The efficacy of the antimicrobial peptides D4E1, VvAMP-1 and Snakin1 against the grapevine pathogen aster yellows phytoplasma

by

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Declaration

By submitting this thesis, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

N Spinas Date

Stellenbosch University

2013

Abstract

Phytoplasma diseases have caused disastrous effects in vineyards around the world. Therefore, the recent discovery of phytoplasmas in South African vineyards could be highly detrimental to the local wine industry. Antimicrobial peptides (AMPs) are small molecules expressed by almost all organisms as part of their non-specific defence system. These peptides can offer protection against a wide variety of bacterial and fungal pathogens in plants. Due to the fact that phytoplasmas lack an outer membrane and cell wall, AMPs are considered to be perfect candidates to confer resistance to this phytopathogen. The current study intends to explore the *in planta* activity of AMPs against the grapevine pathogen aster yellows phytoplasma (AYp) through *Agrobacterium*-mediated transient expression.

The AMPs, Vv-AMP1, D4E1 and Snakin1 (isolated from potato and grapevine) were selected to be tested for their *in planta* effect against AYp. Cauliflower mosaic virus 35S expression vectors containing four different AMP-encoding sequences were therefore constructed. As an alternative method to observe the effect Vv-AMP1 might have on AYp *in planta*, grafting of Vv-AMP1 transgenic *Vitis vinifera* cv 'Sultana' plant material was used. To allow assumptions about AMP efficacy in this transient expression system, attempts were made to describe the spatial distribution and pathogen titre of AYp in *V. vinifera* cv 'Chardonnay' material. Additionally, transmission experiments were carried out to infect *Catharanthus roseus* and *Nicotiana benthamiana* with AYp through the insect vector *Mgenia fuscovaria*. Material was screened for AYp infection by a nested-PCR procedure using universal primers described by Gundersen and Lee (1996). For quantification of AYp infection, a semi-quantitative real-time PCR (qPCR) protocol was optimized, using the SYBR Green-based system.

In total, 86 *V. vinifera* cv 'Chardonnay' plantlets were screened for AYp infection two-, three-, four-, seven- and eleven weeks after introduction into *in vitro* conditions. No AYp infection could however be detected and plantlets displayed a 'recovery phenotype'. To examine the distribution of AYp in canes of an infected *V. vinifera* cv 'Chardonnay' plant, leaf and the corresponding node material from five canes were screened by a nested-PCR procedure. It can be concluded, that AYp was found predominantly in the nodes when compared to leaf material in the late season of the year. It is also highly unlikely for leaf

material to show phytoplasma infection, if in the corresponding node no AYp could be detected. As AYp-infected grapevine material could not be maintained *in vitro*, the effect of VvAMP-1 transgenic grapevine against AYp could not be tested. Infection of *C. roseus* and *N. benthamiana* plants with AYp was successfully achieved by insect vector transmission experiments. Transient expression assays were conducted on AYp-infected *N. benthamiana* material. Quantification of phytoplasma in this material showed a decrease of AYp in both the AMP treatment groups and the control groups.

This study optimized a qPCR procedure to detect and quantify AYp in infected plant material. The *Agrobacterium*-mediated transient expression system used during this study was not reliable, as no significant effect of the AMPs on AYp titre could be observed. This study showed, that AYp cannot be established and maintained in *in vitro* cultured *V. vinifera* cv 'Chardonnay' material, and tissue culture itself might therefore be a way to eradicate AYp in this cultivar. To our knowledge, this study is the first to report on the spatial distribution of AYp in canes of an infected *V. vinifera* cv 'Chardonnay' vine.

Opsomming

Fitoplasma-siektes veroorsaak ramspoedige gevolge in wingerde oor die hele wêreld. Dus kan die onlangse ontdekking van fitoplasmas in Suid-Afrikaanse wingerde baie nadelige gevolge vir die plaaslike wynbedryf beteken. Antimikrobiese peptiede (AMPe) is klein molekules wat in amper alle organismes as deel van hulle nie-spesifieke verdedigingsstelsel tot uitdruk kom. Hierdie peptiede kan beskerming bied teen ŉ wye verskeidenheid van bakteriële en swampatogene in plante. As gevolg van die feit dat fitoplasmas geen selmembraan of selwand het nie, word AMPe oorweeg as middel om weerstand te verleen teen hierdie fitopatogene. Die huidige studie beoog om die *in planta* aktiwiteit an AMPe teen die wingerd-patogeen aster vergeling fitoplasma (AYp) deur middel van Agrobacterium-bemiddelde tydelike uitdrukkingsisteme, te ondersoek.

Die AMPe, Vv-AMP1, D4E1 en Snakin1 (geïsoleer vanuit aartappel en wingerd) is gekies om getoets te word vir hul *in planta* effek teen AYp. Blomkoolmosaïek-virus 35S uitdrukkingsvektore met vier verskillende AMP-koderende volgordes is dus ontwikkel. As ŉ alternatiewe metode om die moontlike effek van Vv-AMP1 op AYp *in planta* te toets, is enting van die Vv-AMP1 transgeniese *Vitis vinifera* cv ‘Sultana’ plantmateriaal gedoen. Om hierdie AMPe se doeltreffenheid in hierdie tydelike uitdrukkingsvektore te toets, is pogings aangewend om die ruimtelike verspreiding en patogeenkonsentrasie van AYp in *V. vinifera* cv ‘Chardonnay’ te beskryf. Verder is transmissie-eksperimente uitgevoer om *Catharanthus roseus* en *Nicotania benthamiana* met AYp dmv die insekvektor, *Mgenia fuscovaria,* te infekteer. Plantmateriaal is getoets vir AYp in ŉ PCR met universele inleiers soos beskyf deur Grundersen en Lee (1996). Vir kwantifisering van die AYp infeksie, is ‘n semi-kwantitatiewe qPCR protokol geoptimiseer, met behulp van die SYBR Groen-gebaseerde stelsel. In totaal is 86 Chardonnay plantjies getoets vir AYp infeksie – twee-, drie-, vier-, sewe- en elf weke na die blootstelling aan die *in vitro* kondisies. Geen AYp infeksie kon egter opgespoor word nie en die plante het ‘n “herstel-fenotipe” vertoon.

Om die verspreiding van AYp in die arms van ŉ geïnfekteerde Chardonnay plant te ondersoek, is blare en ooreenstemmende internode van vyf lote getoets met PCR. Daar kon afgelei word dat, laat in die seisoen, AYp hoofsaaklik in die internode gevind word. In slegs enkele gevalle is fitoplasma-infeksies in blaarmateriaal, waarvan die ooreenstemmende internode negatief getoets het, gevind. Aangesien die AYp-geïnfekteerde wingerdmateriaal nie *in vitro* gekweek kon word nie, kon die effek van VvAMP-1 transgeniese wingerd nie teen AYp getoets word nie. AYp infeksies van *C. roseus* en *N. benthamiana* plante deur transmissie eksperimente met ŉ insekvektor was suksesvol. Toetse met tydelike uitdrukkingsvektore is uitgevoer op die AYp-geïnfekteerde *N. benthamiana* materiaal. Kwantifisering van fitoplasma in hierdie materiaal het die afname van AYp in beide die AMP behandelingsgroep en die kontrole groep getoon.

Hierdie studie het ŉ qPCR-toets geoptimiseer om geïnfekteerde plantmateriaal met AYp op te spoor en dit te kwantifiseer. Die *Agrobacterium*-bemiddelde tydelike uitdrukingsvektore wat in hierdie studie gebruik is, het geen beduidende effek van die AMPe op AYp konsentrasie getoon nie. Hierdie studie het bewys dat AYp nie instand gehou kan word deur *in vitro* kweking van Chardonnay materiaal nie, en dat weefselkultuur dus ŉ manier kan wees om AYp in hierdie kultivar te elimineer. Sover ons kennis strek, is hierdie studie die eerste om die ruimtelike verspreiding van AYp in arms van geïnfekteerde wingerdstokke, te rapporteer.

Abbreviations

bp	base pair
cm	centimetre
cv	cultivar
h	hour
kb	kilo bases
kDa	kilo Dalton
kPa	kilo Pascal
kV	kilo Volt
fg	femtogram
µF	microfarad
µl	microliter
µM	micromolar
min	minute
ng	nanogram
Ω	ohm
sec	second
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
GUS	β-glucuronidase
IWBT	Institute for Wine Biotechnology
KCl	Potassium chloride
KH_2PO_4	Potassium di-hydrogen phosphate
$MgCl_2$	Magnesium chloride
MES	2-(N-morpholino)ethanesulfonic acid

MS	Murashige and Skoog
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Monosodium phosphate
Na_2EDTA	Diaminetetraacetic acid
OD	Optical density
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
SA	South Africa
SDS	Sodium dodecyl sulphate
SN1	Snakin1
Tris-HCL	Tris-hydrochloride
UV	Ultra-violet
qPCR	quantitative real-time PCR
V	Volts
Vv-AMP1	*Vitis vinifera*-antimicrobial peptide 1
W	Watt
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronic acid

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Introduction

1.1 Background and motivation for this study

The importance of grapevine as an agricultural commodity in SA cannot be over emphasized. More than 115 000 hectares of land in SA are planted to grapevine and the South African wine industry contributed 417.5 million gross litres of wine for sale to private and producer cellars in 2011, with an increase of 23.9% estimated for 2012 (http://www.sawis.co.za). Phytoplasma diseases are known to have caused disastrous effects in vineyards in European countries, resulting in significant reductions in fruit yield and wine quality (Lee *et al.*, 2000). Therefore, the recent discovery of phytoplasma infections in SA could be highly problematic to the South African wine industry. It is therefore of high importance to find an approach to control this disease. A long term approach to control this pathogen through the development of resistance is desirable and should be investigated and implemented. The current study intends to explore an approach to induce resistance against the grapevine pathogen aster yellows phytoplasma (AYp), to control this devastating new disease.

Scientists have started employing short peptides, known as antimicrobial peptides (AMPs), to combat plant pathogens. These small molecules of less than 50 amino acids in length are expressed by almost all organisms as part of their non-specific defence system (Montesinos, 2007). Whilst the ultimate aim would be to express AMPs in grapevine, the development of transgenic grapevine is time-consuming and therefore the pre-screening of potential AMPs is necessary. *In vitro* pre-screening of AMP activity is valuable, but is impossible for phytoplasmas since these pathogens cannot be cultured *in vitro*. These limitations can be overcome by using transient expression systems to determine the *in planta* activity of AMPs against phytoplasma pathogens.

In this study, a transient expression system described by Visser *et al.* (2012) was used to test the *in planta* activity of four AMPs against the grapevine pathogen AYp. This system can be used as an *in planta* pre-selection for AMP efficacy and can be performed in a relatively short time period, for a large number of AMPs. To allow assumptions about AMP efficacy in this transient expression system, attempts were made to describe the spatial distribution and pathogen titre of AYp in *Vitis vinifera* cv ‘Chardonnay’ material.

1.2 Project proposal

This study aimed to test the *in planta* activity of AMPs against the grapevine pathogen AYp through a transient expression system.

To achieve the proposed aim, it was necessary to reach the following objectives:

- Test the expression of foreign genes in grapevine using *Agrobacterium*-mediated transient expression vectors containing the GUS control gene
- Construct *Agrobacterium*-mediated transient expression vectors containing AMP genes and test for the expression of these genes
- Identify and establish *in vitro* phytoplasma-infected plants
- Conduct transmission experiments using the vector *Mgenia fuscovaria* on *Nicotiana benthamiana* and *Catharanthus roseus*
- Infiltrate phytoplasma-infected plants with the AMP expression constructs
- Graft phytoplasma-infected plants onto existing Vv-AMP1 transgenic plants
- Test the effects of the AMPs by measuring microbial titres and disease development
- Determine the distribution of AYp in the canes of an infected grapevine plant

1.3 References

Lee IM, Davis RE, Gundersen-Rindal DE (2000) Phytoplasma: phytopathogenic mollicutes. Annual Review of Microbiology 54: 221-255

Montesinos E (2007) Antimicrobial peptides and plant disease control. FEMS Microbiology Letter 270 (1): 1-11

Visser M, Stephan D, Jaynes JM, Burger JT (2012) A transient expression assay for the *in planta* efficacy screening of an antimicrobial peptide against grapevine bacterial pathogens. Letters in Applied Microbiology 54 (6): 543-551

Internet resources

South African Wine Industry Information and Systems (SAWIS): http://www.sawis.co.za

Chapter 2

Literature review

2.1 Introduction

About 1 000 years ago the Chinese were great admirers of the obscure bacteria phytoplasma. They found the symptoms in peonies so attractive, that the Song Dynasty's imperial court was given a special annual tribute consisting of these infected flowers (Strauss, 2009). Most of the effects displayed by these microbes, are however far from pretty. In the European countries alone, phytoplasma infections have caused devastating yield losses in several fruit crops. During only one phytoplasma outbreak in apple trees in 2001, Germany lost €25 million, while Italy made a loss of €100 million (Strauss, 2009). This bacterium is however not only causing effects in the European countries. In Africa and the Caribbean, infected palm trees are causing people to have insufficient nourishment and building materials (Maramorosch, 2011; Strauss, 2009). In grapevine, phytoplasmas are known to have caused disastrous effects in vineyards in European countries, resulting in significant reductions in fruit yield and wine quality (Lee *et al.*, 2000). In 2006, Botti and Bertaccini discovered the first ever mixed phytoplasma infection in South African vineyards. The South African wine industry contributed R2.6 billion to the country's gross domestic product in 2008 and employs over 275 000 people (http://www.sawis.co.za). Due to the importance of the grapevine industry on the South African economy, it is crucial to combat all pathogens including the recently discovered phytoplasma. This chapter will give some background information on phytoplasmas and antimicrobial peptides, which are molecules used for inducing pathogen resistance in plants.

2.2 Phytoplasmas

2.2.1 The discovery of phytoplasmas

In 1926, Kunkel described a disease that destroys crops, orchards and ornamental plants. For several reasons, scientists believed the disease was caused by a virus or viruses, as the pathogen could not be cultured *in vitro*, was transmitted by insects and displayed symptoms similar to a virus infection (Doi *et al.*, 1967). For the next 40 years scientists examined the disease but were unsuccessful in finding a virus.

When Maramorosch (1958) injected insects with the antibiotic tetracycline and the infectious agent phytoplasma, the injected insects did not transmit aster yellows to the plants. Knowing that antibiotics had no effect on viruses, he concluded that the high temperatures in the greenhouse, rather than the drug prevented pathogen transmission. In 1967, Doi and colleagues discovered structures resembling those of mycoplasmas and termed the causal agent mycoplasma-like organisms (MLOs). Mycoplasmas are small groups of typically parasitic bacteria that lack cell walls and can cause diseases in plants, humans and animals. In 1994, this mycoplasma-like organism was given the name phytoplasma by the Phytoplasma Working Team at the 10th Congress of the International Organization of Mycoplasmology.

2.2.2 Classification of phytoplasmas

Phytoplasmas diverged from gram-positive ancestors and belong to the class Mollicutes. They are petite, cell wall-less pleiomorphic bacteria of approximately 500nm in diameter (Lee *et al.,* 1998). Even though phytoplasmas have a smaller genome compared to most bacteria, they manage a very complex life cycle that involves two noticeably different environments – plants and insects. Early diagnostic approaches distinguished phytoplasma infections from other grapevine diseases, by observing the main symptoms that phytoplasma diseases express in plants (Gasparich, 2009). As symptom expression is quite uniform among different phytoplasma species however, symptomatology cannot be used to distinguish one phytoplasma species from another. Focus has therefore shifted to a molecular approach of grouping this pathogen. Phytoplasmas are currently being classified and grouped into different subgroups according to the sequence of their 16S ribosomal RNA (rRNA) genes (Seemüller *et al.,* 1998). The table shown below classifies phytoplasmas into '*Candidatus* Phytoplasma' species based on the nucleotide sequence of the 16S rRNA gene. Each 16S rRNA group represents at least one distinct '*Candidatus* Phytoplasma' species (Table 1). These main groups of phytoplasma species can further be classified into sub-groups, which share ≥97% similarity in their 16S rRNA sequences. Strains found in a specific group are known to have substantial genetic variations and occupy diverse ecological niches (Gundersen *et al.,* 1996; Seemüller *et al.,* 1994; Seemüller *et al.,* 1998).

Table 1: 16S rRNA group-subgroup classification and '*Candidatus* Phytoplasma' species (Dr RE Davis, Unites States Department of Agriculture, Phytoplasma Resource Centre)

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

* Abbreviations are as follows: AY, aster yellows; WB, witches'-broom; Y, yellows; GY, grapevine yellows; Vir, virescence; WL, white leaf.

* The Table lists only phytoplasmas that have been formally named as '*Candidatus* Phytoplasma' species.

2.2.3 Plant hosts

To date, phytoplasmas have been found to infect several dicotyledonous-, cultivated- and wild plant species worldwide (Hollingsworth *et al.*, 2008). Apple, celery, china asters, grapevine, carrots, lettuce, periwinkle, potato and redcurrant are just some examples of plant hosts that phytoplasmas are known to infect (Kuske and Kirckpatrick, 1992; Schneider *et al.*, 1993; Tanne and Orenstein, 1997; Orenstein *et al.*, 1999; Lee *et al.*, 1993; Seemüller *et al.*, 1994; Přibylová *et al.*, 2011). Different phytoplasma species have been shown to infect *Vitis vinifera* including flavescence dorée (FD), bois noir (BN), Australian grapevine yellows phytoplasma (AGYp) and aster yellows phytoplasma (AYp). In South Africa, the phytoplasma strain causing yellows disease in infected vines was found to be AYp (Engelbrecht *et al.*, 2010). AYp is known to infect over 300 plant species from 48 different plant families around the world (Stansbury *et al.*, 2001). To date, AYp infections have been observed in vineyards in the Waboomsrivier area near Rawsonville and in the Olifants River area in the Vredendal district of SA.

2.2.4 Dual life cycle

Phytoplasmas can replicate in two distinctively different hosts - plants and insects (Figure 1). In plants they reside in the cytoplasm of sieve cells of the phloem, and in their insect vectors they are found in various organs inside and outside of the cells (Doi *et al.*, 1967).

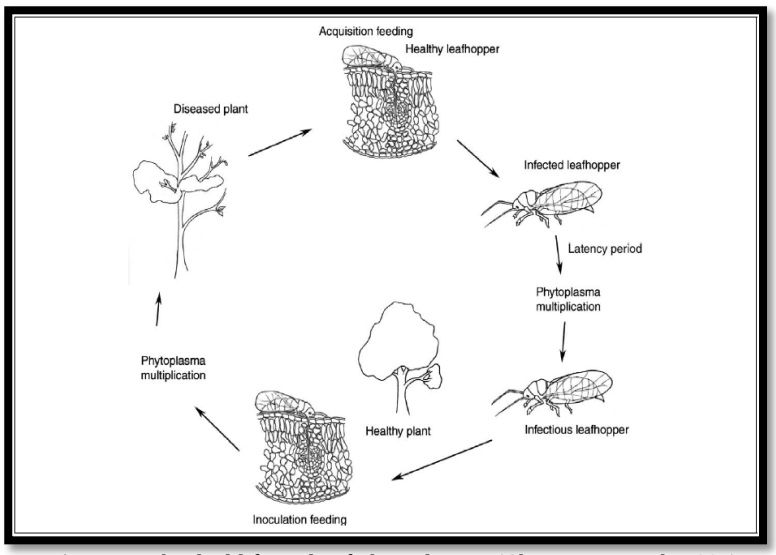

Figure 1: The dual life cycle of phytoplasmas (Christensen *et al.*, 2005).

During the latent period, the insect vector acquires the pathogen from the plant host. It then takes ~ 3 weeks till the phytoplasma titres reach the infectious level.

During the inoculation feeding, the infectious insect introduces the phytoplasma into a healthy plant. This process can take between 7 and 80 days (Murral *et al.*, 1996). Phytoplasmas are transferred with saliva into the punctured sieve element of the plant. From here the pathogen then spreads systematically throughout the plant, using the continuous sieve tube system. As phytoplasmas replicate in both plants and insects and cannot be cultured *in vitro*, they are very challenging pathogens to study.

2.2.5 Insect vector

Insect vectors for phytoplasma transmission include the leafhopper and plant hopper families. In 2011, Krüger and colleagues discovered that the vector for AYp transmission in grapevine in SA was the insect *Mgenia fuscovaria.* Studies have shown that phytoplasma strains in insect vectors and plants vary greatly. The plant host range depends less on the phytoplasma strain, and more on the natural insect vector species that are capable of transmitting the phytoplasma, and by the feeding behaviour of the insect vectors (McCoy *et al.*, 1989; Kunkel, 1926; Grylls, 1979). Phytoplasmas can have a low insect vector specificity or high insect vector specificity, meaning that they can be transmitted by one or more insect vectors at a given time (Christensen *et al.*, 2005). It is also known that insect vectors can transmit more than one type of phytoplasma and that plants can be infected by two or more distinct phytoplasmas at the same time. The geographic distribution of various insect vectors and preferred plant hosts of each vector, are the two major factors that determine whether a specific plant will be infected by one, or by multiple phytoplasmas (Lee *et al.*, 1998).

2.2.6 Symptoms

Grapevine plants show basically the same type of symptoms, regardless of the infecting phytoplasma species (Belli *et al.*, 2010). Some cultivars of grapevine may be more or less tolerant and may therefore show milder symptoms or no symptoms at all. The grapevine cultivar ‘Chardonnay’ is highly susceptible to several different phytoplasma infections, and is thus very useful in the successful identification of affected plants in the vineyard (Gibb *et al.*, 1999; Orenstein *et al.*, 2001). In grapevine, symptoms of phytoplasma infections can be observed in the leaves, canes and bunches (Figure 2).

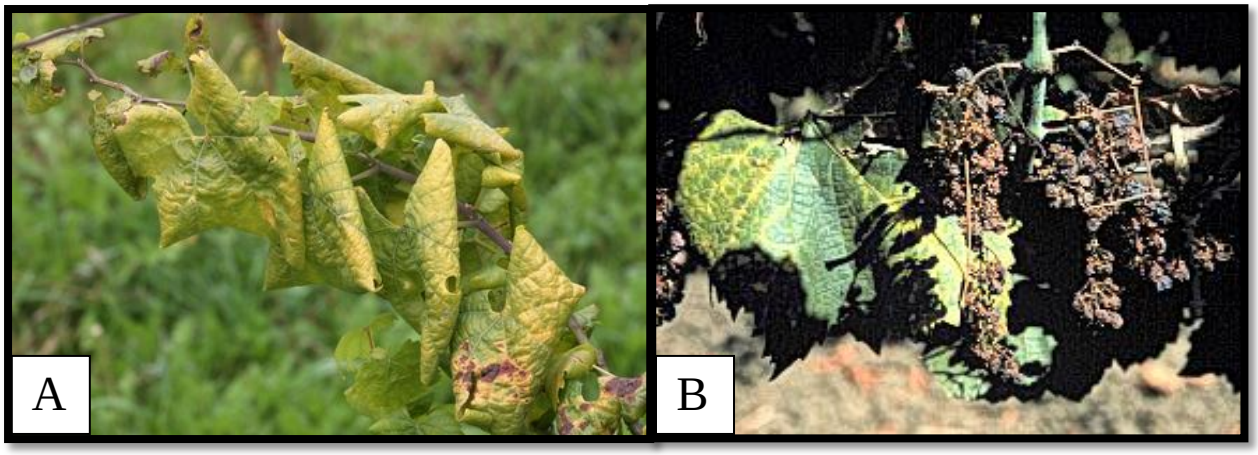

Figure 2: Phytoplasma-associated symptoms in grapevine. (A) A grapevine branch displaying yellowing of the leaves. At the end of the branch, bunch abortion of growth tip can also be observed (Photo taken by J Joubert from VinPro, South Africa). (B) Grapevine showing aborted fruits as well as yellowing and necrosis in leaf veins (Photo taken by Dr RE Davis of the Molecular Plant Pathology Laboratory, Unites States Department of Agriculture).

In early spring, vines may show irregular sprouting and then at the onset of summer, leaves start to roll downwards and become yellow in white-berried cultivars, and purple-reddish in red-berried cultivars (Belli *et al.,* 2010; Gibb *et al.,* 1999; Mitrev *et al.*, 2007; Orenstein *et al.*, 2001; Stansbury *et al.,* 2001; Strauss, 2009). The berries then start to wither and the bunches dry up, while the canes develop irregularly or not at all (Belli *et al.,* 2010; Radonjić *et al.,* 2009; Magarey and Wachtel, 1982). Symptoms of phytoplasma infections can be limited to a sector or a branch, whereas the remaining plant looks normal. Phytoplasmas also promote vegetative growth and dwarfism, but hinder reproductive activities in the infected plant (Strauss, 2009). 'Witches broom' and phyllody is another symptom seen in phytoplasma-infected sink tissues (Bertaccini, 2007; Hogenhout and Loria, 2008). Two or more phytoplasma species can infect the same vine simultaneously. These mixed infections do however not show differences in symptomatology to a single infection, which makes visual evaluation most difficult. The exact interaction of the pathogen with the host plant is still unknown, but the symptoms suggest that phytoplasmas interfere with fundamental cellular and developmental pathways in plants (Hogenhout *et al.,* 2008).

2.2.7 Interaction of phytoplasmas with their hosts

Phytoplasma infections impact the plant negatively; however it may or may not affect the fitness and survival of the insect vector (Hogenhout *et al.,* 2008). Some

of the morphological changes seen in infected plants attract insect vectors, and certain insects live longer and have more progeny on AYp-infected plants (Hogenhout *et al.,* 2008). This suggests that the pathogen doesn't only interfere with the plant's fundamental pathways, but also down-regulates the plant's defence against leafhoppers (Sugio *et al.,* 2011). According to recent studies, phytoplasmas induce phenotypic changes in plants through the production of effector proteins (Bai *et al.,* 2009). To-date, 56 secreted AY-witches' broom proteins, also called SAPs, have been identified that are candidate effector proteins. These proteins are secreted into the plants cytoplasm by the Sec-dependent protein translocation pathway, similar to Gram-positive bacteria. Once the proteins have been discharged into the phloem they target other plant cells by symplastic transport (MacLean *et al.,* 2011). In 2008, Hogenhout and her colleagues discovered SAP11, a protein secreted by AY-witches' broom, which accumulated in the plant cell nuclei and alters plant cell gene activity. More recently, SAP11 has also been shown to destabilize class II *CINCINNATA*- related TCP transcription factors, resulting in the crinkled leaf and witches' broom phenotype (Sugio *et al.,* 2011). Another effector protein discovered in onion yellows phytoplasma, namely TENGU, induces symptoms of witches' broom and dwarfism in plants, and is also thought to interfere with the plants auxin-related pathways, thereby affecting plant development (Hoshi *et al.,* 2009). MacLean and co-workers (2011) discovered SAP45, which has been found to interfere with floral development, another symptom of AY-witches' broom. It is clear that phytoplasmas secrete effector proteins that function inside the hosts cells. The extent to which phytoplasmas rely on these proteins to influence their diverse plant and insect hosts still remains unclear. However, from research done, scientists have discovered new hope for unravelling the pathogenicity mechanism of phytoplasmas.

2.2.8 Detection methods

The importance of being able to reliably distinguish phytoplasmas from similar grapevine diseases, and for discriminating different phytoplasmas from one-another, has attracted the attention of researchers worldwide. This activity has led to the development of a series of detection techniques, which have evolved from biological diagnostic approaches to molecular protocols (Belli *et al.,* 2010).

2.2.8.1 Past – Biological diagnostic approaches

Based on phytoplasma symptoms, one can generally distinguish phytoplasma infections from other grapevine disorders, for example leafroll disease (Belli *et al.,* 2010). Symptom expression is however quite uniform amongst phytoplasma species and can thus not be used to reliably distinguish one phytoplasma species from another. Indexing techniques were therefore applied on the hybrid Baco 22A, but did not help much as the symptomatic response induced by different phytoplasmas in Baco 22A is similar (Belli *et al.,* 2010). Successful transmission to Baco 22A was used to distinguish between FD and BN, but as this type of test is laborious, slow and time-consuming it was dismissed as soon as serological and molecular assays became available,

2.2.8.2 Present – Serological and Molecular assays

From 1982 onwards, monoclonal antibodies and polyclonal antisera were produced for the detection of FD phytoplasma (Caudwell *et al.,* 1982; Schwarz *et al.,* 1989). These antisera were also used for observing phytoplasmas by immunosorbent electron microscopy (ISEM) and fluorescent light microscopy (Lherminier *et al.,* 1989). Successful differentiation between FD and phytoplasmas of the same taxonomic group (16SrV) using monoclonal antibodies was reported by Seddas and co-workers (1993, 1995, 1996). Once the first DNA probe was synthesized on phytoplasma genome sequences, recombinant DNA-based techniques were rapidly developed (Kirkpatrick *et al., 1987*). These techniques were affordable for the detection in herbaceous hosts, but were found to be inaccurate in woody plants (including grapevine), mainly because of the low concentration of the pathogen and erratic distribution in this host (Belli *et al.,* 2010). The availability of the 16S rRNA gene sequences of AYp, FD and BN allowed for the development of universal PCR assays for the detection of all known phytoplasmas (Lim and Sears, 1989; Davis *et al.,* 1993; Daire *et al.,* 1993; Deng and Hiruki, 1991; Lee *et al.,* 2004). These assays were further developed for the reliable identification of grapevine phytoplasma sub-groups, based on restriction fragment length polymorphism and highly sensitive nested-PCRs (Lee *et al.,* 1994; Bianco *et al.,* 1996). For faster and even more specific detection of grapevine

phytoplasmas, real-time RT-PCRs, nanobiotransducers, multiplex nested-PCRs, ligase detection reactions and DNA microarrays were successfully developed (Angelini *et al.,* 2007; Firrao *et al.,* 2005; Clair *et al.,* 2003; Christensen *et al.,* 2004; Frosini *et al.,* 2002) and are currently being used by the industry to accurately detect phytoplasma species.

2.2.9 Seasonal and spatial distribution

Detecting phytoplasmas goes hand-in-hand with the distribution of the pathogen throughout a host plant. Seasonal distribution plays a big role in detecting phytoplasmas. Terlizzi and Credi (2007) reported that the proportion of BN presence was highest in summer throughout five different cultivars of grapevine, located in Italy. In winter, the number of infected grapevines clearly decreased. This seasonal distribution was also described in grapevine infected with AGYp, where detection was most reliable during summer and decreased in autumn (Constable *et al.,* 2003). These results suggest that phytoplasmas are unevenly distributed, seldom spreading systemically through grapevines and rarely infecting them persistently from year to year (Terlizzi and Credi, 2007; Constable *et al.,* 2003; Gibb *et al.,* 1999; Hollingsworth *et al.,* 2008; Seemüller *et al.,* 1994). In *Catharanthus roseus (C. roseus)* plants, the colonization pattern and distribution of two '*Candidatus* P. asteris' subspecies, namely severe AYp and dwarf AYp, were generally similar over a 10 week period (Kuske and Kirkpatrick, 1992). Phytoplasmas are also known to accumulate disproportionately in *Euphorbia pulcherrima* source leaves, and to a lesser extent in the petioles of source leaves, whereas the accumulation of phytoplasmas is lowest in sink organs (Christensen *et al.,* 2004). The infection level of phytoplasmas also differs greatly between host plants. Stolbur phytoplasma showed significant differences in the level of phytoplasma infection between *V. vinifera* cvs 'Cabernet Sauvignon' and 'Sauvignon blanc' (Orenstein *et al.,* 2001). Christensen *et al.* (2004) reported that phytoplasma titres observed in *C. roseus* are significantly higher to pathogen titres seen in *E. pulcherrima.* Despite the long history of research on AYp, no data are available on the spatial pattern of AYp-infected plants and the change in pattern over time as disease incidence increases.

2.2.10 Control strategies

According to Carstens (2008), no control strategy exists to cure a plant infected with phytoplasmas. The Department of Agriculture, Forestry and Fisheries in the Republic of South Africa has thus set aside multiple practices to aid in the prevention of further spread of '*Ca.* P. asteris'. These include weed control and intercropping, chemical control, vineyard sanitation, propagation of material and the marking of infected grapevine in all vineyards. Techniques that are currently being investigated to aid in the control of phytoplasmas are described below.

2.2.10.1 Auxin-induced recovery

In 1968, Davies and his colleagues showed that tetracycline has a bacteriostatic effect on phytoplasmas. Unfortunately, once the treated plants were transferred to antibiotic-free medium, phytoplasma symptoms reappeared. Other substances have been shown to alter phytoplasma ultrastructure. These include β-amino-butyric acid (BABA), polyamines, putrescine, spermidine and spermine (Musetti *et al.,* 1999). Ćurković Perica (2008) discovered that phytoplasma-infected shoots recover better on medium containing auxins, rather than benzyl-aminopurine. This technique is however dependent on the phytoplasma species. For example, '*Ca.* P. asteris' and '*Ca.* P. pruni' were susceptible to the supplementation of endogenous auxins, whereas '*Ca.* P. ulmi' and '*Ca.* P. solani' were not. Phytoplasma-infected *C. roseus* shoots treated with indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) led to the remission of symptoms in *in vitro* grown plants, but did not lead to the elimination of '*Candidatus* P. asteris' (Ćurković Perica *et al.*, 2007; Ćurković Perica, 2008). '*Candidatus* P. ulmi' infected *C. roseus* plants were always symptomatic when grown on medium containing 6-benzylaminopurine (BA) compared to infected shoots grown on IBA, which showed recovery (Leljak-Levanić *et al.*, 2010). Despite the recovery of symptoms, these shoots were still found to be infected by the pathogen through the amplification of its 16S rDNA. These results show that the recovery as a remission of symptoms may or may not involve elimination of the pathogen from the host plant.

2.2.10.2 Natural recovery

The natural remission of symptoms has been observed in several grapevine cultivars worldwide. Recovery was first observed in France and Italy in FD

infected vines, followed by recovery of BN in grapevine (Caudwell, 1961; Belli *et al.*, 1978; Osler *et al.*, 1993). This phenomenon has recently been described in apples infected with apple proliferation phytoplasma and apricots infected with European stone fruit yellows (Musetti *et al.*, 2004). In naturally recovered vines, remission of symptoms is often accompanied by the disappearance of the infection (Osler *et al.*, 2006; Zorloni *et al.*, 2008). Osler and colleagues (1999) suggested that systemic acquired resistance (SAR) might be involved in apple and pear recovery. Recently, an increase of reactive oxygen species (ROS) has been detected in grapevine displaying FD recovery (Musetti *et al.*, 2007). So far, the information available is still insufficient for a clear explanation of recovery, although it seems reasonable that interactions between the pathogen, the host and the environment may play a key role, as well as the involvement of grapevine bacterial or fungal endophytes (Belli *et al.*, 2010; Musetti *et al.*, 2007; Bulgari *et al.*, 2009; Martini *et al.*, 2009).

2.2.10.3 Hot water treatment

Another control strategy to cure dormant woody plant material from phytoplasmas is the use of heat or hot water treatment. Tassart-Subirats *et al.* (2003) used hot water treatment to eliminate FD from grapevine sections. As hot water treatment may interfere with the vitality of woody propagated material, it must be carefully applied under the correct temperature/time regimes and with the proper equipment (Mannini, 2007).

2.2.10.4 Abiotic stresses

Recovery of phytoplasma infections can also be promoted by exposing the grapevine to abiotic stress, such as uprooting followed by immediate transplanting, partial uprooting or pulling and pruning and pollarding (Osler *et al.*, 1993; Romanazzi and Murolo, 2008; Borgo and Angelini, 2002). Partial uprooting has been effective in inducing recovery in almost all grapevine cvs ‘Chardonnay’, ‘Verdicchio’ and ‘Sangiovese’ grafted onto Kober 5BB (Romanazzi and Murolo, 2008). After the first year of recovery from BN obtained by partial uprooting, *V. vinifera* cv ‘Primitivo’ had a similar trend in photosynthesis and respiration compared to healthy plants (Murolo *et al.*, 2009).

2.2.10.5 Expression of antimicrobial peptides in transgenic plants

In recent years, genetic modification has become an option for inducing disease resistance in plants. Du and his colleagues (2005) reported an increase in plant resistance against witches' broom disease in greenhouse transgenic *Paulownia* plants, expressing the antimicrobial peptide Shiva-1. Transgenic tobacco plants expressing a scFv antibody specific for the immunodominant membrane protein of Stolbur phytoplasma showed no significant resistance when the phytoplasma was transmitted to the plants by grafting or by its vector (Le Gall *et al.*, 1998; Malembic-Maher *et al.*, 2005). For engineering genetic resistance to phytoplasmas in grapevine, it could be more beneficial to engineer resistance in rootstocks, rather than individual grapevine cultivars. As phytoplasmas are known to move to the roots during winter, confronting them at this time with resistant rootstock could greatly decrease the chance of recurrence in the following year (Constable *et al.*, 2003). As the knowledge on plant genes inducing phytoplasma resistance is still very scarce, opportunities to select resistant varieties by traditional or molecular assisted breeding is limited (Belli *et al.*, 2010). Keeping the public's acceptance and environmental safety issues of genetically modified plants in mind, transgenic strategies for creating resistance of grapevine towards pathogens, remains challenging. Open and proactive dialogues between the scientific community and the public should be greatly encouraged, as they shed light on the benefits and practical usefulness of this technology.

2.3 Antimicrobial peptides

Grapevines are exposed to many plant pathogens and the resulting diseases may cause major economic losses. Chemical pesticides are being used to combat this global problem. However, pesticide usage has proven to be harmful to the environment and consumers health, and an overuse may lead to pathogen resistance (Keymanesh and Sotani, 2009). Scientists have therefore started looking at elements that present sustainable resistance to a broad range of pests and pathogens and that are safe for the host organism with no side effect on the environment.

2.3.1 General Information

Antimicrobial peptides (AMPs) form part of the innate, non-specific immune system shared by plants, humans and animals and are safe for the host organism with no side effects on the environment (Brown and Hancock, 2006). Rydlo *et al.,* (2006) reported that organisms produce AMPs in response to microbial infection, or they produce the peptides constitutively and store them in large quantities for later use. Antimicrobial peptides are made up of 12-50 amino acid residues and have shown to be effective against Gram-negative and Gram-positive bacteria, fungi, viruses and eukaryotic parasites (Wang *et al.,* 2006). Generally these peptides are cationic, rich in cysteine and amphipatic, giving them a great affinity for the pathogens membrane. Antimicrobial peptides are grouped into two groups based on their electrostatic charge. The positively charged peptides are divided into *β*-sheets, α-helices, extended helices and loop structures (Powers and Hancock, 2003). The second electrostatic group, namely the non-cationic peptides, are grouped into anionic and aromatic peptides and are very scarce. According to Keymanesh and Soltani (2009), some AMPs are produced solely by bacteria and are termed non-ribosomally synthesized peptides, whilst the ribosomally synthesized peptides are made by all organisms. Most peptides are not used in their native form to confer resistance to pathogens due to factors influencing the AMP activity, such as an increase in potency of anti-pathogen activity, reduction of their haemolytic effect or inhibition by host proteases. Scientists are therefore using analogue peptides or derivatives of the original AMPs (Lee *et al.,* 2002). Synthetic peptides are obtained by solid-phase methods and procedures using combinatorial chemistry (Andreu *et al.,* 1983; Monroc *et al.,* 2006). D4E1, a synthetic analogue of the cecropin family is more stable and potent than its native counterpart, and shows minimal cytotoxic activities against mammalian cells. This synthetic peptide demonstrates inhibition of spore germination of various fungal pathogens and also affects bacterial pathogens (Jacobi *et al.,* 2000; Rajaekaran *et al.,* 2009).

2.3.2 Plant AMPs

Plants have two broad mechanisms of pathogen resistance. Firstly, they may use the structures and compounds synthesized throughout their development to confer resistance against pathogens (constitutive resistant factors), or they make use of the induction mechanism which is activated after contact with the pathogen (induced

resistant factors) (Castro and Fontes, 2005). Both of these mechanisms involve the expression of peptides which present direct antimicrobial activity. Plant AMPs are grouped into different families based on their sequence similarity and activity towards certain pathogens. These families include the cyclotides, thionins (now named defensins), snakins, 2S albumins, hevein-type proteins and lipid transfer proteins, among many others (Peligrini *et al.*, 2011). The first plant defensin isolated from *Vitis vinifera* is Vv-AMP1 and was characterized by de Beer and Vivier (2008). Vv-AMP1 shows a strict tissue-specific and developmentally regulated expression pattern and is strongly antifungal. In 2008, de Beer and Vivier proved that Vv-AMP1 showed a very high level of activity against the pathogens *Fusarium oxysporum* and *Verticillium dahlia* in grapevine.

2.3.3 Mechanisms of cell death induced by AMPs

During pathogen infection, the pathogen will utilize substances from the plant host to facilitate its movement through the physical barriers presented by the plant (Castro and Fontes, 2005). The pathogen will also obtain nutrients from the plant for its own survival, while secreting multiple substances into the host which degrade the cell wall, interrupt metabolic functions or pathways, promote imbalance in the plants hormonal system and block the water translocation mechanism throughout the vascular system (Castro and Fontes, 2005).

Once the plant has come into contact with the pathogen, a series of peptides are expressed with some of them showing antimicrobial properties. The cationic peptides are attracted electrostatically to negatively charged molecules found in the pathogen membrane, but they may also interact with membrane lipids by specific receptors at the surface (Sitaram and Nagaraj, 1999). Generally, once the peptide threshold concentration is reached, AMPs accumulate on the membrane surface to direct inner components for cell lyses through pore formation. Three processes of pore formation have been summarized by Pelegrini and colleagues (2011). The barrel-stave mechanism consists of peptide aggregates forming a barrel-ring around an aqueous pore (Figure 3A). Once the peptides have bound to the membrane phospholipids and the threshold concentration has been reached, they start forming a barrel-ring to open a pore. The core of the barrel is made up of the hydrophilic portions, whereas the hydrophobic portion interacts with the membrane phospholipids. The second process of pore formation, namely the toroidal pore, is

very similar to the barrel-stave mechanism. The shape of the pore is similar; however the pore is composed of overlapping peptides and membrane lipids. The last mode of pore formation is the carpet mechanism (Figure 3B) (Pelegrini *et al.*, 2011). Initially the peptides bind to the pathogen membrane electrostatically giving the appearance of a carpet on the bacterial membrane surface. This causes phospholipid displacement that alters the membrane fluidity and reduces barrier properties of the membrane.

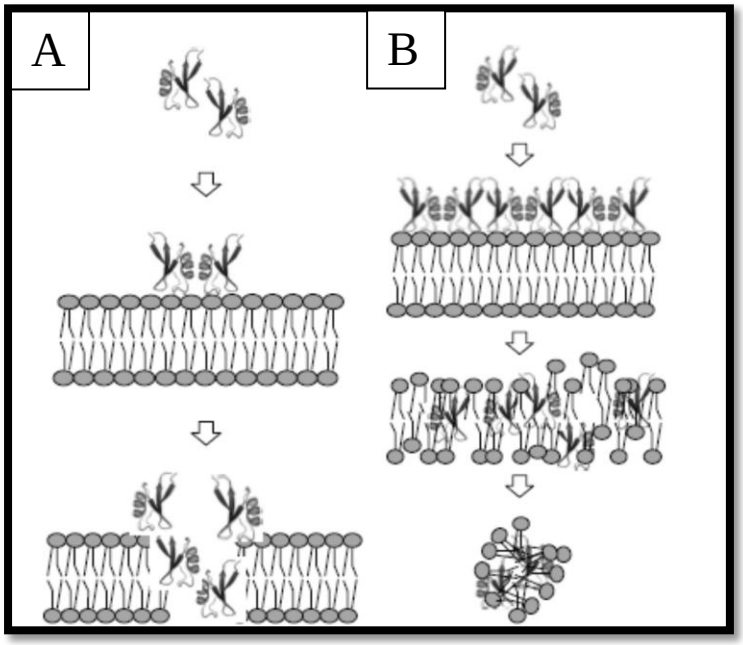

Figure 3: The processes of pore formations by AMPs. A: The barrel-steve mechanism. B: The carpet mechanism (Pelegrini *et al.*, 2011)

Once bound to the pathogen's membrane, AMPs can activate several pathways that will lead to cell death (Figure 4). Some peptides, as mentioned before will form pores. Ions and energy gradients dissipate through these pores and cause cell lysis within minutes (Figure 4A) (Bowman *et al.*, 2003). On the other hand, some peptides do not disrupt the pathogen membrane. Instead, bacteria exposed to these peptides show a decrease in protein synthesis, indicating that the peptide crosses the cell membrane to interact with intracellular targets and inhibit nucleic acid or protein synthesis, leading to cell death. (Figure 4B).

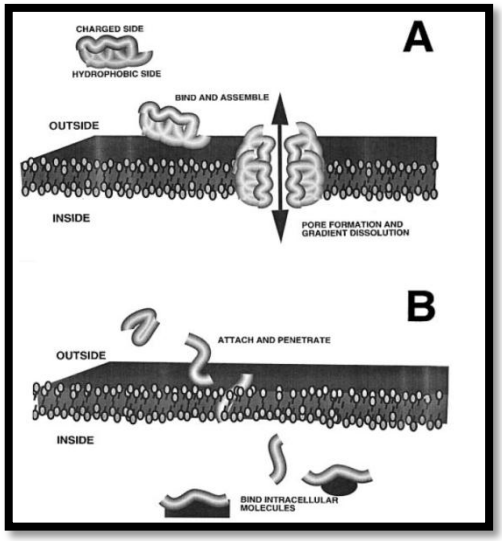

Figure 4: Modes for antimicrobial peptide activity. (Gallo and Huttner, 1998)

2.3.4 Exploiting AMPs in plant disease control

The potential of AMPs as protecting agents against plant pathogens has increased substantially over the last few years, in an effort to minimize pesticide toxicity and other harmful environmental impacts caused by pesticides. Most AMPs show antifungal, antibacterial or antiviral activity, and some are effective even against eukaryotic parasites (Keymanesh and Soltani, 2009).

2.3.4.1 Transgenic plants expressing AMPs

Numerous examples exist for the successful application of AMPs in transgenic plants to induce pathogen resistance. Alan and Earle (2002) reported that the synthetic peptide MSI-99 was the best candidate for the generation of transgenic tomato lines with enhanced resistance to bacterial and fungal disease. Transgenic tobacco plants expressing a magainin analogue is another example displaying both bacterial and fungal resistance (de Gray *et al.,* 2001). Transgenic grapevine expressing MSI-99 showed increased resistance to powdery mildew and crown gall development (Vidal *et al.,* 2006). This study was recently extended to include the expression of the AMPs Cecropin B, Shiva-1 and EsF-12 in transgenic grapevine, showing different levels of resistance against *Agrobacterium tumefaciens*, *Agrobacterium vitis* and *Botrytis cinerea* (Rosenfield *et al.,* 2010). Great success has been shown in transgenic tobacco plants where the synthetic peptide CEMA conferred resistance against the highly virulent fungus *Fusarium solani* (Yevtushenko *et al.,* 2005). The peptide Shiva-1 has been

expressed in transgenic *Paulownia* and resulted in an improved resistance to witches' broom disease (Du *et al.,* 2005). Overexpression of Snakin1 (SN1) in transgenic potatoes showed significant protection against *Rhizoctiona solani* and *Erwinia carotovora* (Almasia *et al.,* 2008). In 2001, a US patent by Smith and colleagues (patent number 7119262) describes the *in planta* activity of certain peptide classes against phytoplasmas in transgenic poinsettia. The effect of different AMPs against other members of the class *Mollicutes,* illustrates the potential use of AMPs to be active against phytoplasmas (Béven *et al.,* 2003; Borth *et al.,* 2001; Béven and Wroblewski, 1997).

2.3.4.2 Transient expression of AMPs

The generation of transgenic crops is however a very expensive technique and it can take months or several years to establish before AMP efficacy screening can be performed. To overcome this problem, the pre-screening of possible AMP candidates by means of expression vectors used to transform plant cells, which allow for the transient expression of foreign genes can be used. Transient expression systems have the advantage that they are much faster, more flexible and can be applied to fully differentiated plant tissue (Fischer *et al.,* 1999; Voinnet *et al.,* 2003). SN1 isolated from potato tubers showed activity against bacterial and fungal pathogens (Seguro *et al.,* 1998). In 2008, Kovalskaya and Hammond demonstrated that the production of functionally active SN1 proteins is suitable for antimicrobial activity in *in vitro* assays, using a prokaryotic expression system. Santos-Rosa *et al.* (2008) implemented a transient expression system to examine the function of stilbenes in a grapevine leaf environment against the fungus, *Plasmopara viticola* through over-expression of stilbene synthase. The defence role of glyoxal oxidase from *Vitis pseudoreticulata* against the grapevine pathogen *P. viticola* was also investigated in a recent study (Guan *et al*., 2010). This was achieved by applying *Agrobacterium*-mediated transient expression of *VpGLOX* in susceptible plants. The synthetic peptide D4E1 was recently shown to induce resistance against the grapevine pathogens *A. vitis* and *X. ampelinus* through transient expression (Visser *et al.,* 2012). These results illustrate the value of transient expression systems as a pre-screening

method of AMP activity *in planta* in economically important crops like grapevine.

2.3.5 Factors influencing AMP expression

Several intrinsic and extrinsic parameters have been reported to affect the threshold peptide concentration. The concentration of the AMP, the time of exposure and the bacterial density may affect the *in vitro* and *in vivo* action of the peptide (Fassi Fehri *et al.,* 2007). pH, salt concentration and the cationic nature of the medium may also have an effect on the activity of plant-derived peptides (Osborn *et al.,* 1995). The phospholipid membrane composition, membrane fluidity and head group size form part of the extrinsic factors (Yeamn and Yount, 2003). Maisnier-Patin *et al.* (1996) reported that different AMPs each work best at an optimal temperature. All of these factors need to be taken into consideration when working with AMPs, as they may lead to differences observed in peptide efficacy.

2.4 Conclusion

Antimicrobial peptides are active against a broad range of bacterial and fungal pathogens, and have also shown to be active against grapevine pathogens (Rosenfield *et al.,* 2010). As phytoplasmas lack a cell wall, AMPs are perfect candidates for resistance against this phytopathogen. Santos-Rosa *et al.* (2008) reported on the use of a transient expression system as a reliable and time-effective method for the expression of foreign genes in agricultural crops, including grapevine. The transient expression system described by Visser and co-workers (2012), will be used during the current study to induce resistance against the grapevine pathogen, aster yellows phytoplasma. A previously established qPCR procedure will be used to facilitate the quantification of AYp titres *in planta* (Angelini *et al.,* 2007; Visser *et al.,* 2012). As only preventative measures are currently available for the control of phytoplasma diseases, these applications can play an important role in the development of plant resistance to this pathogen. Despite the long history of research on AYp, no data are available on the spatial pattern of AYp-infected plants. The current study observes the spatial distribution of AYp along canes of an infected *Vitis vinifera* cv ‘Chardonnay’ plant.

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Internet resources

South African Wine Industry Information and Systems (SAWIS): http://www.sawis.co.za

Chapter 3

Establishing aster yellows phytoplasma-infected plant material

3.1 Introduction

The aim of this study is to induce resistance to aster yellows phytoplasma (AYp) infected plant material through the transient expression of antimicrobial peptides (AMPs). The establishment of AYp-infected plant material will be described during this chapter.

Aster yellows phytoplasma is phloem-limited and infects several dicotyledonous -, cultivated - and wild plant species worldwide (Hollingsworth *et al.,* 2008). The pathogen is known to infect multiple hosts including; apple, celery, china asters, grapevine, carrots, lettuce, periwinkle, potato, redcurrant and many more (Kuske and Kirckpatrick, 1992; Schneider *et al.,* 1993; Tanne and Orenstein, 1997; Orenstein *et al.,* 1999; Lee *et al.,* 1993; Seemüller *et al.,* 1994; Přibylová *et al.,* 2011).

Symptom expression is quite uniform amongst phytoplasma species and can thus not be used to reliably distinguish one phytoplasma species from another (Belli *et al.,* 2010; Radonjić *et al.,* 2009). Aster yellows phytoplasma-infected grapevine displays several symptoms. Dwarfism, necrosis of young shoots, shortening of internodes and clustering of branches has been observed (Kozina *et al.,* 2011; Nejat *et al.,* 2010; Strauss, 2009). Vines may display a lack of lignification and canes may seem droopy. Towards the end of the season, phytoplasma-infected leaves tend to roll downwards and canes mature irregularly or not at all (Belli *et al.,* 2010; Radonjić *et al.,* 2009). Infected vines decline and die eventually (Carstens, 2008).

Phytoplasmas are transmitted by several phloem-feeding insect vectors belonging to the Cicadellidae (leafhoppers), Fulgomorpha (planthoppers) and Psyllidae (psyllids) families (Weintraub and Beanland, 2006). With phytoplasma strains, host ranges in insect vectors and plants vary greatly. The plant host ranges that can be infected by phytoplasmas depends less on the phytoplasma strain, and more on the natural insect vector species that are capable of transmitting the disease (McCoy *et al.,* 1989; Kunkel, 1926; Grylls, 1979). It is also known that insect vectors can transmit more than one type of phytoplasma and

that plants can be infected by two or more distinct phytoplasmas at a given time. In the Western Cape of South Africa, AYp was detected in grapevine in 2010 in the vineyards of the Olifants River Valley (Engelbrecht *et al.,* 2010). The insect vector for AYp in grapevine in South Africa is the leafhopper *Mgenia fuscovaria,* belonging to the Cicadellidae family (Krüger *et al.,* 2011).

Phytoplasma-infected material can be maintained *in vitro* under controlled conditions. Apple proliferation phytoplasma - (APp) and European stone fruit yellows phytoplasma (ESFYp) infected shoot tip cultures have been maintained *in vitro* since 1985 and 1991 (Jarausch *et al.,* 1996; Jarausch *et al.,* 1994). Bois noir (BN) phytoplasma was maintained in micro-propagated grapevine plants cultivated in Murashige and Skoog (MS) medium (Gribaudo *et al.,* 2007). In paulownia plants, a five time increase of phytoplasma concentration was detected in tissue culture material (Wang *et al.,* 1994). On the other hand, mulberry plants severely infected with mulberry dwarf phytoplasma were found to be disease free after being micro-propagated in MS medium containing no phytohormones (Dai *et al.,* 1997). The same phenomenon was seen in phytoplasma-infected almond varieties, sugarcane and *C. roseus* (Chalak *et al.,* 2005; Parmessur *et al.,* 2002; Möllers and Sarkar, 1989). Therefore, tissue culture techniques can be used for maintaining a pathogen, but also for eliminating phytoplasma from a plant. During the current study, AYp-infected *Vitis vinifera (V. vinifera)* cv 'Chardonnay' plants will be micro-propagated and used for the transient expression of the AMPs. The cultivar Chardonnay was chosen due to the fact that it is the most susceptible grapevine cultivar to phytoplasma infections and due to its importance in the South African wine industry (Gibb *et al.,* 1999; Orenstein *et al.,* 2001; Jeff Joubert, VinPro).

Phytoplasma can be transmitted through vegetative propagation and natural transmission by the insect vector, but there are several other ways to confer and maintain phytoplasmas in plant material. Phytoplasmas can also be transmitted through dodder transmissions, grafting and transmission experiments using the respective insect vector. Dodder transmissions of AYp by *Cuscuta campestris* to *C. roseus* were successful, and symptoms could be seen after four month (Přibylová *et al.,* 2011). During a different study, successful dodder transmission was also seen in *C. roseus*, using the infected medical plant *Rehmannia glutinosa* and the redcurrant plant *Rubus rubrum* (Přibylová and Ŝpak, 2013). Using *in vitro* grafting as a pathogen-inoculation method has been

successfully described by several scientists. Apple proliferation phytoplasma-infected *Malus pumila* MM106 cultures were grafted onto healthy plants, and a successful graft transmission was seen in 90-94% of plants after three months of graft contact (Jarausch *et al.,* 1999). *Catharanthus roseus* plants infected with phytoplasma successfully transmitted the disease to healthy *C. roseus* plants through grafting (Kamińska and Śliwa, 2005; Nejat *et al.,* 2010). Transmission of bois noir and flavescence dorée from infected *C. roseus* to grapevine through grafting is efficient and is far easier than dodder transmission (Tanne and Orenstein, 1997). Using the insect vector in transmission experiments has also proven to be a successful technique in inoculating a plant with phytoplasma. Transmission experiments using the insect vector for FD showed that the insect vector is capable of transmitting FD from clematis to grapevine (Filippin *et al.,* 2009). Watercress yellows phytoplasma was successfully transmitted to watercress, plantain and lettuce through vector transmission (Borth *et al.,* 2006). Successful transmission experiments using the insect vector *M. fuscovaria* have been described on grapevine for AYp (Krüger *et al.,* 2011). During the current study, transmission of AYp from infected to healthy grapevine was performed using the insect vector *M. fuscovaria*. Once infected, these plants were to be used to test the effect of AMPs on AYp.

Catharanthus roseus is a very well-known plant and is common in tropical and sub-tropical regions worldwide. It is also a very valuable experimental host (Nejat *et al.,* 2010). Kamińska and Ŝliwa (2005) used this decorative plant to maintain phytoplasma cultures during their study. This plant is susceptible to AYp infection and the phytoplasma is known to accumulate in high concentration throughout the plant (Berges *et al.,* 2000). *C. roseus* can also be grown throughout the whole year and thus offers the possibility of all year round testing and experimentation. Therefore, during the current study *C. roseus* plants were to be infected with AYp through natural transmission of the disease and used as an alternate host to maintain AYp in a controlled greenhouse environment.

3.2 Materials and methods

3.2.1 *Vitis vinifera* plant material

In order to establish AYp-infected *V. vinifera* cv 'Chardonnay' plants *in vitro*, plant material was collected from a farm near Vredendal in the Western Cape, South Africa, throughout January 2011 till April 2012. Leaf material was screened for AYp infection through the nested-PCR described in Section 3.2.4 before being placed *in vitro*. Infected plants were cut into 3-5cm pieces containing one node each. These cuttings were then sterilized by shaking them in 70% Ethanol for 2 min, followed by a washing step using sterile water for 2min. They were then shaken for 12min in 2% Bleach and rinsed in sterile water (four times for 2min). The sterilized cuttings were then cultured in tissue culture flasks (Lasec, South Africa) containing agar-solidified Murashige and Skoog (MS) media (0.5X MS Macro, 0.5X MS Micro, 0.5X B5 Vitamins, 0.5X Fe/EDTA, 0.75% Sucrose and 6g Phytagel^{TM} filled to 1L with distilled water) Tissue culture flasks were kept in a growth chamber with a 16h light and 8h dark photoperiod, at 23°C and 19°C respectively (Figure 5).

Figure 5: Tissue culture *V. vinifera* cv 'Chardonnay' plants cultured in MS media. Plantlets were kept at a 16h light and 8h dark photoperiod at 23°C and 19°C.

After two-, three-, four-, seven-, and eleven weeks, phloem scrapings and leaf material was collected from *in vitro* material using a scalpel blade (Figure 6).

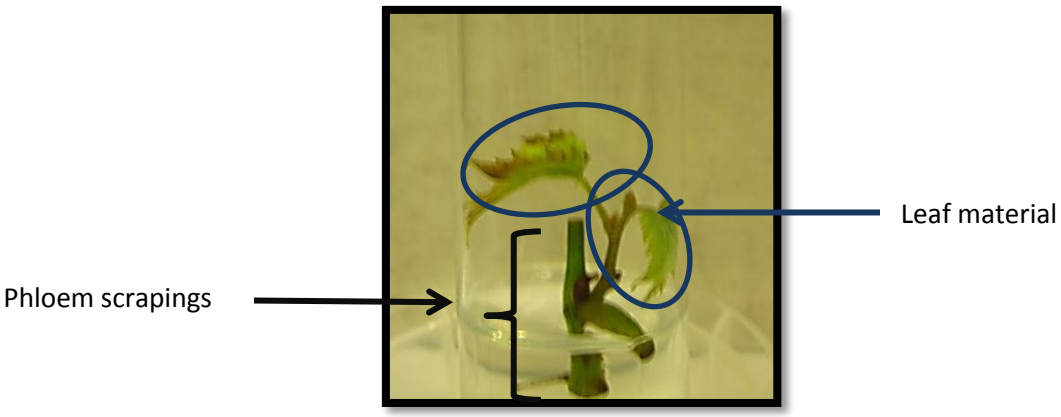

Figure 6: Phloem scrapings and leaf material collected from *in vitro V. vinifera* cv 'Chardonnay' plants. Phloem scrapings were taken using a scalpel blade.

This material was ground up to a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted according to the manufacturers' protocol using the NucleoSpin® Plant II kit (Macherey-Nagel) and stored at -20°C until used for screening by the nested-PCR, described in Section 3.2.4.

3.2.2 *Nicotiana benthamiana* plant material

To establish AYp-infected *N. benthamiana* as an alternative host, transmission experiments were carried out on six plants using the insect vector *Mgenia fuscovaria* in March 2012. Due to quarantine regulations, the vector was not allowed to be brought to Stellenbosch and all transmission experiments were conducted in a field laboratory in Vredendal, in collaboration with Professor Krüger (University of Pretoria). *M. fuscovaria* was collected in a severely AYp-infected vineyard in Vredendal, using an insect net and an insect cage for transport. Field-collected insects were used due to the difficulties experienced in establishing cultures (Krüger *et al.*, 2011). Insects were not tested for infection prior to transmission, and thus five randomly collected insects were placed on one plant. These plants were then kept in a cage for 2 days before being sprayed with the insecticide Confidor, and were then placed under controlled greenhouse conditions for further analysis (Figure 7).

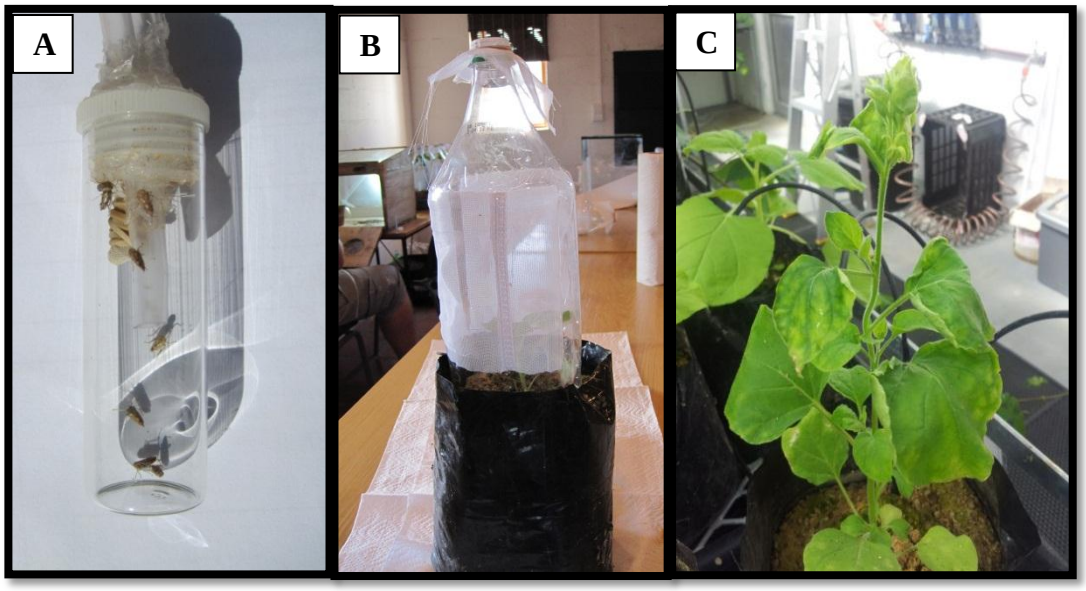

Figure 7: A) Insect vector *M. fuscovaria*. B) *N. benthamiana* containing five insects kept in a cage for two days. C) *N. benthamiana* plant 4 weeks after the transmission experiment.

Healthy *N. benthamiana* plants were grown in a greenhouse under controlled conditions.

3.2.3. *Catharanthus roseus* plant material

In January 2011 and 2012, *C. roseus* plants were placed into a severely AYp-infected vineyard in Vredendal where the insect vector for AYp, *M. fuscovaria*, was found. Once plants displayed symptoms of phytoplasma (~ one year) they were sprayed with the insecticide Confidor and infection was confirmed by nested-PCR (Section 3.2.4). The infected plants were then transferred to greenhouse conditions to maintain AYp-infected plant material. Healthy *C. roseus* material was placed *in vitro* using the technique described in Section 3.2.1.

3.2.4. Diagnostic nested-PCR used to detect AYp

All plant material was screened for AYp infection using a nested-PCR procedure. Universal diagnostic primers R16mF2 (CATGCAAGTCGAACGGA) and R16mR1 (TGACGGGCGGTGTGTACAAACCCCG) (Gundersen and Lee, 1996) were used in the first PCR reaction. The reaction mix contained 1X KapaTaq buffer A, 1X Cresol, 0.2mM dNTPs, 0.2µM of each primer and 0.05U/µl KapaTaq DNA polymerase. A final reaction volume of 20µl was used, of which 1µl was the template DNA (DNA concentrations ranged from 10ng/µl - 40ng/µl). The PCR conditions were as follows: 2min at 94°C, 35 cycles of 30sec at 94°C, 45sec at

55°C and 1min at 72°C. This was then followed by a final elongation step of 5min at 72°C. The PCR products were then diluted 30 times and used in the nested-PCR reaction with the primers R16vdal-F (GGAAACTACTGCTAAGACTGGATA) (modified R16F2N primer) and R16R2 (TGACGGGCGGTGTGTACAAACCCC G) (Gundersen and Lee, 1996). The reaction mix was the same as for the first PCR and the PCR conditions were as follows: 2min at 94°C, 35 cycles of 30sec at 94°C, 45sec at 62°C and 1min at 72°C, followed by a final elongation step of 5min at 72°C. PCR products were run on a 1% agarose gel for 30min at 120V. The first PCR reaction produced a 1.432kb amplicon and a 1.247kb amplicon was expected after the nested-PCR reaction when AYp was present.

3.3 Results

3.3.1 Establishment of AYp-infected *V. vinifera* material

All grapevine canes collected from the farm in Vredendal were screened for AYp infection before being placed *in vitro* and the expected amplicon sizes were detected on a 1% agarose gel after the diagnostic nested-PCR. Three-hundred-and-ninety *V. vinifera* cv 'Chardonnay' plants were put *in vitro*. Three-hundred-and-four of these plants developed endophytic fungi contamination before leaf material could develop and therefore could not be screened for AYp infection. The contamination rate was higher in plants collected in January 2012 compared to plants collected in April 2012, starting at 59% contamination rate and ending at 100% (Figure 8). In January, 61 AYp-infected Chardonnay plants were placed *in* vitro. A total of 25 plantlets remained after 2 months and could be screened for AYp infection. During February, a total of 188 *V. vinifera* cv 'Chardonnay' plants were placed *in vitro*. On the 8th of February, 56 plants were collected and after two months, 35 of these plants were disposed of due to contamination. One-hundred-and-thirty-two plants were collected on the 23rd of February of which 40 remained and could be tested for AYp infection. In March, 78 plants were put *in vitro* and all of these were taken out after four weeks due to fungal contamination. Lastly, 63 AYp-infected Chardonnay plants were placed *in vitro* in April. All 63 plants developed fungal contamination after two weeks and were disposed of.

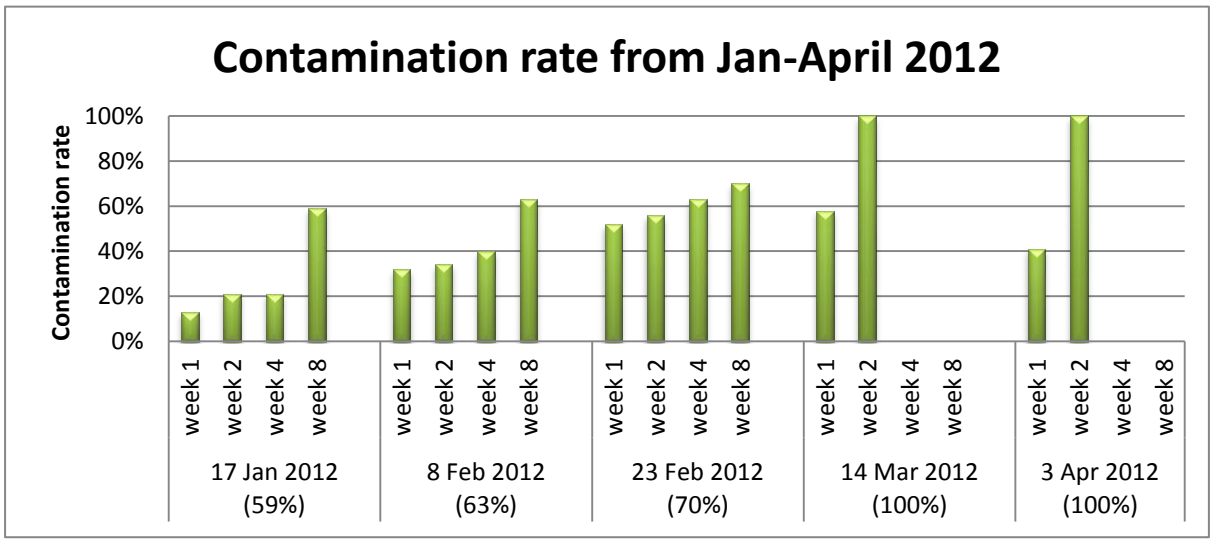

Figure 8: Contamination rate seen in Chardonnay plants once placed *in vitro*, collected from the vineyard during five different time intervals. Plants collected in January showed a 59% contamination rate after 2 months. This contamination rate increased to 63% and 70% when plants were collected in February. From March onwards, all plants placed *in vitro* developed 100% contamination after 2 weeks.

In total, 86 *in vitro* plantlets remained contamination free throughout all five time intervals (Jan-Apr) after 2 months and could be screened for AYp infection. All *in vitro* plantlets displayed no AYp symptoms once placed into the incubator after 2 months. After screening the respective phloem and leaf material from each plantlet (86 X 2 = 172) by the nested-PCR, the expected amplicon of 1.247kb could not be detected, indicating that AYp was not present (Figure 9). Healthy plant material collected from Vredendal was also screened prior to being placed *in vitro.*

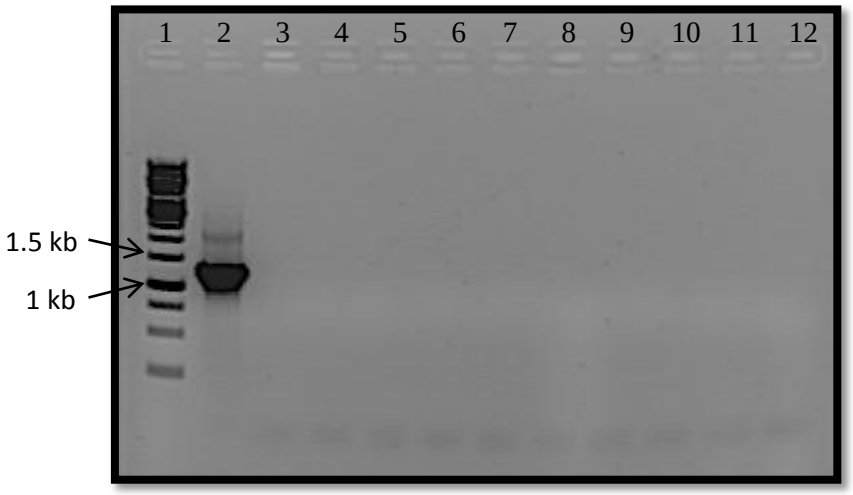

Figure 9: Agarose gel-electrophoresis of nested-PCR products. Lane 1: 1kb Molecular marker. Lane 2: Positive control. Leaf material collected from a *V. vinifera* cane before being placed *in vitro.* Lane 3, 5, 7, 9: Phloem scrapings from *in vitro* material. Lane 4, 6, 8, 10: Leaf material from *in vitro* material. Lane 11: Healthy *V. vinifera* leaf material. Lane 12: No-template control

3.3.2 Establishment of AYp-infected *N. benthamiana* material

After four weeks of being kept in a controlled greenhouse environment, leaf material was collected from all six *N. benthamiana* plants out of the insect vector transmission experiment, and ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA was then extracted according to the manufacturers' protocol using the NucleoSpin® Plant II kit (Macherey-Nagel) and stored at -20°C until needed. Samples were then screened for AYp infection using the nested-PCR procedure described in Section 3.2.4. Three out of the six samples displayed the expected amplicon size of 1.247kb after being run on a 1% agarose gel (Figure 10, lane 5, 7 and 8). Sequencing was also performed on these three samples to confirm infection by AYp. BLAST results confirmed a 97% maximum identity with the AYp strain: SA-Vdal 16S rRNA gene (GO365729.1). The remaining three plants (Figure 10: lanes 4, 6 and 9) did not display the expected amplicon size of 1.247kb after being run on a 1% agarose gel, and were thus not successfully infected by the insect vector *M. fuscovaria*. AYp-infected *N. benthamiana* plants did not display specific symptoms when compared to the healthy plants.

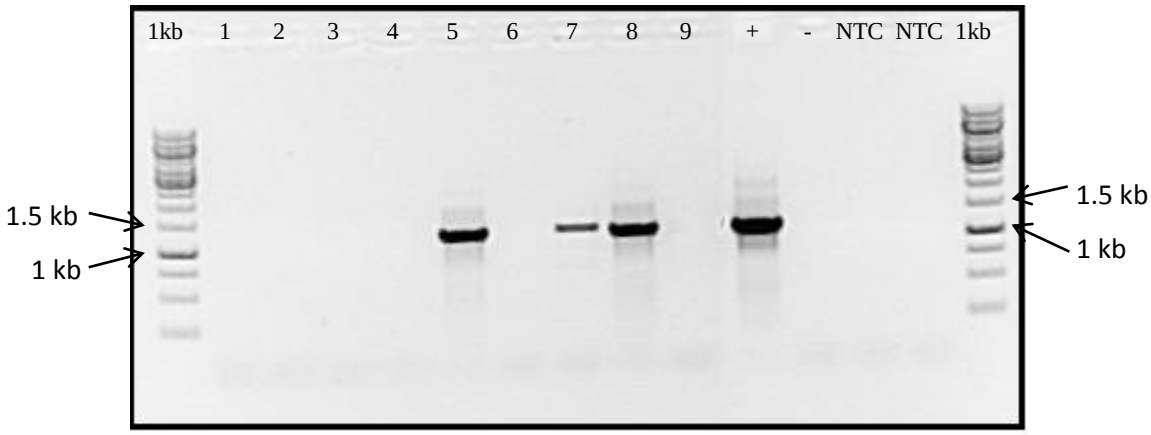

Figure 10: Agarose gel electrophoresis of nested-PCR products. Lane 0 and last lane: 1kb Molecular marker. Lane 1-3: Healthy *N. benthamiana*. Lane 4, 6 and 9: Healthy *N. benthamiana* after transmission experiment. Lane 5, 7 and 8: AYp -infected *N. benthamiana* after transmission experiment. +: Positive control. -: Negative control. NTC: no-template control after the first PCR and nested-PCR.

3.3.3 Establishment of AYp-infected *C. roseus* material

In January 2011 twenty *C. roseus* plants were placed into a severely AYp-infected vineyard in Vredendal. In March 2012 two of these plants showed symptoms of phytoplasma infection. Compared to healthy plants, AYp-infected *C. roseus* plants

displayed yellowing of the leaves, flower abortion, shortened internodes and the leaves were curling downwards (Figure 11).

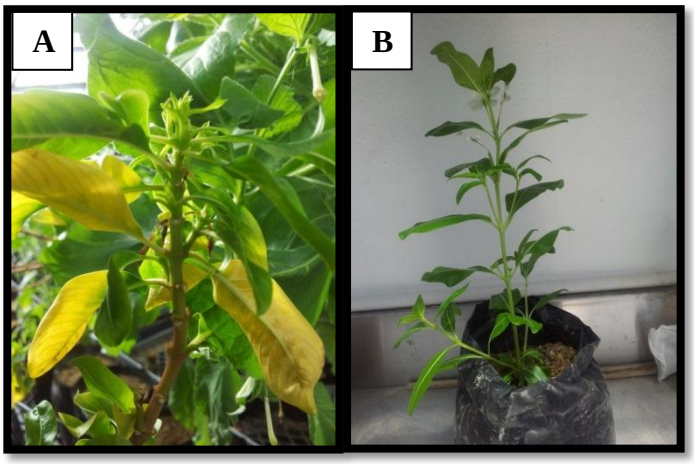

Figure 11: A) Aster yellows phytoplasma-infected *C. roseus* plant infected through natural transmission by the insect vector *M. fuscovaria*. B) Healthy *C. roseus* plant grown in the greenhouse.

Leaf material was collected from these two plants and AYp infection was confirmed by the nested-PCR and sequencing of amplicons. BLAST results of the sequenced amplicons showed a maximum identity of 95% with the AYp strain SA-Vdal 16S rRNA gene (GO365729.1), but showed a higher maximum identity (98%) against periwinkle phyllody phytoplasma genes for 16S rRNA (AB646267.1) As this study focused on AYp specifically, the two sequences were blasted against one another and displayed a 100% maximum identity, indicating that the two strains belong to the same subgroup of phytoplasmas, namely '*Ca.* P. asteris'. In January 2012 fifty more *C. roseus* plants were placed into the AY-infected vineyard in Vredendal. Unfortunately the plants all died due to unforeseen weather conditions and thus no testing could be done on these plants. Healthy *C. roseus* material was successfully grown *in vitro*.

3.4 Discussion

The establishment of AYp-infected *N. benthamina* plants through transmission experiments using the insect vector *M. fuscovaria* was successful and these infected plants can now be used to test the efficacy of antimicrobial peptides through transient expression. AYp-infected *C. roseus* was also successfully established by natural infection through the insect vector *M. fuscovaria* and will be used as an alternate host, as

phytoplasmas are known to accumulate in high concentrations throughout the plant (Berges *et al.,* 2000; Christensen *et al.,* 2004).

None of the AYp-infected *V. vinifera* material placed *in vitro* showed detectable infection by the nested-PCR after two-, three-, four-, seven- and eleven weeks, and also displayed no phytoplasma symptoms once micro-propagated. It is known that phytoplasma species are randomly distributed throughout an infected plant (Hollingsworth *et al.,* 2008). Due to this uneven distribution, it might be that cuttings taken from the AYp-infected Chardonnay vine and placed *in vitro* had lowered or no phytoplasma present at all. As no data are available for the distribution of AYp in grapevine, this uncertainty led to the observation of AYp distribution along five canes of an infected *V. vinifera* cv 'Chardonnay' vine, discussed in Chapter 4.

Recovery from phytoplasma infection through micro-propagation has also been observed in *V. vinifera* cv 'Chardonnay' and 'Barbera' infected with FD grown in MS medium (Gribaudo *et al.,* 2007). Stem cultures of mulberry dwarfism-infected mulberry plants, grown in MS media containing no hormones, showed no phytoplasma infection after three years of continuous testing on stem and leaf material (Dai *et al.,* 1997). The recovery of phytoplasma-infected plant material placed *in vitro* has also been seen in sugarcane material infected with sugarcane yellows phytoplasma and Lebanese almond varieties infected with '*Candidatus* P. phoenicium' (Parmessur *et al.,* 2002; Chalak *et al.,* 2005). Factors involved in the phytoplasma recovery of naturally-, vineyard- or orchard-grown plants are not completely understood (Ćurković Perica, 2008). Attempts have been made to understand natural-recovery of phytoplasma-infected plants. Musetti and colleagues (2004, 2005, 2007) suggested that the H_2O_2 accumulation is higher in phytoplasma-recovered grapevine, apple and apricot when compared to infected or healthy plant material. This accumulation of H_2O_2 reduces pathogen multiplication and disease symptom expression in infected material (Musetti *et al.,* 2007). Agronomical stresses were also shown to induce recovery of '*Ca.* P. solani'-infected grapevine (Romanazzi and Murolo, 2008). In *in vitro* grown cultures, phytoplasma remission was induced in *C. roseus* plants by adding the auxins indole-3-butyric-acid (IBA) and indole-3-acetic-acid (IAA) to the MS medium (Ćurković Perica, 2008). During the current study however, no exogenously supplemented auxins were added to the MS medium.

On the other hand, studies have also shown an increase in phytoplasma infection in micro-propagated grapevine material (Petrovic *et al.,* 2000). Shekari *et al.* (2011) had great success in preserving lime witches' broom phytoplasma in key lime by tissue culture using agar-solidified Murashige and Tucker medium. Apple proliferation phytoplasma-infected material has also been successfully maintained *in vitro* since 1985 (Jarausch *et al.,* 1996).

It is still unclear why some cultivars infected with phytoplasma can be maintained in *in vitro* conditions while others recover from phytoplasma infection. The current study however shows that AYp cannot be maintained in *in vitro* cultured Chardonnay material, and tissue culture itself might therefore be considered a way to eradicate AYp in this cultivar.

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Chapter 4

Spatial distribution of AYp in *Vitis vinifera* cv 'Chardonnay'

4.1 Introduction

As no aster yellows phytoplasma (AYp) infected *Vitis vinifera* cv 'Chardonnay' material could be established and maintained *in vitro* (Chapter 3), the question arose whether plant material might have been taken from a part of the cane where phytoplasma titre was very low or totally absent. Therefore, the spatial distribution of AYp along five canes of an infected *V. vinifera* cv 'Chardonnay' plant was investigated and will be discussed in this chapter.

In general it is believed that phytoplasma species are unevenly distributed throughout their plant hosts (Gundersen and Lee, 1996; Osler *et al.,* 1995; Seemüller *et al.,* 1984). Bois noir (BN) phytoplasma is unevenly distributed in the grapevine cultivars 'Ancellotta', 'Lambrusco Salamino', 'Sangiovese' and 'Trebbiano Romagnolo', and rarely infects the grapevine persistently from year to year (Terlizzi and Credi, 2007). Australian grapevine yellows phytoplasma (AGYp) shows an uneven distribution of pathogen titre throughout *V. vinifera* cv 'Chardonnay' plants, and detectable levels of AGYp fluctuated from season to season (Constable *et al.,* 2003). During a study on stolbur phytoplasma (Stolp)–infected *V. vinifera* cv 'Cabernet Sauvignon' and 'Sauvignon blanc' plants, the phytoplasma levels were found to be significantly different between the two cultivars and within different growing regions, having higher levels of infection in the warmer sub-regions (Orenstein *et al.,* 2001). Seemüller and colleagues (1984) reported that phytoplasma populations peak during summer and start decreasing from autumn onwards, making accurate detection of this pathogen challenging during the late season. In poinsettia and *Catharanthus roseus* plants, large differences in phytoplasma infection levels were seen between the two plant species, with *C. roseus* having a much higher pathogen titre compared to infected poinsettia (Christensen *et al.,* 2004). To our knowledge, no research has been done on the spatial distribution of AYp in *V. vinifera* cv 'Chardonnay' material. The geographical and field level distribution of AYp in wheat, oat and barley production fields showed no apparent spatial pattern between or within three years (Hollingsworth *et al.,* 2008). Lettuce plants naturally infected with AYp were found to be clustered in commercial and experimental fields, and

the degree of aggregation of disease incidence showed an increase over time within twelve fields (Madden *et al.*, 1995). In *C. roseus* plants, the colonization pattern and distribution of two '*Candidatus* P. asteris' subspecies - severe AYp and dwarf AYp - were generally similar over a 10 week period (Kuske and Kirkpatrick, 1992). It is also known that AYp titres in *C. roseus* and clover phyllody titres in strawberry are lowest in the roots and highest in symptomatic pedicels, followed by sepals, petals and leaves (Kuske and Kirkpatrick, 1992; Clark *et al.*, 1983).

Multiple studies have used quantitative real-time PCR (qPCR) for the accurate quantification of plant pathogens. qPCR is based on the same principle as conventional PCR, but differs in that it can quantify the amount of DNA in a reaction after each PCR cycle, thus enabling the monitoring of increasing PCR product in real-time. During qPCR a fluorescent signal is measured which gives an indication of the amount of amplicon. The fluorescent-based system used during the current study made use of the fluorescent molecule SYBR Green. During the qPCR, SYBR Green binds to the double stranded products at the end of each elongation step. As the amount of product amplifies, the amount of bound SYBR Green in the reaction increases, resulting in an increase in the total fluorescent signal detected. During the first few qPCR cycles, the amount of fluorescence in the reaction resulting from the template is shielded by the amount of background fluorescence. The threshold cycle (Ct) is defined as the number of cycles required for the fluorescent signal to rise above this background fluorescence (Wilhelm *et al*., 2001). The more initial template there is in the reaction the smaller the Ct value will be, and based on this principle the concentration of the pathogen can be deducted (Gibson *et al.*, 1996). Although detection of phytoplasma is challenging due to fluctuating seasonal pathogen titre and the irregular distribution in infected vines, several studies have reported on the successful quantification of phytoplasmas. Phytoplasma titres have been measured in *C. roseus* and *Euphorbia pulcherrima* plants using qPCR (Christensen *et al.*, 2004). The relative quantification of chrysanthemum yellows phytoplasma in its plant host and insect vector was performed using qPCR and was expressed as genome units of phytoplasma DNA per nanogram of host DNA (Marzachi and Bosco, 2005). Based on this system, quantification of '*Candidatus* Phytoplasma prunorum' in its natural plant host was also successful (Martini *et al.*, 2007). During the current study, qPCR was used to measure the AYp titres in five canes of an infected *V.*

vinifera cv 'Chardonnay' plant, and will be expressed in genome units of phytoplasma DNA per nanogram of host DNA.

4.2 Materials and methods

4.2.1 Plant material

Five canes from a previously tested AYp-infected *V. vinifera* cv 'Chardonnay' plant were collected in a vineyard in Vredendal (South Africa) in the late season (April) of 2012. Phloem scrapings together with leaf material were sampled using a scalpel blade and tweezers, and stored at -80°C until needed (Figure 12).

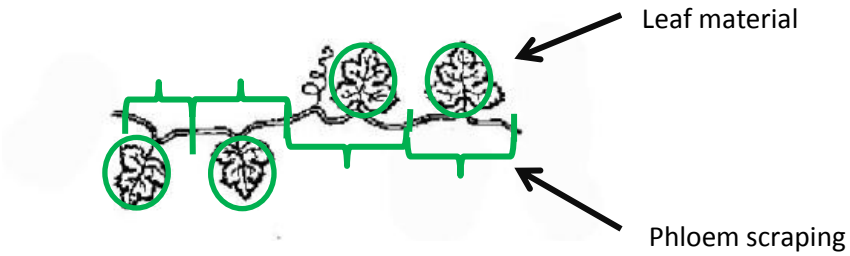

Figure 12: Leaf material and respective phloem scrapings taken from all five canes and stored at -80°C.

4.2.2 Diagnostic PCR

Phloem scrapings of each node together with the respective leaf were removed from storage and ground up separately in liquid nitrogen using a mortar and pestle. DNA extractions were done according to the manufacturers' protocol using the NucleoSpin® Plant II kit (Macherey-Nagel) and samples were marked clearly before being stored at -20°C. These samples were then screened for AYp infection using the nested-PCR procedure described in Section 3.2.4 of Chapter 3.

To show that DNA quality was optimal for the detection of AYp by the nested-PCR, an internal control using the 18S rDNA of *V. vinifera* was used. Primers and their sequences used for the internal control can be seen in Table 2 (Section 4.2.3). The reaction mix contained 1X KapaTaq buffer A, 1X Cresol, 0.2mM dNTPs, 0.2µM of each primer and 0.05U/µl KapaTaq DNA polymerase. A final reaction volume of 20µl was used, of which 1µl was template DNA. The PCR conditions were as follows: 2min at 94°C, 30 cycles of 20sec at 94°C, 20sec at 60°C and 30sec at 72°C. This was then followed by a final elongation step of 10min at 72°C. PCR products were run on a 1% agarose gel for 30min at 120V and an amplicon of 184bp was expected.

4.2.3 Quantitative analysis

Once the presence of AYp was confirmed in phloem scrapings and leaf material as described in Section 4.2.2, the phytoplasma titre was determined in these samples by quantitative real-time PCR. Concentrations of all DNA extracts were measured using the Nanodrop® ND-1000 spectrophotometer and all samples were diluted in MilliQ water to a final concentration of 20ng/µl. A Rotor-Gene Q (Qiagen) thermal cycler was used to perform all qPCRs and the Rotor-Gene Q Series Software 1.7 was used for run setup and analysis. Primers were designed by Visser (2011) and were constructed based on primers described by Hollingsworth *et al.* (2008) and Angelini *et al.* (2007). Primer sequences can be seen in Table 2 below.

Table 2: Primers used for the detection of the 18S rDNA of *V. vinifera* plants and for the quantitative real-time PCR analysis to determine AYp titre.

Primer name	Organism	Sequence	Tm (°C)
18S rDNA-f	Vitis species 18S rDNA	CTTCGGGATCGGAGTAATGA	60
18S rDNA-r	Vitis species 18S rDNA	TGGTTGAGACTAGGACGGTA	60
AY_F	Phytoplasma	AAACCTCACCAGGTCTTG	51.9
AY_R	Phytoplasma	AAGTCCCCACCATTACGT	53.4

To determine the efficiency of the qPCR, a standard curve was set up. Total DNA extractions following the manufacturers' protocol using the NucleoSpin® Plant II kit (Macherey-Nagel) were performed on leaf material from an infected AYp *V. vinifera* cv 'Chardonnay' plant in October 2011. This DNA was screened by the nested-PCR and the product was run on a 1% agarose gel and visualized under UV light. The amplified 1.247kb AYp fragment was cloned into the pGem®-T Easy plasmid (Promega) and termed pAY61. For the construction of the standard curve, a 7-fold dilution series (1ng to 1fg) was established by diluting pAY61 in 20ng/µl of total DNA from a healthy *V. vinifera* cv 'Chardonnay' plant. One fg of pAY61 contains 228 molecules of plasmid each containing a single copy of the AYp 16S rDNA gene. This was calculated by determining the molecular weight in Daltons of the double stranded DNA (www.changbioscience.com/genetics/mw). As the AY 16S rDNA gene is present in two copies in phytoplasma genomes, one fg of pAY61 corresponds to 114 AYp genome units (GU). All reactions were performed in triplicate for each pAY61 concentration. Threshold levels, threshold cycles and

standard curves were automatically calculated by the Rotor-Gene Q Series Software 1.7. Absolute quantification of AYp DNA in infected phloem and leaf material was achieved by comparison with dilution series of the pAY61 plasmid. For each quantitative run, the reaction volume was 20µl and contained 1X SYBR Buffer, 0.1µM of each primer and 20ng/µl of sample DNA. Cycling conditions were as follows: 3min at 95°C followed by 45 cycles at 95°C for 5sec and 62°C for 20sec. For each qPCR run, phloem scrapings together with the corresponding leaf material were run in triplicate, together with at least one standard dilution. DNA from a healthy host plant (at 20ng/µl) was used as a negative control, and a PCR mix with water instead of DNA was used as a no-template control. After each run, melting curve analysis were performed to determine the specificity of the amplified products.

4.3 Results

4.3.1 Spatial distribution of AYp

In total, 249 phloem and leaf samples from five canes of an AYp-infected *V. vinifera* cv 'Chardonnay' vine were collected. DNA was extracted and screened for the presence of AYp by the nested-PCR procedure. Table 3 below shows the number of leaf and node samples collected and also gives an indication of how many node samples had no leaf material available, compared to node samples with corresponding leaf material.

Table 3: Number of leaf and node samples collected from all five canes from one AYp-infected *V. vinifera* cv 'Chardonnay' plant.

Total samples available = 249			
Leaf material	**Node material**	**Node with corresponding leaf**	**Node with no leaf material**
115/249 **(46%)**	134/249 (**54%**)	223/249 (**90%**)	26/249 (**10%**)

Out of 134 nodes screened, 82 (61%) were found to be AYp-infected, whereas only 38 out of the 115 (33%) leaf samples screened, showed presence of AYp. Twenty-seven samples (12%) showed an infection in the node and corresponding leaf material. In most cases however, we did not detect AYp in the leaf if the node DNA displayed an amplicon after the nested-PCR. It was less likely to detect AYp in the

leaf if the corresponding node was not AYp-infected, and was only seen in 18 out of the 249 samples (circled in blue in Figure 13). This data is summarized in Table 4.

Table 4: AYp infection detected in leaf and node material in five canes from one AYp-infected *V. vinifera* cv 'Chardonnay' plant.

Leaf material	Node material	Node and corresponding leaf infected	Node infected and corresponding leaf healthy	Node healthy and corresponding leaf infected	Node and corresponding leaf healthy
38/115 (**33%**)	82/134 (**61%**)	27/223 (**12%**)	148/223 (**66%**)	18/223 (**8%**)	30/223 (**13.5%**)

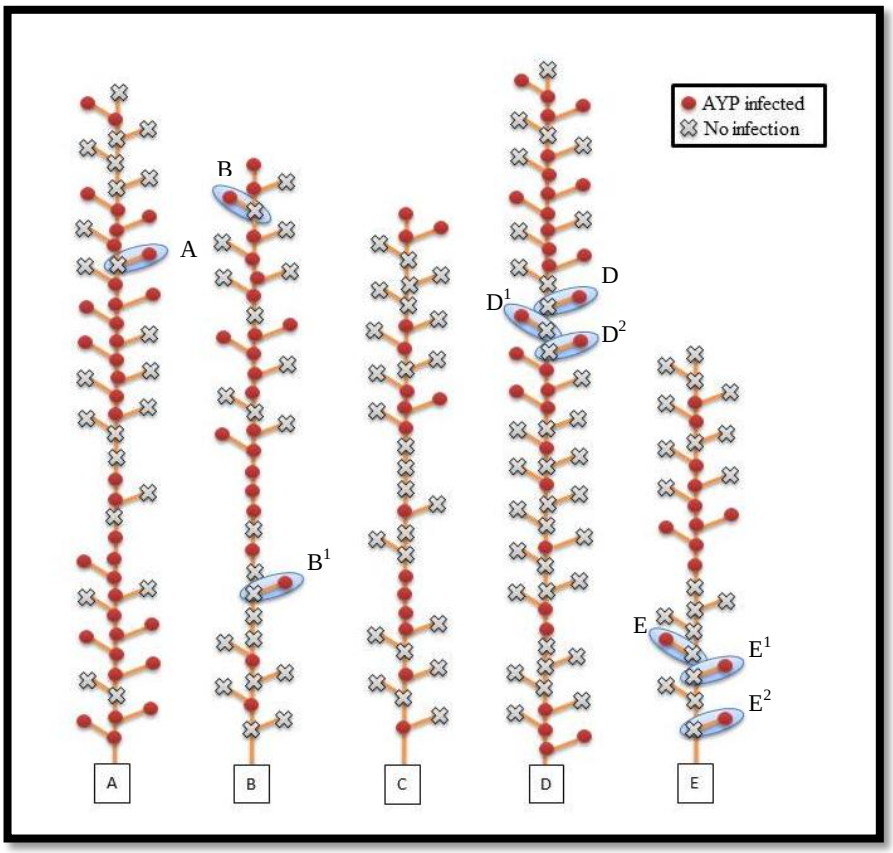

Figure 13: Spatial distribution of AYp in five canes (A-E) of the same *V. vinifera* cv 'Chardonnay' plant from Vredendal, South Africa. Node and leaf samples were tested on each cane for the presence of AYp. Samples labelled (A, B, B^1, D, D^1, D^2, E, E^1, E^2) were run on a PCR as an internal control for the 18S rDNA of *V. vinifera*.

As the canes were collected during the late season (April) the leaf material available on the five canes was in a suboptimal condition for DNA extractions. Once DNA was extracted and concentrations were analysed by the Nanodrop® ND-

1000 spectrophotometer, the 260/280 and 260/230 ratios were lower than the optimal values of 1.8 and 2.0-2.2. This may indicate that contaminants such as proteins, phenols, salts and EDTA were present, that absorb strongly at or near 280nm. An internal control using the 18S rDNA of *V. vinifera* was therefore used to ensure the nested-PCR was functional and not giving negative results due to suboptimal DNA quality. All 18 samples (circled in blue, Figure 13) in which the node was negative and the leaf positive for AYp infection were analysed as described in Section 4.2.2 and displayed the 184bp amplicon after being run on a 1% agarose gel (Figure 14).

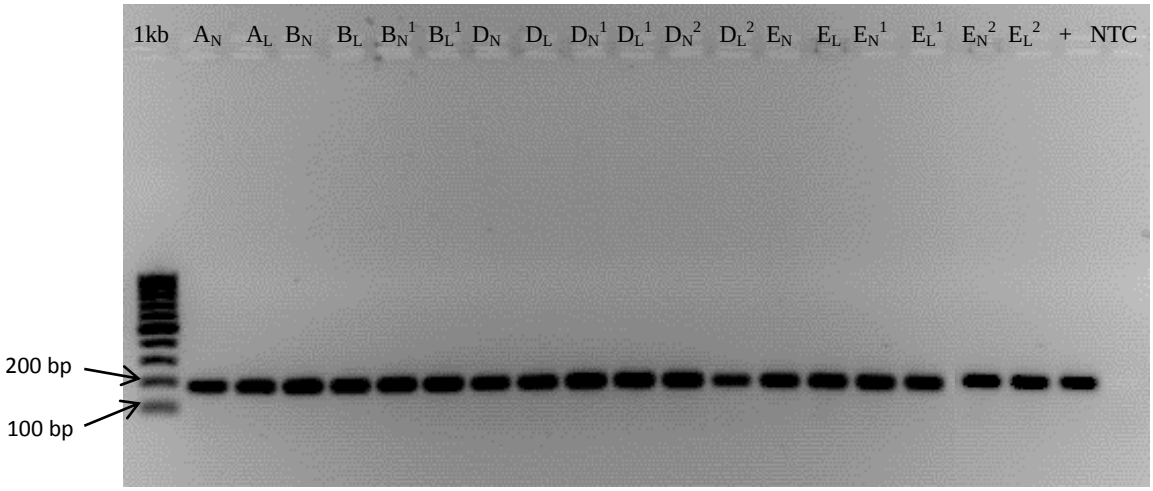

Figure 14: Agarose gel electrophoresis of *V. vinifera* 18S rDNA PCR products. Lane 0: 1kb molecular marker. Lane 1-18: 18 samples of *V. vinifera* cv 'Chardonnay' material circled in blue from Figure 13. The subscripts L and N stand for leaf and node material. + : Positive control. NTC: no-template control.

The DNA extracted was not 'pure' DNA as the ratio of absorbance at 260nm and 280nm was lower than the optimal value. However, the 18S rDNA was amplified in all 18 *V. vinifera* samples, indicating that the DNA quality was suitable enough for the accurate detection of AYp by the nested-PCR procedure.

4.3.2 Quantitative analysis of AYp

A standard curve was constructed by plotting the mean C_T value of each standard dilution versus the logarithm of its concentration. When the C_T values were plotted against their relative concentrations the efficiency of the standard was 1.00. The slope (M value) was -3.327, and the regression correlation efficient (R^2) was 0.99687 (Figures 15 and 16). Therefore, the reactions proved to be sufficient for the

accurate quantification of AYp. The mean threshold cycles for the standard curve dilution series can be seen in Table 5 below.

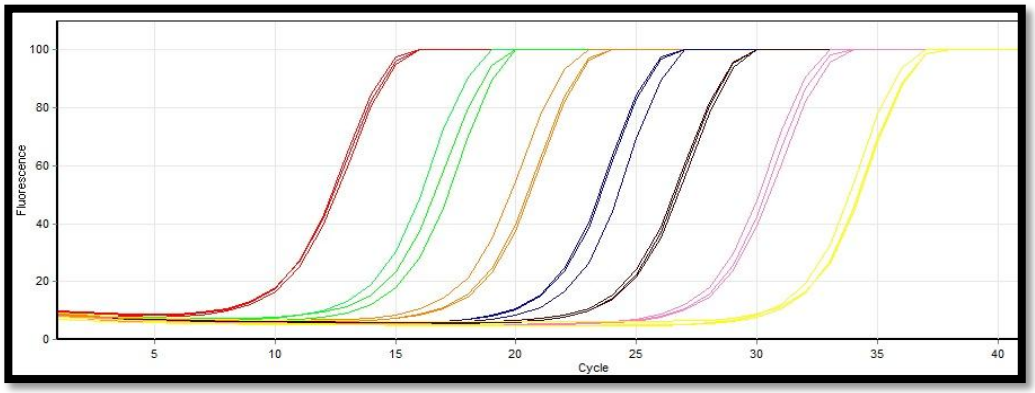

Figure 15: Amplification profile of the dilution series. 1ng 0.1ng 0.01ng $1X10^{-3}$ng $1X10^{-4}$ng $1X10^{-5}$ng $1X10^{-6}$ng

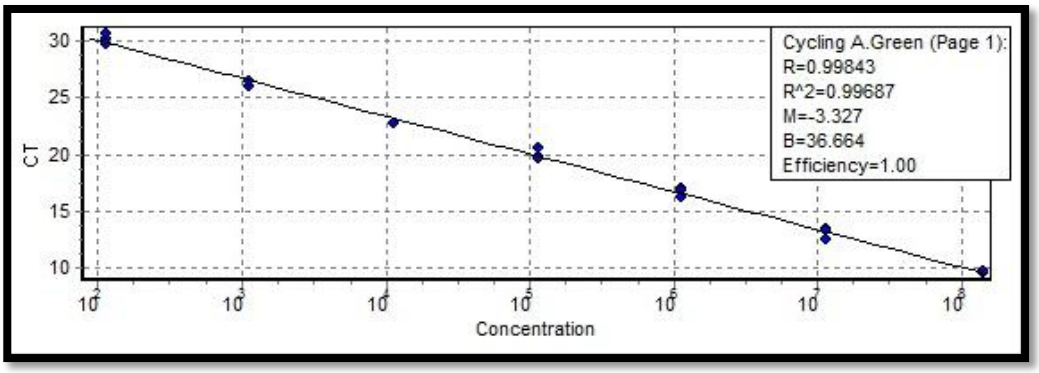

Figure 16: The standard curve resulting from the C_T values of each triplicate plotted against the concentrations of each sample.

Table 5: Mean threshold cycles (C_T) of standard pAY61 seen in all seven dilutions run in triplicate. The genome unit (GU) for each dilution are also shown together with the C_T standard deviation calculated for each sample run in triplicate.

	Standard dilutions	**C_T value**	**Std dev**
pAY61	1ng = $114X10^6$ GU	9.66	±0.07
		9.56	
		9.68	
	0.1ng = $114X10^5$ GU	12.50	±0.53
		13.48	
		13.33	
	0.01ng = $114X10^4$ GU	16.92	±0.41
		16.85	
		16.19	
	$1X10^{-3}$ng = $114X10^3$ GU	20.51	±0.47
		19.78	
		19.56	
	$1X10^{-4}$ng = 11 400 GU	22.78	±0.02
		22.75	

		22.79	
	$1X10^{-5}$ng = 1 140 GU	25.99	±0.24
		26.44	
		26.35	
	$1X10^{-6}$ng (1fg) = 114 GU	30.18	±0.43
		29.75	
		30.60	

Absolute quantification of node and leaf DNA was achieved by comparison of infected samples with the pAY61 dilution series. Figures 17 and 18 below show the differences seen in the amplification profiles and melt curves of samples collected from the same vine during the summer season in 2011, compared to the samples analysed for quantification of AYp titre collected in April 2012. The amplification profile showed a clear distinction between the cycles at which the fluorescence of the standard control and the positive control rose above the background fluorescence, compared to samples analysed for the quantification of AYp titre. A unique melting peak at 85°C was observed after real-time PCR with DNA from plasmid and infected node and leaf material. In Figure 17 it is evident that the standard control (pAY61) is amplified first and thus has the highest melt curve peak (Figure 18) and a C_T value of 8.23. The sample collected in October 2011 ( ) displays a slightly lowered melt curve peak and a C_T value of 32.04. Samples collected from the same grapevine in April 2012 ( ) had such minute concentrations of AYp, that no C_T values could be determined and accurate quantification on this material could thus not be done. The Rotor-Gene Q Series Software 1.7 was used to analyse all data represented above. As no C_T values could be determined for the samples collected in April, significant differences could not be determined when compared to the standard dilutions.

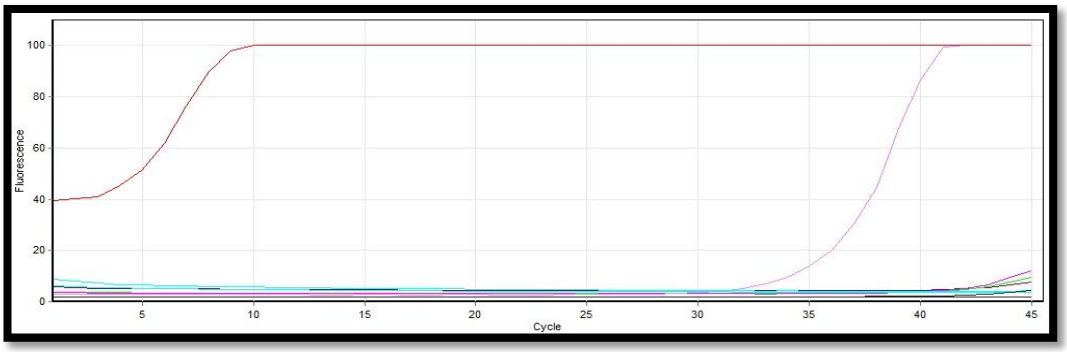

Figure 17: Amplification curve of AYp-infected *V. vinifera* plant material collected from the same vine in different seasons. pAY61. *V. vinifera* collected in October 2011. AYp-infected *V. vinifera* collected in April 2012. Healthy *V. vinifera*. No-template control

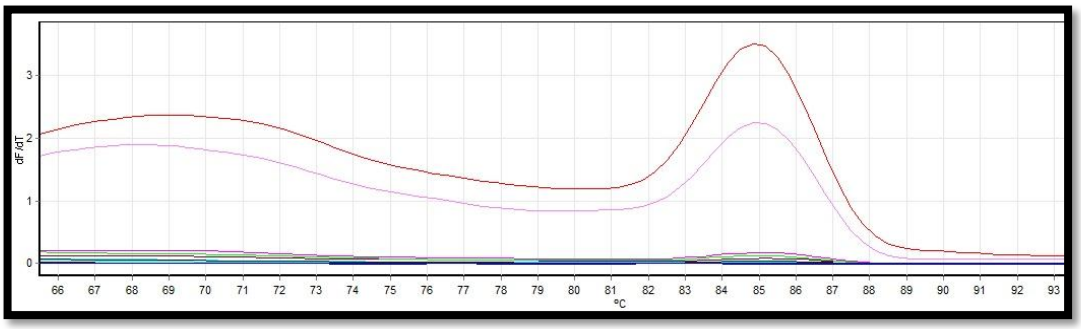

Figure 18: Melt curve of AYp-infected *V. vinifera* plant material collected from the same vine in different seasons. pAY61. *V. vinifera* collected in October 2011. AYp-infected *V. vinifera* collected in April 2012. Healthy *V. vinifera*. No-template control

4.4 Discussion

As we were unable to establish and maintain AYp *in vitro* in grapevine material, we examined the distribution of AYp in five canes of an infected *V. vinifera* cv 'Chardonnay' plant. To our knowledge, this is the first study to report on the spatial distribution of AYp in grapevine material. As can be seen in Table 3 (Section 4.3.1), 10% of the nodes tested had no corresponding leaf material, probably as a result of the uneven bud development in phytoplasma-infected vines (Constable *et al.*, 2003). After leaf and the corresponding node material from five canes were screened by a nested-PCR, it can be concluded that AYp is found predominantly in the nodes (66%) when compared to leaf material in the late season of the year. It is also evident that there is a very slight chance of leaf material showing infection if in the corresponding node no AYp could be detected. From Figure 13 (Section 4.3.1) it is also evident that AYp infection was mostly detected in the upper part of the canes compared to the lower sections. These results coincide with findings in periwinkle plants where AY strains were consistently detected in the expanding shoots of infected plants (Kuske and Kirkpatrick, 1992). In strawberries, clover phyllody phytoplasma (CPp) titre was also highest in the pedicels followed by the sepals, petals and then the leaves (Clark *et al.*, 1983). As a norm, material for micro-propagation and accurate detection of phytoplasmas, should be collected from symptomatic expanding shoots during the growing season (summer).

A qPCR using the SYBR® Green I chemistry was optimized during the current study to detect and quantify the AYp titre in *V. vinifera* cv 'Chardonnay' material. Absolute quantification of AYp DNA was achieved by comparing it with a standard curve of dilutions of a plasmid containing a single copy of the AYp 16S rDNA gene. Samples

collected during the late season that were found to be infected by AYp by the nested-PCR were quantified by the qPCR. C_T values of these samples could not be determined and quantification of AYp titre could therefore not be calculated. It is known that qPCR is highly sensitive to DNA quality and that contaminants such as proteins, phenol/chloroform, salts and EDTA can interfere with amplification and fluorescent detection. Demeke and Jenkins (2010) reported that these PCR inhibitors are a major obstacle for efficient amplification in qPCR. As the DNA collected during the late season showed suboptimal 260/280 ratios when analysed using the Nanodrop® ND-1000 spectrophotometer, it might be possible that quantification on this material was not reliable due to the presence of PCR inhibitors. This could also explain why no C_T values could be determined after absolute quantification on this material. It is therefore of high importance to have pure DNA for the accurate quantification of pathogens by the quantitative real-time PCR.

We were able to observe the spatial distribution of AYp in five canes of an infected *V. vinifera* cv 'Chardonnay' vine through screening leaf and node material by a nested-PCR procedure using universal 16S rDNA primers. Despite the long history of research on AYp, little quantitative information on its epidemiology is known (Madden *et al.,* 1995). During the current study, an assay for quantifying AYp has been optimized and it would be of interest to compare the AYp titre throughout a whole vine, taking different seasons into consideration and also different cultivars of grapevine. Such studies could help in understanding plant-phytoplasma relationships better, help in determining efficient sampling procedures to accurately detect AYp and could help to describe the multiplication and movement of phytoplasmas in their plant hosts.

4.5 References

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Internet resources

Chang Bioscience: http://www.changbioscience.com/genetics/mw

Chapter 5

Antimicrobial peptides and their *in planta* activity against AYp

5.1 Introduction

When a plant comes into contact with a pathogen, it may express a series of peptides, some of which may show antimicrobial activity (Rosenfield *et al.*, 2010). Antimicrobial peptides (AMPs) form part of the plants non-specific defence system and in the case of bacterial pathogens, interact with lipid molecules on the bacterial cell surface causing the membrane to collapse (Yeaman and Yount, 2003; Sitaram and Nagaraj, 1999). Once bound to the bacterial membrane, the peptide activates one of several pathways that will cause cell death to the pathogen (Figure 19).

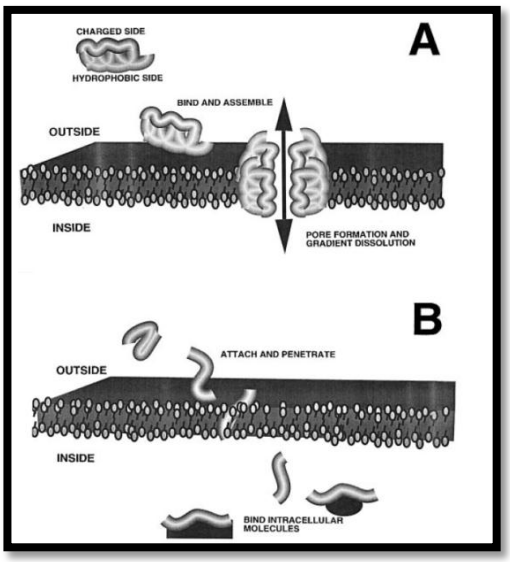

Figure 19: Modes for antimicrobial peptide activity (Gallo and Huttner, 1998). A: AMPs may form pores through which ions leak out, causing the energy gradients to dissipate and leading to cell lysis (Bowman *et al.*, 2003). B: AMPs bind to intracellular targets within the bacterial cell which causes a decrease in protein synthesis, leading to cell death (Park *et al.*, 1998).

Plants are incapable of producing linear amphipathic AMPs. Therefore, synthetic linear AMPs have been produced that are more stable and potent than their native counterparts, without the concomitant toxicity to host cells (Rajasekaran *et al.*, 2001).

As phytoplasmas lack a cell wall, AMPs are considered to be perfect candidates to confer resistance to this phytopathogen. Multiple studies have been done to confer pathogen resistance in transgenic plants overexpressing AMPs. Great success has been seen in

transgenic tobacco plants, where the synthetic peptide CEMA conferred resistance against the highly virulent fungus *Fusarium solani* (Yevtushenko *et al.*, 2005). The peptide Shiva-1 has been expressed in transgenic *Paulownia* which resulted in an improved resistance to witches' broom phytoplasma (Du *et al.*, 2005). In 2001, a US patent (patent number 7119262) by Smith and colleagues described the *in planta* activity of certain peptide classes against phytoplasma in transgenic poinsettia. The generation of transgenic crops is however a very time consuming and expensive technique. Santos-Rosa *et al.* (2008) reported the use of a transient expression system as a reliable and time-effective method for the expression of foreign genes in agricultural crops, including grapevine.

The first AMP used during this study will be a *Vitis vinifera* antimicrobial peptide, namely Vv-AMP1. Vv-AMP1 was isolated from *V. vinifera* berries and is a heat stable peptide encoding 77 amino acids (de Beer and Vivier, 2008). In *V. vinifera*, Vv-AMP1 showed significant activity against the wilting disease-causing pathogens *Fusarium oxysporum* and *Verticillium dahlia*, decreasing fungal growth by 50% (de Beer and Vivier, 2008). Transgenic Vv-AMP1 *V. vinifera* plants infected with *Botrytis cinerea* showed enhanced resistance towards the disease, which confirms that the peptide is both present and active in transgenic plants, and that overexpression of AMPs in transgenic lines may lead to a phenotype with enhanced resistance (Tredoux, 2011). The exact target range of Vv-AMP1 is still unknown, but this peptide does form part of the subgroup B1 of plant defensins, which show activity against both bacterial and fungal pathogens (de Beer and Vivier, 2008). Inducing resistance through grafting has been proven in several studies (Guan *et al.*, 2012; Jenns and Kuć, 1979; Tam and Mitter, 2010). To observe the effect which Vv-AMP1 might have on AYp *in planta*, grafting of Vv-AMP1 transgenic *V. vinifera* cv 'Sultana' and AYp-infected *V. vinifera* cv 'Chardonnay' plants will be tested during the current study.

The second AMP used during this study will be Snakin1 (SN1), an AMP comprising of 63 amino acids that was initially isolated from potato tubers, and that shares motif similarities with disintegrin hemotoxic venoms from various snakes (Segura *et al.*, 1998). SN1 has been shown to confer resistance against the fungus *Rhizoctonia solani* and the bacterium *Erwinia carotovora* by overexpression of the peptide in transgenic potatoes (Almasia *et al.*, 2008). Overexpression of SN1 in transgenic wheat plants also lead to an

enhanced resistance against *Blumeria graminis* f.s.p. *tritici* (Faccio *et al.*, 2011). Kovalskaya and Hammond (2008) reported that functionally active SN1 peptides are suitable for antimicrobial *in vitro* assays, using *Escherichia coli* expression systems. During these assays, SN1 was shown to have activity against the bacterial pathogen *Clavibacter michiganensis* subsp. *sepedonicus,* as well as the fungal pathogens *Clostridium coccoides* and *B. cinerea.* Due to SN1's broad range of activity against fungal and bacterial pathogens, the peptide will be used during this study to test its effect against the grapevine pathogen AYp through transient expression. For that, a grapevine SN1 homologue was identified *in silico* using the BLASTn search tool (www.ncbi.nlm.nih.gov). The identified sequence was then amplified by PCR, cloned and sequenced. To our knowledge, this is the first study to report on the isolation of SN1 from grapevine material.

The final AMP used during this study will be the synthetic peptide D4E1. During transient expression assays to determine the *in planta* effect of D4E1, a clear reduction in pathogen titre could be seen towards the pathogens *Xylophilus ampelinus* and *Agrobacterium vitis* in grapevine (Visser *et al.,* 2012). De Lucca and colleagues (1998) showed that D4E1 inhibits the growth of the mycotoxin-producing fungi *Aspergillus* and *Fusarium*. Transgenic tobacco plants expressing D4E1 demonstrated increased resistance to *Aspergillus flavus, V. dahlia* and *Colletotrichum destructivum* (Cary *et al.,* 2000). In transgenic poplar, D4E1, showed significant reduction in disease symptoms caused by the bacterial pathogens *Agrobacterium tumefaciens* and *Xanthomonas populi* (Mentag *et al.,* 2003).

The current study focuses on boosting the plant's defence mechanism against AYp by overexpressing the AMPs Vv_AMP1, SN1 from potato and grapevine and D4E1 through an *Agrobacterium*-mediated transient expression system.

5.2 Materials and Methods

5.2.1 Candidate antimicrobial peptides

The cloned genes of the AMPs D4E1 and Vv-AMP1 were both available during the current study and have previously been described by Visser (2011). All AMPs used, were brought under control of an enhanced Cauliflower mosaic virus (CaMV)

35S and termination signal in binary vectors, described below. The procedure for obtaining the genomic sequences of the peptides Snakin1 from potato and *V. vinifera* cv 'Chardonnay' material is described below.

5.2.2 Isolation of Snakin1

5.2.2.1 Snakin1 isolation from potato tubers

Potato tubers were ground to a fine powder in liquid nitrogen using a mortar and pestle, and total DNA was extracted according to the manufacturer's protocol using the NucleoSpin® Plant II kit (Macherey-Nagel). DNA was then stored at -20°C until needed for further screening. Primers were designed for the amplification of SN1-Potato using CLC Main Workbench 6, and can be seen in Table 6 below. The sequence information of SN1-Potato was available on the National Center for Biotechnology Information's website (www.ncbi.nlm.nih.gov).

Table 6: Primers used to amplify Snakin1 from potato. Restriction enzyme recognition sequences (underlined) and translation enhancer sequence (bold) are indicated.

Primer name	Sequence	Size
SN1_Pot_s	AGAGCTC**ATCGATTAGGAGATATAACA**ATGAAG TTATTTCTATTAAC	47bp
SN1_Pot_as	ATTTTTGGATCCTTAAGGGCATTTAGACT	29bp

Designed primers included restriction enzyme recognition sequences for cloning purposes, and the forward primer SN1_Pot_s included a translational enhancer sequence (Lütcke *et al.,* 1987). The PCR reaction mix contained 1X KapaTaq buffer A, 1X Cresol, 0.1mM dNTPs, 0.6µM of each primer, and 0.04U/µl KapaTaq DNA polymerase. The final volume was 25µl, of which 1µl was total DNA extracted from the potato tubers. PCR conditions were as follows: 2min at 94°C, 35 cycles of 20sec at 94°C, 30sec at 60°C and 45sec at 72°C, followed by a final elongation step of 10min at 72°C. PCR products were run on a 1% agarose gel for 30min at 120V, and a 770bp amplicon was visible. Using *in silico* analysis, the consensus sequence (EF206290) confirmed that a 770bp fragment containing the entire open reading frame was expected, including an intron sequence of around

500bp (Almasia *et al.*, 2008). Sequencing was performed to verify that SN1 was amplified.

5.2.2.2 Snakin1 isolation from Chardonnay

Healthy *V. vinifera* cv 'Chardonnay' leaf material was collected from a farm near Vredendal, South Africa. The material was ground to a fine powder in liquid nitrogen using a mortar and pestle and total DNA was extracted according to the manufacturer's protocol using the NucleoSpin® Plant II kit (Macherey-Nagel). DNA was stored at -20°C until used for further screening. The sequence for SN1 from *V. vinifera* was obtained by executing a homology search using available SN1 from potato in the BLASTn function on the National Center for Biotechnology Information's website (www.ncbi.nlm.nih.gov) against the grapevine genome and available EST databases. Using the identified SN1 homologous sequence from grapevine, primers were designed using CLC Main Workbench 6 (Table 7) and the sequence was amplified by PCR, as described below.

Table 7: Primers used to amplify Snakin1 from *V. vinifera* cv 'Chardonnay'. Restriction enzyme recognition sequences (underlined) and translational enhancer sequence (bold) are identified.

Primer name	Sequence	Size
SN1_Ch_s	AGAGCTC**ATCGATTAGGAGATATAACA**ATGAAG CCCCTCTTGGCAAC	47bp
SN1_Ch_as	AGGATCCTTAAGGGCACTTGGGTTGG	26bp

The PCR reaction mix contained 1X KapaTaq buffer A, 1X Cresol, 0.1mM dNTPs, 0.6µM of each primer, and 0.04U/µl KapaTaq DNA polymerase. The final volume was 25µl, of which 1µl was total DNA extracted from the Chardonnay leaf material. PCR conditions were as follows: 2min at 94°C, 35 cycles of 20sec at 94°C, 30sec at 60°C and 45sec at 72°C, followed by a final elongation step of 10min at 72°C. PCR products were run on a 1% agarose gel for 30min at 120V. Using *in silico* analysis, a 310bp amplicon excluding an intron was expected.

5.2.3 AMP expression vector constructs

For the transient expression experiments, all AMP sequences were brought under control of a CaMV 35S promoter and terminator signal and subsequently transferred at T-DNA between the right and left border sequences in the available binary vectors pBIN61S (Silhavy *et al.,* 2002) or pCB301 (Xiang *et al.,* 1999). As indicated above, all primers used were designed to contain the restriction enzyme recognition sequences required for the cloning of the fragments into the respective vectors. All expression constructs were electroporated into *A. tumefaciens* as described in Section 5.2.4. The control vector 35S:GUSi (Vaucheret, 1994) was provided by Pere Mestre (Laboratoire de Ge'ne'tique et Ame'lioration de la Vigne, France) and was used to test and optimize foreign gene expression in grapevine and *Catharanthus roseus* leaf tissue.

5.2.3.1 Vv-AMP1 and D4E1 expression vectors

Vv-AMP1 and D4E1 expression vectors were constructed during a previous study by Visser (2011). Briefly, D4E1 and Vv-AMP1 PCR fragments were cloned into pGem®-T Easy (Promega). They were then excised and cloned into the *Bam*HI and *Sac*I sites of the binary vector pBin61S resulting in the vectors pBin61S-D4E1 and pBin61S-Vv-AMP1 (Visser, 2011). Table 8 displays the primers used with their respective restriction enzyme sites.

Table 8: List of primers used during Vv-AMP1 and D4E1 expression vector construction. The restriction enzyme recognition sites (underlined) and translational enhancer sequences (bold) are indicated.

Primer name	Sequence
SacI_35S_D4E1_s	A<u>GAGCTC</u>**ATCGATTAGGAGATATAACA**ATGTTTAAGTTGAGA
BamHI_35S_D4E1_as	A<u>GGATCC</u>TTACAACTTAATCTTAGCTCTCA
SacI_35S_VvAMP1_s	A<u>GAGCTC</u>**ATCGATTAGGAGATATAACA**ATGAGGACCTGTGAGAGT
BamHI_35S_VvAMP1_as	<u>AGGATCC</u>TTAACAATGCTTAGTGCAGAAG

5.2.3.2 SN1-Chardonnay and SN1-Potato expression constructs

SN1 was amplified from *V. vinifera* cv 'Chardonnay' and potato DNA, using the primers described in Sections 5.2.2.1 and 5.2.2.2. The PCR fragments were then cloned into the pGem®-T Easy cloning vector. The

AMP sequence was confirmed by sequencing. From here the PCR fragments were excised using the restriction enzymes *Ecl*136II (Fermentas) and *Bam*HI (Fermentas) and were cloned into the same sites of the p442_pe35Stu_pA cloning vector (Hasan, 2004) between the enhanced CaMV 35S promoter and termination signal, resulting in p442_pe35Stu_pA_SN1-Chardonnay and p442_pe35Stu_pA_SN1-Potato. From both constructs, the AMP-containing 35S expression cassette was cloned into the binary vector pCB301 using the restriction enzymes *Sac*I (Fermentas) and *Pst*I (Fermentas), resulting in pCB_SN1-Chardonnay and pCB_SN1-Potato.

5.2.4 Transformation of *Agrobacterium* cells

Electrocompetent *A. tumefaciens* cells (strain C58C1), containing the helper plasmid pCH32 (Santos-Rosa *et al.,* 2008), were prepared during a previous study according to a protocol by Annamalai and Rao (2006). The already available and newly cloned expression constructs from Sections 5.2.3.1 and 5.2.3.2 were electroporated into *A. tumefaciens* using electroporator (Biorad) settings of 25µF, 100Ω, 1.5kV and 25W.

5.2.5 Agro-infiltration of plants

Recombinant *A. tumefaciens* cells containing the respective AMP or GUSi (β-glucuronidase gene) expression constructs were grown on selective Luria Bertani (LB) agar media (50mg/l kanamycin/ 5mg/l tetracyclin) at 28°C for two days. Cells were then transferred into liquid LB containing selective antibiotics and shaken overnight at 28°C. These cultures were then pelleted by centrifugation at room temperature for 5min at 6000rpm. Once the supernatant was completely removed, pellets were re-suspended in 40ml re-suspension buffer (10mM $MgCl_2$, 10mM MES and 0.1mM acetosyringone) and incubated at room temperature for 2-3 hours.

5.2.5.1 Agro-infiltration of 35:GUSi

Infiltration of healthy *V. vinifera* and *C. roseus* material with the GUSi expression construct was conducted by vacuum-infiltration. Using a scalpel blade, several small cuts were made on the leaves of *in vitro* cultured *V. vinifera* cvs ‘Chardonnay’ and ‘Chenin blanc’ plantlets, and *in vitro* cultured *C. roseus* plantlets. Whole plantlets were then placed into an ultrasonic

chamber (Labotec, SA) for two seconds and were then fully immersed in a cell suspension of *Agrobacterium*, which had an OD_{600} of 0.05. The cell suspension containing the plantlets was placed into the vacuum chamber and three different vacuum procedures were applied twice (30kPa, 50kPa and 90kPa) for 15min, 10min and 2 minutes respectively, to achieve infiltration. The vacuum was quickly released between the two steps. *V. vinifera* material underwent two successive vacuums (50kPa) of 10min each. The vacuum was again quickly released between the two steps. The plantlets were then rinsed in distilled water and transferred into a tissue culture flask containing perlite, and watered with distilled water. After six days of being in controlled incubator conditions (16h light and 8h dark photoperiod at 23°C and 19°C) the GUS assay was conducted on the infiltrated leaves (Section 5.2.6).

5.2.5.2 Agro-infiltration of the AMPs

Agrobacterium-mediated vacuum infiltration with the AMP expression constructs were not carried out in grapevine material as no AYp-infected *V. vinifera* cv 'Chardonnay' material could be established and maintained *in vitro* (Chapter 3). From the three *Nicotiana benthamiana (N. benthamiana)* plants that were successfully infected with AYp (Chapter 3, Section 3.3.2) one infected plant was agro-infiltrated with the AMP expression vectors using a method described by Visser (2011). This plant was chosen on the basis of showing the highest phytoplasma titre after quantitative analysis. A 5ml syringe with a needle was used to aspirate the *Agrobacterium* suspension containing the AMP expression vectors (OD_{600} of 0.5). The needle was then removed and the syringe pressed against the lower surface of the leaf on one side of the main vein. The suspension was slowly injected into the leaves by applying a constant, low pressure. As a control, *Agrobacterium* cells containing an empty binary vector lacking the AMP genes was used on each leaf, on the opposite side of the main vein (Figure 20).

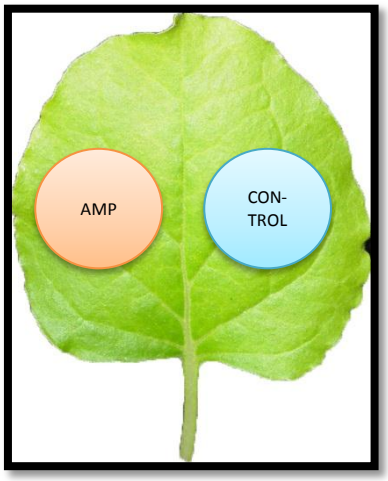

Figure 20: Agro-infiltration on *N. benthamiana* using an AMP expression vector and a control. The AMP expression vector was infiltrated on one side of the main vein and the control vector on the opposite side.

5.2.6 GUS assay

Agro-infiltrated leaf material of *V. vinifera* cvs 'Chardonnay' and 'Chenin blanc' plants together with *C. roseus* plants were detached from the plantlets 6 dpi (days post infiltration) and placed into a 50ml centrifuge tube (Corning® Incorporated, NY). 6ml of GUS substrate buffer (50 mM NaH_2PO_4, 0.5 mM K-Ferrocyamid, 0.5 mM K-Ferricyamid, 0.1% Triton X100, 100 mM Na_2EDTA, pH 7) with freshly added X-Gluc (12.5µl/100ml of buffer) was then added to the tubes containing the plant material. The centrifuge tubes were placed into the vacuum chamber and vacuum was applied twice at 90kPa for 2 minutes to achieve infiltration. The vacuum was quickly released between the two steps. The tubes were then closed and incubated overnight at 37°C. Leaves were decoloured by rinsing in 96% ethanol for an extended period of time, replacing the ethanol regularly. Areas of GUS expression were visually assessed after two days as blue areas on the leaves.

5.2.7 Screening the *in planta* activity of AMPs against AYp in *N. benthamiana*

Multiple leaves on the AYp-infected *N. benthamiana* plant were infiltrated with the Vv-AMP1, D4E1, SN1-Chardonnay and SN1-Potato expression constructs. Infiltrated areas were cut out of the leaf (approximately 2cm in diameter) 6dpi and DNA was extracted according to the manufacturers' protocol using the NucleoSpin® Plant II kit (Macherey-Nagel) and stored at -20°C. The concentration of the DNA was determined by means of the Nanodrop® ND-1000 spectrophotometer. Phytoplasma titres were determined using the qPCR protocol

described in Chapter 4 and were compared between AMP-treated and untreated control plants. All samples were run in triplicate.

5.2.8 Peptide expression

In order to determine if the peptide is expressed in the agro-infiltrated leaf tissue, protein extractions were performed on *N. benthamiana* plants infiltrated with pBin61S_Vv-AMP1, pCB_SN1-Chardonnay and pCB_SN1-Potato. Plants infiltrated with pBin61S served as a negative control. Western blot analysis was used to visualize the expressed peptide. The primary antibody against Vv-AMP1 detection was raised in mice and provided by Abre de Beer (IWBT, Stellenbosch University). Detection of Vv-AMP1 was achieved with an anti-mouse IgG alkaline phosphatase (AP) secondary antibody raised in goats (Sigma-Aldrich®, SA). The expected size of the Vv-AMP1 peptide was ~ 5.5 kDa. Six primary antibodies against SN1 detection were commercially designed (Abmart, China) in mice, using the Abmart monoclonal seal library design kit. Briefly, the provided SN1-Chardonnay amino acid sequence was evaluated for potential antigenic regions. These different regions were identified (Table 9) and short peptide sequences were synthesised and used to immunization of BALB/C mice. After quality control, six antibodies based on three potential antigenic regions were provided. All six antibodies were tested in Western blot experiments. Detection of SN1 was achieved with the same anti-mouse IgG AP secondary antibody raised in goats, used for the detection of Vv-AMP1 (Sigma-Aldrich®, SA). The expected size of the SN1 peptide was ~10kDa.

Table 9: The original SN1-Chardonnay amino acid sequence sent to Abmart for antibody production. The potential antigenic regions used by Abmart (China) to design six primary antibodies for the detection of Snakin 1 are listed below.

Original SN1-Chardonnay amino acid sequence	
MKDRCLKYCGICCEECKCVPSGTYGNKHECPCYKDKKNSKGQPKCP	
Potential antigenic regions	**Antibodies designed per potential antigenic region**
PSGTYGNKHE	1
KDKKNSKGQP	4
EECKCVPSGT	1

No antibody was available for the detection of the D4E1 peptide, therefore no expression tests were performed.

5.2.8.1. Protein extractions

Six days post-infiltration (dpi), 300mg of infiltrated leaf material was cut out using a scalpel blade. This material was ground in 750µl pre-heated (95°C) Berger buffer (750mM Tris-HCL (pH 8.8), 4% SDS, 4% 2-Mercaptoethanol, 40% Saccharose - adapted from Berger *et al.*, 1989) using a mortar and pestle. 500µl of cold Berger buffer was then added to the plant material and transferred to a 2ml reaction tube. All tubes were incubated at 95°C for 10min after which they were centrifuged for 10min at 12 110*g*. The supernatant was then transferred to a clean 1.5ml reaction tube and stored at -20°C until needed.

5.2.8.2 Western blot

The proteins were separated on a 15% (w/v) Tris-tricine gel (Schägger and von Jagow, 1987) together with a low molecular marker (Thermo Scientific, SA, Cat. # 26628). After this the gel was stained using Coomassie Blue R250 dye and de-stained for 48h in 30% methanol and 5% acetic acid, to observe protein separation. As protein concentrations were unknown, 15µl of each protein extract was loaded. A nitrocellulose membrane was then soaked in methanol for 1 minute before the gel was electroblotted to the membrane by soaking the membrane and gel in blotting buffer (25mM Tris, 192 mM Glycin, 20% Methanol, pH 8.3) for 1 hour at 100V. After this, the membrane was left in 5% skim milk overnight to block unspecific bindings. The membrane was then incubated in PBS (8g NaCL, 0.2g KCL, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, made up to 1L, pH 7.4) for 15min and washed in PBS-T (0.05% Tween, 1X PBS) (3 times for 5min each). The membrane was incubated overnight in a 1:500 dilution of primary Vv-AMP1 antibody and 1:200 dilution of primary SN1 antibodies prepared in PBS-T. The following day the membrane was washed for 5min in PBS-T, which was repeated three times. Detection of Vv-AMP1 and SN1 was achieved by incubating the membrane for 1 hour in a 1:10000 dilution of anti-mouse IgG AP antibody prepared in PBS-T. The membrane was washed in PBS-T for 5min and this washing step was repeated three times. An alkaline phosphate (AP) staining solution (20ml AP buffer, 132µl NBT, 66µl BCIP) was added

and after the wanted fragment was seen, the reaction was stopped by washing the membrane in distilled water.

5.2.9 The effect of Vv-AMP1 on AYp through *in vitro* grafting

One Vv-AMP1 transgenic *V. vinifera* cv 'Sultana' plant was obtained from the IWBT in April 2011 and kept under controlled greenhouse conditions. The expression of the transgene was confirmed by Northern blot analysis performed by Martha Tredoux at the IWBT (2011). The plant was propagated *in vitro* following the procedure described in Chapter 3 (Section 3.2.1) and used in grafting experiments using AYp-infected *V. vinifera* cv 'Chardonnay' material. While working under the microscope, a scalpel blade and tweezers were used to cut the apex of the rootstock plant into a longitudinal cleft of 5-10mm. The basal part of a scion of similar size was cut into a wedge and fixed into the recipient plantlet cleft (Figure 21).

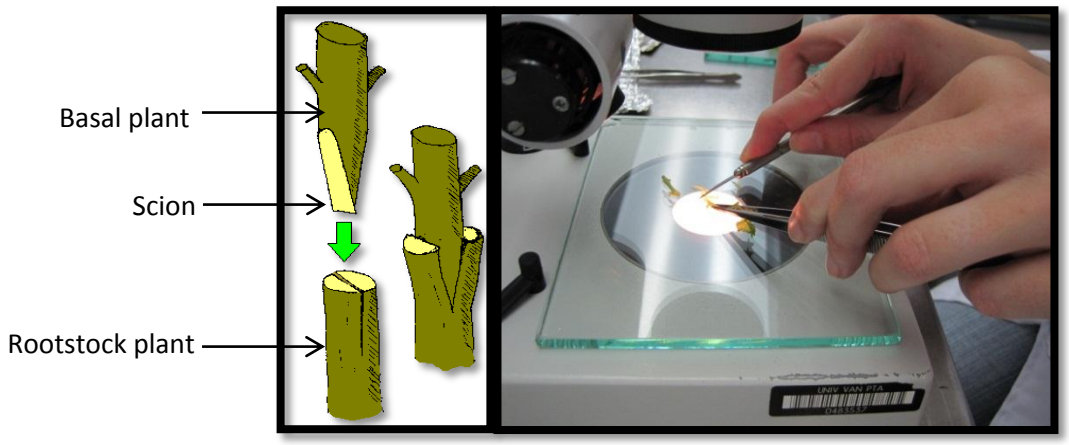

Figure 21: Grafting procedure under the microscope using a scalpel blade and tweezers.

5.3 Results

5.3.1 Snakin1 isolation from potato and grapevine

The 770bp fragment amplified from potato material was consistent with the *in silico* analysis including an intron of approximately 500bp. Sequencing results confirmed a 97% maximum identity for the Solanum tuberosum Snakin-1 (SN1) gene (EF206290). The homologous SN1 sequence from grapevine material was identified, by executing a search using available SN1 from potato in the BLASTn function on the National Center for Biotechnology Information's website (www.ncbi.nlm.nih.gov) against the grapevine genome and available EST

databases. The amplified SN1 from *V. vinifera* cv 'Chardonnay' material was approximately 310bp in size, which was consistent with the *in silico* analysis. Therefore, the wanted SN1 fragments from potato and grapevine were successfully amplified and could be used to construct expression vectors for transient expression assays.

5.3.2 Construction of AMP expression vectors

35S expression vectors containing the foreign genes were constructed in order to conduct AMP *in planta* activity screening. The constructs included vectors for the expression of SN1-Chardonnay and SN1-Potato. Sequencing results confirmed the integrity of the inserted foreign genes. The Vv-AMP1, D4E1 and 35S:GUSi vectors were previously designed by Visser (2011). Figure 22 below depicts the 35S:SN1 expression vectors constructed during the current study.

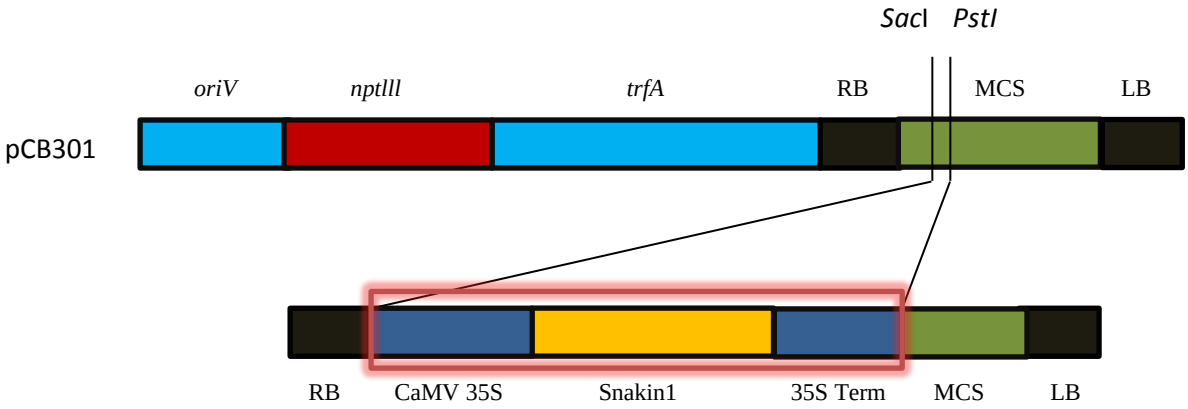

Figure 22: The binary vector pCB301. *oriV*: origin of replication. *nptIII*: neomycin phosphotranferase gene. *trfA*: part of the origin of replication. RB: right border. MCS: multiple cloning site. LB: left border. CaMV 35S: 35S promoter from cauliflower mosaic virus. Snakin1: Snakin1-Chardonnay or Snakin1-Potato. 35S Term: 35S termination signal. The AMP-containing 35S expression cassette (outlined in red) was cloned into the pCB301 binary vector using the restriction enzymes *Sac*I and *Pst*I (indicated in the MCS of pCB301).

5.3.3 GUS expression in *V. vinifera* and *C. roseus*

To evaluate foreign gene expression in leaf tissues and to optimize the transient expression procedure, both grapevine and *C. roseus* plants were subjected to a GUS expression assay after infiltration with the marker gene 35S:GUSi. An empty pBin61S vector was used as the negative control. Foreign gene expression in *N. benthamiana* leaf material was evaluated during a previous study (Visser, 2011) and was thus not repeated during the current study. As a result of GUS expression, blue coloration could be seen in the infiltrated leaves (Figure 23 and 24)

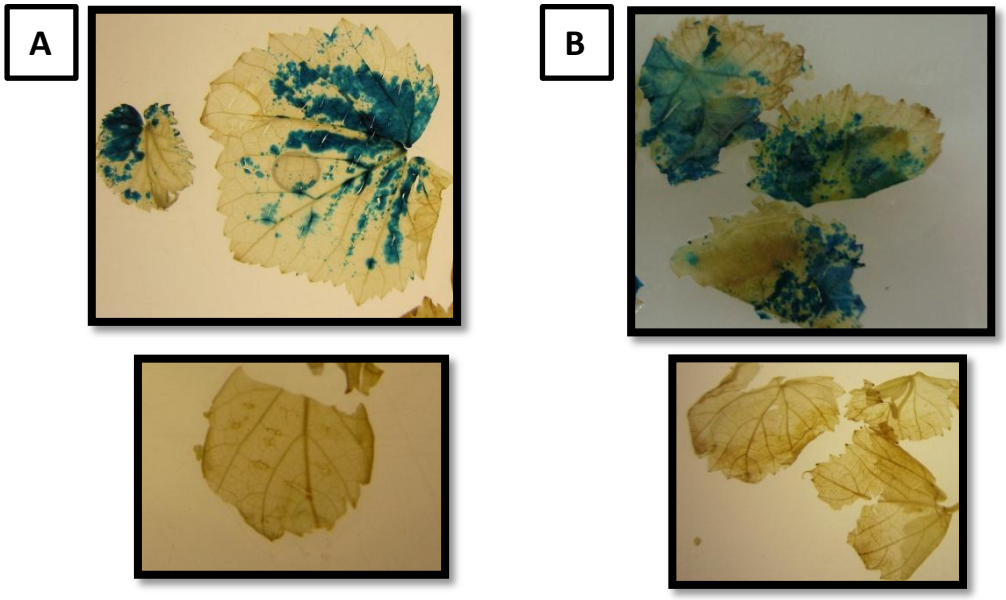

Figure 23: GUS expression observed in *V. vinifera* cvs 'Chardonnay' and 'Chenin blanc' leaf material. A: *V. vinifera* cv 'Chenin blanc' leaves infiltrated with 35:GUSi 6dpi (top) and the negative control (bottom). B: *V. vinifera* cv 'Chardonnay' leaf material leaves infiltrated with 35:GUSi 6dpi (top) and the negative control (bottom).

An estimated GUS expression of 60-70% was seen in infiltrated *V. vinifera* cv 'Chenin blanc' leaves, whereas the percentage of GUS expression in *V. vinifera* cv 'Chardonnay' material was estimated to be 40-50%. In both grapevine cultivars, GUS expression was most prominent around cut sites, but also visible throughout the whole leaf. This was similar to previous observations by Visser (2011). In *C. roseus* infiltrated tissue, only small spots of GUS staining were detected around the cutting sites in spite of three different vacuum procedures used (Figure 24). GUS staining was barely visible in leaves placed under vacuum at 90kPa for 2min (Figure 24C), whereas leaves placed under vacuum at 30kPa for 15min showed similar GUS expression to leaves placed under vacuum at 50kPa for 10min (Figures 24A and 24B). Light blue coloration between cutting sites are most probably due to diffusion of the GUS staining solution and do not represent zones of real transient expression. Leaves of the negative control plants showed no blue areas of GUS expression (Figure 24D).

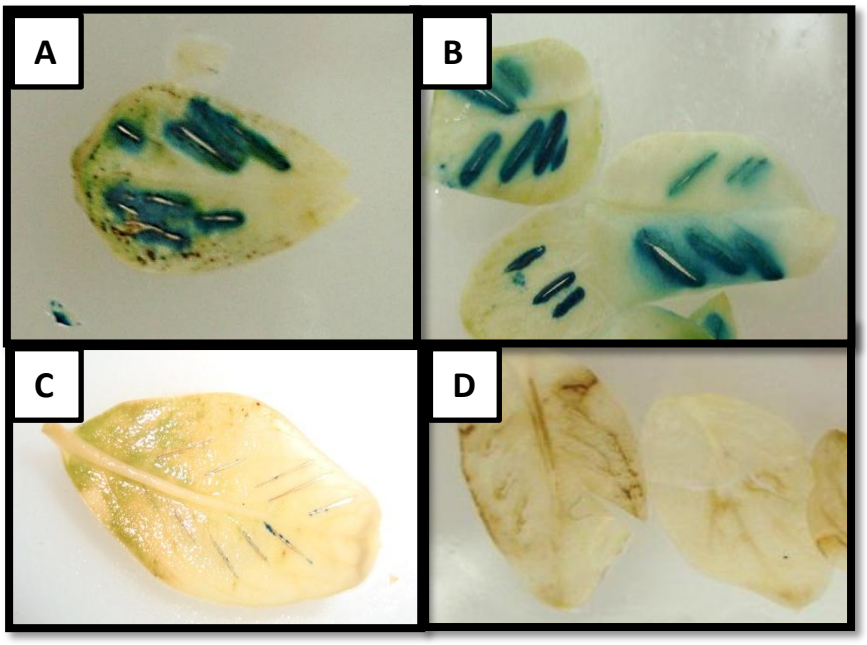

Figure 24: GUS expression observed in *C. roseus* leaf material. A: *C. roseus* leaves infiltrated with 35:GUSi 6dpi at 30kPa for 15min. B: *C. roseus* leaves infiltrated with 35:GUSi 6dpi at 50kPa for 10min. C: *C. roseus* leaves infiltrated with 35:GUSi 6dpi at 90kPa for 2min. D: Negative control

5.3.4 *In planta* activity of AMPs against AYp

A 35S transient expression system was used to express the AMPs D4E1, Vv-AMP1, SN1-Chardonnay and SN1-Potato in AYp-infected *N. benthamiana* plants. After extraction of DNA from agro-infiltrated areas, the AYp titre was determined by qPCR (as described in Chapter 4) to examine the inhibitory effect of the AMPs on this pathogen. The *in planta* effect of AMPs was not screened against AYp infection in *V. vinifera* cv 'Chardonnay' material as no infected material could be established and maintained *in vitro* (as described in chapter 3). Real-time PCR protocols were optimised for the detection and quantification of AYp using a SYBR Green-based system (Chapter 4, Section 4.2.3). Up to nine leaves per AMP treatment were tested for AYp titre and compared to the control infiltrations on the same leaf (Table 10).

Table 10: Antimicrobial peptides used for the transient expression in an AYp-infected *N. benthamiana* plant. AY titres were detected and analysed by quantitative PCR.

AMP used for transient expression	Nr of leaves infiltrated
SN1-Chardonnay	9
SN1-Potato	5
D4E1	8
Vv_AMP1	5

pAY61 was used as the standard control and *N. benthamiana* leaf material collected before infiltration served as a positive control. Table 11 below lists the C_T values obtained from the amplification run by means of the Rotor Gene Software Series 1.7, for each sample infiltrated with the respective AMP and the control. The nine leaves treated with SN1-Chardonnay did not show a significant reduction in AYp titre when compared to the control samples (p-value = 0.25). Five leaves infiltrated with SN1-Potato and Vv-AMP1 also showed no significant reduction in AYp titre when compared to the control groups (p-value = 0.48 and p-value = 0.29 respectively). The remaining eight leaves infiltrated with D4E1 and the control construct showed similar results, with a p-value of 0.40.

As DNA extracted from both the control – and AMP-infiltrated areas showed a decrease in phytoplasma titre with no significant difference (p-value > 0.05), we were unable to reliably determine if the transient expression of AMPs induces resistance to AYp infection.

Table 11: C_T values obtained from qPCR profiles of *N. benthamiana* plants infected with AYp that were treated with Vv-AMP1, D4E1, SN1-Chardonnay and SN1-Potato and the untreated control plants. The statistical differences between the two treatment groups are shown by the p-value. C_T values obtained from AMP infiltrated leaf areas are represented as the GOI.

Samples	GOI	Control	p-value
SN1-Chardonnay 1	40.55	39.84	
SN1-Chardonnay 2	39.98	41.37	
SN1-Chardonnay 3	41.33	41.50	
SN1-Chardonnay 4	40.39	39.70	
SN1-Chardonnay 5	40.36	42.10	0.25
SN1-Chardonnay 6	39.76	38.57	
SN1-Chardonnay 7	41.76	41.34	
SN1-Chardonnay 8	42.89	41.35	
SN1-Chardonnay 9	39.99	38.67	
SN1-Potato 1	42.78	42.65	
SN1-Potato 2	41.99	41.96	
SN1-Potato 3	42.10	42.21	0.48
SN1-Potato 4	43.26	42.34	
SN1-Potato 5	40.91	38.71	
Vv-AMP1 1	42.71	42.12	
Vv-AMP1 2	42.95	41.66	
Vv-AMP1 3	41.35	41.69	0.29
Vv-AMP1 4	37.97	38.76	
Vv-AMP1 5	40.06	40.89	
D4E1 1	39.68	39.99	
D4E1 2	39.16	39.58	
D4E1 3	39.60	41.00	
D4E1 4	41.99	40.02	
D4E1 5	40.97	39.66	0.40
D4E1 6	41.13	43.62	
D4E1 7	43.71	42.98	
D4E1 8	41.05	42.57	

5.3.5 Peptide expression

Crude protein extractions of leaf material infiltrated with pBin61S_Vv-AMP1, pCB_SN1_Chardonnay, pCB_SN1_Potato and pBin61S were separated on a SDS-PAGE and stained overnight with Coomassie Blue R250 (Figure 25). After de-staining for two days, protein separation was visible on the gel. In Figure 25 below, no protein separation can be seen below the 25kDa size marker (Lane 1). This gives

an indication that smaller sized proteins may have been lost during the crude protein extraction performed on infiltrated leaf material. Furthermore, gel conditions might not have been optimal for the separation of smaller sized proteins.

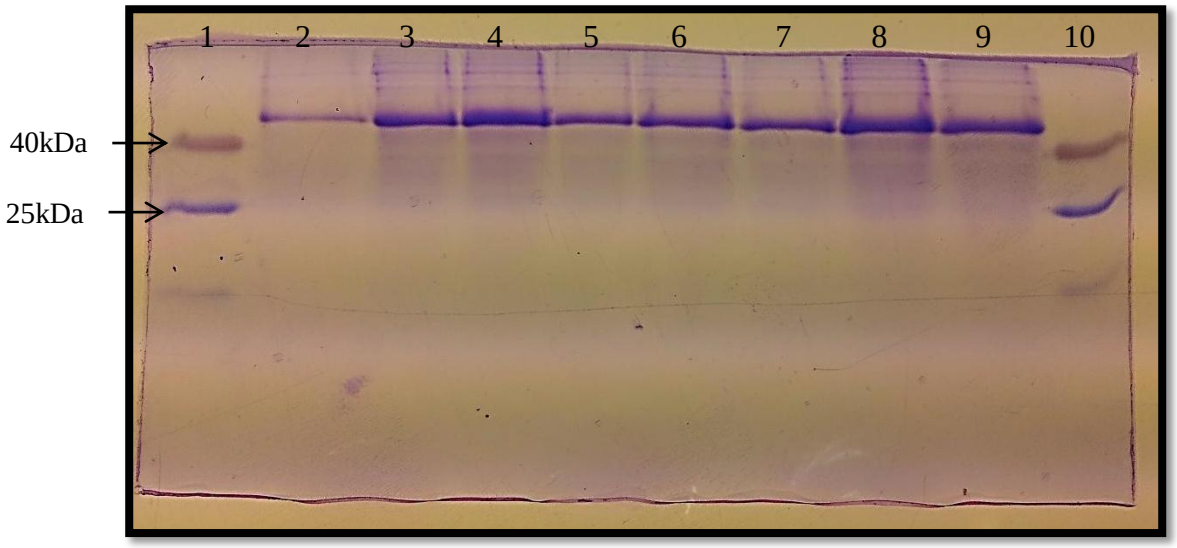

Figure 25: SDS-PAGE stained with Coomassie blue. Lanes 1+10: Low weight molecular marker. Lanes 2-4: *N. benthamiana* leaf material infiltrated with Vv-AMP1. Lanes 5+6: *N. benthamiana* leaf material infiltrated with SN1-Chardonnay. Lanes 7+8: *N. benthamiana* leaf material infiltrated with SN1-Potato. Lane 9: *N. benthamiana* leaf material infiltrated with the control construct pBin61S.

Western blot analysis was performed using the same protein extractions to test for the expression of Vv-AMP1, SN1-Chardonnay and SN1-Potato at 6dpi. The vector pBin61S served as the negative control (Figure 26). As no antibody was available for D4E1, no blots could be performed to test its expression in *N. benthamiana* leaf material.

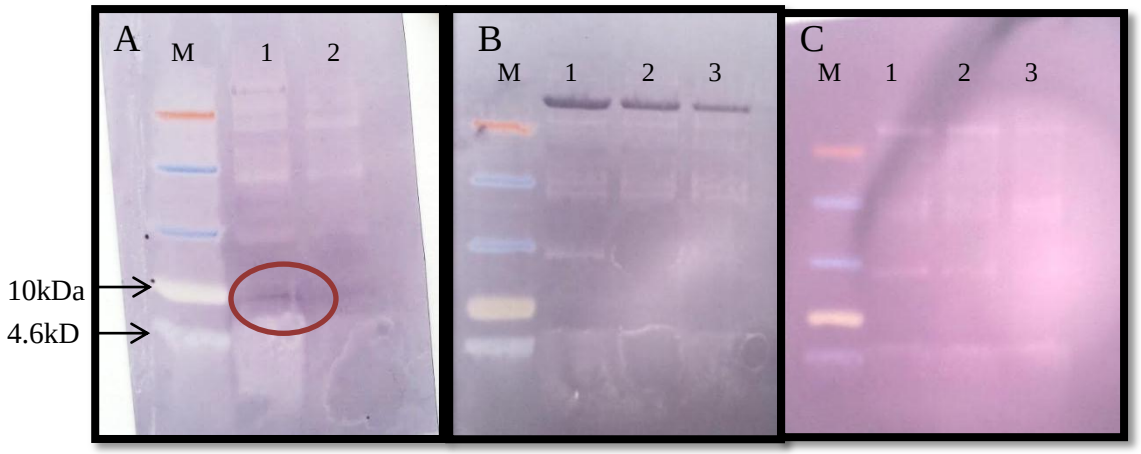

Figure 26: Western blot results for Vv-AMP1, SN1-Chardonnay and SN1-Potato expression in *N. benthamiana* plants. (A) M: Low weight molecular marker. Lane 1: Vv-AMP1 expression in infiltrated *N. benthamiana* leaves. Lane 2: Control infiltration using pBin61S. (B) M: Low weight molecular marker. Lane 1: SN1-Chardonnay expression in infiltrated *N. benthamiana* leaves. Lane 2: SN1-Potato expression in infiltrated *N. benthamiana* leaves. Lane 3: Control infiltration using pBin61S. The four antibodies designed to recognize the antigen region KDKKNSKGQP, displayed these results. (C): M: Low weight molecular marker. Lane 1: SN1-Chardonnay expression in infiltrated *N. benthamiana* leaves. Lane 2: SN1-Potato expression in infiltrated *N. benthamiana* leaves. Lane 3: Control infiltration using pBin61S. The two antibodies designed to recognize the antigen regions PSGTYGNKHE and EECKCVPSGT, displayed these results.

Expression of Vv-AMP1 (~5.5kDa) can be seen in Figure 26A (circled in red). The expected size of SN1 is ~10kDa. The four SN1 antibodies designed for the potential antigen region KDKKNSKGQP all bound to a fragment bigger than 40kDa. As these results were the same amongst the four antibodies, only one image is shown (Figure 26B). This was seen in *N. benthamiana* material infiltrated with SN1-Chardonnay and SN1-Potato and in the control infiltrations. Therefore, no significant difference could be observed between the AMP treated plant material compared to the control infiltrated material. The two SN1 antibodies designed to recognize the potential antigen regions PSGTYGNKHE and EECKCVPSGT, displayed similar patterns of antibody binding and failed in detecting peptide expression in infiltrated *N. benthamiana* material. These results can be seen in Figure 26C (Only one image is shown for both antibodies as the results were the same).

5.3.6 The effect of Vv-AMP1 on AYp through *in vitro* grafting

Compatibility to graft *V. vinifera* cvs 'Chardonnay', 'Sultana' and 'Chenin blanc' has been tested and the procedure has been optimized (Figure 27, the graft site is circled in blue).

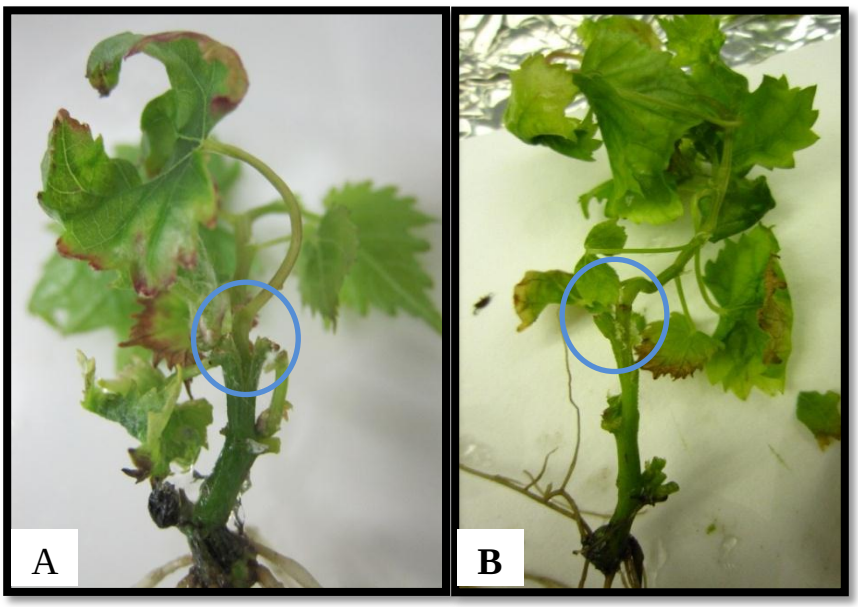

Figure 27: Chenin blanc and Chardonnay shoots grafted onto sterile Vv-AMP1 transgenic Sultana. A: Healthy Chenin blanc grafted onto Vv-AMP1 transgenic Sultana. B: Healthy Chardonnay grafted onto Vv-AMP1 transgenic Sultana

After 4 weeks of being kept in controlled incubator conditions, grafts were tested by trying to pull the two canes apart at the graft site. *V. vinifera* cvs 'Chenin blanc' and 'Chardonnay' scions were strongly attached to the transgenic rootstock and produced new buds after 4 weeks of incubation. Fifty *V. vinifera* cv 'Sultana' X 'Chenin blanc' plants were grafted with a success rate of 22% (11 out of 50 successful grafts). Fifty-one *V. vinifera* cv 'Sultana' X 'Chardonnay' plants were grafted with a success rate of 25.5% (13 out of 51 successful grafts). As no AYp-infected *V. vinifera* cv 'Chardonnay' material could be established and maintained *in vitro*, we were unable to test if grafted Vv-AMP1 transgenic rootstock material could have an effect on AYp-infected scion material.

5.4 Discussion

The current chapter determined the *in planta* efficacy of four AMPs against the grapevine pathogen AYp using an *Agrobacterium*-mediated transient expression system. For this purpose, four 35S vectors expressing Vv-AMP1, D4E1, SN1-Chardonnay and SN1-Potato, respectively, were successfully constructed and confirmed by sequencing.

To confirm the expression of Vv-AMP1 and SN1 in the transient expression system, western blots were performed on crude protein extracts from agro-infiltrated *N. benthamiana* leaves. As no antibody was available for the detection of D4E1, no expression confirmation was done in the current study. Western blot analysis carried out showed faint expression of Vv-AMP1 in *N. benthamiana* material. De Beer (2008) was unable to detect Vv-AMP1 expression in Vv-AMP1 transgenic *N. benthamiana*, even after enriching for cationic peptides from crude leaf extracts. *N. benthamiana* plants are known to express a peptide highly homologous to Vv-AMP1, resulting in the down-regulation of both these peptides. This may result in the Vv-AMP1 concentration being too low for optimal Western blot detection. Another model plant should therefore be considered for future studies to determine the expression of Vv-AMP1. Moreover, peptide expression could potentially be boosted by adding a plant viral suppressor of RNA silencing in future agroinfiltration experiments (Stephan *et al*., 2011). Because of high costs for antibody production, only antibodies raised against SN1-Chardonnay were produced by a commercial company. The six antibodies which were designed based on three potential antigenic regions (Table 9) did not detect SN1-Chardonnay or SN1-Potato in infiltrated *N. benthamiana* material. The antibody based on the selected potential antigenic sequence KDKKNSKGQP, detected protein products larger than 40kDa in SN1-Chardonnay and SN1-Potato agro-infiltrated leaf tissues. These results were, however, also observed in the control infiltrations and were thus not of significance. The low concentration of the extracted proteins may have caused the expressed SN1-Chardonnay concentration to be too low for Western blot detection. Even if the expression of SN1-Chardonnay, SN1-Potato, VvAMP1 and D4E1 was not conclusively confirmed, all AMP containing expression constructs were still tested in the transient expression assay for their efficiency against AYp.

To test the efficacy of the AMPs, the efficiency of the transient expression system was determined in *V. vinifera* and *C. roseus* plants using the 35S:GUSi construct. GUS expression was observed in both *V. vinifera* cvs 'Chenin blanc' and 'Chardonnay' leaf material. The results for GUS expression in grapevine material obtained during this study, were similar to previous observations by Santos-Rosa *et al.* (2008) and Visser (2011) using the same 35S expression vector. GUS expression in *N. benthamiana* leaf material is known to show more prominent expression throughout the agro-infiltrated leaf areas, when compared to GUS expression in grapevine material (Visser, 2011). As *C. roseus* is known to maintain phytoplasma infections under *in vitro* conditions (Ćurkovic-Perica and Ježić, 2010; Ćurkovic Perica and Šeruga Musić, 2005) this plant host was tested for its suitability in the *Agrobacterium*-mediated transient expression system. This was done by using the GUS-marker gene. In infiltrated *C. roseus* leaf material, GUS expression was limited to tissue directly at the cutting sites, even after increasing the vacuum to 30min at 30kPa. Therefore, the applied infiltration procedure on *C. roseus* material was not optimal for the transient expression of AMPs. The leaf morphology of *C. roseus* might also not be favourable for infiltration of *Agrobacterium*. Additionally, efficiency of transient expression in specific plant species is largely dependent on virulence factors carried by the respective *A. tumefaciens* strain (Santos-Rosa *et al.,* 2008). Therefore, future studies should test different *Agrobacterium* strains for their efficiency in transient gene expression of *C. roseus* leaf material.

This study aimed to test the effect of selected AMPs against AYp-infected *in vitro* grapevine plantlets, by using a transient expression system. It was earlier shown that transient gene expression was more consistent using *in vitro*-grown plants compared to greenhouse-grown plants (Santos-Rosa *et al.,* 2008). Nevertheless, AYp-infected *V, vinifera* cv 'Chardonnay' material could not be established and maintained *in vitro* during this study (discussed in Chapter 3). Therefore, the alternative AYp host plant species *N. benthamiana* was used to test the effect of AMPs against AYp.

The *in planta* activity of Vv-AMP1, SN1-Chardonnay, SN1-Potato and D4E1 against AYp was tested in infected *N. benthamiana* material. A qPCR procedure was used to determine the difference in phytoplasma titres between two treatment groups, one expressing an AMP by a 35S expression vector, and the other infiltrated with an empty 35S expression vector. The result of the transient expression assay showed a decrease in phytoplasma titre in all AMP treatment groups. As the control infiltrations showed a

similar decrease of AYp titre in infiltrated material, no significant AYp titre reduction could be seen when compared to AMP infiltrated material. Initially the reduction in phytoplasma titre throughout the plant was believed to have resulted from stress caused by agro-infiltration. Recent studies have however shown that *Agrobacterium* sp. infection of plants, leads to an increase of indole-3-acetic acid (IAA) concentration in plant material (Bulgari *et al.*, 2012). This exogenous application of IAA on phytoplasma-infected periwinkle plants induced symptom remission, or completely eliminated the pathogen from the plant (Ćurković Perica, 2008). Due to the presence of *A. tumefaciens*, IAA concentrations might have increased in the AYp-infected host plant during the current study, leading to a decrease of phytoplasma titre throughout this plant. This may explain why a decrease in AYp titre was detected in both the AMP treatment groups and the control groups. Therefore, the transient expression method used during this study is not reliable, as no significant differences could be observed in AYp titre between AMP infiltrated material and material infiltrated with the control construct.

Lastly, this chapter focused on grafting AYp-infected *V. vinifera* cv 'Chardonnay' material onto Vv-AMP1 transgenic plant material, to observe the effect Vv-AMP1 may have on AYp. Potentially, Vv-AMP1 produced by the transgenic rootstock moves across the graft junction and could be able to confer resistance in the AYp-infected scions. Inducing resistance through grafting has been proven in several studies (Guan *et al.*, 2012; Jenns and Kuć, 1979; Tam and Mitter, 2010). As we were unable to establish and maintain AYp-infected grapevine material *in vitro*, this method of induction could not be tested. Once AYp-infected grapevine material can be maintained *in vitro*, future research should focus on the effect that Vv-AMP1 transgenic grapevine may have on AYp through *in vitro* grafting.

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Internet resources

National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/

Chapter 6
General conclusion

In this study we validated the use of transient expression systems which express antimicrobial peptides (AMPs), to study the *in planta* effect of these peptides against the grapevine pathogen aster yellows phytoplasma (AYp). We focused on four AMPs: Vv-AMP1, D4E1, SN1 isolated from potato and SN1 isolated from grapevine. The *Agrobacterium*-mediated expression system used during the current study, was successfully used for the *in planta* expression of D4E1 against *Agrobacterium vitis* and *Xylophilus ampelinus,* resulting in a reduction of both bacterial titres (Visser *et al.,* 2012).

In the present work, no AYp-infected *Vitis vinifera* cv 'Chardonnay' material could be established by micro-propagation, starting from AYp-infected vineyard-growing plants as source material. Difficulties to establish and maintain phytoplasma infections in micro-propagated material has been observed in *V. vinifera* infected with flavescence dorée, in mulberry plants infected with mulberry dwarfism phytoplasma, in sugarcane infected with sugarcane yellows phytoplasma and in Lebanese almonds infected with '*Candidatus* P. phoenicium' (Gribaudo *et al.,* 2007; Caudwell, 1961; Dai *et al.,* 1997; Parmessur *et al.,* 2002; Chalak *et al.,* 2005). It is however no problem to maintain phytoplasmas through micro-propagation in paulownia, key lime and apple plants, and success has also been observed in *V. vinifera* infected with bois noir (Gribaudo *et al.,* 2007; Shekari *et al.,* 2011; Jarausch *et al.,* 1996; Wang *et al.,* 1994). Factors involved in the natural recovery of phytoplasma-infected plants are not fully understood yet, although it seems reasonable that the interactions between the pathogen, the host and the environment may play a key role, as well as the involvement of grapevine bacterial or fungal endophytes (Musetti *et al.,* 2007; Bulgari *et al.,* 2009). As no AYp-infected *V. vinifera* cv 'Chardonnay' material could be established and maintained *in vitro,* the question arose whether plant material might have been taken from a part of the cane where phytoplasma titre was very low or totally absent. Therefore, the spatial distribution of AYp in five canes of an infected *V. vinifera* cv 'Chardonnay' plant was investigated. Aster yellows phytoplasma was found predominantly in the nodes when compared to leaf material collected in the late season. Lastly, AYp infection was mostly detected in the upper, expanding parts of the cane when compared to lower sections. Further analysis on a bigger cohort of plants is needed to fully understand the spatial distribution of AYp throughout

grapevine, taking different seasons and cultivars into consideration. Transmission experiments using the insect vector *Mgenia fuscovaria* were successful in establishing AYp-infected *Nicotiana benthamiana* and *Catharanthus roseus* plants. A quantitative real-time PCR assay, using SYBR-Green® I chemistry, was optimized during the current study for the quantification of AYp. When the *in planta* effects of the four AMPs were screened by the qPCR, a significant reduction of AYp titre was observed when compared to the positive control. This reduction in pathogen titre was also observed in the control treatment group. Therefore, no significant AYp titre differences could be seen in the AMP treatment group when compared to the control treatments. It is known that bacterial endophytes have an influence on the natural recovery of phytoplasma-infected plant hosts. A recent study has shown that infection by *Agrobacterium* sp. can increase the indole-3-acetic acid (IAA) concentration in AYp-infected plant material (Bulgari *et al.*, 2012). This increase in IAA is known to decrease the phytoplasma concentration in plants and possibly explains why a decrease in AYp titre for both treatment groups was observed. These results show that the *Agrobacterium*-mediated transient expression assay used during the current study, was possibly the wrong choice for the *in planta* screening of AMPs against the grapevine pathogen AYp.

Phytoplasmas lack an outer membrane and cell wall, making this pathogen an ideal target for AMPs. Developing alternate transient expression systems to reliably determine the effect of AMPs on AYp is therefore of great importance in future studies. The use of transient expression systems has the potential to play an important role in future disease resistant studies and in the improvement of grapevine, which is an economically important crop worldwide. To our knowledge, this study is the first to report on the distribution of AYp in infected grapevine material and serves as a pilot study for future research. Such studies could help in understanding plant-phytoplasma relationships better and help in determining efficient sampling procedures for accurate diagnostics.

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