months after inoculation in grafted vines (Angelini, 2008). According to Hogenhout (2009) the latent period can vary between 7 and 80 days and symptoms can develop in plants after approximately 7 days,

but it can also take much longer (6-24 months) depending on the plant species and the specific phytoplasma. The infections in the above mentioned Pinotage and Chenin blanc vineyards could have been caused by insect transmission from nearby infected vineyards as leafhoppers had been found in these vineyards (De Klerk, personal communication) and both vineyards had GY-affected vineyards bordering two sides of the vineyard. However, this suggestion can only be confirmed when the latent period of the vector, *Mgenia fuscovaria* (Stal), had been determined. If the latent period turns out to be longer than three months, the infection in these two vineyards were caused by infected plant material and not by insect vectors.

The different sites show varied patterns of yearly and cumulative disease incidences. Some of these patterns can be ascribed to the pollarding of vines (Chardonnay, site 4) where vines were pollarded by the producer from 2011 onwards and the Chenin blanc vineyard (site 5) that was pollarded in 2011 only. This removal of symptomatic plants could contribute to the higher yearly disease incidence at site 4 from 2011 to 2013; and at site 5 in 2011, as vines that were pollarded were recorded as disease-affected, while it might not have been positive for AY. However, symptoms reappeared on some of these pollarded vines the next year. Unfortunately vines were pollarded by the producer without determining beforehand if the vines were AY affected and no conclusions on the effect of pollarding on AY disease could therefore be drawn from this experiment. Trials will have to be executed to compare different pruning methods in order to determine if pruning practices can be used to reduce the presence of AY, and therefore minimise the chance of transmission of the phytoplasma. The Pinotage at site 12 was also infected with leafroll virus and this could perhaps affect the visual assessments, since virus symptoms and some AY symptoms are sometimes difficult to distinguish. This was especially the case in the beginning of the survey when the technical team was not so familiar with the symptoms of AY disease.

After the first survey in 2010 producers were made aware of the presence of AY disease and to reduce the disease inoculum, symptomatic parts of plants were removed. This limited the number of vineyards that could be used for the survey. An insect vector, *Mgenia fuscovaria* (Stal), was identified (Douglas-Smit et al., 2010) in the same year and

producers immediately treated vines with the systemic neonicotinoid insecticide, imidacloprid, which was found to be more effective than organophosphates in preventing transmission of chrysanthemum yellows phytoplasma (CYP) by the leafhopper *Macrostelus quadripunctulatus* Kirschbaum (Saracco et al., 2008). Treatments of vineyards were performed in the spring of 2010 and after harvest in March 2012. These treatments probably influenced the disease incidences recorded in 2012 and 2013 considering that only 3.1% and 1.2% new infections occurred. Disease incidence of AY could therefore be potentially higher than what was found in this study if no control of vectoring insects was performed and natural spread of the disease was allowed.

Varied disease incidences were recorded for the different cultivars studied, namely Chardonnay, Chenin blanc and Pinotage, and incidences also varied between the different sites. The mean yearly disease incidence in the first year of the survey was 7.24% and it increased to 19.47% over the 4 year survey period. Pinotage showed a mean yearly disease incidence of 5.8%, which was lower than Chenin blanc (16.64%) or Chardonnay (29.95%). However, statistically there was no significant difference ( $p \leq 0.05$ ) between the mean yearly disease incidences of the three cultivars. Disease incidence of five vineyards showed an increasing pattern and in two of the vineyards yearly disease incidence fluctuated. These fluctuating patterns could be contributed to pollarding and objectivity of the evaluating team. The disease incidence of AY is not as high as that reported for FD, where 'epidemic' incidences (95%) occurred in some vineyards (Bressan et al., 2006) nor as low as that reported for NAGY, where yearly incidences of approximately 4% occurred (Wolf, 2000). Yearly disease incidences were all below 20%, except for two vineyards. In one case (Chardonnay, site 4) a higher initial disease incidence (32.7%) possibly skewed the incidence pattern. Likewise, pollarded vines (Chenin blanc, site 5), which were assumed to be positive for AY, could have affected the incidence at this site. AY disease yearly disease incidence is also lower than that reported for AGY, where incidences of 20%, 55%, 20%, 44%, 46% and 44% occurred (Constable et al., 2004). Cumulative incidences indicated that new records of AY infected grapevines occurred in every vineyard in every year, except for Pinotage (site 6), which had no new infections in 2012. There was no significant difference between the new AY infections occurring in the three different cultivars. New infections of the different cultivars varied between 2.7% and 9.2%.

Cumulative incidences of AY followed the same pattern except for the vineyards where high incidence could be attributed to factors such as initial high incidence, pollarding of infected vines or the objectivity of the evaluating team. The mean cumulative disease incidence of 37.77% recorded at the end of the 4-year survey period for AY affected Chardonnay in the Vredendal region is much lower than that of AGY where cumulative incidences of 77.3%, 71.5% and 53.7% after a 6-year period and 55.7% after a 5-year period were reported for Chardonnay (Constable et al., 2004). Although the mean cumulative disease incidence of Pinotage (10.87%) was lower than that of Chenin blanc (32.31%) and Chardonnay (37.77%) the three cultivars did not differ significantly. Depending on the initial disease incidence this results indicate that Chardonnay vineyards can be 100% infected in 10 years at the current tempo of infection.

This study contributes significantly to our understanding of grapevine yellows in the Vredendal region, but is not necessarily a true reflection of the situation in all grape growing regions in South Africa. High incidence of AGY was reported in the warmer inland districts of Australia compared to other regions (Constable et al., 2004), but temperature is not the only factor that has an effect on the transmission of the disease by insect vectors. Factors such as vector efficiency, vector numbers, AY inoculum levels and possible host plants can all have an effect on disease incidence. Results of this study can therefore not be extrapolated to other wine producing areas and epidemiological studies should also be performed in other areas where AY occurs.

PCR was optimised and used to confirm visual assessments of AY-affected vineyards. Samples that had been scored as phytoplasma-positive based on symptomology, but showed up negative in the first round of nPCR were re-tested using 1 µl of undiluted, 2 µl of a 1/10 dilution and 2 µl of a 1/20 dilution of the total extracted nucleic acid as template. In most cases, DNA was re-isolated from plant material and then subjected to nPCR. In addition, to rule out the possibility of false negatives due to PCR-inhibitors in the DNA samples, 1 µl of the 1/10 dilution of the total nucleic acid was spiked with the positive control DNA.



Symptomless AY phytoplasma infections were found to occur in South African grapevines (4.5% of asymptomatic plants tested positive for AY) as reported in other parts of the world for phytoplasma diseases (Gibb et al., 1999; Constable et al., 2003a). In Italy however, no evidence could be found for the presence of AY in asymptomatic plants (Bianco et al., 1996). The spatial distribution of visually infected vines is therefore not an accurate indication of the spatial distribution of AY phytoplasma. Molecular detection methods will be more accurate when determining disease status for disease incidence studies. AY was detected in 74.2% of the symptomatic vines. The fact that AY has not been detected in all symptomatic vines indicates that uneven distribution of the AY phytoplasma occurs in vines as previously described by Constable (2003a) and Spinis (2013).

Molecular genetic analyses showed that all vines sampled in the Vredendal vicinity contained AY only, which confirms the results found by Engelbrecht et al, 2010. Weeds and other possible host plants collected in and around AY-infected vineyards were tested for the presence of phytoplasma using the universal primers. Some samples yielded an approximately 1200 bp band following nPCR and gel electrophoresis, indicating possible phytoplasma infection. Group-specific PCR and PCR-RFLP of the samples showed that phytoplasmas in the weed samples were not AY, nor did it belong in Phytoplasma Groups I, III, V or X. PCR products for the weed samples were cloned and sequenced to determine the origin of the amplicons. BLAST analysis showed the ~1200 bp DNA fragment to have a high sequence similarity to the 16S rRNA genes of two Gram-positive bacteria belonging to the genera *Bacillus* and *Friedmaniella*. This demonstrates the importance of conducting additional tests such as PCR-RFLP and sequencing when using the universal phytoplasma primers in PCR. Although weeds such as *Convolvulus arvensis*, *Solanum nigrum*, *Chenopodium album*, *Urtica* species and *Conyza canadensis*, all which occur regularly in and around vineyards in the Vredendal region, were mentioned in literature to host phytoplasmas (Batlle et al., 2000; Weaver, 2001; Mori et al., 2008; Berger et al., 2009; Longone et al., 2011), no phytoplasmas were found present in any weeds or other possible host plants during this study. However, limited numbers of each possible host were collected, which makes the chances of finding phytoplasma infection very slim. Disease patterns displaying clustering of phytoplasma-infected vines on the sides of a vineyard could therefore not be attributed to the presence of alternative host



plants, but rather to the presence of infected vineyards next to surveyed blocks. The Chardonnay at site 3 is the only vineyard where infection started at a specific point where there is no infected vineyard adjacent. This infection started opposite a tap where water is collected for spraying. Movement of people at this spot could possibly have carried phytoplasma-infected vectoring insects from nearby diseased vineyards.

The analysis of the disease incidence maps of vineyards during the survey suggests an increase in incidence and clustering over the years. Young vineyards initially show random disease incidence patterns which change to non-random patterns as the disease progresses. Similar results were reported by Magarey et al. (2005), who noted that clustering was more likely to occur in vineyards with high AGY incidence, while in vineyards with lower disease incidence, the disease was more likely to be randomly distributed. Non-random disease incidence patterns are typical of transmission of disease by an insect vector (Beanland et al., 2006). Aggregation along plant rows could also indicate the spread of disease by viticultural practices such as pruning or mechanical harvesting, which is the case with a bacterial disease like grapevine bacterial blight. The spread of phytoplasma diseases by pruning has not been observed (Angelini, personal communication) but it is important that this be confirmed by scientific experiments in order to eliminate this mode of transmission.

## 6 CONCLUSION

The aim of this study was to conduct surveys in phytoplasma disease-affected vineyards in the Vredendal area to determine the incidence and spatial distribution of grapevine yellows disease in a variety of cultivars. A survey was also conducted in and around AY-affected vineyards in search of possible alternative host plants of the phytoplasma.

During this study, a rapid decline of AY-infected Chardonnay, which eventually leads to the death of vines, was observed. This indicates that Chardonnay is very sensitive to AY phytoplasma infection, confirming the severe sensitivity of Chardonnay to infection by a wide variety of phytoplasma diseases.

Symptomless AY phytoplasma infections were found to occur in South African grapevines, as reported worldwide for other phytoplasma diseases. AY has not been detected in all symptomatic vines, which indicate that uneven distribution of the AY phytoplasma occurs in vines as previously described by Spinaz (2013). Molecular analyses showed that all vines sampled in the Vredendal vicinity contained AY only, which confirms the results found by Engelbrecht et al, 2010. No phytoplasmas were found to be present in any weeds or other possible host plants during this study. Limited numbers of possible host plants were surveyed and tested, which makes the chances of identification very slim.

The analysis of the disease incidence maps of vineyards during the survey suggests an increase in incidence over the years. Yearly disease incidence of AY was mostly lower than 20% but with cumulative disease incidence of 37.77% at the end of the 4-year study the possibility exist that Chardonnay vineyards can be 100% infected with AY in 10 years time. Spatial distribution patterns were non-random with clustering occurring along and across vine rows in most of the vineyards surveyed. Non-random patterns are typical of transmission of disease by an insect vector (Beanland et al., 2006). Aggregation of infected vines mostly occurs on the side of vineyards adjacent to infected vineyards.

In conclusion, the four-year survey of wine grape vineyards in the Vredendal area showed that there is no statistically significant difference in disease incidence between the cultivars studied and that spatial distribution patterns are mostly clustered.

The high incidence and progression of AY disease in some vineyards indicate a need for control of the disease in the form of an integrated management strategy such as the use of clean planting material, chemical control of the insect vectors and the execution of sanitation practices and methods to reduce disease inoculum in vineyards. Pruning of infected shoots or cordon arms, or pollarding as a method to reduce the phytoplasma inoculum in vineyards has been used to reduce disease inoculum (Riedle-Bauer et al., 2010), but for these practises to be effective, further research to determine the spatial distribution of the phytoplasmas in individual plants will be important. It will also be necessary to determine if AY can be transmitted mechanically during pruning, which might contribute to transmission of disease.

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