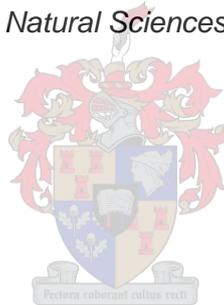


Investigating the recovery phenotype phenomenon in Aster Yellows phytoplasma-infected grapevine

by
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Genetics in the Faculty of Natural Sciences at Stellenbosch University*



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Declaration

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Abstract

South Africa is ranked as the seventh largest wine producing country in the world. Grapevine is one of the most important crops, which warrants extensive research on pathogens and diseases that impact vine health. Aster Yellows (AY) phytoplasma was first identified in South African vineyards in 2010, and poses a major threat to local vineyards. The pathogen symptomatology results in substantial grape yield loss and in many cases death. Though no treatment for AY-infections have been commercialised, a common practice among farmers have been to inflict physiological and chemical stresses on infected plants resulting in the induction of a recovery phenotype. It is unknown whether this recovery is permanent.

The aim of this study was to identify an AY-infected vineyard and induce a recovery in half of the sample group, after which the AY-infection status of the plants was monitored over two years. Furthermore, the AY genetic diversity of isolates in the vineyard were investigated to ensure that any observed recovery is not due to false negative diagnostics. The effect of possible viral pathogens on recovery phenotype induction in AY-infected vines was also investigated.

A triple-nested PCR assay allowed for the identification of 40 AY-infected and 40 healthy plants in February 2016, after which half of each experimental group was coppiced to induce a recovery phenotype. A large-scale remission in AY-infection was observed throughout the vineyard, both in coppiced and uncoppiced plants. Through RFLP assays and Sanger sequencing, a single genetic variant was observed in the studied vineyard, thereby suggesting that the observed recovery was a true one. Grapevine viruses were found in almost all of the AY-positive plants before coppicing, with all healthy plants being virus free. This changed after coppicing however, where a large remission in virus infections was seen post coppicing in AY-positive plants. Additionally, viruses were identified in a small number of AY-negative plants after coppicing. The presence of viruses seemed to have no effect on recovery phenotype induction. This study contributes to our understanding of recovery phenotype induction, reporting a large-scale remission of the pathogen even in the absence of coppicing.

Opsomming

Suid-Afrika word as die sewende grootste wynproduserende land in die wêreld beskou. Druive wingerdstokke is een van die belangrikste gewasse, wat navorsing oor wingerdpatogene en -siektes dus sterk motiveer. Die voorkoms van Astervergeeling (AY) fitoplasma is vir die eerste keer in 2010 in Suid-Afrika aan geteken en is 'n groot bedreiging tot plaaslike wingerde. Simptome van die patogeen sluit in aansienlike opbrengsverlies en in baie gevalle terugsterf van plante. Alhoewel geen kommersiële chemiese behandelings vir AY-infeksies beskikbaar is nie, is 'n algemene praktyk onder boere om fisiologiese of chemiese stresse toe te pas op siek plante, wat lei tot die induksie van 'n herstellings-fenotipe (RP). Dit is tans onbekend of hierdie herstelling permanent is.

Die doel van hierdie projek was om 'n AY-geïnfekteerde wingerd te identifiseer en 'n RP in die helfde van die eksperimentele groep te bewerkstellig, waarna die AY infeksiestatus oor twee jaar gemonitor is. Verder is die AY genetiese diversiteit binne die wingerd ook ondersoek om te verseker dat enige waargenome herstelling nie te wyte is aan vals negatiewe diagnoses nie. Die moontlike effek van virus patogene op die RP indusering in AY-besmette wingerdstokke is ook ondersoek.

'n Drievoudige PKR toets is gebruik vir die identifisering van 40 AY-positiewe en 40 gesonde plante in Februarie 2016, waarna die helfde van elke groep net bo die entlas afgesny is om 'n RP te induseer. 'n Groot skaalse remissie in AY-infeksie was waargeneem, beide in afgesnyde en ongesnyde plante. Beperkingsfragmentlengte Polimorfisme-toetse en Sanger-volgordebepaling het bevestig dat 'n enkele genetiese variant in die wingerd voorgekom het, wat vals negatiewe diagnoses elimineer het, en dus daarop dui dat die waargenome RP 'n ware een was. Virusse is gevind in byna al die AY-positiewe plante voor die afsny van die plante, terwyl alle gesonde plante virus-vry was. 'n Remissie in virus infeksies in AY-positiewe plante na afsny het voorgekom. Daarbenewens is virusse in 'n klein aantal AY-negatiewe plante geïdentifiseer na afsny. Hierdie studie dra by tot ons kennis van RP-indusering deurdat 'n groot skaalse remissie in die voorkoms van die patogeen waargeneem was, ongeag van of plante afgesny was of nie.

List of Abbreviations

AAP	Acquisition access period
AMP	Antimicrobial peptide
APSIM	Agricultural Productions Systems sIMulator
AY	Aster Yellows
BA	6-benzylaminopurine
BLASTn	Basic Local Alignment Search Tool (nucleotide)
BN	Bois noir
Ca	<i>Candidatus</i>
cv	Cultivar
CVYV	<i>Cucumber vein yellowing virus</i>
CYP	Chrysanthemum yellows phytoplasma
CYSDS	<i>Cucurbit yellow stunting disorder virus</i>
ELISA	Enzyme-linked immunosorbent assay
ESFY	European stone fruit yellows
FD	Flavescence dorée
GFKV	<i>Grapevine fleck virus</i>
GFLV	<i>Grapevine fanleaf virus</i>
GLD	Grapevine leafroll disease
GLRaV-1	<i>Grapevine leafroll associated virus-1</i>
GLRaV-2	<i>Grapevine leafroll associated virus-2</i>
GLRaV-3	<i>Grapevine leafroll associated virus-3</i>
GLRaV-4	<i>Grapevine leafroll associated virus-4-like</i>
GLRaV-7	<i>Grapevine leafroll associated virus-7</i>
GRSPaV	<i>Grapevine rupestris stem pitting-associated virus</i>
GVA	<i>Grapevine virus A</i>
GVB	<i>Grapevine virus B</i>
GVE	<i>Grapevine virus E</i>
GVF	<i>Grapevine virus F</i>
GY	Grapevine yellows
GYSVd	<i>Grapevine yellow speckle viroid</i>
H ₂ O ₂	Hydrogen peroxide
HSVd-g	<i>Hop stunt viroid</i>
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid

IMPs	Immunodominant membrane proteins
ICVG	International Council for the Study of Virus and Virus-like Diseases of the Grapevine
LP	Latent period
MLOs	Mycoplasma-like organisms
PCR	Polymerase Chain Reaction
qPCR	Quantitative real time PCR
RBDV	<i>Raspberry bushy dwarf virus</i>
RFLP	Restriction Fragment Length Polymorphism
RLMV	<i>Raspberry leaf mottle virus</i>
ROS	Reactive oxygen species
RP	Recovery phenotype
RSP	Rupistris stem pitting
RT-PCR	Reverse transcription PCR
SAWIS	South African Wine Industry Information and Systems
ScYLV	<i>Sugarcane yellow leaf virus</i>
ScYP	Sugarcane yellows phytoplasma
sp.	Species
spp.	Species (plural)
UV	Ultra Violet
YLS	Yellow leaf syndrome

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Chapter 1: Introduction

1.1 General Introduction

South Africa is currently ranked seventh globally with regards to wine production. The wine industry contributes greatly to the country's economy. The local production totalled 898.4 million litres of wine during the 2016 season, resulting in a producer's income of R5.03 billion. Furthermore, the industry contributed R36.15 billion to the South African GDP in 2013, and provided employment for 289 151 individuals (VinPro Cost Guide 2017).

Phytoplasma are known to cause three economically important diseases in grapevines, namely Bois noir (BN), Flavescence doreé (FD) and Grapevine yellows (GY). With symptoms that often result in the abortion of immature berries (Engelbrecht *et al.* 2010), these diseases often lead to high levels of yield loss. Though these diseases have had a negative impact on wine production throughout Europe and Australia (Lee *et al.* 2000) recent advances in disease control methods have significantly decreased yield loss. The damage caused by symptoms demonstrates the need for research focussing on pathogen control (Smyth 2015). '*Candidatus* Phytoplasma asteris', or Aster Yellows (AY) phytoplasma is the causative agent of GY. AY was first confirmed to infect vineyards in South Africa in the Vredendal region of the Olifantsriver Valley (Engelbrecht *et al.* 2010), and has since been reported in Montague, Rawsonville and Robertson (Carstens 2014). This led to the need for research to develop more effective control strategies, as well as a possible treatment.

To date, no permanent cure for AY has been proven. One method, which has shown some promise recently, is recovery phenotype (RP) induction (Musetti *et al.* 2007, Romanazzi and Murolo 2008, Smyth 2015). This treatment entails placing infected plants under a chemical or physiological stress, which ultimately results in the remission of both the infection and its symptoms. The permanence of the RP induced remission is not known and will therefore be a main focus of this study. This study aimed to identify vines in a single vineyard that were AY-positive and AY-negative. Subsequently, half of the AY-positive and half of the AY-negative plants were stressed to induce an RP. The vines were then monitored over time in order to examine the permanence of the induced RP, as well as what the underlying mechanisms of RP induction may be. Additionally, this study inspected the presence of different genetic variants of AY in the experimental vineyard. This confirmed that any observed recovery is a true recovery and not a false negative diagnosis caused by certain variants not being detected by the 16S rDNA PCR assay employed. We also investigated the viral status in both RP-induced, healthy and diseased

plants, in order to deduce whether virus infections in grapevine plants influenced RP induction in AY-positive plants.

1.2 Project Proposal

This research project aimed to validate the efficacy of RP induction in AY-infected grapevine as a permanent cure for AY.

It also aimed to identify different genetic variants of AY in the vineyard, and to establish the correlation between the AY and viral status of individual plants.

These aims will be achieved by means of the following objectives:

- To identify 40 AY-positive plants and 40 healthy control plants in a vineyard in Vredendal cv. Columbar
- To coppice 20 AY-positive plants and 20 healthy plants, in order to induce an RP, with the remaining plants acting as uncoppiced controls
- To monitor the AY status of the 80 plants over a two-year period
- To determine the identity of genetic variants of AY in AY-positive plants using a multigene RFLP analysis approach
- To determine the viral status of 12 known grapevine viruses in the vineyard and across the four sample groups using established RT-PCR assays

1.3 Chapter Layout

Chapter 1: Introduction, aims and objectives of the research project is stated, chapter layout is presented.

Chapter 2: A summation of previous literature pertaining to AY phytoplasma, RP induction and grapevine viruses is presented.

Chapter 3: The monitoring of the AY-status of AY-positive and -negative, as well as coppiced and uncoppiced vines over three time-points is discussed.

Chapter 4: The genetic diversity of the AY phytoplasma infecting the vineyard is determined using restriction fragment length polymorphism analysis of four AY reference genes to ensure that any observed recovery is a true recovery.

Chapter 5: Determining the virus species present in the experimental vineyard using RT-PCR assays, and determining whether RP-induction in AY-infected vines is affected by co-infection with viruses.

Chapter 6: General Conclusion.

1.4 References

Carstens, R., 2014 The incidence and distribution of grapevine yellows disease in South African vineyards. MSc thesis. Stellenbosch University

Engelbrecht, M., J. Joubert and J.T. Burger, 2010 First report of Aster Yellows phytoplasma in grapevines in South Africa. *Disease Notes* 94(3):373

Lee, I-M., R.E. Davis and D.E. Gunderson-Rindal, 2000 Phytoplasma: Phytopathogenic Mollicutes. *Annual Review of Microbiology* 54:221-255

Musetti, R., R. Marabottini, M. Badiani, M. Martini, L. Sanità di Toppi *et al.*, 2007 On the role of H₂O₂ in the recovery of grapevine (*Vitis vinifera* cv. Prosecco) from Flavescence dorée disease. *Functional plant Biology* 34:750-758

Romanazzi, G., and S. Murolo, 2008 Partial uprooting and pulling to induce recovery in Bois noir-infected grapevines. *Journal of Phytopathology* 156:747-750

Smyth, N., 2015 The determination of the spatial and temporal distribution of aster yellows phytoplasma in grapevine. MSc thesis. Stellenbosch University

VinPro Cost Guide 2017/18, 2017 VinPro Agricultural Economic Services, Picardi Farm, Cecilia Street, Southern Paarl, Paarl, 7646

Chapter 2: Literature review

2.1 Introduction

'*Candidatus Phytoplasma spp.*' are plant pathogenic bacteria that were first chanced upon over 1000 years ago in China. Deliberate infection was used to induce a coveted colour change in the leaves and flowers of peonies (Strauss 2009). Phytoplasmas have since grown much less desirable as a result of the devastating effects that infections have had on a wide range of economically important crops. Peanut, soybean and sesame seed deterioration has been reported in phytoplasma-infected plants throughout Asia (Bertaccini 2007, Smyth 2015). The pathogen has also spread to North America and Europe leading to the death of apple and pear trees (Strauss 2009). '*Candidatus Phytoplasma asteris*', or Aster Yellows (AY) phytoplasma is one of the causal agents of Grapevine Yellows (GY) disease, which leads to abortion of grape bunches and even vine death in extreme cases (Hogenhout *et al.* 2008, Engelbrecht *et al.* 2010, Smyth 2015). AY was first observed in South Africa in 2010 (Engelbrecht *et al.* 2010). With the South African wine industry currently ranked seventh globally regarding production (VinPro Cost Guide 2017), the country's economy is at risk, especially considering that phytoplasma infections may lead to a yield loss of up to 80% in an infected vineyard (Magarey 1986).

Early research on phytoplasmas progressed slowly, mainly due to its small size and low titre. This led researchers to initially conclude that it was a virus, since no one had been able to visualise it using a microscope (Kunkel 1926). This was supported by the organism's infectious nature as well its capability of infecting both plants and insect vectors (Bertaccini 2007).

Currently, no commercial treatment for phytoplasma infections exists (Smyth 2015). Though the induction of a recovery phenotype through chemical or physiological stresses has proved to be an effective treatment (Musetti *et al.* 2007, Romanazzi and Murolo 2008), little is known about the permanence of the resulting remission. Even less is known about the underlying genetic and physiological mechanisms that cause the remission to occur. It is therefore of paramount importance to investigate these underlying mechanisms. Once these mechanisms are understood, it may be determined whether recovery can be achieved without stress induction, thereby being less time consuming and labour intensive as well as preventing yield loss.

2.2 Phytoplasmas

2.2.1 Discovery

The first phytoplasma disease was described as early as 1000 years ago in China, in the form of slight green colouration of the Yao's yellow peonies (Maramorosh 2011). Though this colouring was extremely sought-after, it was only attributed to phytoplasma infection some eight centuries later.

Initially phytoplasmas were hypothesized to be viral organisms due to the lack of any visible bacterial or fungal entities in infected plant tissue (Kunkel 1926). It was in the late 1960s that Japanese researchers were able to describe phytoplasmas as “mycoplasma-like organisms” (MLOs) (Doi *et al.* 1967) within phloem tissue. Phytoplasmas are comparable to mycoplasmas owing to their obligate intracellular existence, as well as the pathogens lacking cell walls.

Later developments in molecular assays, such as group-specific PCR primers, have led to the identification and classification of a myriad of phytoplasmas associated with diseases in hundreds of economically significant plant species (Lee *et al.* 1998).

2.2.2 Characteristics

Morphology: As stated, phytoplasma morphology strongly resemble that of mycoplasmas. These pathogens are pleomorphic, polymorphic, gram-positive bacteria lacking cell walls (Lee *et al.* 2000). They are miniscule in size, ranging between 300nm and 500nm in diameter (Figure 2.1) (Lee *et al.* 1998, Costanzo 2012).

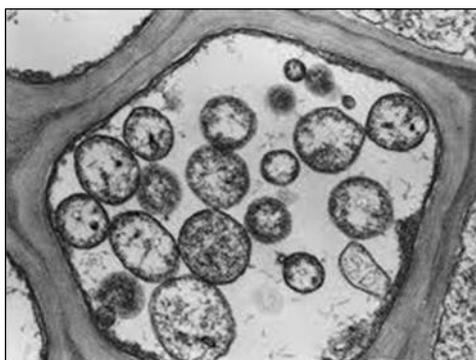


Figure 2.1: Electron micrograph of a plant phloem cell infected with phytoplasmas, source: http://dna-barcoding.blogspot.com/2012_12_01_archive.html.

Genome: Organisms in the genus *Phytoplasma* may have genome sizes ranging between 500 and 1200kb with a GC-content between 23 and 29% (Tran-Nguyen *et al.* 2008). Like their mycoplasma counterparts, the phytoplasma genome is minimalistic. This is likely a consequence of genome reduction that resulted from the organisms' obligate parasitic existence. Polygenic analysis of conserved genes found across all species of phytoplasma revealed that they belong to a

monophyletic clade descending from a single common ancestor in the class Mollicutes (Lee *et al.* 2000). This clade is divided into 15 distinct subclades (16Srl to 16SrXV). One of these subclades is the Aster Yellows phytoplasma group, 16Srl, which is divided into subgroup 16Srl-A to 16Srl-Y (Acosta *et al.* 2015). AY phytoplasma is the species with the highest genetic diversity based on 16S rDNA sequence analysis (Lee *et al.* 2000).

2.3 Phytoplasma Host Organisms

Phytoplasmas make use of a dual host life cycle (Figure 2.2) in which it may colonise either a plant- or an insect host. This enables phytoplasma infections to spread between plants using an insect vector.

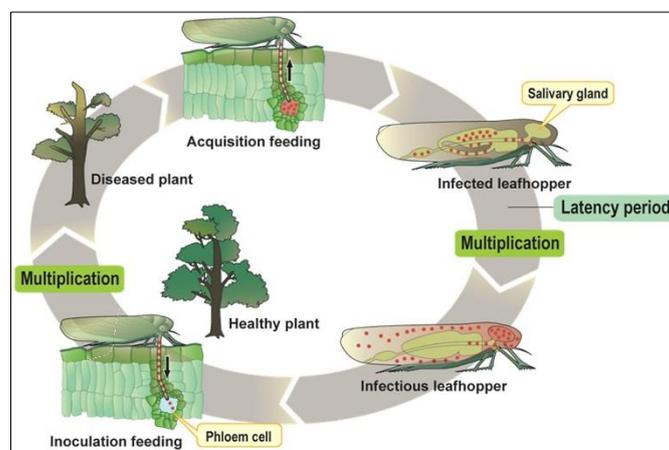


Figure 2.2: Phytoplasmas use a dual host life cycle in order to move between plant hosts. The pathogens colonise plant phloem tissue, as well as the hemocoel and salivary glands of insect hosts. Taken from Oshima *et al.* (2011).

2.3.1 Plants as Hosts

Phytoplasmas can colonise the phloem sieve tissue of over 100 different economically important crop species, and result in a myriad of complex diseases (Lee *et al.* 2000). Since phytoplasmas lack flagella, these pathogens have been suggested to move throughout the plant using the phloem stream (Christensen *et al.* 2005). Though not much is known about the exact interaction between phytoplasmas and its plant host, it has been reported that phytoplasmas cause alterations in the levels of phytohormones in the plant (Ehya *et al.* 2013). In addition, phytoplasma infection was shown to hamper photosynthesis by inhibiting ribulose-1,5-bisphosphate carboxylase and photosynthetic pigment production (Bertamini *et al.* 2002, Rusjan *et al.* 2012). The titres of phytoplasmas reportedly vary significantly amongst host plants (Bertaccini and Duduk 2009). Titres in grapevine can be extremely low (Berges *et al.* 2000); this frequently lead to inaccurate diagnosis. In grapevine, phytoplasma infections result in devastating diseases such as GY, Bois noir (BN) and Flavescence dorée (FD) (Bertaccini and Duduk 2009).

2.3.2 Insect Hosts

Phytoplasma infections are spread between plants by an insect vector. These vectors mostly comprise of psyllids and plant- or leafhoppers (Weintraub and Beanland 2006). These insects all belong to the order Hemiptera and possess many favourable characteristics for phytoplasma transmission (Weintraub and Beanland 2006). Firstly, the insects are effective as vectors during all developmental stages. Secondly, the vectors and pathogens are reported to have a positive propagative relationship, meaning that the insect host is able to reproduce more effectively when carrying phytoplasmas (Beanland *et al.* 2000). Finally, all identified insect vectors only feed on plant phloem tissue, making transmission of phytoplasma to a new host plant more likely to occur (Weintraub and Beanland 2006).

Krüger *et al.* (2011) confirmed the AY vector in South African vineyards to be the leafhopper *Mgenia fuscovaria*. This was determined by surveying a vineyard infected by AY for plant- and leafhoppers over the course of two years. All insects collected were screened for AY, after which transmission experiments were performed. To date, no other insect vectors for AY in South Africa have been identified. It is not unheard of for a single vector to transmit more than one species of phytoplasma (Weintraub and Beanland 2006).

Acquisition of the phytoplasma occurs from the phloem by way of the insect's stylet when feeding. The insect must feed on the phloem of an infected plant for at least a certain time-period in order for the phytoplasma to colonise a vector. This timeframe is termed the acquisition access period (AAP) (Weintraub and Beanland 2006). Following the AAP, there is a latent period (LP) before the phytoplasmas can be transferred from the vector's salivary glands to the phloem of the next plant. During the LP, the phytoplasmas replicate and colonise the insect's salivary glands, and only when the phytoplasmas have moved through the salivary glands can the vector be infectious. When the phytoplasmas are transmitted to a new plant, the cycle is completed. Transmission occurs in a persistent manner (Weintraub and Beanland 2006).

Interestingly, it has been reported that leafhoppers exposed specifically to AY tend to live up to 73% longer, and produce close to twice the number of offspring compared to uninfected leafhoppers (Beanland *et al.* 2000). This was determined by rearing the leafhopper *Macrostelus quadrilineatus* Forbes on asters infected with AY, and comparing them to control leafhoppers reared on uninfected asters.

2.4 Aster Yellows Phytoplasma in South African Vineyards

2.4.1 First Reports of AY in South African vineyards

Symptoms associated with GY led to the first observation of phytoplasmas in South Africa (Botti and Bertaccini 2006). Diagnostic procedures indicated the presence of Stolbur Phytoplasma as well as '*Candidatus Phytoplasma aurantifolia*' in samples collected from the symptomatic vineyard. These results were however contrasted by subsequent studies that failed to find any traces of phytoplasma in the plant material. It was only in 2010 that Engelbrecht *et al.* verified the presence of AY in the Vredendal region of the Olifants River Valley. AY-infections have since been reported in vineyards in Robertson, Montagu and Rawsonville (Carstens 2014).

2.4.2 Symptomatology in Grapevines

Grapevine plants infected with phytoplasma express essentially identical symptoms, regardless of the specific species of phytoplasma that infects the host (Belli *et al.* 2010). These symptoms (Figure 2.3) include yellowing and downward curling of the leaves (Costanzo 2012), shoots displaying shortened internodes and incomplete lignification (Botti and Bertaccini 2006, Engelbrecht *et al.* 2010, Smyth 2015) and the abortion of immature grape bunches and growth tips as well as stunting (Engelbrecht *et al.* 2010, Smyth 2015).



Figure 2.3: Images (a) and (b) taken by author from AY-infected vineyard in Vredendal, depicting (a) shortened internodes in the cane on the right and (b) incomplete cane lignification. Image (c) depicts yellowing and curling of leaves, taken from <http://www.omafra.gov.on.ca/IPM/english/grapes/diseases-and-disorders/phytoplasmas.html>.

The severity of symptom expression in GY tends to show seasonal trends (Belli *et al.* 2010). For example, phytoplasma infected grapevines may show abnormal sprouting, after which the leaf pigmentation and rolling will begin early in the summer. This is then followed by abortion of the berries, and incomplete cane lignification (Belli *et al.* 2010). Symptoms are also often restricted to certain parts of the plant, where only one branch or a few canes may display symptoms. In highly susceptible grapevine varieties, phytoplasma infection may lead to death within a few years (Belli *et al.* 2010, Carstens 2014).

2.5 Diagnosis of AY Infected Grapevines

2.5.1 Simple diagnostic procedures

Until recently, phytoplasmas were generally understood to be unculturable *in vitro*, though recently complex solid and liquid media has resulted in promising results (Contaldo *et al.* 2015, Contaldo *et al.* 2016). As a result, initial diagnosis usually relied on symptomatology (Lee *et al.* 2000). A shortcoming of diagnoses made based on symptom expression is that it does not distinguish between different species of phytoplasma. Symptoms of phytoplasma infections in grapevine are also easily confused with that of viral diseases, such as Grapevine leafroll disease (GLD). In cases where symptoms are not irrefutable, electron microscopy (Figure 2.1) can allow visualisation of the pathogens in cross sections of phloem tissue (Bertaccini and Duduk 2009). The flaw however remains that no distinction can be made between phytoplasma species. An additional pitfall is that the specific phloem sample may not contain any phytoplasma due to erratic distribution within the plant, resulting in a false negative diagnosis.

2.5.2 ELISA Tests

Enzyme-linked immunosorbent assays (ELISAs) were previously used for phytoplasma detection. These serological tests made use of very specific, monoclonal- or polyclonal antibodies, reared from immunodominant membrane proteins (IMPs) or *secA* membrane proteins (Naghmeh and Vadamalai 2013). It was, however, only able to identify certain strains of phytoplasma, leaving room for the possibility of a false negative diagnosis (Lee *et al.* 2000). Additionally, ELISAs lacked the needed specificity to distinguish between different subgroups of phytoplasma (Naghmeh and Vadamalai 2013). Developing specific antibodies is also laborious and time-consuming, as the antibodies often cross-react with plant proteins which in turn cause false positive diagnoses (Adams *et al.* 2001).

2.5.3 Polymerase Chain Reaction Based Assays

Polymerase Chain Reaction (PCR) primers are predominantly designed to amplify the 16S rDNA region of the phytoplasma genome (Lee *et al.* 2000). PCR based methods have become preferable to those previously described due to increased sensitivity and specificity (Naghmeh and Vadamalai 2013). Quantitative PCR (qPCR) assays have been widely popular, not only for phytoplasma detection but also for analysis of fluctuations of phytoplasma throughout plant tissues and over time, and for differentiation between subgroups (Christensen *et al.* in 2004, Angelini *et al.* 2007, Hodgetts *et al.* 2009). Accurate phytoplasma detection, however, remains problematic due to the extremely low concentrations of the pathogen in specific infected plant material, especially in grapevine. False negative diagnoses therefore remain a pitfall (Smyth 2015). At present, nested

PCR assays are the preferred method for phytoplasma detection. This is owed to the increased sensitivity, which is gained by the repeated PCR runs (Smyth 2015). Nested PCR assays involve reamplification steps following the initial PCR reaction (Figure 2.4). Additional sets of primers designed to bind to the target sequence between the original primer pairs are utilised in a subsequent PCR reaction, in which the template is the amplicon created in the initial reaction. This method increases the sensitivity of the assay (Gunderson *et al.* 1996). It therefore addresses the main shortcoming of conventional qPCR methods, detecting phytoplasmas even at low infection titres.

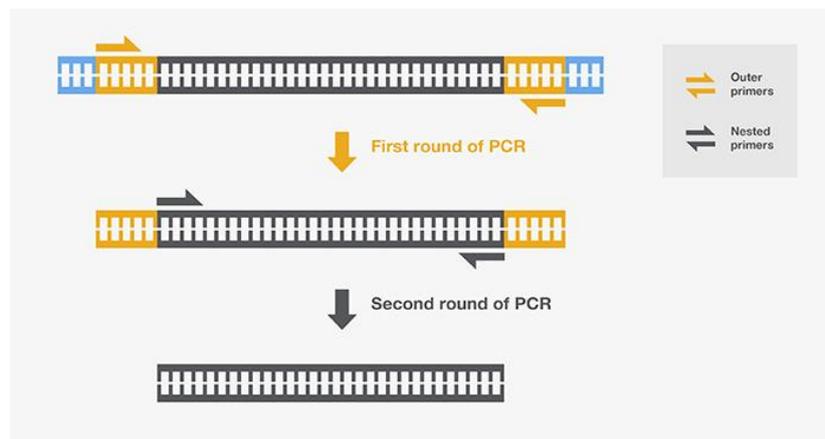


Figure 2.4: Diagram illustrating how nested PCR primers bind to the target DNA sequence. Retrieved from https://www.thermofisher.com/content/dam/LifeTech/global/life-sciences/Cloning/Images/0616/pcr-methods-WE41324_Fig03.jpg.

Currently, the preferred method for phytoplasma detection entails three sequential PCR reactions, of which the final two are nested. A third primer pair and nested reaction is used to further increase sensitivity and aid in differentiation between phytoplasma subspecies. This assay is referred to as a triple-nested PCR. In 2015, Smyth compared the accuracy of AY detection using a triple-nested PCR assay to that of qPCR in grapevine, and found the triple-nested PCR method to be effective in samples diluted to 0.0001 ng/ μ L, where the Taqman qPCR assay was ineffective in samples diluted to lower than 0.1 ng/ μ L (Smyth 2015). This can be ascribed to the low titres of phytoplasmas in the plant tissues rather than the inefficacy of qPCR assays.

2.5.4 Genetic Diversity of Phytoplasmas

Phytoplasma species are classified into different genetic groups and sub-groups based on their 16S rDNA gene sequences, with AY falling into the 16Srl group. Restriction Fragment Length Polymorphism (RFLP) analysis of PCR amplicons can be utilised to determine the specific 16S group and subgroup of the phytoplasma in question (Hodgetts and Dickinson 2012). Although these groups and subgroups were originally assigned based on 16S rDNA data, subsequent studies have found that this gene does not provide enough distinction between closely related

strains (Gundersen *et al.* 1996). Subsequently, additional reference genes have been utilised for finer molecular differentiation.

Gundersen *et al.* (1996) performed RFLP analysis on 16S rDNA and *ribosomal protein* (*rp*) gene PCR fragments to discern the differentiation between phytoplasma species within subgroup 16Srl. Through these results, the group proved to be more genetically diverse than previously stated (Gundersen *et al.* 1996). Lee *et al.* (2004) confirmed that AY-associated phytoplasmas belonged to a single taxonomic group by RFLP analysis of the *rp* and *tuf* gene (which encodes elongation factor Tu) sequences. This allowed for them to differentiate between 10 distinct AY subgroups. Shortly after Lee *et al.* (2006) studied the genetic diversity of AY using RFLP analysis of the *secY* gene, as it showed greater variability than *rp*, allowing for a more comprehensive diversity analysis. The *secY* gene encodes a subunit of a eubacterial protein secreting ATPase complex. Once again 10 distinct subgroups were observed corresponding to those reported by Lee *et al.* (2004). Another gene, *groEL*, which encodes a bacterial chaperonin aiding in protein folding, was utilised by Mitrović *et al.* (2011) for finer differentiation between strains in the taxon '*Candidatus Phytoplasma asteris*'. A nested PCR assay was designed to ensure that only the *groEL* gene sequence of '*Candidatus Phytoplasma asteris*' would be amplified, thereby presenting a diversity analysis assay tailored to this specific taxonomic group. In this study, 27 '*Candidatus Phytoplasma asteris*' strains were identified of which 11 strains have not been studied before. However, analysis of the *groEL* gene did not allow for differentiation between strains belonging to the 16Srl-L and 16Srl-M subgroups (Mitrović *et al.* 2011).

Zambon *et al.* (2015) followed a multigene approach for phytoplasma diversity analysis. RFLP analysis of the *rp*, *groEL*, *secY* and *antigenic membrane protein* (*amp*) genes of phytoplasmas generated non-identical profiles, which could be attributed to more than one genetic variant of AY being present throughout the samples (Zambon *et al.* 2015). This study also highlighted the differences in RFLP patterns generated for strains originating from South Africa and an Italian strain.

2.5.5 Spatial and Temporal Distribution of Phytoplasmas in Grapevine

A previous study reported on the combined spatial and temporal distribution of AY in grapevine plants over three growing seasons in South Africa (Smyth 2015). This allowed for an estimation of the most accurate plant tissue and time-point to sample for accurate detection. Final results from this study suggested phloem scrapings of grapevine canes to result in the most accurate diagnosis of AY, and the month of February resulting in the most accurate detection in infected samples in South Africa. These temporal results echo an earlier study performed by Constable *et al.* (2003) in Australia, in which infected grapevine plants were monitored over the course of three years. Again,

February (with regards to the southern hemisphere) was identified as being the month in which the highest number of positive diagnoses was obtained (Constable *et al.* 2003). It has been suggested that the temporal fluctuations of phytoplasma may be attributed to the effects that changes in temperatures and climate may have on the rate of nutrient circulation throughout the host plant (Gibb *et al.* 1999). An alternative explanation may be that seasonal changes may affect the insect vector rather than the host plants, which may also lead to fluctuations of phytoplasma titres over time (Elder *et al.* 2002).

2.6 Phytoplasma Control

Once an AY infection has been introduced into a vineyard it is difficult to eradicate, mostly due to the dual host life cycle of the pathogen. Both the vectors and infected plants must be controlled to completely rid the vineyard of the infection. Since no permanent treatment for phytoplasma infections currently exists (Smyth 2015), control strategies are mostly aimed at preventing infections. Control strategies usually centre around the methods listed below:

Vector control: Known insect vectors could be eradicated from the site through the application of pesticides. Though chemical control is often used (Weintraub and Beanland 2006) some biological vector control methods include the use of mycoinsecticides or the introduction of insects that act as natural antagonists to the insect vector (Laimer *et al.* 2009). It is also of importance to maintain the vineyard by constantly removing weeds which may grow between the rows of plants. These weeds can either serve as alternative phytoplasma hosts or attract possible vectors.

Reduce disease inoculum: Symptomatic plants are often completely removed from the vineyard, a practice referred to as roguing (Uyemoto *et al.* 1998). This prevents the spread of phytoplasma infections to neighbouring plants either through insect vectors or through mechanical transmission.

Breeding resistant plant varieties: This method suggests either a resistance to phytoplasma infection, or vector feeding (Thomas and Mink 1998, Nagadhara *et al.* 2003). Research on this topic is limited, and remains unsuccessful in grapevine (Carstens 2008, Laimer *et al.* 2009).

Antibiotics: Attempts have been made in the past to treat phytoplasma infections using antibiotics (McCoy 1982, Magarey *et al.* 1986). Though both tetracycline and oxytetracycline have been reported to lead to the remission of the infection, the plants could never be cured permanently (McCoy 1982). Additional drawbacks include the possible emergence of antibiotic resistant phytoplasmas in plants that were not subjected to proper treatment, as well as the probability of the antibiotics entering human food supplies (Magarey 1986).

Transgenic resistance: Another treatment which has been investigated makes use of introducing antimicrobial peptide (AMP) genes into the host plant. Rufo *et al.* (2017) reports on the efficacy of transforming hosts with the synthetic AMP BP100 against '*Candidatus Phytoplasma rubi*' and '*Candidatus Phytoplasma solani*'. The AMP was 100% effective when used preventatively. When administered as a treatment, disease symptomatology disappeared completely, though they were still able to detect '*Candidatus Phytoplasma rubi*' and '*Candidatus Phytoplasma solani*' in 75% and 50% of the plants respectively. However, there is concern for the possible effects of transgenic grapes on human consumption, with the general public being wary of ingesting transgenic foods (Laimer *et al.* 2009).

2.7 Recovery Phenotype

2.7.1 Recovery Phenotype Induction as a Control Strategy

A recovery phenotype (RP) is defined as the abrupt decline or disappearance of phytoplasma symptoms (Caudwell 1961). A plant can only be classified as "recovered" once it has proved to be free of phytoplasma symptoms for three sequential years, with no phytoplasmas being detected in any plant tissues using nested PCR (Musetti *et al.* 2013). Several RP-induction methods have been reported, all pertaining to inducing either a chemical or a physiological stress in the plant.

A previous study by showed the full recovery of AY-infected grapevines after treatment with the auxins indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) (Ćurković 2008). Though both auxins led to a recovery, IBA had a markedly higher efficacy. When this treatment was repeated on plants infected with '*Candidatus Phytoplasma solani*', a remission in phytoplasma symptoms was also observed. The pathogen, however, remained detectable by nested PCR assays (Ćurković 2008).

Physiological stresses, such as replanting, grafting or coppicing (cutting back), are also often utilised to induce an RP (Osler *et al.* 1993). Romanazzi and Murolo (2008) showed that the partial uprooting and pulling of grapevines cv. Chardonnay could be applied to treat BN infection. Almost all the plants included in this study remained disease-free and recovered over the two years in which this study was performed (Romanazzi and Murolo 2008). These methods are currently preferred among producers. In Vredendal, producers induce an RP by coppicing the infected plants a few centimetres above the graft union and allowing a single bud to regrow.

In addition to the severity of the phytoplasma infection, the success of stressing the plants to induce an RP depends significantly on the age of the grapevine (Riedle-Bauer 2010). No scientific evidence exists at this time for the permanence of disease remission through RP induction in grapevines (Smyth 2015).

2.7.2 Underlying Mechanisms of RP Induction

Different theories on the underlying mechanisms of RP-induction have been proposed. In a prior study Musetti *et al.* (2007) reported on the association between grapevines that have recovered from phytoplasma infections and the levels of hydrogen peroxide (H_2O_2) in the phloem plasmalemma of the leaves. The study presented the presence of cerium perhydroxide localised in the plasmalemma of the leaves of recovered vines, which is indicative of the presence of H_2O_2 in the cells. In contrast, both FD infected leaves and healthy leaves did not contain detectable levels of cerium perhydroxide (Musetti *et al.* 2007).

Leljak-Levanić *et al.* (2010) investigated the methylation levels in periwinkle plants infected with AY after being transferred from a medium containing 6-benzylaminopurine (BA) to one supplemented with IBA. The infected plantlets became recovered, which is concurrent to the findings of Ćurković (2008). AY-infected shoots had significantly lower levels of genome methylation when grown on BA, with AY shoots on an IBA medium having increased genome methylation levels (Leljak-Levanić *et al.* 2010). However, it is still unclear whether the changes in genome methylation caused the remission in AY infection, or whether the elimination of AY from the periwinkle plants resulted in the increase in methylation.

Osler *et al.* (2014) explored the possibility of tolerance acquired by RP induction being transferred to clones propagated from recovered plants through epigenetics. Apricots afflicted by the phytoplasma disease European stone fruit yellows (ESFY) that were found to spontaneously show symptom remission were monitored over a 12-year period. The recovery was hypothesised to have an epigenetic origin. Two plants that were stably recovered and two which were stably symptomatic were clonally grafted onto a peach rootstock known to transfer ESFY susceptibility to their scion through epigenetic mechanisms, and planted in an orchard known to have a high ESFY incidence. The propagated clones of the recovered plants showed an acquired tolerance, whereas a high ESFY incidence was observed in clones propagated from symptomatic plants (Osler *et al.* 2014). In a similar study by the same group, recovered apricots were grafted onto both heat-treated and non-heat-treated rootstocks known to relay ESFY sensitivity to the scion (Osler *et al.* 2016). The plants were monitored for 10 years and all but one plant remained symptom free for the duration of the study (Osler *et al.* 2016).

Another hypothesis is that bacterial endophytes play a role in RP-induction. A study by Bulgari *et al.* (2011) describes the difference between the endophytic bacterial community of GY-diseased vines and recovered vines. A much higher endophytic bacterial diversity was observed in healthy plants than was observed for recovered and symptomatic vines. Notably, *Bacillus pumilus*,

Burkholderia spp., *Paenibacillus pasadenensis* and an uncultured *Bacillus* sp. were identified only in recovered plants. *Burkholderia* species are known to produce antifungal molecules which aid in protecting host plants against pathogens (el-Banna and Winkelmann 1998). Many *Bacillus* species are also implicated in playing a role in induced systemic resistance (Bulgari *et al.* 2009). These bacterial strains also have an increased resistance to reactive oxygen species (ROS), which is of interest when considering the possible role of H₂O₂ accumulation in RP-induction.

A recent study by Naor *et al.* (2015) investigated the endophytic bacterial communities of infected, recovered and healthy grapevines cv. Cabernet-Sauvignon, as well as the endosymbionts in AY insect vectors. Endophytes of the genera *Bacillus* and *Xanthomonadaceae* were isolated, of which four (only named isolates C, D, H and X) were administered to phytoplasma infected *ex vitro* grapevine and periwinkle plantlets as well as healthy controls. GY symptomatology noticeably decreased in diseased plants (Naor *et al.* 2015). The endophytes were introduced by stem injection, root dip and by smearing pricked leaves, demonstrating multiple means by which endophytes may be introduced into diseased plants as a bio-control method in an agricultural context (Naor *et al.* 2015).

2.8 Grapevine viruses

2.8.1 Co-occurrence of Phytoplasma and Viruses.

Grapevines are susceptible to more than 70 infectious agents according to the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG) (Martelli 2014). Of these pathogens, 65 are viruses, five are viroids and eight are phytoplasmas (Martelli 2014). Margaria *et al.* (2009) screened grapevine plants for the presence of FD and BN phytoplasmas, as well as *Grapevine associated virus-1* and *-3* and *Grapevine virus A*, since all of these agents have been implicated in generating symptoms consistent with GY. The study reported that 30% of the samples were simultaneously infected with phytoplasmas and viruses, whereas 69% of the plants had mixed viral infections. This attested to the high frequency of multiple infections in field samples of grapevines, suggesting that viruses and phytoplasmas often co-contribute to the pathology of GY-diseased vines (Margaria *et al.* 2009). It is therefore important that the disease dynamics between viruses and phytoplasmas infecting grapevine are further investigated in order to surmise the synergistic effects co-infections may have on the pathogens, and in what way these effects may influence RP-induction in phytoplasma infected grapevines.

2.8.2 Common Viruses in South African Vineyards

Grapevines are vulnerable to the highest number of plant viruses for any crop plant (Martelli and Boudon-Padieu 2006). Viral complexes, rather than single infections, cause many significant

grapevine diseases; up to nine different virus species have been identified in individual plants (Prosser *et al.* 2007).

GLD is considered to be the most economically important grapevine disease globally (Maree *et al.* 2013). GLD is associated with up to 10 different virus species (Coetzee *et al.* 2010). Of these, *Grapevine leafroll associated virus 3* (GLRaV-3) is the most prominent and widespread virus associated with GLD. Other viral species of interest in South Africa are *Grapevine leafroll associated virus-1* (GLRaV-1), *Grapevine leafroll associated virus-2* (GLRaV-2), *Grapevine leafroll associated virus-4-like* (GLRaV-4). Symptoms associated with GLD include red colouration of the interveinal leaf surface in red berried cultivars, with only a slight yellowing in some white berried cultivars. This is accompanied by the downward rolling of leaf edges (Maree *et al.* 2013, Martelli 2014, Naidu *et al.* 2014). In some white berried cultivars, symptoms may be extremely subtle, or even completely absent (Maree *et al.* 2013, Naidu *et al.* 2014). Berries of infected vines take longer to mature, yielding irregular final products of a lower quality and quantities as well as a lower sugar yield (Naidu *et al.* 2008, Martelli 2014) Another related virus, *Grapevine leafroll associated virus-7* (GLRaV-7) has not yet been observed in South Africa and only causes mild leafroll symptoms (Martelli 2014).

Another virus disease complex found in South African vineyards is the Rugose wood complex. Symptoms associated with diseases in this complex include a swelling above the graft union with the bark above the union appearing corky with a sponge-like texture. Pitting and/or grooving may typically occur across the cambial face of the stem in ranging severity depending on scion and root stock combinations. Vines also appear to be less vigorous than healthy vines, and in some cases leaf rolling may occur (Martelli 2014).

Grapevine rupestris stem pitting-associated virus (GRSPaV), which belongs to the family *Foveavirus*, is the causative agent of Rupistris stem pitting (RSP) disease (Gambino *et al.* 2012, Martelli 2014), and has also been identified in vines suffering from “Syrah Decline” (Martelli 2014). *Grapevine virus A* (GVA) is the type species representing the genus *Vitivirus*, and is the presumed causative agent in Grapevine Kober stem grooving disease (Garau *et al.* 1994, Chevalier *et al.* 1995, Martelli 2014). GVA has also been associated with “Shiraz disease” in South Africa (Coetzee *et al.* 2010, Martelli 2014).

Grapevine virus B (GVB), a member of the *Vitivirus* genus, is considered to be one of the main causal agents in the Rugose Wood Complex disease Grapevine corky bark (Martelli 2014).

Two additional *Vitivirus* species of interest in a South African context are *Grapevine virus E* (GVE) and *Grapevine virus F* (GVF). GVE is serologically distinct from GVA and GVB. The virus has been associated with stem pitting symptoms in grapevine, though no direct relationship between the virus and RSP has been established (Martelli 2014). GVF has been reported to induce graft incompatibility in grapevines of the cultivar Cabernet Sauvignon (Martelli 2014).

Fanleaf degeneration disease, caused by *Grapevine fanleaf virus* (GFLV) is another grapevine disease of interest in South Africa. It is considered a severe grapevine disease and causes great economic losses in Europe (Andret-Link *et al.* 2004, Martelli 2014). Symptoms of this disease include asymmetrical, wrinkled leaves that display chlorotic mottling, shortened internodes and abnormal shoot formation. The disease also leads to a decrease in berry yield, with extreme cases causing up to 80% yield loss (Andret-Link *et al.* 2004, Martelli 2014). The virus belongs to the genus *Nepovirus*.

Lastly, *Grapevine fleck virus* (GFKV) is a virus mainly causing symptoms in *V. rupestris*, while infections in *V. vinifera* are considered to be symptomless (Martelli 2014). Symptoms of Grapevine fleck disease in *V. rupestris* are the appearance of localised translucent spots on the leaves, with severely spotted leaves becoming wrinkled and curling upward. Some severe cases may also lead to stunted growth (Goussard 2013, Martelli 2014). The virus belongs to the genus *Maculavirus*.

2.8 References

Acosta, K., F.N. Silva, L. Ramona, M. Quiñones, R. Martínez *et al.*, 2015 Detection and molecular characterization of an Aster Yellows group phytoplasma associated with soybean and evidence of two new 16Srl subgroups in Cuba. *Journal of Plant Pathology* 97(2)

Adams, A.N., D.L. Davies and M.J. Kirby, 2001 Virus and phytoplasma detection in fruit trees. *Outlook on Agriculture* 30:45-54

Andret-Link, P., C. Schmitt-Keichinger, G. Demangeat, V. Komar and M. Fuchs, 2004 The specific transmission of Grapevine fanleaf virus by its nematode vector *Xiphema index* is solely determined by the viral coat protein. *Virology* 320(1):12-22

Angelini, E., G. L. Bianchi, L. Filippin, C. Morassutti and M. Borgo, 2007 a new Taqman® method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. *Journal of Microbial Methods* 68:613-622

Beanland, L., C. W. Hoy, S. A. Miller and L. R. Nault, 2000 Influence of Aster yellows phytoplasma on the fitness of aster leafhopper (Homoptera: Cicadellidae). *Annual Entomological Society of America* 93(2):271-276

Belli, G., P. A. Bianco and M. Conti, 2010 Grapevine yellows in Italy: past, present and future. *Journal of Plant Pathology* 92(2):303-326

Bertaccini, A., 2007 Phytoplasmas: diversity, taxonomy, and epidemiology. *Frontiers in Bioscience* 12:673-689

Bertaccini, A. and B. Duduk, 2009 Phytoplasma and phytoplasma diseases: a review of recent research. *Phytopathologia Mediterranea* 48:355-378

Berges, R., M. Rott and E. Seemüller, 2000 Range of phytoplasma concentrations in various plant hosts as determined by competitive polymerase chain reaction. *Bacteriology* 90(10):1145-1152

Bertamini, M., N. Nedunchezian, F. Tomasi and M.S. Grando, 2002 Phytoplasma [Stolbur-subgroup (Bois noir-BN)] infection inhibits photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase and photosynthetic activities in field grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves. *Physiological and Molecular Plant Pathology* 61:357-366

Botti, S. and A. Bertaccini, 2006 First report of phytoplasmas in grapevine in South Africa. *Plant Disease* 90:1360

Bulgari, D., P. Casati, P. Crepaldi, D. Daffonchio, L. Brusetti *et al.*, 2009 Endophytic bacterial diversity in grapevine (*Vitis vinifera* L.) leaves described by 16S rRNA gene sequence analysis and length heterogeneity-PCR. *Journal of Microbiology* 47:393-401

Bulgari, D., P. Casati, P. Crepaldi, D. Daffonchio, F. Quaglino *et al.* 2011 Restructuring of endophytic bacterial communities in grapevine yellows-diseased and recovered *Vitis vinifera* L. plants. *Applied and Environmental Microbiology* 77(14):5018-5022

Carstens, R., 2008 Aster yellows disease in vineyards in South Africa. *Winelands* 228:90-91

Carstens, R., 2014 The incidence and distribution of grapevine yellows disease in South African vineyards. MSc thesis. Stellenbosch University

Caudwell, A., 1961 Les phénomènes de rétablissement chez la flavescence dorée de la vigne. *Annales des Epiphyties* 12:347-354

Chevalier, S., C. Greif, J. M. Clauzel, B. Walter and C. Fritsch, 1995 Use of an immunocapture-polymerase chain reaction procedure for the detection of grapevine virus A in Kober stem grooving-infected grapevines. *Journal of Phytopathology* 143(6):369-373

Christensen, N. M., M. Nicolaisen, M. Hansen and A. Schulz, 2004 Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Molecular Plant Microbe Interactions* 17(11):1175-1184

Christensen, N. M., K. B. Axelsen, M. Nicolaisen and A. Schulz, 2005 Phytoplasmas and their interactions with hosts. *Trends in Plant Science* 10(11):526-535

Coetzee, B., M. J. Freeborough, H. J. Maree, J. M. Celton, D. J. G. Rees *et al.*, 2010 Deep sequencing analysis of viruses infecting grapevines: virome of a vineyard. *Virology* 400:157-163

Contaldo, N., Y. Zambon, E. Satta, S. Paltrinieri and A. Bertaccini, 2015 Cultivation in chemically defined media of phytoplasmas from field-infected grapevine plants showing yellows symptoms. *Proceedings of the 18th Congress of ICVG, Ankara, Turkey* 121-123

Contaldo, N., E. Satta, Y. Zambon, S. Paltrinieri and A. Bertaccini, 2016 Development and evaluation of different complex media for phytoplasma isolation and growth. *Journal of Microbiological Methods* 127:105-110

Constable, F. E., K. S. Gibb and R. H. Symons, 2003 Seasonal distribution of phytoplasmas in Australian grapevines. *Plant Pathology* 52:267-276

Costanzo, S., 2012 Selected '*Candidatus* Phytoplasma spp.' of apple, grape and peach. *New Pest Response Guidelines*. United States Department of Agriculture

Ćurković, P.M., 2008 Auxin-treatment induces recovery of phytoplasma-infected periwinkle. *Journal of Applied Microbiology* 105(6):1826-1834

Doi, Y. M., M. Teranka, K. Yora, and H. Asuyama, 1967 Mycoplasma or PLT-group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows or paulownia witches' broom. *Annals of the Phytopathological Society of Japan* 33:259-266

Ehya, F., A. Monavarfeshani, E.M. Fard, L.K. Farsad, M.K. Nekouei *et al.*, 2013 Phytoplasma-responsive microRNAs modulate hormonal, nutritional, and stress signalling pathways in Mexican lime trees. *PLOS ONE* 8(6)

- El-Banna, N. and G. Winkelmann, 1998 Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity against fungi and novel activities against streptomycetes. *Journal of Applied Microbiology* 85(1):69-78
- Elder, R. J., J. R. Milne, D. J. Reid, J. N. Guthrie and D. M. Persley, 2002 Temporal incidence of three phytoplasma-associated diseases of *Carica papaya* and their potential hemipteran vectors in central and south-east Queensland. *Australasian Plant Pathology* 31:165-176
- Engelbrecht, M., J. Joubert and J. T. Burger, 2010 First report of Aster yellows phytoplasma in grapevines in South Africa. *Disease Notes* 94(3):373
- Gambino, G., D. Cuozzo, M. Fasoli, C. Pagliarani, M. Vitali *et al.*, 2012 Co-evolution between *Grapevine rupestris stem pitting-associated* and *Vitis vinifera* L. leads to decreased defence responses and increased transcription of genes related to photosynthesis. *Journal of Experimental Botany* 63(16):5919-5933
- Garau, R., V. A. Prota, R. Piredda, D. Boscia and U. Prota, 1994 On the possible relationship between Kober stem grooving and grapevine virus A. *Vitis* 33:161-163
- Gibb, K. S., F. E. Constable, J. R. Moran and A. C. Padovan, 1999 Phytoplasmas in Australian grapevines – detection, differentiation and association. *Vitis* 38(3): 107-114
- Goussard, P., 2013 A guide to grapevine abnormalities in South Africa: virus and virus-like diseases – fleck and enation (part4.6). *WineLand Magazine*. Retrieved from <http://www.wineland.co.za/a-guide-to-grapevine-abnormalities-in-south-africa-virus-and-virus-like-diseases-fleck-and-enation-part-4-6/>
- Gunderson, D.E., I.-M. Lee, D.A. Schaff, N.A. Harrison, C.J. Chang *et al.*, 1996 Genomic diversity and differentiation among phytoplasma strains in 16S rRNA group I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). *International Journal of Systemic Bacteriology* 46:64-75
- Hodgetts, J., N. Boonham, R. Mumford and M. Dickinson, 2009 Panel of 23S rRNA gene-based real-time PCR assays for improved universal and group-specific detection of phytoplasmas. *Applied and Environmental Microbiology* 75(9):2945-2950
- Hodgetts, J. and M. Dickinson, 2012 T-RFLP for detection and identification of phytoplasmas in plants. *Methods in Molecular Biology* 938:233-244

- Hogenhout, S. A., K. Oshima, E. D. Ammar, S. Kakizawa, H. N. Kingdom and S. Namba, 2008 Phytoplasmas: bacteria that manipulate plants and insects. *Molecular Plant Pathology* 9:403-423
- Krüger, K., A. de Klerk, N. Douglas-Smit, J. Joubert, G. Pietersen *et al.* 2011 Aster yellows phytoplasma in grapevines: identification of vectors in South Africa. *Bulletin of Insectology* 64(suppl.):S137-S138
- Kunkel, L.O., 1926 Studies on aster yellows. *American Journal of Botany* 23:646-705
- Laimer, M., O. Lemaire, E. Herrbach, V. Goldschmidt, A. Minafra *et al.*, 2009 Resistance to viruses, phytoplasmas and their vectors in the grapevine in Europe: a review. *Journal of Plant Pathology* 91(1):7-23
- Lee, I-M., D. E. Gundersen-Rindal and A. Bertaccini, 1998 Phytoplasma: ecology and genomic diversity. *Phytopathology* 88:1359-1366
- Lee, I-M., R. E. Davis and D. E. Gunderson-Rindal, 2000 Phytoplasma: Phytopathogenic Mollicutes. *Annual Review of Microbiology* 54:221-255
- Lee, I-M., D. E. Gunderson-Rindal, R. E. Davis, K. D. Bottner, C. Marcone *et al.*, 2004 '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. *International Journal of Systematic and Evolutionary Microbiology* 54:1037-1048
- Lee, I-M., Y. Zhao and K. D. Bottner, 2006 SecY gene sequence analysis for finer differentiation of diverse strains in the Aster yellows phytoplasma group. *Molecular and Cellular Probes* 20(2):87-91
- Leljak-Levanić, D., M. Ježić, V. Cesar, J. Ludwig-Müller, H. Lepaduš *et al.*, 2010 Biochemical and epigenetic changes in phytoplasma-recovered periwinkle after indole-3-butyric acid treatment. *Journal of Applied Microbiology* 109(6):2069-2078
- Magarey, P. A., 1986 Grapevine yellows – aetiology, epidemiology and diagnosis. *South African Journal of Enology and Viticulture* 7(2):90-100
- Maramorosh K., 2011 Historical reminiscences of phytoplasma discovery. *Bulletin of Insectology(Suppl.):S5-S8*

- Maree, H. J., R. P. P. Almeida, R. Bester, K. M. Chooi, D. Cohen *et al.*, 2013 *Grapevine leafroll-associated virus 3*. *Frontiers in Microbiology* 4(82):1-21
- Margaria, P., M. Turina and S. Palmano, 2009 Detection of Flavescence dorée and Bois noir phytoplasmas, *Grapevine leafroll associated virus-1* and *Grapevine virus A* from the same crude extract by reverse transcription-RealTime taqman assays. *Plant Pathology* 58:838-845
- Martelli, G.P. and E. Boudon-Padieu, 2006 Directory of infectious diseases of grapevine. International centre for advanced Mediterranean agronomic studies. *Options Méditerran., Ser. B, Stud. Res* 55:59-75
- Martelli, G. P., 2014 Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology* 96(1-Suppl.)
- McCoy, R.E., 1982 The use of tetracycline antibiotics to control yellow diseases. *Plant Disease* 66:539-542
- Mitrović, J., S. Kakizawa, B. Duduk, K. Oshima, S. Namba *et al.*, 2011 The *groEL* gene as an additional marker for finer differentiation of ‘*Candidatus* Phytoplasma asteris’-related strains. *Annals of Applied Biology* 159(1):41-48
- Musetti, R., R. Marabottini, M. Badiani, M. Martini, L. Sanità di Toppi *et al.*, 2007 On the role of H₂O₂ in the recovery of grapevine (*Vitis vinifera* cv. Prosecco) from Flavescence dorée disease. *Functional plant Biology* 34:750-758
- Musetti, R., K. Farhan, F. De Marco, R. Polizzotto, A. Paolacci *et al.*, 2013 Differentially-regulated defence genes in *Malus domestica* during phytoplasma infection and recovery. *European Journal of Plant Pathology* 136(1):13-19
- Nagadhara, D., S. Ramesh, I.C. Pasalu, Y.K. Rao, N.V. Krishnaiah *et al.*, 2003 Transgenic indica rice resistant to sap-sucking insects. *Plant Biotechnology Journal* 1:231-240
- Naghme, N. and G Vadamalai, 2013 Diagnostic techniques for detection of phytoplasma diseases: past and present. *Journal of Plant Diseases and Protection* 120(1):16-25
- Naidu, R.A., S. O’Neil and D. Walsh, 2008 Grapevine leafroll disease. *WSU Extension Bulletin*. Retrieved from: <http://cru.cahe.wsu.edu/CEPublications/eb2027e/eb2027e.pdf>

- Naidu, R.A., A. Rowhani, M. Fuchs, D. Golino and G. P. Martelli, 2014 Grapevine leafroll: a complex viral disease affecting a high-value fruit crop. *Plant Disease* 98(9):1172-1185
- Naor, V., L. Iasur-Kruh, R. Barkai, R. Bordolei, S. Rodoy *et al.*, 2015 Introduction of beneficial bacteria to grapevines as a possible control of phytoplasma associated diseases. *Phytopathogenic Mollicutes* 5(1 Suppl.): S111-S112
- Oshima, K., Y. Ishii, S. Kakizawa, K. Sugawara, Y. Neriya *et al.*, 2011 Dramatic transcriptional changes in an intracellular parasite enable host switching between plant and insect. *PLOS ONE* 6(8):e23242
- Osler, R., L. Carraro, N. Loi and E. Refatti, 1993 Symptom expression and disease occurrence of a yellows disease of grapevine in northeastern Italy. *Plant Disease* 77:496-498
- Osler, R., S. Borselli, P. Ermacora, A. Loschi, M. Martini *et al.*, 2014 Acquired tolerance in apricot plants that stably recovered from European stone fruit yellows. *Plant Disease* 98(4):492-496
- Osler, R., S. Borselli, P. Ermacora, F. Ferrini, A. Loschi *et al.*, 2016 Transmissible tolerance to European stone fruit yellows (ESFY) in apricot: cross-protection or a plant mediated process? *Phytoparasitica* 44(2):203-211
- Prosser, S., D. Goszczynski and B. Meng, 2007 Molecular analysis of double-stranded RNAs reveals complex infection of grapevines with multiple viruses. *Virus Research* 124:151-159
- Reidle-Bauer, M., K. Hanak, F. Regner and W. Tiefenbrunner, 2010 Influence of pruning measures on recovery of Bois Noir-infected grapevines. *Journal of Phytopathology* 158:628-632
- Romanazzi, G., and S. Murolo, 2008 Partial uprooting and pulling to induce recovery in Bois noir-infected grapevines. *Journal of Phytopathology* 156:747-750
- Rufo, R., A. Battle, A. Camprubi, E. Montesinos and C. Calvet, 2017 Control of rubus stunt and stolbur diseases in Madagascar periwinkle with mycorrhizae and a synthetic antibacterial peptide. *Plant Pathology* 66(4):551-558
- Rusjan, D., H. Halbwirthj, K. Stich, M. Mikulič-Petkovšek and R. Veberič, 2012 Biochemical response of grapevine variety 'Chardonnay' (*Vitis vinifera* L.) to infection with grapevine yellows (Bois noir). *European Journal of Plant Pathology* 134(2):231-237

Smyth, N., 2015 The determination of the spatial and temporal distribution of Aster yellows phytoplasma in grapevine. MSc thesis. Stellenbosch University

Strauss, E., 2009 Phytoplasma research begins to bloom. *Science* 325:388-390

Thomas, P.E. and G.I. Mink, 1998 Tomato hybrids with nonspecific immunity to viral and mycoplasma pathogens of potato and tomato. *HortScience* 33:764-765

Tran-Nguyen, L.T., M. Kube, B. Schneider, R. Reinhardt and K.S. Gibb, 2008 Comparative genome analysis of "*Candidatus* Phytoplasma australiense" (subgroup tuf-Australia I; rp-A) and "*Ca. Phytoplasma asteris*" strains OY-M and AY-WB. *Journal of Bacteriology* 190(11):3979-3991

Uyemoto, J.K., R.E. Bethell, B.C. Kirkpatrick, K.W. Brown, G.P. Munkvold *et al.*, 1998 Eradication as a control measure for X-disease in California cherry orchards. *Acta Horticulturae* 472:715-721

VinPro Cost Guide 2017/18, 2017 VinPro Agricultural Economic Services, Picardi Farm, Cecilia Street, Southern Paarl, Paarl, 7646

Weintraub, P.G. and L. Beanland, 2006 Insect vectors of phytoplasmas. *Annual Review of Entomology* 51:91-111

Zambon, Y., N. Contaldo, R. Souza Richards, A. Bertaccini and J.T. Burger, 2015 Multigene characterization of Aster yellows phytoplasmas infecting grapevine in South Africa. *Phytopathogenic Mollicutes* 5(1-Suppl.):S21-S22

Chapter 3: Aster Yellows infection status confirmation and monitoring

3.1 Introduction

Aster Yellows (AY) was first confirmed to infect South African vineyards in the Vredendal region of the Olifants River Valley by Engelbrecht *et al.* in 2010. AY-incidence has since spread to Rawsonville, Robertson and Montagu (Carstens 2014). These regions encompass 25.96% of all vines across South Africa (SAWIS booklet 41, 2017). With the high yield loss associated with phytoplasma disease in grapevine, it is of importance to investigate possible control measures.

Many treatment methods have been proposed, with antibiotic applications and transgenic resistance showing promise (McCoy 1982, Magarey *et al.* 1986, Rufo *et al.* 2017). However, a lingering concern is the emergence of antibiotics and antimicrobial peptides in the human food supply, as well as the development of resistant strains if treatments are not administered properly. Additionally, some forms of antibiotic treatment proved to be unsustainable from an economic standpoint, as a continuous treatment is required to prevent the resurfacing of the phytoplasma infections (McCoy 1982).

Some phytoplasma infected plants show signs of spontaneous remission in symptoms. This spontaneous recovery phenotype (RP) was first observed in Flavescence dorée (FD) afflicted grapevines in France (Caudwell 1961), and defined as an abrupt decline or disappearance of symptoms. The same phenomenon was also reported in FD-infected vines in Italy (Belli *et al.* 1973), as well as for Bois noir (BN) infected vines (Osler *et al.* 1993). A plant is classified as recovered, and no longer a source of inoculum, after being free of symptoms and detectable levels of phytoplasma for three sequential years (Musetti *et al.* 2013). It has been shown that the observed RP may be induced through either chemically or physiologically stressing afflicted vines (Musetti *et al.* 2007, Romanazzi and Murolo 2008). Physiological stresses, which are of interest to this study, include grafting, replanting, and coppicing. Coppicing is an extreme pruning method in which the vine is cut down above the graft union, allowing a single shoot to regrow. This is the preferred method among farmers in Vredendal. Research on the topic of RP-induction is, however, still limited; the underlying biochemical, physiological, and genetic mechanisms of this phenomenon remains unclear. It is still unknown whether the induced RP is of a permanent nature or whether it is only a temporary state (Smyth 2015). This may be established by inducing a recovery in a phytoplasma-infected vineyard and monitoring the infection status of the vineyard over several consecutive growing seasons.

Many methods for phytoplasma detection have been developed. Electron microscopy (Jacoli and Ronald 1974) and ELISA methods (Chen and Jiang 1998, Lee *et al.* 2000) were initially employed, though PCR-based methods are preferred due to an increase in sensitivity, as well as being able to detect a wider range of phytoplasmas. Even when methods such as qPCR are employed, the accurate diagnosis of the plants are hampered by low phytoplasma titres, as well as by the uneven spatial and temporal distribution of the pathogen in its host (Margarita *et al.* 2009, Smyth 2015). The current preferred diagnostic method for AY in grapevine is a PCR-based method, in which three sequential PCR reactions are performed, with the final two being nested reactions (hereafter referred to as triple-nested PCR, described in section 3.2.2). When comparing a triple-nested PCR assay to a Taqman qPCR assay, Smyth (2015) found the former to be able to detect AY at a 1000 times higher dilution than the latter. Smyth also investigated the temporal and spatial distribution of AY in grapevine to optimise diagnostics with regards to sampling time-point and plant tissue. Results indicated that the warmer month of February (with reference to plants in the southern hemisphere) resulted in the most positive diagnoses, with phloem cane scraping yielding the most accurate results (Smyth 2015). This paralleled previous results by Constable *et al.* (2003), who also reported most positive results in plants sampled at the height of the Australian summer.

This chapter focusses on the induction of an RP in an AY-positive vineyard in Vredendal, and the monitoring of the RP and AY-status over two grape growing season.

3.2. Materials and Methods

3.2.1. Sample Collection and DNA extraction

Samples were collected from a Colombar vineyard in the Vredendal region of the Western Cape, South Africa. Initial sampling in February 2016 comprised of the cane material from 60 plants displaying distinct AY symptoms, as well as from 40 asymptomatic plants. DNA extraction was performed utilising a CTAB method (Addendum A), after which the purity and concentration of the DNA was determined with a Nanodrop® ND-2000 spectrophotometer. The extracted DNA was separated on a 1% agarose gel at 100V to ensure that the DNA was not degraded (Figure 3.1). After screening all the initial samples for phytoplasma infection, 80 plants (40 AY-positive and 40 AY-negative) were monitored biannually for its AY-infection status. Subsequent sampling dates were November 2016 and February 2017.

3.2.2 Sample Screening

A triple-nested PCR method was employed. Primers used for this assay are listed in Table 3.1. PCR reactions comprised of 50ng extracted DNA, 0.2 μ M of each primer, 10mM dNTP mix

(Thermo Fisher), 1X KapaTaq Buffer B with Mg and 0.9 U/ μ L KapaTaq DNA polymerase in a final volume of 20 μ L (Smyth 2015). The first PCR products were diluted 30X and 1 μ L was used as the template for the first nested PCR reaction, after which the resulting product was diluted 10X and again 1 μ L was used in the second nested PCR reaction. Every PCR assay included one positive control, one plant negative control and one no-template control. The reaction conditions for all three reactions are listed in Table 3.2. After the final nested reaction, 10 μ L of the amplicon was separated on a 1% agarose gel alongside 4 μ L GeneRuler 1kb ladder (Thermo Fisher), and visualised with ethidium bromide staining and a UV transilluminator. When false negative results were suspected, samples were screened using a nested PCR assay amplifying the AY-phytoplasma *secY* gene sequence. *secY* PCR reactions comprised of 50ng extracted DNA, 0.2 μ M of each primer, 10mM dNTP mix (Thermo Scientific), 1X KapaTaq Buffer B with Mg and 0.9 U/ μ L KapaTaq DNA polymerase in a final volume of 20 μ L (Zambon 2015) The PCR products were diluted 30X and used as template for the nested reaction. The amplicons generated were separated on a 1% agarose gel at 100V.

Table 3.1: Primers used in triple-nested PCR assay.

Primer	Sequence	Position	Amplicon size	Reference
P1	5' AAG AGT TTG ATC CTG GCT CAG GAT T-3'	16S rDNA	1792 bp	Deng and Hiruki 1991
P7	5'-CGT CCT TCA TCG GCT CTT-3'	23s rDNA		Schneider <i>et al.</i> 1995
R16F2n	5'-GAA ACG ACT GCT AAG ACT GG-3	16S rDNA		Gunderson <i>et al.</i> 1996
R16R2	5'-TGA CGG GCG GTG TGT ACA AAC CCC G-3'	16S rDNA	1244 bp	Lee <i>et al.</i> 1994
R16(I)F1	5'-TAA AAG ACC TAG CAA TAG G-3'	16S rDNA	1100 bp	Lee <i>et al.</i> 1994
R16(I)R1	5'-CAA TCC GAA CTG AGA CTG T-3'	16S rDNA		

Table 3.2: PCR reaction conditions of the triple-nested assay and *secY* PCR assay

PCR reaction	Primers	First hold	Cycle 35 X						Final hold		
			Denaturation	Annealing	Extension						
16S 1	P1 (Deng and Hiruki 1991)	5'	94°C	20''	94°C	30''	55°C	45''	72°C	7'	72°C
	P7 (Schneider <i>et al.</i> 1995)										
16S 2	R16F2n (Gunderson <i>et al.</i> 1996)	2'	94°C	1'	94°C	2'	58°C	3'	72°C	10'	72°C
	R16R2 (Lee <i>et al.</i> 1994)										
16S 3	R16(I)F1 (Lee <i>et al.</i> 1994)	2'	94°C	1'	94°C	2'	50°C	3'	72°C	10'	72°C
	R16(I)R1 (Lee <i>et al.</i> 1994)										
secY 1	secYF1 (Lee <i>et al.</i> 2006)	3'	94°C	1'	94°C	2'	52°C	3'	72°C	7'	72°C
	secYR1 (Lee <i>et al.</i> 2006)										
secY 2	secYAYF (This study)					2'	57°C				
	secYAYR(This study)										

3.2.2 Coppicing samples

In the final week of July 2016 (year one), every third row of the vineyard was cut down due to farming practices. This unintended coppicing affected 32 of the plants included in this study. Within three weeks of the coppicing, on the 18th of August 2016, those coppiced plants were inspected for any signs of physical damage, which could prevent sprouting. An additional eight plants were then coppiced. The sample groups from this point on consisted of 20 AY-symptomatic, coppiced vines, 20 AY-symptomatic, uncoppiced vines, AY-asymptomatic, coppiced vines and 20 AY-asymptomatic, uncoppiced vines.

3.3 Results

3.3.1 AY-infection status determination

Intact genomic DNA was extracted for every sample (Figure 3.1). The results of the triple-nested PCR assay for phytoplasma detection are summarised in Table 3.3. AY was detected in 43 of the 60 symptomatic plants in February 2016 (Figure 3.2). Forty AY-positive plants were selected for continued monitoring. Three of the 40 asymptomatic plants (A4, A11 and A23) were also found to be AY-positive. Six additional asymptomatic plants were sampled and screened in November 2016 to replace A4, A11 and A23. All six plants were AY-negative, consequently plants A41, A42 and A43 were selected to replace A4, A11 and A23. Twenty of the original symptomatic plants, as well as 20 asymptomatic plants, were coppiced during July and August of 2016 to induce an RP. Two AY-positive, coppiced plants were removed from the vineyard during farming practices. Therefore only 18 AY-positive, coppiced plants could be monitored for the remainder of the study.

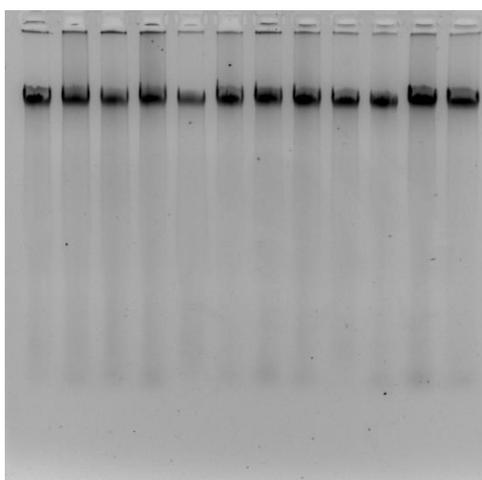


Figure 3.1: Example of a 1% agarose gel with the total DNA extracted from cane phloem scrapings.

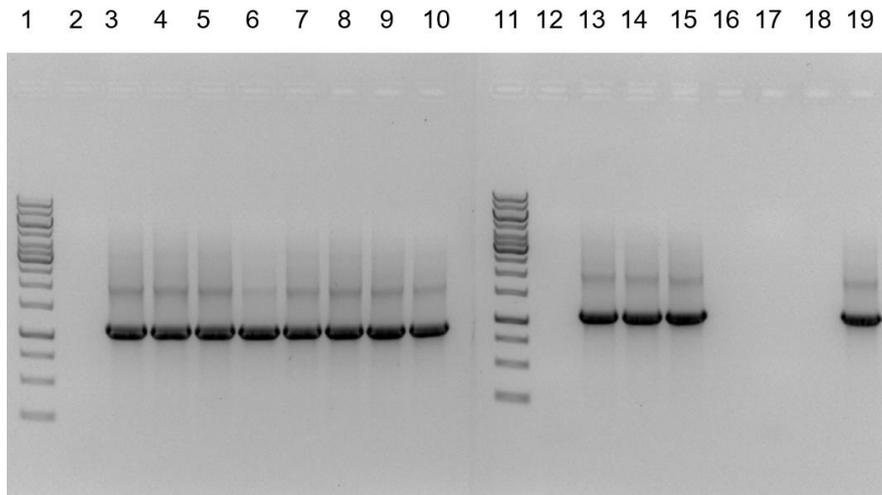


Figure 1.2: Example of a 1% agarose gel of amplicons generated by the triple-nested PCR assay. Lane 1 and 11: 1kb ladder, lane 3 to 9 and 13 to 15: PCR amplicons indicating that the sample is AY-positive, lane 17: negative control, lane 18: no-template control, lane 9 and 19: positive control.

The second time-point for AY screening was in November 2016. Of the original 40 AY-positive plants, only five coppiced plants and two uncoppiced plants tested positive for AY. These results were validated by repeating the DNA extractions, as well as by screening the samples with a second nested PCR assay which amplifies the phytoplasma *secY* gene sequence (described in Section 4.2.1). All the original 37 AY-negative plants, as well as the three replacement plants, tested negative at this time-point.

The final sampling time-point for diagnostics was February 2017. Only four previously AY-positive, coppiced plants remained AY-positive, namely S1, S18, S44 and S51. No AY symptoms were visible in any of the previously AY-positive plants, except for three of the four AY-positive plants. Of these four plants, only S1 and S51 tested AY-positive at all three time-points. Again, all original AY-negative plants remained free of AY.

Table 3.3: AY- and coppicing status of all plants across three time-points. Coordinate in vineyard should be read row/bay/plant. Positive diagnoses highlighted in yellow. Black cells represent plants that were uprooted from the vineyard.

Symptomatic samples					Asymptomatic samples				
Sample and coordinate	Feb-16	Nov-16	Feb-17	Coppicing	Sample and coordinate	Feb-16	Nov-16	Feb-17	Coppicing
S2 16/3/2	AY+	AY-	AY-	Uncoppiced	A1 16/8/4	AY-	AY-	AY-	Uncoppiced
S4 15/5/4	AY+	AY-	AY-	Uncoppiced	A2 16/9/4	AY-	AY-	AY-	Uncoppiced
S6 13/25/3	AY+	AY-	AY-	Uncoppiced	A3 15/10/3	AY-	AY-	AY-	Uncoppiced
S15 13/37/1	AY+	AY-	AY-	Uncoppiced	A5 15/19/1	AY-	AY-	AY-	Uncoppiced
S17 12/22/3	AY+	AY-	AY-	Uncoppiced	A6 16/21/3	AY-	AY-	AY-	Uncoppiced
S19 9/6/2	AY+	AY-	AY-	Uncoppiced	A7 16/24/1	AY-	AY-	AY-	Uncoppiced
S23 10/19/2	AY+	AY-	AY-	Uncoppiced	A9 13/6/2	AY-	AY-	AY-	Uncoppiced
S24 10/39/4	AY+	AY-	AY-	Uncoppiced	A10 13/8/2	AY-	AY-	AY-	Uncoppiced
S26 7/8/1	AY+	AY-	AY-	Uncoppiced	A14 12/8/2	AY-	AY-	AY-	Uncoppiced
S30 7/45/2	AY+	AY-	AY-	Uncoppiced	A18 12/10/2	AY-	AY-	AY-	Uncoppiced
S31 6/1/1	AY+	AY-	AY-	Uncoppiced	A20 12/13/2	AY-	AY-	AY-	Uncoppiced
S39 1/1/2	AY+	AY+	AY-	Uncoppiced	A22 10/4/4	AY-	AY-	AY-	Uncoppiced
S40 1/08/03	AY+	AY+	AY-	Uncoppiced	A24 10/8/1	AY-	AY-	AY-	Uncoppiced
S43 18/9/2	AY+	AY-	AY-	Uncoppiced	A25 10/8/4	AY-	AY-	AY-	Uncoppiced
S47 18/13/1	AY+	AY-	AY-	Uncoppiced	A26 9/9/2	AY-	AY-	AY-	Uncoppiced
S49 18/15/1	AY+	AY-	AY-	Uncoppiced	A27 10/10/01	AY-	AY-	AY-	Uncoppiced

S56 18/31/3	AY+	AY-	AY-	Uncoppiced	A36 6/2/2	AY-	AY-	AY-	Uncoppiced
S57 18/33/2	AY+	AY-	AY-	Uncoppiced	A41 1/1/4	AY-	AY-	AY-	Uncoppiced
S59 19/13/1	AY+	AY-	AY-	Uncoppiced	A42 1/2/2	AY-	AY-	AY-	Uncoppiced
S60 19/14/3	AY+	AY-	AY-	Uncoppiced	A43 1/4/3	AY-	AY-	AY-	Uncoppiced
S1 16/1/1	AY+	AY+	AY+	Coppiced	A8 14/1/3	AY-	AY-	AY-	Coppiced
S5 14/10/4	AY+	AY-	AY-	Coppiced	A12 11/7/2	AY-	AY-	AY-	Coppiced
S7 14/26/4	AY+	AY-	AY-	Coppiced	A13 11/7/3	AY-	AY-	AY-	Coppiced
S8 14/28/1	AY+	AY-	AY-	Coppiced	A15 11/8/3	AY-	AY-	AY-	Coppiced
S9 14/30/3	AY+	AY-	AY-	Coppiced	A16 11/9/1	AY-	AY-	AY-	Coppiced
S14 14/36/3	AY+	AY-	AY-	Coppiced	A17 11/9/4	AY-	AY-	AY-	Coppiced
S18 11/30/2	AY+	AY-	AY+	Coppiced	A19 11/11/3	AY-	AY-	AY-	Coppiced
S29 8/30/3	AY+	AY-	AY-	Coppiced	A21 9/2/3	AY-	AY-	AY-	Coppiced
S32 6/1/4	AY+	AY-	AY-	Coppiced	A28 7/1/2	AY-	AY-	AY-	Coppiced
S33 5/5/3	AY+	AY-	AY-	Coppiced	A29 8/1/1	AY-	AY-	AY-	Coppiced
S34 5/18/1	AY+				A30 7/2/4	AY-	AY-	AY-	Coppiced
S35 5/34/3	AY+				A31 7/4/3	AY-	AY-	AY-	Coppiced
S44 17/10/1	AY+	AY-	AY+	Coppiced	A32 8/4/4	AY-	AY-	AY-	Coppiced
S46 17/12/3	AY+	AY+	AY-	Coppiced	A33 8/6/1	AY-	AY-	AY-	Coppiced
S48 17/14/1	AY+	AY+	AY-	Coppiced	A34 6/1/2	AY-	AY-	AY-	Coppiced

S51 17/27/2	AY+	AY+	AY+	Coppiced	A35 5/2/1	AY-	AY-	AY-	Coppiced
S52 17/28/3	AY+	AY-	AY-	Coppiced	A37 6/3/1	AY-	AY-	AY-	Coppiced
S54 17/31/1	AY+	AY+	AY-	Coppiced	A38 5/3/4	AY-	AY-	AY-	Coppiced
S55 17/38/3	AY+	AY-	AY-	Coppiced	A39 5/6/3	AY-	AY-	AY-	Coppiced
S58 20/11/3	AY+	AY-	AY-	Coppiced	A40 5/7/4	AY-	AY-	AY-	Coppiced

3.3.2 Coppicing of Samples

All plants regrew new buds after coppicing. Two AY-symptomatic, coppiced plants (S34, S35) were removed from the vineyard during farming practices, leaving only 18 vines in the AY-positive, coppiced sample group. Figure 3.3 is an aerial photograph of the vineyard, with coppiced rows indicated. Figure 3.4 provides a layout of all the plants which were included in this study.

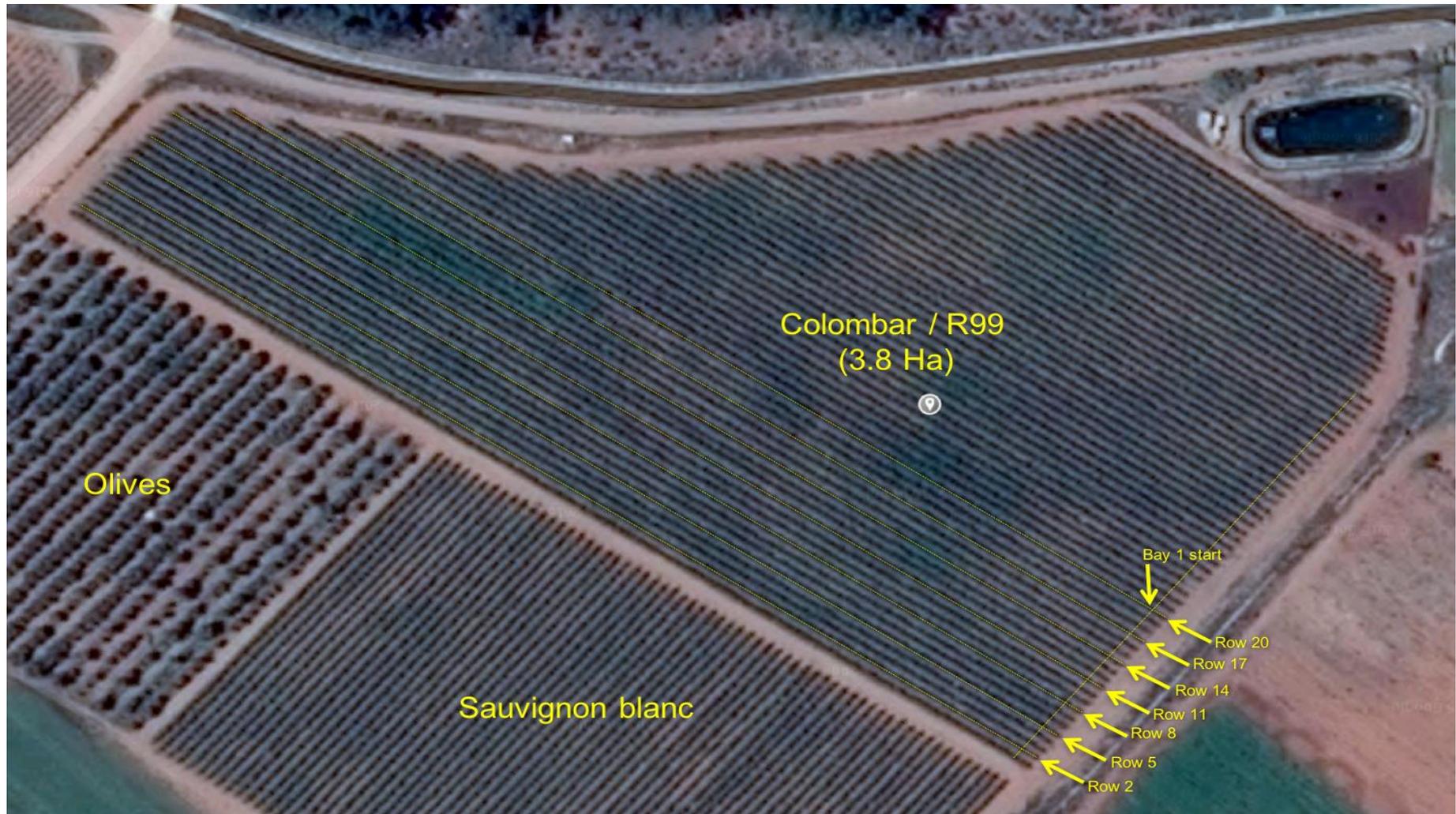


Figure 3.3: Colombar vineyard which is the subject of this study. Coppiced rows are indicated. The bay which was considered “bay 1” in every row has also been indicated.

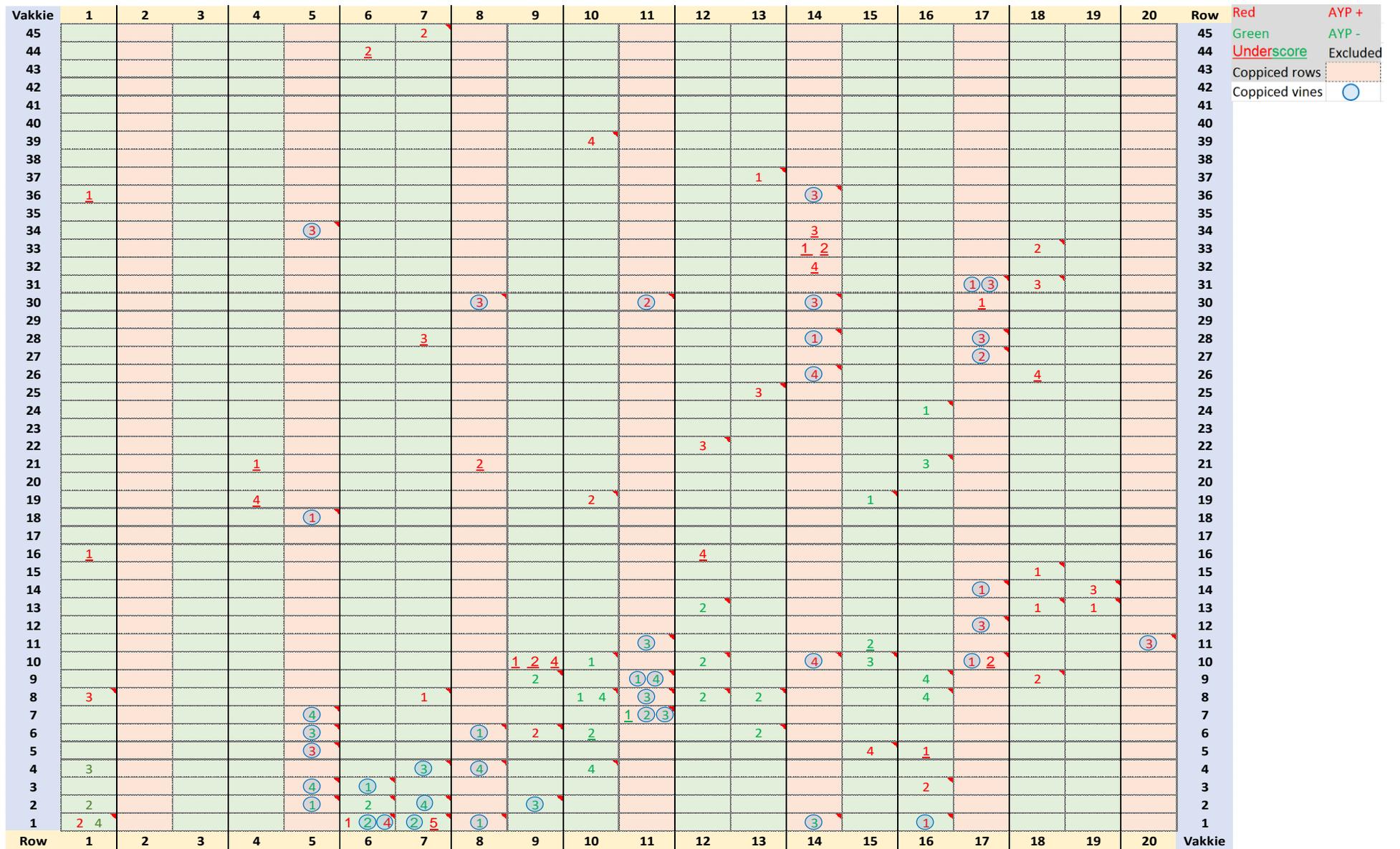


Figure 3.4: A map of the vineyard. Coordinates of individual vines are in the format: row/bay/vine, e.g. the address of the only vine in row 20 is: 20/11/3.

3.4 Discussion and Conclusion

Of the 60 symptomatic vines sampled initially, 43 were shown to be AY-positive (71.66%). This coincides with previous studies, which report positive diagnostic testing ranging between 70% and 78% of symptomatic plants for January and February sampling events in the southern hemisphere (Gibb *et al.* 1999, Constable *et al.* 2003). Thirty-seven of the 40 asymptomatic plants proved to be AY-negative. The initial diagnostics therefore largely concurred with symptom observation in the field. Additional AY-negative plants were sampled and the 40 AY-positive plants were selected to monitor over the study period. After the coppicing of half of each experimental group in July and August 2016 (year one), all coppiced plants regrew, except for two symptomatic plants, which were uprooted and removed from the vineyard. At the two subsequent sampling time-points (November 2016 and February 2017), all AY-negative plants tested negative. This can be attributed to vector control, as the insecticide Imidacloprid was administered to all the vines through a dripping irrigation system since February 2016. This most likely prevented further spread of the disease in the vineyard. Of the AY-positive plants collected in November 2016, recovery was observed in the coppiced sample group where only five of the 18 plants tested AY-positive. Substantial recovery was, however, also observed for the uncoppiced, AY-positive sample group, since only two of the 20 plants tested AY-positive. These diagnostic results could not be related to symptomatology, since vines do not display clear symptoms at that point in the growing season. It was suspected that the recovery observed was true, and not a result of ineffective diagnostic assays, as repeated extractions and diagnostics delivered the same results as confirmed when the *secY* nested diagnostic assay was employed. The recovery of the previously AY-infected vines persisted into the February 2017 sampling round when none of the uncoppiced vines were AY-positive, and only four coppiced vines testing positive at that time. These results therefore excluded the possibility that the previous results were caused by seasonal fluctuations of AY in grapevines. With the exception of three of the four remaining positive vines, the vineyard showed no GY symptoms. Diagnostics therefore coincided with symptom expression in the vineyard.

When the RP was first observed, it was described as a spontaneous recovery from phytoplasma infection and symptoms (Caudwell 1961). Recently, after identifying the correlation between stressing plants and RP induction, some cultivars of grapevine have displayed particularly high rates of recovery without being stressed. These cultivars are thought to not be persistently infected and must be reinfected every season (Belli *et al.* 2010). In a study, performed on phytoplasma-infected cv. Barbera in Italy by Belli *et al.* (1978), plants were treated with an insecticide that is particularly effective against the local vector. Over five years, 50% of the vines recovered each year, with only two of the original 37 phytoplasma-infected plants testing positive throughout the duration of the study. No previous studies have investigated the persistence of phytoplasma infection in vines of the cv. Colombar in the absence of a phytoplasma insect vector. It is therefore

possible that the large-scale remission in infection, regardless of whether or not the plant was physically stressed, may be as a result of the elimination of the insect vector *M. fuscovaria* from the vineyard, thereby preventing reinfection. The vector was controlled in the vineyard by using the systemic insecticide Imidacloprid as of February 2016. In this scenario, the fact that some of the coppiced plants remained AY-positive can be explained by the coppicing which occurred in July 2016 due to farming practices. During this coppicing, instruments were not sterilised between plants, thereby possibly inadvertently mechanically reinfected some of the coppiced plants.

Alternative theories to a recovery due to a lack of reinfection should also be considered. One possibility is that an unintentional abiotic stress, such as a drought, may have induced the large-scale RP observed in the Colombar vineyard. In May 2017, serious drought conditions caused the Western Cape government to declare the region, in which the experimental vineyard is located, a drought disaster area. Araujo *et al.* (2016) reported on the negative effect which droughts have on grape yields in vineyards in South Africa. In a farm scale simulation using the Agricultural Production Systems sIMulator (APSIM) tool, they found a strong negative correlation ($r \approx -0.9$) between grape berry yield and the occurrence of droughts. Drought was in this case defined as a significant decrease in rainfall, accompanied by a significant increase in temperature (Araujo *et al.* 2016). An article published by de Villiers (2017) supports these findings, stating that the current drought has cost Western Cape grape farmers up to R500 million by the month of May. The increased sensitivity of grapevine plants in times of drought, coupled to strict irrigation limitations (ranging on average between 30% and 100% (de Villiers 2017)), which have been placed on farms in the Western Cape during the latest drought, may have significant effects on the AY-infection status of affected vines. The irrigation allowance for vineyards in Vredendal specifically was reduced by 23% in the 2017 season. Drought conditions could therefore possibly have resulted in sufficient abiotic stress to induce an RP in the experimental vineyard.

Additionally, previous studies have investigated how changes in endophytic communities in phytoplasma infected plants may play a role in RP induction (Bulgari *et al.* 2011, Noar *et al.* 2015). These studies report that differences are observed between the endophytic communities of AY-positive, AY-negative and AY-recovered plants. Endophytes often partake in mutualistic relationships with the host plants. They assist in handling, among others, abiotic stresses such as salinity, extreme temperature and droughts (Hardoim *et al.* 2015). The endophyte *Burkholderia phytofirmans* strain PsJN has specifically been linked to aiding drought resistance in maize (Naveed *et al.* 2014). This same endophyte has also been implicated in resistance against chilling in grapevine plants grown at 4°C (Ait Barka *et al.* 2006). *Burkholderia* spp. has previously been implicated in RP induction in grapevines (Bulgari *et al.* 2011). It may be possible that similar bacterial endophytes may have played a role in RP induction in the experimental vineyard, conceivably through a restructuring in the endophytic community as a response to the drought conditions in the Western Cape.

Of the four plants that were positive at the February 2017 time-point, two consistently tested positive at each time-point. The other two plants were negative at the November 2016 time-point. The uneven distribution of the pathogen throughout the plant, combined with the low titres of phytoplasma in host plants may explain these inconsistent diagnostic results (Smyth 2015). This, however, does not explain the negative diagnoses made in November 2016 and February 2017. The disappearance of GY symptoms suggests that the diagnostics are accurate, and that a true recovery has occurred.

In conclusion, of 40 initially identified AY-positive plants, 34 adopted an RP within a year of the first sampling excursion. Plants recovered from the AY-infection regardless of whether they were coppiced or not, with only four coppiced individuals remaining positive throughout the duration of the study. Currently, it is impossible to determine what the exact cause of the observed recovery was. Many factors, such as the drought status of the Western Cape as well as the elimination of AY insect vectors, might have played a role in the observed AY-recovery. Continuous observation of the AY-infection status of the experimental vineyard is suggested to determine whether the RP persists. Subsequent work investigating the endophytic communities of the recovered plants is also recommended to determine whether a restructuring, possibly brought on by the drought conditions and irrigation limitations, may be implicated in the observed AY-recovery.

3.5 References

Ait Barka, E., J. Nowak and C. Clément, 2006 Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. *Applied and Environmental Microbiology* 72(11):7246-7252

Araujo, J.A., B.J. Abiodun and O. Crespo, 2016 Impacts of drought on grape yields in Western Cape, South Africa. *Theoretical and Applied Climatology* 123(1-2):117-130

Belli, G., A. Fortusini, R. Osler and A. Amici, 1973 Presenza di una malattia del tipo "Flavescence dorée" in vigneti dell'Oltrepò pavese. *Rivista di Patologia Vegetale Ser IV* 9(suppl.):50-56

Belli, G., A. Fortusini and R. Osler, 1978 Present knowledge on diseases of the type "Flavescence doree" in vineyards of Northern Italy. *Proceedings of the 6th Meeting of ICVG, Cordoba, Spain, 1976 (Monografias INIA No. 18, 1978):7-13*

Belli, G., P. A. Bianco and M. Conti, 2010 Grapevine yellows in Italy: past, present and future. *Journal of Plant Pathology* 92(2):303-326

Bulgari, D., P. Casati, P. Crepaldi, D. Daffonchio, F. Quaglino *et al.* 2011 Restructuring of endophytic bacterial communities in grapevine yellows-diseased and recovered *Vitis vinifera* L. plants. *Applied and Environmental Microbiology* 77(14):5018-5022

Carstens, R., 2014 The incidence and distribution of grapevine yellows disease in South African vineyards. MSc thesis. Stellenbosch University

Caudwell, A., 1961 Les phénomènes de rétablissement chez la flavescence dorée de la vigne. *Annales des Epiphyties* 12:347-354

Chen, T.A., X.F. Jiang 1988, Monoclonal antibodies against the maize bushy stunt agent. *Canadian Journal of Microbiology* 34(1):6-11

Constable, F. E., K. S. Gibb and R. H. Symons, 2003 Seasonal distribution of phytoplasmas in Australian grapevines. *Plant Pathology* 52:267-276

Deng, S. and C. Hiruki, 1991 Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods* 14:53-61

De Villiers, J., 2017 Drought already cost Western Cape grape farmers R500m. News 24. Retrieved from <http://www.news24.com/SouthAfrica/News/drought-already-cost-western-cape-grape-farmers-r500m-mec-20170524>

Engelbrecht, M., J. Joubert and J. T. Burger, 2010 First report of Aster yellows phytoplasma in grapevines in South Africa. *Disease Notes* 94(3):373

Gibb, K. S., F. E. Constable, J. R. Moran and A. C. Padovan, 1999 Phytoplasmas in Australian grapevines – detection, differentiation and association. *Vitis* 38(3): 107-114

Gunderson, D.E., I.-M. Lee, D.A. Schaff, N.A. Harrison, C.J. Chang *et al.*, 1996 Genomic diversity and differentiation among phytoplasma strains in 16S rRNA group I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). *International Journal of Systemic Bacteriology* 46:64-75

Hardoim, P.R., L.S. van Overbeek, G. Berg, A.M. Pirttilä, S. Compant *et al.*, 2015 The hidden world in plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews* 79(3):293-320

Jacoli, G.G., W.P. Ronald, 1974 Electron microscopic studies of plant tissue cultures with the aster yellows disease. *Journal of Ultrastructure Research* 46(1):34-42

Lee, I-M., D.E. Gunderson, R.W. Hammond and R.E. Davis, 1994 Use of mycoplasma-like organism (MLO) group specific oligonucleotide primers for nested-PCR assays to detect mixed MLO infections in a single host plant. *Phytopathology* 84:559-566

Lee, I-M., R. E. Davis and D. E. Gunderson-Rindal, 2000 Phytoplasma: Phytopathogenic Mollicutes. *Annual Review of Microbiology* 54:221-255

Lee, I-M., Y. Zhao and K. D. Bottner, 2006 *SecY* gene sequence analysis for finer differentiation of diverse strains in the Aster yellows phytoplasma group. *Molecular and Cellular Probes* 20(2):87-91

Magarey, P. A., 1986 Grapevine yellows – aetiology, epidemiology and diagnosis. *South African Journal of Enology and Viticulture* 7(2):90-100

Margaria, P., M. Turina and S. Palmano, 2009 Detection of Flavescence dorée and Bois noir phytoplasmas, Grapevine leafroll associated virus-1 and Grapevine virus A from the same crude extract by reverse transcription-RealTime taqman assays. *Plant Pathology* 58:838-845

McCoy, R.E., 1982 The use of tetracycline antibiotics to control yellow diseases. *Plant Disease* 66:539-542

Musetti, R., R. Marabottini, M. Badiani, M. Martini, L. Sanità di Toppi *et al.*, 2007 On the role of H₂O₂ in the recovery of grapevine (*Vitis vinifera* cv. Prosecco) from Flavescence dorée disease. *Functional plant Biology* 34:750-758

Musetti, R., K. Farhan, F. De Marco, R. Polizzotto, A. Paolacci *et al.*, 2013 Differentially-regulated defence genes in *Malus domestica* during phytoplasma infection and recovery. *European Journal of Plant Pathology* 136(1):13-19

Naveed, M., B. Mitter, T.G. Reichenauer, K. Wieczorek and A. Sessitsch, 2014 Increased drought stress resilience of maize through endophytic colonization by *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17. *Environmental and Experimental Botany*

Naor, V., L. Iasur-Kruh, R. Barkai, R. Bordolei, S. Rodoy *et al.*, 2015 Introduction of beneficial bacteria to grapevines as a possible control of phytoplasma associated diseases. *Phytopathogenic Mollicutes* 5(1 Suppl.): S111-S112

Osler, R., L. Carraro, N. Loi and E. Refatti, 1993 Symptom expression and disease occurrence of a yellows disease of grapevine in northeastern Italy. *Plant Disease* 77:496-498

Romanazzi, G., and S. Murolo, 2008 Partial uprooting and pulling to induce recovery in Bois noir-infected grapevines. *Journal of Phytopathology* 156:747-750

Rufo, R., A. Batlle, A. Camprubi, E. Montesinos and C. Calvet, 2017 Control of rubus stunt and stolbur diseases in Madagascar periwinkle with mycorrhizae and a synthetic antibacterial peptide. *Plant Pathology* 66(4):551-558

Smyth, N., 2015 The determination of the spatial and temporal distribution of Aster yellows phytoplasma in grapevine. MSc thesis. Stellenbosch University

South African Wine Industry Statistics nr 41 (2017) South African Wine Industry Information & Systems, SAWIS, PO Box 238, Paarl 7620

Zambon, Y., 2015 Caratterizzazione molecolare multigenica di fitoplasmi associati a giallumi della vite in Italia e Sud Africa. MSc thesis. Università di Bologna

Chapter 4: Aster Yellows phytoplasma genetic diversity analysis

4.1 Introduction

The genus '*Candidatus Phytoplasma*' is divided into 20 subclades based on 16S rDNA sequencing data (Seemüller *et al.* 1990) with Aster Yellows (AY) phytoplasma belonging to subclade 16Srl. Within this subclade, AY subgroups 16Srl-A to 16Srl-Y have been identified to date (Acosta *et al.* 2015), a list that may expand as phytoplasma research continues. AY phytoplasma is the species with the highest genetic diversity based on 16S rDNA sequence analysis (Lee *et al.* 2000). The genetic diversity observed even within subgroups poses a possible problem when investigating recovery phenotype (RP) induction in phytoplasma infected plants. With PCR diagnostic assays being sequence specific, genetic diversity of the phytoplasma infecting a vineyard may lead to false negative diagnostic results, thereby resulting in the wrongful assumption that a plant is recovered. It is therefore important to investigate whether possible genetic diversity may be present in the AY infecting the experimental vineyard, in order to ensure that the recovery suggested by the 16S rDNA triple-nested PCR results is a true recovery. Multigene analyses are used to discern the specific subgroup or strain of phytoplasma infecting host plants (Hodgetts and Dickinson 2012). Utilizing 16S rDNA gene sequences often does not provide sufficient differentiation between the closely related strains in the aster yellows group (Mitrović *et al.* 2011). This highlights the need to use other genes when looking to distinguish between strains of AY. Previous studies have employed assays utilising reference genes such as *groEL* (Mitrović *et al.* 2011), *rp* (Gunderson *et al.* 1996, Lee *et al.* 2004), *secY* (Lee *et al.* 2006, Lee *et al.* 2010) and *amp* (Kakizawa *et al.* 2006).

Recently, Zambon *et al.* (2015) exploited a multigene protocol in which *rp*, *secY*, *groEL* and *amp* PCR amplicons were digested with the restriction enzymes *AluI* and *Tru1I*. The Restriction Fragment Length Polymorphism (RFLP) patterns generated from AY-infections in vineyards in Robertson and Vredendal in South Africa were compared to positive samples from Italy. All South African isolates generated identical RFLP pattern for *rp*, with only a single sample generating a unique profile for *groEL*. Some variability was observed with *amp* amplicon digestion, although not all samples generated amplicons for this gene. *secY* RFLP analysis generated five distinct patterns in the South African samples (Zambon *et al.* 2015). These results suggest that *secY* is the most informative gene to use when determining the genetic diversity of AY in a South African context. Different RFLP patterns were generated for South African and Italian isolates for every reference gene (Zambon *et al.* 2015).

In this chapter, we report on the AY diversity observed in a South African Colombar vineyard, and how the observed diversity may affect conclusions about an induced RP throughout the vineyard. We also present the sequencing data generated for *groEL*, *secY*, *amp*, *rp* and 16S rDNA amplicons isolated from this vineyard.

4.2 Materials and Methods

4.2.1 PCR amplification of Phytoplasma Genes

The analysis was performed on DNA extracted from the February 2016 samples mentioned in Chapter 3. Four phytoplasma reference genes were amplified using nested PCR assays: *rp*, *groEL*, *amp*, and *secY*. The primers used can be seen in Table 4.1, with PCR conditions listed in Table 4.2. Some of the primer pairs were designed for the purpose of this study (refer to Table 4.1) with Oligo Explorer. PCR reactions comprised of 50ng extracted DNA, 0.2 μ M of each primer, 10mM dNTP mix (Thermo Scientific), 1X KapaTaq Buffer B with Mg and 0.9 U/ μ L KapaTaq DNA polymerase in a final volume of 20 μ L (Zambon 2015). The PCR products were diluted 30X and used as template for the nested reaction. The amplicons generated were separated on a 1% agarose gel at 100V.

Table 4.1: Primer sequences and amplicon size generated for genes *groEL*, *rp*, *amp* and *secY*.

Gene region	Primer	Sequence	Amplicon size	Reference
<i>rp</i> direct	rpF1	GGACATAAGTTAGGTGAATTT	1245 – 1389 bp	Martini <i>et al.</i> 2007
	rpR1	ACGATATTTAGTTCTTTTTGG		
<i>rp</i> nested	rp(l)F1 A	TTTTCCCCTACACGTA	1200 bp	Martini <i>et al.</i> 2007
	rp(l)R1 A	GTTCTTTTTGGCATTAAACAT		
<i>groEL</i> direct	groEL gene F	ATCAGAAAAAGAAAAATCCT	2100 bp	Mitrović <i>et al.</i> 2011
	groEL gene R	GCAACAGCAGCAAATAAAAC		
<i>groEL</i> nested	groELF 1	GGCAAAGAAGCAAGAAAAG	1500 bp	Mitrović <i>et al.</i> 2011
	groELR 1	TTTAAGGGTTGTAAAAGTTG		
<i>amp</i> direct	ampC1	AAGAGCTCGAGTTTATTGTTTTG TTTTTTTAAAC	700 bp	Kakizawa <i>et al.</i> 2006
	ampN1 cor	AAGAATTCCATATGCAAATCAAA AAACTCA		
<i>amp</i> nested	ampAY f	GAAAGGAGAACAACAATGC	611 bp	This study
	ampAF r	AGAACCACACTGTTTTGTAC		
<i>secY</i> direct	secYF1	CAGCCATTTTAGCAGTTGGTGG	1400 bp	Lee <i>et al.</i> 2006
	secYR1	CAGAAGCTTGAGTGCCTTACC		

secY nested	secYA	TGTTTAGGAACTTCTTGGC	1223 bp	This study
	Yf			
	secYA	TTGAGTGCCTTTACCAATTC		
	Yr			

Table 4.2: PCR reaction conditions for *groEL*, *rp*, *amp* and *secY*.

Gene	First hold		Cycle 35X				Final hold	
			Denaturation	Annealing	Extension			
<i>rp</i> reactions			94°C	1'	50°C	2'	72°C	3'
<i>groEL</i> reactions					55°C			
<i>amp</i> reactions	94°C	3'			50°C		72°C	7'
<i>secY</i> direct					52°C			
<i>secY</i> nested					57°C			

4.2.2 Amplicon Digestion and RFLP Analysis

All amplicons generated were digested with restriction enzymes *AluI* and *TruI*. Three to 8µL of the amplicons were added to 2µL Thermo Scientific 10X Tango Buffer, 0.25µL restriction enzyme and adjusted with MilliQ water to 15µL. *AluI* and *TruI* reactions were incubated for 16 hours at 37°C and 65°C respectively. Following digestion with *AluI*, 6µL of the digest was separated on a 3% agarose gel at 80V, whereas 5µL of the *TruI* digest was separated on an 8% polyacrylamide gel at 70V. All digests were separated alongside 4µL O'Generuler 100 bp ladder (Thermo Fisher) and visualised with ethidium bromide staining and a UV transilluminator. A representative of every unique RFLP pattern generated for the four reference genes was subjected to Sanger sequencing at the Central Analytical Facilities, Stellenbosch University. Sequencing was performed from both ends of the amplicon, after which the sequences were assembled using the CLC Main Workbench (version 7). Virtual RFLP patterns were also generated for amplicons with unique RFLP patterns from the sequencing data using the Biolabs NEBcutter online tool.

4.3 Results

4.3.1 PCR Amplification of Genes

The results of the PCR assays of the four respective genes are listed in Table 4.3. Not all PCR reactions were successful.

Table 4.3: Diagnostic results of *groEL*, *rp*, *amp* and *secY* in AY-positive samples. An X indicates that a PCR amplicon was successfully generated.

	<i>groEL</i>	<i>rp</i>	<i>amp</i>	<i>secY</i>		<i>groEL</i>	<i>rp</i>	<i>amp</i>	<i>secY</i>
S39	X	X	X	X	S14				
S40	X	X	X	X	S15		X		
S31		X	X		S1		X	X	
S32	X	X	X		S2	X		X	
S33	X	X	X	X	S4	X	X	X	X
S34	X	X	X	X	S43	X	X	X	X
S35	X	X	X		S44	X	X	X	X
S26					S46	X	X	X	
S29		X	X		S47		X		X
S30		X	X	X	S48	X	X	X	X
S19	X	X	X	X	S49		X	X	X
S23		X	X	X	S51	X	X	X	X
S24	X	X	X	X	S52		X	X	X
S17	X	X	X	X	S54	X	X	X	X
S18	X	X	X	X	S55	X	X	X	X
S5	X	X	X	X	S56	X	X	X	X
S6	X	X	X	X	S57		X	X	X

S7	X	X	X		S58	X	X	X	
S8	X	X	X	X	S59	X	X	X	X
S9	X	X	X	X	S60	X	X	X	X

4.3.2 Amplicon Digestion

Figures 4.1 and 4.2 show the results of the RFLP digestion of the four genes. A single example pattern is included per gene. All reference genes produced single RFLP patterns for every amplicon when digested with *AluI* and *TruI* respectively. The virtual gels generated with NEBcutter from the sequencing data generated similar RFLP patterns to the gel images in Figure 4.1 and 4.2. The sequencing data for the representative amplicons, as well as the 16S rDNA amplicon is presented in addendum B to F.

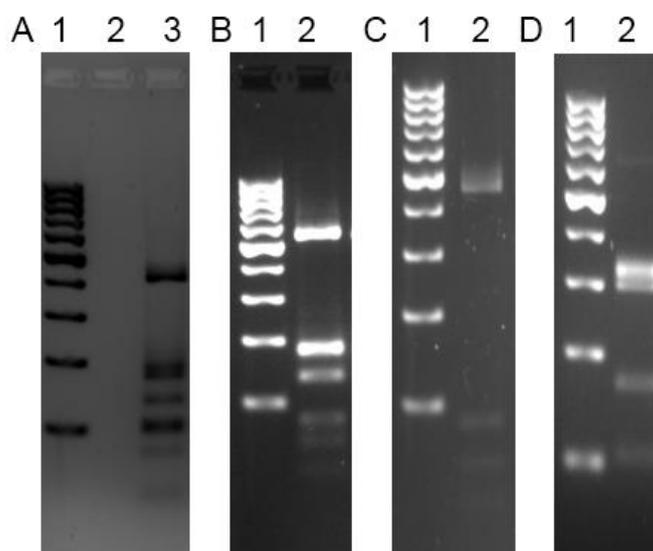


Figure 4.2: 3% Agarose gels depicting the RFLP patterns of (A) *groEL*, (B) *rp*, (C) *amp* and (D) *secY* genes digested with *AluI*. Lane 1 in every case is a 100bp ladder.

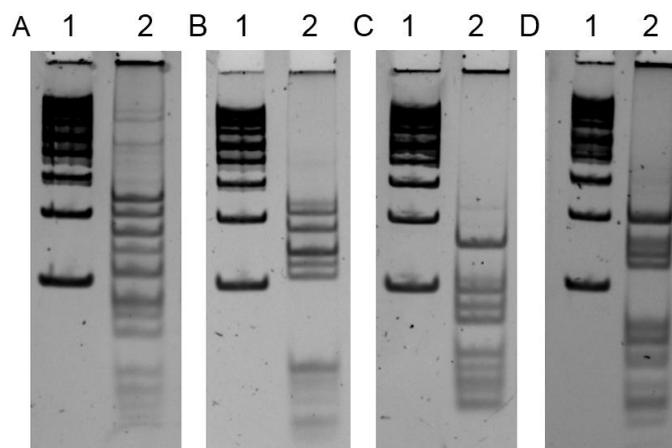


Figure 4.3: 8% Polyacrylamide gels depicting the RFLP patterns of (A) *groEL*, (B) *rp*, (C) *amp* and (D) *secY* genes digested with *TruI*. Lane 1 in every case is a 100bp ladder.

4.3.4 Classifying Observed AY Genetic Variant

When compared to previous genetic diversity experiments, all generated patterns could be matched to the RFLP patterns of known AY reference strains. Refer to Table 4.4 for the names of those strains and the sources from which the RFLP patterns were obtained. By matching RFLP patterns to known reference strains, the sub-group or sub-groups of the isolates could be determined. The majority of the RFLP patterns correspond to reference strains of subgroup 16Srl-B with only three patterns corresponding to strains of subgroup 16Srl-C.

Table 4.4: RFLP patterns generated in this project matched up with those generated from reference strains in previous publications.

Gene	Enzyme	Reference strain	Reference strain subgroup	Source
groEL	<i>Alul</i>	AY-J	Srl-B	(Mitrović <i>et al.</i> 2011)
	<i>Tru1I</i>	AY-J	Srl-B	
rp	<i>Alul</i>	MBS	Srl-B	(Lee <i>et al.</i> 2004)
	<i>Tru1I</i>	MBS	Srl-B	
secY	<i>Alul</i>	MBS	Srl-B	(Lee <i>et al.</i> 2006)
	<i>Tru1I</i>	KVG	Srl-C	
amp	<i>Alul</i>	KVE	Srl-C	(Zambon 2015)
	<i>Tru1I</i>	KVE	Srl-C	

4.4 Discussion and Conclusion

No genetic diversity was observed when performing RFLP analysis on *groEL*, *amp*, *rp* and *secY* genes with enzymes *Alul* and *Tru1I* in the experimental vineyard. The sequencing data and NEBcutter virtual gels corresponded to the RFLP gel images.

RFLP patterns corresponding to known AY genetic variants were observed. *groEL* RFLP patterns generated by both *Alul* and *Tru1I* matched those of reference strain AY-J that belongs to subgroup 16Srl-B (Mitrović *et al.* 2011). Patterns generated for *rp* by *Alul* and *Tru1I*, as well as the *Alul* pattern generated for *secY* corresponds to that of reference strain MBS (Lee *et al.* 2004), which belongs to the 16Srl-B subgroup of phytoplasmas (Harrison *et al.* 1996). *Tru1I* digestion of the *secY* amplicons generated RFLP patterns matching the reference strain KVG that belongs to subgroup 16Srl-C (Lee *et al.* 2006). Both *Alul* and *Tru1I* patterns generated for *amp* corresponded to reference strain KVE (Zambon 2015) that forms part of the 16Srl-C subgroup (Marcone *et al.*

2000). The genetic variant present in the vineyard is therefore suspected to either fall in subgroup 16Srl-B or 16Srl-C.

These results partly correspond to those of a recent study by Zambon (2015) that investigated the genetic diversity of AY in three vineyards in Vredendal, as well as a vineyard in Robertson. Identical *groEL* RFLP patterns across samples corresponding to the reference strain AY-J was reported. Profiles generated for *rp* almost exclusively matched that of reference strain NJ-AY, with only a single sample originating in Vredendal having a profile matching reference strain MBS. The majority of *secY* profiles generated corresponded to reference strains MBS and AVUT, with a few novel profiles being observed. Most of the *amp* profiles matched the reference strain KVE, with three novel RFLP profiles observed in three of the samples. All the reference strain matches observed in this study corresponds to at least one of the observations made by Zambon (2015). A much lower genetic diversity was observed in this study. This is expected, since only a single vineyard was surveyed in this study.

Not all the AY-positive plants generated amplicons for all of the reference genes. This also corresponds to findings by Zambon *et al.* (2015), in which certain genes generated amplicons more readily than others. Though it is unclear exactly why, there are many possible explanations for this. It is possible that the phytoplasma titres in some of the samples are too low to be detected by the nested PCR assays used for the four reference genes, with a triple-nested reaction being necessary to generate an amplicon. Sub-optimal primer design or PCR reaction conditions may also be the cause. It is most likely that it was a combination of these issues that resulted in poor amplification.

In conclusion, the Colombar vineyard was found to be infected by a single genetic variant of AY, likely belonging to either sub-group 16Srl-B or 16Srl-C. This may be further investigated in the future by including additional genes of interest. As only a single genetic variant was observed, it is unlikely that the observed recovery is the result of a false negative diagnosis.

4.5 References

Acosta, K., F.N. Silva, L. Ramona, M. Quiñones, R. Martínez *et al.*, 2015 Detection and molecular characterization of an Aster Yellows group phytoplasma associated with soybean and evidence of two new 16Srl subgroups in Cuba. *Journal of Plant Pathology* 97(2)

Gunderson, D.E., I.-M. Lee, D.A. Schaff, N.A. Harrison, C.J. Chang *et al.*, 1996 Genomic diversity and differentiation among phytoplasma strains in 16S rRNA groups I (Aster Yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). *International Journal of Systemic and Evolutionary Microbiology* 46:64-75

Harrison, N.A., P.A. Richardson, J.H. Tsai, M.A. Ebbert and J.B. Kramer, 1996 PCR assay for the detection of the phytoplasma associated with maize bushy stunt disease. *Plant Disease* 80:263-269

Hodgetts, J. and M. Dickinson, 2012 T-RFLP for detection and identification of phytoplasmas in plants. *Methods in Molecular Biology* 938:233-244

Kakizawa, S., K. Oshima and S. Namba, 2006 Diversity and functional importance of phytoplasma membrane proteins. *Trends in Microbiology* 14(6):254-256

Lee, I-M., R.E. Davis and D.E. Gunderson-Rindal, 2000 Phytoplasma: Phytopathogenic Mollicutes. *Annual Review of Microbiology* 54:221-255

Lee, I-M., D. E Gunderson-Rindal, R. E. Davis, K. D Bottner, C. Marcone *et al.*, 2004 'Candidatus Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. *International Journal of Systematic and Evolutionary Microbiology* 54:1037-1048

Lee, I-M., Y. Zhao and K. D. Bottner, 2006 *SecY* gene sequence analysis for finer differentiation of diverse strains in the Aster yellows phytoplasma group. *Molecular and Cellular Probes* 20(2):87-91

Lee, I.-M., K.D. Bottner-Parker, Y. Zhao, R.E. Davis and N.A. Harrison, 2010 Phylogenetic analysis and delineation of phytoplasmas based on *secY* gene sequences. *International Journal of Systemic and Evolutionary Microbiology* 60:2887-2897

Marcone, C., I.-M. Lee, R.E. Davis, A. Ragozzino and E. Seemüller, 2000 Classification of aster yellows-group phytoplasmas based on combined analyses of rRNA and *tuf* gene sequences. *International Journal of Systematic and Evolutionary Microbiology* 50:1702-1713

Martini, M., I-M. Lee, K.D. Bottner, Y. Zhao, S. Botti *et al.*, 2007 Ribosomal protein gene-based phylogeny for finer differentiation and classification of phytoplasmas. *International Journal of Systematic and Evolutionary Microbiology* 57:2037-2051

Mitrović, J., S. Kakizawa, B. Duduk, K. Oshima, S. Namba *et al.*, 2011a The *groEL* gene as an additional marker for finer differentiation of 'Candidatus Phytoplasma asteris'-related strains. *Annals of Applied Biology* 159(1):41-48

Mitrović, j., N. Contaldo, S. Paltrinieri, J.F. Mejia, N. Mori *et al.*, 2011b The use of groEL gene for characterisation of aster yellows phytoplasmas in field collected samples. *Bulletin for Insectology* 64(Suppl.):S17-S18

Seemüller, E., C. Marcone, U. Lauer, A. Ragozzino and M. Göschl, 1990 Current status of molecular classification of the phytoplasmas. *Journal of Plant Pathology* 80(3-26)

Zambon, Y., 2015 Caratterizzazione molecolare multigenica di fitoplasmi associati a giallumi della vite in Italia e Sud Africa. MSc thesis. Università di Bologna

Zambon, Y., N. Contaldo, R. Souza Richards, A. Bertaccini and J.T. Burger, 2015 Multigene characterization of aster yellows phytoplasmas infecting grapevine in South Africa. *Phytopathogenic Mollicutes* 5(1-Suppl.):S21-S22

Chapter 5: Virus species present in Aster Yellows infected vineyards

5.1 Introduction

Grapevines are susceptible to a myriad of pathogenic agents. According to the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG), up to 75 infectious agents of grapevine have been identified, of which 65 are viruses, five are viroids and eight are phytoplasmas (Martelli 2014). Many important diseases of grapevine result from infection by a complex of pathogens (Prosser *et al.* 2007, Coetzee *et al.* 2010).

Grapevine leafroll disease (GLD), which is considered the most important disease of grapevine in South Africa, has been associated with up to 10 different viruses (Coetzee *et al.* 2010). Grapevine yellows (GY), another important disease of grapevine has been ascribed to infections by viruses, phytoplasma, or even a complex consisting of members from both groups. Margaria *et al.* (2009) screened grapevine plants for the presence of Flavescence doreé (FD) and Bois noir (BN) phytoplasmas, as well as *Grapevine leafroll associated virus-1* (GLRaV-1), *Grapevine leafroll associated virus-3* (GLRaV-3) and *Grapevine virus A* (GVA). The study reported that 30% of the samples were simultaneously infected with phytoplasmas and viruses, whereas 69% of the plants had mixed virus infections. This attested to the high frequency of multiple infections in grapevine, suggesting that viruses and phytoplasmas often co-contribute to the pathology of GY-diseased vines (Margaria *et al.* 2009). It is important to investigate the possible synergism created by co-infection with multiple viruses and phytoplasmas in grapevine plants as well as its effect on GY disease aetiology. Aljanabi *et al.* (2001) explored sugarcane infection with sugarcane yellows phytoplasma (ScYP) and *Sugarcane yellow leaf virus* (ScYLV), both single, as well as co-infections. Both pathogens are implicated in causing yellow leaf syndrome (YLS). The study found that a mixed infection was associated with symptom expression in 85.5% of the sampled plants. When considering samples singly infected with ScYLV, the virus was detected in 71% of symptomless sugarcane plants. This indicates a possible synergism between the phytoplasma and the virus in which co-infection may enhance the severity of the syndrome. It is important to determine whether a similar situation may occur in GY-afflicted grapevines.

Another virus complex afflicting grapevine in South Africa, and possibly affecting GY aetiology, is the Rugose Wood Complex, which results in diseases such as Rupestris stem pitting (RSP), Syrah Decline, Grapevine Kober stem grooving, Shiraz disease, and Grapevine corky bark (Martelli 2014). Additional grapevine virus diseases of local interest include Fanleaf degeneration disease

and Grapevine fleck disease. It is currently unknown what the synergistic properties are of a mixed infection with Aster Yellows (AY) and the viruses causing the abovementioned grapevine diseases. If a synergism exists between AY phytoplasma and grapevine viruses in individual vines, it is possible that this synergism may affect the success rate of recovery phenotype (RP) induction, as well as affecting the permanence of the recovery. The possible implications of co-infections between viruses and phytoplasmas on RP induction in AY-infected vines, was therefore investigated.

5.2 Materials and Methods

5.2.1 RNA extraction

RNA was extracted from samples all AY-symptomatic and AY-symptomatic vines included in this study at two time-points, February 2016 (before coppicing) and February 2017 (after coppicing). Cane phloem scrapings were collected, which were homogenised mechanically in liquid nitrogen with a mortar and pestle. The RNA extractions were performed using a CTAB protocol (Addendum G) that makes use of a lithium chloride RNA precipitation step. Quantity and quality of the RNA was determined using a Nanodrop® ND-2000 spectrophotometer. To check for degradation, 10µL of the extracted RNA was separated on a 2% agarose gel at 100V alongside 4µL GeneRuler 1kb DNA ladder (Thermo Fisher) (Figure 5.1).

5.2.3 Reverse Transcription PCR assay

Two-step reverse transcription (RT) PCR assays were performed for virus detection. The 12 viruses screened for were GLRaV-1, *Grapevine leafroll associated virus-2* (GLRaV-2), GLRaV-3, *Grapevine leafroll associated virus-4-like* (GLRV-4), GVA, *Grapevine virus B* (GVB), *Grapevine virus E* (GVE), *Grapevine virus F* (GVF), *Grapevine rupestris stem-pitting associated virus* (GRSPaV), *Grapevine fanleaf virus* (GFLV) and *Grapevine fleck virus* (GFKV). The reference gene encoding actin served as an internal control. Following RNA extraction, 500ng RNA from each sample was pooled into four symptomatic and four asymptomatic pools. Each pool consisted of RNA extracted from 10 of the plants. Five hundred nanogram of RNA from each pool was subsequently subjected to a primer annealing step with 150ng random primers in a final volume of 13µL. The reaction was incubated at 65°C for five minutes, followed by incubation on ice for two minutes. The RNA was reverse transcribed in a reaction mix containing 1X Kapa RT buffer, 1mM dNTP's, 100 units Kapa RT enzyme and 20 units Kapa Ribolock enzyme. The reaction was incubated at 25°C for 10 minutes, followed by incubation at 50°C for 30 minutes. The PCR reaction mix consisted of 1X Kapa PCR buffer A, 0.4µM of each primer, 0.2mM dNTP's, 2.5µL cDNA, 1 unit Kapa Taq polymerase and 18.3µL milliQ water. Table 5.1 lists the primers and PCR conditions utilised to screen for each of the viruses, as well as for the actin PCR assay. Amplicons were

separated on a 2% agarose gel at 100V. If a pooled sample tested positive for any of the listed viruses, RT-PCR assays were performed for the specific virus for each of the individual plants in the pool. Amplicons generated from individual plant RT-PCR assays were purified from a 2% agarose gel, and sent to the Central Analytical Facilities at Stellenbosch University for Sanger sequencing. To confirm the virus infection status, sequencing data was subjected to BLASTn analysis.

Table 5.1: Table summarising the primers and PCR conditions utilised for every PCR assay. “In-house primer” refers to an unpublished primer previously designed and optimised in the research group for diagnostic purposes.

Virus	Primers	First hold		Cycle X35						Final hold	
		95°C'	5'	Denaturation	Primer Annealing	Extension	72°C	7'			
GLRaV-1	ACTACGGTGRTTCATATTATTA (In-house primer)	95°C	5'	95°C	30"	51°C	20"	72°C	20"	72°C	7'
	WATAATYTCGTGTCCGAA (In-house primer)			95°C	30"	62°C	30"	72°C	30"		
GLRaV-2	RAAACGTTGGGTGGGTTG (In-house primer)	95°C	5'	95°C	30"	53°C	20"	72°C	20"	72°C	7'
	KCCTCCAGAAGCKKCGCAC (In-house primer)			95°C	30"	58°C	30"	72°C	30"		
GLRaV-3	ATGAAYGARAARGTYATGGC (Bester <i>et al.</i> 2014)	95°C	5'	95°C	30"	54°C	20"	72°C	20"	72°C	7'
	CTAAACGCYTGTYCTAG (Bester <i>et al.</i> 2014)			95°C	30"	54°C	20"	72°C	20"		
GLRaV-4-like	ATGGCATTGTCTGCGACTAG (Jooste <i>et al.</i> 2010)	95°C	5'	95°C	30"	54°C	20"	72°C	20"	72°C	7'
	TAAACACAGACTTCGGAGTAGC (Jooste <i>et al.</i> 2010)			95°C	30"	54°C	20"	72°C	20"		
GLRaV-7	GTGCAAGAATCAGCTATCTG (In-house primer)	95°C	5'	95°C	30"	52°C	20"	72°C	20"	72°C	7'
	TCACGTTTAGTTGAATTGGTT (In-house primer)			95°C	30"	52°C	20"	72°C	20"		
GRSPaV	AACVAAAGCWGAGATGGT (In-house primer)	95°C	5'	95°C	30"	51°C	20"	72°C	20"	72°C	7'
	AGTACGGTATTCCAGCGA (In-house primer)			95°C	30"	51°C	20"	72°C	20"		
GVA	GATACCCTAGTTATGCCAGA	95°C	5'	95°C	30"	51°C	20"	72°C	20"	72°C	7'

	(In-house primer) CGATGTACCCGAAGAGAG						
GVB	(In-house primer) CGAGACAATAAGCAAGCA	95°C	30"	53°C	20"	72°C	20"
	(In-house primer) GGGTCCTGAAGTACATGG						
GVE	(In-house primer) ATGATTTGATGCTCAGTCACAGG	95°C	30"	60°C	20"	72°C	20"
	(In-house primer) GGGTTCTTATGGCCTGCTTA						
GVF	(In-house primer) CCGGACAGATTATGARCA	95°C	30"	57°C	20"	72°C	20"
	(In-house primer) GATGCTACTCACCTTAGGTGG						
GFLV	(In-house primer) ATGAGATATTGCTTCAAGAA	95°C	30"	51°C	20"	72°C	20"
	(In-house primer) CTGGAATAGTGGAAAGAGA						
GFKV	(In-house primer) CCATCCAGAAGGACACGATC	95°C	30"	57°C	20"	72°C	20"
	(In-house primer) GAAGTTTGAGGCCGATTT						

5.3 Results

5.3.1 Virus detection February 2016

For this time-point, as well as February 2017, RNA was successfully extracted from all of the samples (Figure 5.1). Of the 12 viruses screened for, only two viruses were detected during February 2016, namely GLRaV-3 and GVE (Figures 5.1 and 5.2). Twenty-eight AY-symptomatic plants tested positive for GLRaV-3, and 19 for GVE. BLASTn similarity searches confirmed the RT-PCR results. Only five AY-symptomatic plants were completely virus-free, whereas all AY-asymptomatic plants were free of the 12 viruses.

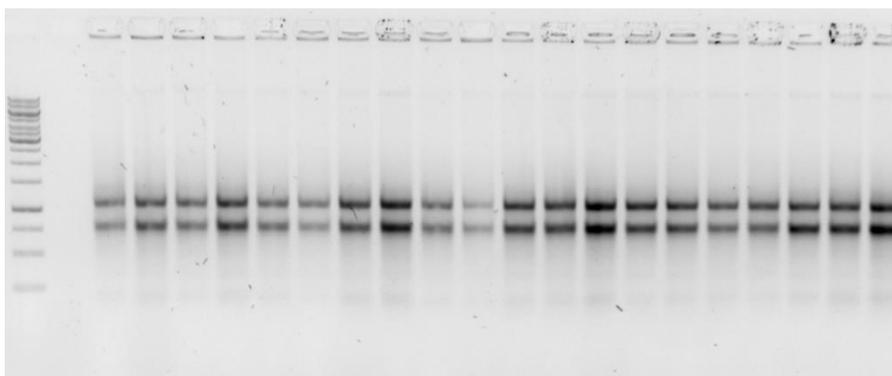


Figure 5.1: Example of a 2% agarose gel of the total RNA extracted from cane phloem scrapings. The first lane is a 1kb with the remainder of the lanes containing the extracted RNA.

5.3.2 Virus detection February 2017

Four viruses were detected during February 2017 (Figures 5.1 and 5.2). GLRaV-3 was detected in both AY-symptomatic and -asymptomatic samples. Fewer samples tested positive for GLRaV-3 compared to the previous time-point, with five AY-symptomatic, uncoppiced, three AY-symptomatic, coppiced, two AY-asymptomatic, uncoppiced and two AY-asymptomatic, coppiced plants (12 in total) testing positive. Figure 5.3 illustrates the change in GLRaV-3 incidence in AY-symptomatic vines over the two timepoints. GLRaV-3 incidence reduced by 64.7% in uncoppiced vines and 83,3% in coppiced vines. GVA was detected in three plants, all belonging to the AY-symptomatic, uncoppiced sample group. GVB was detected in two AY-symptomatic, coppiced plants. GFKV was detected in three AY-asymptomatic, uncoppiced plants. Again, sequencing data confirmed the RT-PCR results. No GVE was detected in any of the plants at this time-point.

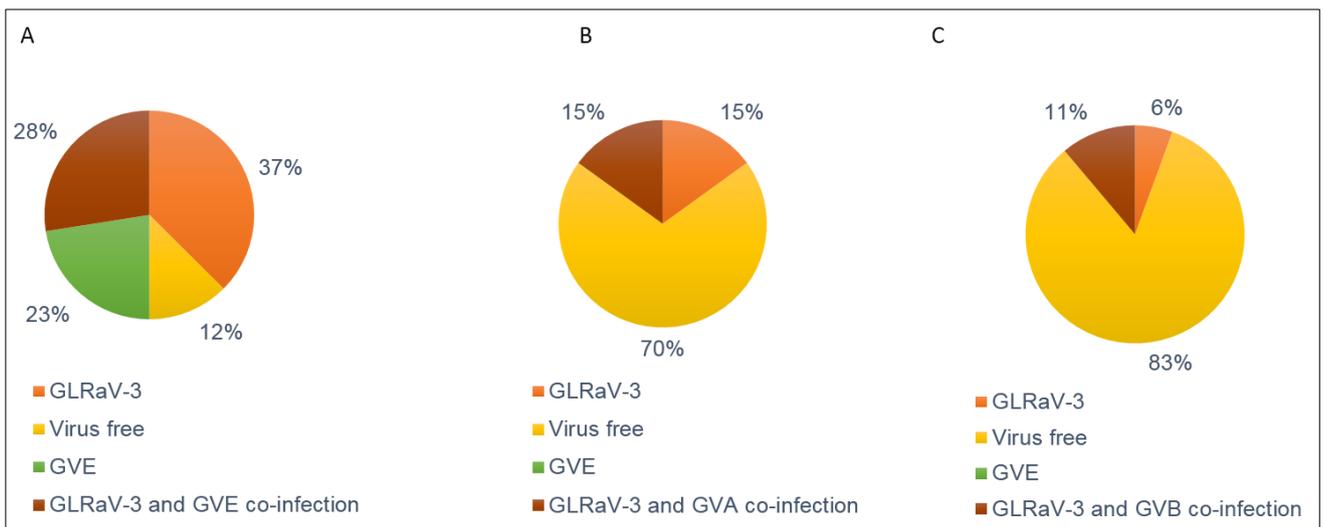


Figure 5.2: Pie charts summarising the virus populations in (A) AY-symptomatic samples February 2016, (B) AY-symptomatic, uncoppiced samples February 2017 and (C) AY-symptomatic, coppiced samples February 2017.

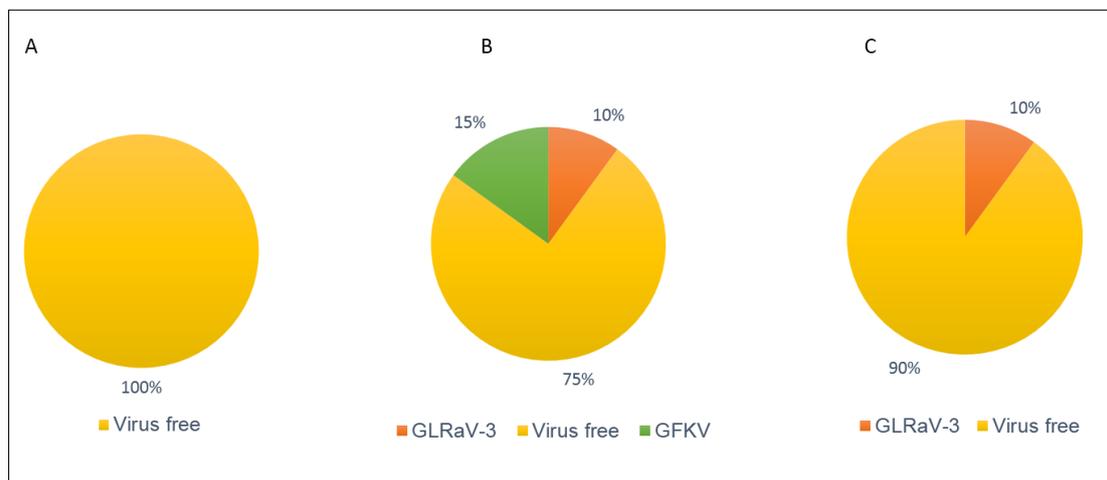


Figure 5.3: Pie charts summarising the virus populations in (A) AY-asymptomatic samples February 2016, (B) AY-asymptomatic, uncoppiced samples February 2017 and (C) AY-asymptomatic, coppiced samples February 2017.

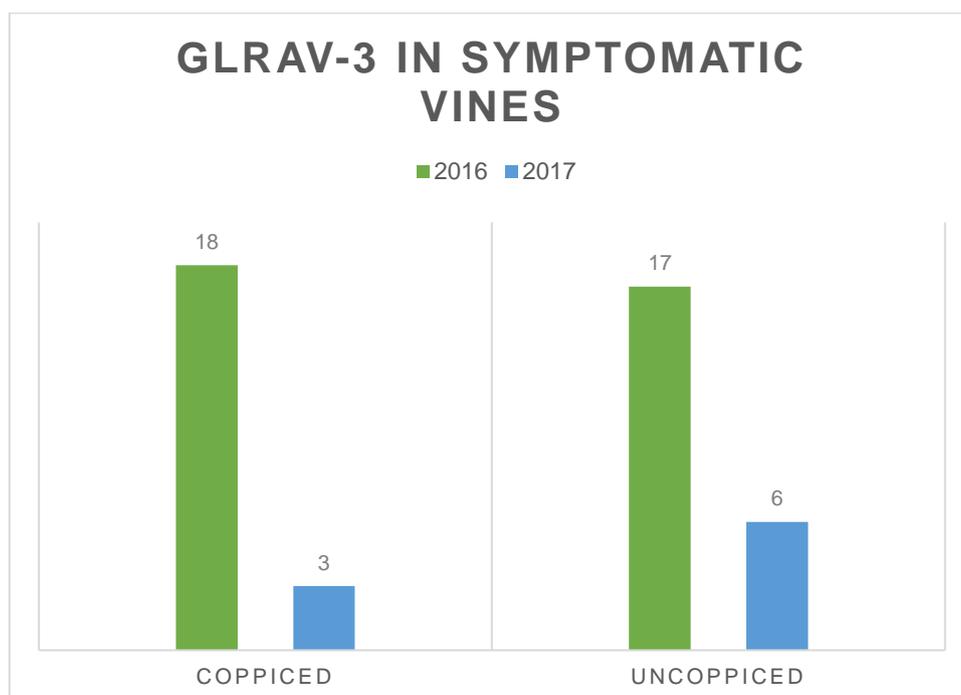


Figure 5.4: Diagram illustrating the decline in GLRaV-3 incidence in AY-symptomatic, coppiced and -uncoppiced vines from February 2016 to February 2017.

5.4 Discussion and Conclusion

Initially a high incidence of GLRaV-3 and GVE was recorded in the AY-symptomatic sample group, whereas no viruses were detected in AY-asymptomatic plants. This may be the result of a weakened line of defence against secondary infections in AY-infected plants. However, in sampling symptomless plants for the AY-asymptomatic experimental group, a sampling bias may have been introduced, as symptoms of grapevine virus infections often overlap with symptoms of AY infections. Therefore, there may be many AY-negative plants in the experimental vineyard with virus infections, but they simply were not included in the study as they were suspected of being AY-positive.

Echoing the AY-infection status of the plants over time, a remission in virus infection was observed in the vineyard. An 83.3% and a 64.7% decline in GLRaV-3 incidence was recorded in the AY-symptomatic, coppiced and –uncoppiced sample groups respectively. Additionally, a 100% remission in GVE incidence is reported. The results were validated by repeated RNA extractions and RT-PCR screening for both time-points. The recorded remission is unusual in virus-infected plants. Previous studies mostly demonstrate an increase in virus incidence in healthy and infected vineyards over time (Cabeleiro and Seruga 2006, Habili and Nutter 1997, Charles *et al.* 2009). The remission which was observed in the Colombar vineyard is therefore uncommon. Though RP-induction in plants with virus infections is a sparsely investigated topic, one possible explanation may be that the same stressor which caused the large scale AY remission in the vineyard induced

a similar remission in GLRaV-3 and GVE. Abiotic stressors, such as extreme temperatures, soil salinity or drought conditions, have been implicated in the decrease in virus infections. Virus-infected plants have previously been subjected to heat treatments as a control measure. Kassanis (1950) reported on the efficacy of storing potato tubers infected with potato leafroll virus in humid atmospheres at 37.5°C. It was found that plants which still retained their germinating power at 25 days would produce virus-free plants once planted, thereby being cured of the initial infection. The major downfall of this approach remained however in that not all of the tubers survived the heat treatment. A similar study by Gifford and Hewitt (1961) induced GFLV recovery in grapevine cv Colombar via heat stress. Plantlets excised from GFLV positive vines were grown at 37.7°C for 60 to 90 days. The combination of shoot tip culture and heat treatment freed the plants of GFLV infection. Mannini *et al.* (1998) also applied heat treatment methods to induce GLRaV-3, GVA, GFLV and GFKV recovery in grapevine cv Grignolino, Nebbiolo and Nebbiolo Michet. It is therefore not inconceivable that the abiotic stress caused by the drought discussed in Chapter 3 may have led to the observed remission in GLRaV-3 and GVE. The possibility that the remission is stress-induced is supported by the fact that a notably higher number of coppiced plants was recovered (83.33% recovery) compared to uncoppiced plants (64.7% recovery).

Three viruses were observed in February 2017 that were not present in February 2016. *Grapevine virus B* (GVB) was detected in two AY-symptomatic, coppiced plants. *Grapevine fleck virus* (GFKV) was detected in three AY-asymptomatic, uncoppiced plants. Interestingly, all plants that remained GLRaV-3 positive at the February 2017 time-point were either co-infected with GVE in February 2016, or were co-infected with GVA or GVB at the February 2017 time-point. It is possible that the synergistic effects of co-infection may have prevented these specific vines from recovering from the initial infection. Synergism here refers to the increase in the reproduction rate of one or both virus species that co-infect a single plant (Syller 2012). Many previous studies have investigated the possible synergism brought on by multiple virus infections in a single plant. Gil-Salas *et al.* (2012) studied cucumber plants infected with *Cucumber vein yellowing virus* (CVYV) and *Cucurbit yellow stunting disorder virus* (CYSDV). Utilising an RT-qPCR approach, these authors showed that the co-infection resulted in an increase in CYSDV titre, although the CVYV titre remained stable (Gil-Salas *et al.* 2012).

A similar study by Quinto-Avila and Martin (2012) explored the possible synergism of *Raspberry bushy dwarf virus* (RBDV) and *Raspberry leaf mottle virus* (RLMV). The study revealed a 400-fold increase in the titre of RBDV when co-infected with RLMV in raspberry plants when compared to single infections.

The same trend seems to be true for virus-infected grapevine plants. Szychowski *et al.* (1995) investigated the synergistic relationship of GFLV and several grapevine viroids in causing vein-banding disease syndrome. The study showed that both symptomatic and asymptomatic plants contained the two viroids *Grapevine yellow speckle viroid* (GYSV-d) and *Hop stunt viroid* (HSVd-g).

Symptomatic vines however exhibited higher viroid titres when co- infected with GFLV. These results therefore point to a synergistic interaction between the viroids and GFLV in vein-banding disease aetiology (Szychowski *et al.* 1995).

It can be argued that the presence of GVE, GVA and GVB in some of the GLRaV-3 infected vines may have resulted in an increase in the GLRaV-3 titre, thereby preventing GLRaV-3 recovery from occurring. Since only one virus may benefit from the synergistic effects of a co-infection (Syller 2012), it is plausible that GVE was eradicated regardless of being part of a co-infection, while only GLRaV-3 titres may have increased. To support this hypothesis, further investigation into the synergism between the observed viruses and the possible induction of a virus RP is required.

In conclusion, there was a discrepancy between the virus infection status of AY-symptomatic and AY-asymptomatic grapevine plants. It is unknown whether this difference is a result of a weakened stress response due to AY-infection, or whether it is caused by a sampling bias which resulted from sampling plants based on their symptom expression. RP-induction in AY-infected vines was successful in spite of the presence of grapevine viruses. This suggests that the synergistic effects of co-infections with viruses and AY phytoplasma does not interfere with RP-induction. An apparent large-scale remission in virus infections was recorded in the vineyard. Though the cause of this remission is unclear, the possibility of RP induction in virus-infected vines justifies further investigation.

5.6 References

Aljanabi, S.M., Y. Parmessur, Y. Moutia, S. Saumtally and A. Dookun, 2001 Further evidence of the association of a phytoplasma and a virus with yellow leaf syndrome in sugarcane. *Plant Pathology* 50:628-636

Bester, R., P.T. Pepler, J.T. Burger and H.J. Maree, 2014 Relative quantitation goes viral: an RT-qPCR assay for a grapevine virus. *Journal of Virological Methods* 210:67-75

Cabaleiro, C. and A. Segura, 2006 Temporal analysis of grapevine leafroll associated virus 3 epidemics. *European Journal of Plant Pathology* 114:441-446

Charles, J.G., K.J. Froud, R. van den Brink and D.J. Allan, 2009 Mealybugs and the spread of grapevine leafroll-associated virus 3 (GLRaV-3) in a New Zealand vineyard. *Australian Plant Pathology* 38(6):576-583

Coetzee, B., M. J. Freeborough, H. J. Maree, J. M. Celton, D. J. G. Rees *et al.*, 2010 Deep sequencing analysis of viruses infecting grapevines: virome of a vineyard. *Virology* 400:157-163

Gifford Jr., E.M. and W.M.B. Hewitt, 1961 The use of heat therapy and in vitro shoot tip culture to eliminate fanleaf virus from the grapevine. *American Journal of Enology and Viticulture* 12:129-130

Gil-Salas, F.M., J. Peters, N. Boonham, I.M. Cuadrado and D. Janssen, 2012 Co-infection with Cucumber vein yellowing virus and Cucurbit yellow stunting disorder virus leading to synergism in cucumber. *Plant Pathology* 61:468-478

Habili, N. and F. W. Nutter Jr., 1997 Temporal and spatial analysis of grapevine leafroll-associated virus 3 in pinot noir grapevines in Australia. *Plant Disease* 81(6):625-628

Jooste, A.E.C., H.J. Maree, D.U. Bellstedt, D.E. Goszczynski, G. Pietersen *et al.*, 2010 Three genetic grapevine leafroll-associated virus 3 variants from South African vineyards show high variability in their 5'UTR. *Archives of Virology* 155(12):1997-2006

Kassanis, B., 1950 Heat inactivation of leaf-roll virus in potato tubers. *Annals of Applied Biology* 37(3):339-341

Mannini, F., V. Gerbi and R. Credi, 1998 Heat-treated v. virus-infected grapevine clones: agronomical and enological modifications. *Acta Horticulturae (ISHS)* 473:155-164

Margarita, P., M. Turina and S. Palmano, 2009 Detection of Flavescence dorée and Bois noir phytoplasmas, Grapevine leafroll associated virus-1 and Grapevine virus A from the same crude extract by reverse transcription-RealTime taqman assays. *Plant Pathology* 58:838-845

Martelli, G. P., 2014 Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology* 96(1-Suppl.)

Prosser, S., D. Goszczynski and B. Meng, 2007 Molecular analysis of double-stranded RNAs reveals complex infection of grapevines with multiple viruses. *Virus Research* 124:151-159

Quito-Avila, D.G. and R.R. Martin, 2012 Real-time RT-PCR for detection of *Raspberry bushy dwarf virus*, *Raspberry leaf mottle virus* and characterizing synergistic interactions in mixed infections. *Journal of Virological Methods* 179(1):38-44

Syller, J., 2012 Facilitative and antagonistic interactions between plant viruses in mixed infections. *Molecular Plant Pathology* 13(2):204-216

Szychowski, J.A., M.V. McKenry, M.A. Walker, J.A. Wolpert, R. Credi *et al.*, 1995 The vein-banding disease syndrome: a synergistic reaction between grapevine viroids and fanleaf virus. *Vitis* 34:229-232

Chapter 6: General conclusion

This research project aimed to validate the efficacy of recovery phenotype (RP) induction in Aster Yellows (AY) phytoplasma infected grapevine as a permanent cure for AY. Additionally, it aimed to identify different genetic variants of AY in the vineyard, and to establish the possible effects of co-infections between AY phytoplasma and grapevine viruses on RP induction. To be able to do this, two grapevine control groups (AY-symptomatic and AY-asymptomatic) had to be identified in a single vineyard.

Forty AY-symptomatic and 40 AY-asymptomatic grapevine plants were identified in a vineyard in Vredendal using a triple-nested PCR assay in February 2016. Twenty plants from both control groups were coppiced just above the graft union to induce an RP. All plants were sampled and screened for AY at two subsequent time-points, namely November 2016 and February 2017. Upon screening the November 2016 samples, a large-scale remission in AY-infection was observed not only in AY-symptomatic, coppiced samples (only five AY-symptomatic plants), but also in AY-symptomatic, uncoppiced samples (only two AY-symptomatic plants). These results could not be correlated to AY-symptoms in the plants, as symptoms are not apparent at that time in the grapevine growing season. All plants in the AY-asymptomatic control groups still tested AY-negative. The observed remission persisted into February 2017, where all AY-symptomatic, uncoppiced plants tested AY-negative, with four AY-symptomatic, coppiced plants testing positive. It was possible to correlate diagnostics results to symptom expression; AY-symptoms had all but disappeared from the vineyard except for three of the four plants which screened positive. Again, all AY-asymptomatic plants tested negative. Although it is rare for grapevine plants to experience an AY-remission in the absence of physiological stresses, it is not unheard of. Though most grapevine cultivars are persistently infected, some cultivars need to be re-infected by their insect vector every season. Since the insecticide Imidacloprid was administered to the vineyard in February 2016, it is possible that a lack of reinfection allowed for the observed RP in the vineyard regardless of coppicing status. It is also important to consider the abiotic stress experienced by the vines during the current drought throughout the Western Cape. The current drought, coupled to water restrictions leading to irrigation limitations ranging between 30% and 100%, may have caused sufficient abiotic stress in the AY-infected vines to induce an RP.

The genetic diversity of AY in the vineyard was investigated and found to be limited to only one genetic variant. Four reference genes, namely *secY*, *amp*, *rp* and *groEL* were amplified from AY-symptomatic phloem tissue sampled in February 2016. The generated amplicons were then subjected to RFLP analysis. Representative amplicons for each of these genes, as well as the 16s

rDNA gene fragment amplified by the triple-nested PCR assay was subjected to Sanger sequencing. Since no AY phytoplasma genetic diversity was observed in the experimental vineyard, it is concluded that the observed recovery is a true recovery and not a false negative diagnosis.

Finally, how AY-infections in grapevine and RP induction may be influenced by the presence of grapevine viruses were investigated. Twelve viruses were screened for at two time-points throughout this study, namely February 2016 (pre-coppicing) and February 2017 (post-coppicing). For February 2016 samples, *Grapevine leafroll associated virus-3* (GLRaV-3) and *Grapevine virus E* (GVE) were identified in the AY-symptomatic samples, either as single infections or as co-infections. Only five AY-symptomatic plants were virus free, whereas all AY-asymptomatic plants were virus free. The large difference in viral communities of AY-symptomatic and AY-asymptomatic sample groups may indicate a weakened immune system in AY-infected plants. Another likely explanation may be that this resulted from a sampling bias, as many symptoms of virus infections in grapevines overlap with AY-infection symptoms. Similar to the AY-infection status, a large-scale recovery of GLRaV-3- and GVE-infection was observed between February 2016 and February 2017. GLRaV-3 incidence decreased with 64.7% and 83.3% in uncoppiced and coppiced vines respectively, with GVE disappearing completely from the vineyard. It is suspected that this recovery may have been induced by abiotic- and physiological stress, as there is a much higher rate of recovery in coppiced vines. Additionally, three viruses that were not present in February 2016 were detected in February 2017. *Grapevine virus A* (GVA) was detected in three plants, all belonging to the AY-symptomatic, uncoppiced experimental group. *Grapevine virus B* (GVB) was detected in two AY-symptomatic, coppiced plants. *Grapevine fleck virus* (GFKV) was detected in three AY-asymptomatic, uncoppiced plants. Interestingly, in every case that GLRaV-3 was still present at the February 2017 time-point, the plant had either previously been infected by both GLRaV-3 and GVE, or was co-infected with GLRaV-3 and GVA or GVB at the second time-point. It is suspected that synergistic interactions between these viruses may have benefited GLRaV-3 in a way that allowed the infection to persist. As AY-recovery was induced regardless of the presence of viruses in the plant, it is suspected that infection by viruses does not hamper RP induction in AY-infected grapevines.

This project contributes to what is understood about RP induction in AY-infected grapevine. It speculates on the possibility of naturally occurring abiotic stresses being able to induce an RP in infected vines. The possibility of grapevine cv. Colombar not being persistently infected is also suggested, though further research is warranted to verify this. Additionally, this study proves that a single genetic variant of AY was present in the vineyard, thereby eliminating the possibility of the observed RP being a false negative diagnosis. Finally, the study reports evidence of a remission in virus infections in grapevine plants that may have been induced by physiological and abiotic

stresses, indicating the possibility for RP induction to treat grapevine viral infections. Further research is suggested on this topic is suggested to determine whether it is a recurring phenomenon. The study also demonstrated that virus infections do not prevent RP induction in AY-infected grapevines.

Addendum A

DNA Extraction Protocol

- Grind 100mg material in liquid nitrogen and transfer to a 2mL eppi, add 25mg PVP-10 and shake to mix
- Let liquid nitrogen evaporate before adding 1mL preheated CTAB extraction buffer mixed with 3% (30 μ L) β -mercaptoethanol and incubate in a water bath for 15 minutes @ 60°C
- Centrifuge at 13200 rpm for 10 minutes (4°C)
- Retain aqueous phase (\pm 800 μ L) into a new 2mL eppi
- Add 5 μ L RNase A (Stock 10mg/uL)
- Invert and incubate in a water bath/oven for 15min at 37°C
- Add equal volume cold C:I (24:1) and invert
- Centrifuge at 13200 rpm for 10 minutes (4°C)
- Retain aqueous phase and transfer to a new eppi
- Add 0.8 volume cold Isopropanol and invert
- Centrifuge at 13200 rpm for 5 minutes (4°C), remove supernatant – use pipette
- Wash with 500 μ L cold 70% ethanol – centrifuge at 13200 rpm for 5 minutes
- Remove the 70% ethanol with pipette and spin down for another 2 minutes and pipette the ethanol that is left – repeat step if necessary
- Allow the pellet to air dry for 10 minutes
- Resuspend in \pm 25-40 μ L H₂O (volume dependent on pellet size and concentration) – store in fridge until pellet is dissolved

Addendum B

Aster Yellows 16S rDNA Partial Gene Sequence

TTTGTAACAGCCATTGTATCACGTTTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTC
GTCCCCACCTTCCTCCARTTTATCACTGGCAGTCTTGCTAAAGTCCCCACCATTACGTGCTGG
TAACTAACAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGA
CGACAACCATGCACCACCTGTGCAACTGATAACCTCCACTGTGTTTCTACAGCTTTGCAGAAG
CATGTCAAGACCTGGTGAGGTTTTTCGGGTACCTTCGAATTAACAACATGATCCACCGCTTG
TGCGGAGTCCCGTCAATTCTTTAAGTTTCATACTTGCGTACGTACTACTCAGGCGGAGTACT
TAATGTGTTAACTTCAACACTGGTTTTACCCAACGTTTAGTACTCATCGTTTACGGCGTGGACT
ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGCGTCAGTAAAGACCCAG
CAAGCCGCCTTCGCTACTGGTGTTCCCTCCATATATTTACGCATTTTACCACTACACATGGAATT
CCACTTGCCTCTATCTTACTCTAGCTAAACAGTTTTTATAGCATCACAATGTTGAGCATTGCAC
TTAGACCATAAACTTATTTAACCGCCTACGCACCCTTACGCCCAATAATTCGGATAACGCTT
GCCCCCTATGTATTACCGCGGCTGCTGGCACATAGTTAGCCGGGGCTTATTCATTAGGTACC
GTCAGAATGATTTTTCCATCATTTATTCTTCCCTAATAAAAGAACTTTACGTACCGAAATACTTC
ATCGTTCACGCGGCGTTGCTCGGTGAGAGTTTCCTCCATTGCCGAAAATTCCTACTGCTGCC
TCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGTTCAACCTCTCAGCCCGGCT
ACACATCATAGTCTTGGTAGGCCTTTACCCCACCAAC

Addendum C

Aster Yellows *rp* Partial Gene Sequence

GTCTGTTAGGAGTGTTAGAAGATTGCGAAGTAAACGGTTTTCTAGTGTCTAGAATGGTTTGTC
CTGGTAAAACCTTCACCGTGGAATCCATACTTTAATTCCTAAAACCTCCATAAGTAGTGTGAGC
TTCAAGAGCAGCGTAATCGATGTCTGCTCTTAGAGTGTGTAGAGGAACTCTGCCTTCGGCATG
TCCTTCGCTACGAGCTATTTACGACCCGCCAAACGACCAGAAATTAAGTTTTTACTCCTTTG
GCACCAGCTTTTAGGGCTTTTTGGATTGCCATTTTTGAACACGGCGGAAAAACATACGATTTT
CTAGTTGTTACGCCATATTTGAGCAATTAATAAAGCAATTTTATCAGAGTTTTTAACTTCTAAC
ACGTTAAGATTAACGTCTTTTTGGGTAAGTTCTTTGAGTTTGTCAACTAATTTGTTGCGTGTATC
GCCATCTTTCCAATAATAACGCCTGGTTTAGCGGTGTGGACAGAAATAGTGATACGGTTTTTA
TTTTTTCTTTAGGCGTTCAATGTCAATTTGACTGATAGCACTTTTTTTAGTAAAATTATTGATT
AGTTTACGAATTAATAAATCTTCTTTAATTAATTAAGGAATTTCTTTATCATTAAACACACCATTGA
GATTCCCAAGTTCTAATAATGCCTAATCTTAAGCCGTTAGGATTAGTTTTTTGACCCACTTTGTT
CTTCCTCCTTTGATGTTTGCAAGTTTGTGCTAGAAGTTATTACTAAAGTAATGTGGCTGGTTYT
TTTTTTAATCATATCACCAGAACCTTTAGCTCTTGAAACATACGTTTTTAAACGCAAACCTTCGT
TGACAAAACTTCTTTAACATAAAGTTGTTTCGCGGTTTAATTTTAAATTATTAACAGCATTGGAA
ACAGCACTGTTTTAAAGTTTTAAATAACGGGAGCAGCTACTTTAGGGGTAAAAGTTAAAATGG
CTTGAGCTTGTGCAATATTTTTCTCGAATTAATCAACAATAAACGTGCTTTTCGAGGGGC
GATTGAACTTTTCTAGCAATCGCTTTGGCGTTTTTGGTTTCCATAGTTATTCCTTCCCATTA

Addendum D

Aster Yellows *groEL* Partial Gene Sequence

TGACTTTAGGACCTAAAGGACGTAATGTTATTTTAGAAAAAGCCWATGATTCACCTGCTATTGT
AAATGATGGTGTCTTATTGSTAAAGAAATTGAATTAATAAATCCTTATCAAAATATGGGAGCAA
AGTTAGTATATGAAGTAGCTTCCAAAACCTAACGATAAAGCAGGAGATGGAACAACCTACAGCAA
CTGTTTTGGCACAAAGTATGATTCATCGTGGGTTTGGATGCAATTGATGCAGGAGCTAATCCTG
TTTTAGTAAAAGAAGGAATTGAGTTAGCAGCATTAAACAGTTGCCAAAAAATTTTTAGTTAAATCT
AAAAAAGTAGACGCCCAAGAAGATATTCAAAATGTGGCTGCTGTTTCATCAGGTAGTCAAGAA
ATTGGTAAAATCATTGCCCAAGCGATGCAAAAAGTAGGAAAAGATGGAGTTATTAATGTTGAT
GAATCCAAAGGTTTTGAAACAGAATTAGAAGTTGTTGAAGGATTGCAGTACGATAAAGGATAT
GCTTCTCCTTATTTGTCTCTGATAGAGAAAGTATGACAGTACAGTTAGAAAATGCGTTAGTTT
TAGTAACTGATCATAAAATTAGTACTGTGCAAGAAATTGTACCTATTTTGGGAAGAAGTAGTAAA
AGCATCTAGACCTTTATTAATTGTAGCTGAAAGCTGTGGGAAAATGAAAGTTTTAGGGGTTTTG
GTAGCTAATAMAATTAAGAGGAACTTTTAATGTAGTTGTAACCTAATGCTCCTGGTTTTGGTGAT
AATCAAAAAGAAATGTTACAAGATATTGCAGTACTTACAAAAGCTAATTTTGTCTTAAAGAACT
TAATATGAAATTAGCAGATTTAAAAATGGATGATTTAGGA

Addendum E

Aster Yellows *amp* Partial Gene Sequence

AAAATCAAAAACTCAAAAATCTTTAGTTGCTAAAGTTTTAGTTTTATTTGCTGCCGTTGCTTTA
ATGTTTGTTGGCGTTCAAGTTTTTGCTGATGATAAACTAGATTTAAGCACTTTAGAATGTAAAG
ACGCTCTTGAACCTTACTGCTACTGATGCTGCTGATGCAGAAAAAGTTGTTAAACAATGGAAAG
TTCAAAACACTTCATTGAATGCAAAAGTAACAAAAGATTCTGTAAAAGTATTGGTTTCTACTGAT
AGTAAACAGTAACAGTTTCACCTGCAGATGGTGATGCTGGAAAACTTTATCAGGCGCAAAA
GTATTAAATTTAGTAGGCGTATGTGAATTAGATAAATTAACCTTAGGCAAAGACAAAAACTTAC
ACTTACAGTTAAAAATGGCAAAGTAGATGCAGAAGCTGGTTTAAAAGCTTTAAAAGAAGCTGG
AGCTAAAGTTCCTGCAACCGTAAACAAAGACGACGTAACCTTTCACAGTTGGTAAAGACGACGA
TGCTAATAAAGTTACTGTTAAAGCTGTAGATGGTAAAGATACTGTTTCAGGACAAGTTGTCTTT
GAATTTAATGTAGCTAAAACACCTTGGTACAAAAC

Addendum F

Aster Yellows *secY* Partial Gene Sequence

TGGCCTCTTCCTTTTATTAATACTAAATCCCTTGATTTGTCCAAACTTTTTGGGGTTTTTTCCAT
AAATGCTGGTACTCTTTTTGGATTGGGAATCACTCCTTACATCACTGCTTCCATTGTAGTGCAA
TTTTTGCAAAAACCTTCTTCCTATTTGTCGCGAATGGAAAGACCAAGGACAAATGGGCAAACGC
AACTTAATCTTTTAACACGTAGTCTTGCCTTATTGTTTGCTTTTGGGCAATCTTTTGCTTTTTT
GAACAGTTATTCAAACCTTTTTGTCACATCAATAAGTACAAGCCAACCTGTTTTTGTTAGCTTTAA
TTGCTACTGCAGGAGTTGCTATTTTAATTTGGTTTGCTGACCTTATCAATTCCAAAGGTATTGG
AAACGGGACTTCTATTTTAATTGTTGTTTCGATGAGCCACAGTCTAATTAATCTATTTGTAAATC
TGAACGAATCATATTTATCTCAAAAAAATTTTTAACTTTGAAAACCTTTAATTTTGCATGTATTG
TTCTTTTACTTCTCTTATTTTTAATTTTTACTGTAGTTGTGCAAATAACATCTTTAAAAATACCTAT
CAATTATGCGCGCAATCAAGTGCAAGGAAAAAGCTACATTCCATTAATAAATTAATAGTGCAGG
AGTTATGCCAGTTATTTTGGCATCTGCTTTATTGCAACCTTTCCAGATGTTATCAGGAGTTATT
GGGAATACAAAATTTACAGAAGTAGTAGATTTTTTTGCCAAAACCTTTTCTGGAACCAAAA
TTAACTTTTTTTGCCATAGGCTTTTTAGTCTTGTTAGTAATTGTTTTTTCTTTCTTTTCTGCTTTTAT
GAATGTCAATCCTGAAGATATTTTACAGAACATTTATCCAAACAAGATGCCTATATTGCAGGTTTA
AGACCAGGTGAACAACTACTCGTTATTTAGCTAATACCTTATTTAAAATCACCGTTTTAGGAA
CTGTTTTTATTGCTGCTCTTGTTGTAACACCTATTCTTATGGAACATTTTTTLAGGTTTGAAAGAT
ATGAAATTAGGAGGAACCAGTTTGCTTATTATTGTTAGTGTAGCCCTTGAAAACCTATCCAACGCA
TCAAAGCTACTGCCAACAAAAAGAATATCAAAAATTATTTAATTAAGCAAACAAGACAATATG
ATACTAATATTATTAGGACCGCCCGGAATTGGTAAA

Addendum F

Total RNA Extraction Protocol

- Add 3% β -mercaptoethanol to 2% CTAB buffer before use and heat to 65°C
- Homogenize 200mg plant material in liquid nitrogen using a mortar and pestle
- Add 1.2mL of preheated CTAB/ β -mercaptoethanol mixture to the homogenized plant material
- Vortex and incubate at 65°C for 30 minutes
- Work on ice from this point
- Centrifuge at maximum rcf (4°C) for 10 minutes and transfer the supernatant to a new tube
- Add an equal volume (1.2mL) of Chloroform:Isoamylalcohol (C:I) (premixed in a 24:1 ratio) to the supernatant, vortex and spin down for 10 minutes at maximum rcf and transfer the supernatant to a new tube
- Repeat the C:I step
- Add LiCl (8M) to a final concentration of 2M
- Incubate overnight at 4°C
- Centrifuge at maximum rcf (4°C) for 60 minutes and remove the supernatant
- Add 500 μ l 70% ethanol to the pellet and centrifuge again for 10 minutes at 4°C
- Remove all ethanol
- Quick spin to collect all remaining ethanol, and carefully pipette it out
- Air-dry the pellet for at least 15 min, until no ethanol is visible
- Resuspend each pellet in 50 to 100 μ L dH₂O (MilliQ)
- Pool the 3 eppi's per sample (final volume +/- 100 μ l per sample)