Application of tissue culture and molecular techniques in disease resistance breeding of grapevine

By

Sithandiwe Buhlebemvelo Desiree Shange

Thesis submitted in fulfilment of the requirements for the degree

Master of Technology: Agriculture

In the
Faculty of Applied Sciences

At the
Cape Peninsula University of Technology

Supervisor: Prof F.B. Lewu
Co-supervisor: Dr Y. Petersen & Ms P. Burger

Wellington
March 2021

CPUT copyright

The thesis may not be published either in part (in scholarly, scientific or technical journals), or as a whole (as a monograph), unless permission has been obtained from the University.

Declaration

I, Sithandiwe Shange, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

SBD Shange	**November 2020**
Signed	**Date**

Abstract

Grapevines (*Vitis vinifera* Linnaeus.) are susceptible to a number of diseases which lead to reduced yields and shortened lifespans of vines. This study concerned two important diseases of grapevine, namely bacterial blight caused by *Xylophilus ampelinus* and powdery mildew caused by *Erysiphe necator.* The study had two aims, firstly it aimed to develop an *in vitro* phenotypic screening method for resistance to bacterial blight of grapevine using three varieties, namely Dauphine, Redglobe and C-3229, which is a cross between G4-682 (a breeding line) and Regal Seedless, to reduce the time required for disease resistance screening. Secondly, the study aimed at selecting individuals raised from self-pollinated Kishmish Vatkana (KV) which are homozygous for *Ren1*. *Ren1* is a single, dominant gene conferring resistance to powdery mildew (PM) and is heterozygous in KV. These selected homozygous donors of *Ren1* will ensure the transfer of resistance to powdery mildew to all progeny derived from these lines.

Grape plants of two cultivars, Dauphine and Redglobe known to be tolerant and susceptible respectively, to bacterial blight were inoculated with VS20 isolate of *X. ampelinus* (1 x 10^8 CFU/ml) using scalpel and needle pricking methods for screening. The results revealed that both inoculation methods were successful in discerning resistance to grapevine bacterial blight. Both methods caused plants to develop necrosis at the point of inoculation (IP). However, the needle pricking method showed disease progression over time became worse as necrosis progressed to other parts of the plant and eventually resulted in wilting of plants. On the basis of these results, the needle pricking method was selected to assess C3229, a selection whose resistance reaction was unknown at the start of the study. C3229, like Redglobe, showed susceptibility to bacterial blight. Efficacy of the inoculation was verified with nested PCR analysis. Analysis of macerated samples inoculated with the bacteria confirmed the presence of *X. ampelinus* in the inoculation point and in areas away from the IP for all three varieties.

This indicates some useful traits in Dauphine that have been linked with tolerance to bacterial blight and can be considered as basis for breeding tolerant varieties. The second part of the study involved developing homozygous *Ren1* donors for breeding

through self-pollinating KV. The population comprised 36 offspring. Genomic DNA from the leaves of the offspring were screened using SSR markers (UDV020, UDV124, VMC9h4.2, VMCNg4e10.1 and VVIP10) to identify offspring homozygous for the *Ren1* gene. Among the 36 offspring, four were found to be off types, in other words, they were not from the self-pollinated KV, and rather KV crossed with another cultivar. Some individuals, 18 in total, were similar to the parent since they were heterozygous for *Ren1,* while five were found to be homozygous, for the recessive alleles. The latter individuals, although homozygous are excluded from the breeding programme as they will be susceptible to powdery mildew. Ten offspring were found to be homozygous for the *Ren1* gene. These individuals were identified as suitable parents for breeding. These individuals will be planted out, thereafter, the plants showing the best fruits for breeding will be selected.

Keywords: Bacterial blight, *Erysiphe necator*, Grape breeding, Powdery mildew, *Ren1, Xylophilus ampel*

Acknowledgments

Firstly, I would like to thank God for giving me grace and good health throughout the study period. I would like to express my special thanks of gratitude my supervisors Prof. Francis Lewu, Dr. Yolanda Petersen and Ms Phyllis Burger for their guidance throughout this study. My sincere gratitude to Prof. Lewu for seeing the potential in me and opening doors and offering me the opportunity to come and study at CPUT. Am also grateful for the encouragement and gentle correction. To Dr. Petersen and Phyllis Burger, thank you for making all plant material available for study. Thank you for your valuable support and patience throughout this study and writing period. Your ability to craft my muddled thoughts into scientific prose is amazing. To Dr. Nompumelelo Nyembe, thanks for helping me through the research journey from my baby step to where I am now. I could not have done it without you sis! I am also grateful for the financial support received from the ARC and for providing the work space and all necessary equipment. I would like to thank the Technology and Human Resources for Industry Programme that covered different aspects of the study. To the lab members at Cengen lab in Woester, thank you for training and kindly helping with sample preparation and genotypic scoring. To my wonderful colleagues at Crop Development both in tissue culture lab and molecular biology lab – thank you all for the support, encouragement, readiness to help and concern. My sincere thanks to Christelle Cronje for her help and support since I started my journey in my studies. I am also deeply grateful to my daughter Mvelo for her support, encouragement and understanding as she had to miss out on mom – daughter quality time because I had to be in the lab. To my family, mom, dad, my brother and sisters, thank you for your support and prayers over the years.

Lastly, I would like to thank my spiritual family for their continued prayers and anyone who supported me during this study period.

Table of Contents

Declaration i

Abstract ii

Acknowledgments iv

List of Figures viii

LIST OF TABLES xi

LIST OF ABREVIATIONS xii

Chapter 1: Introduction 1

1.1. Introduction **1**

Chapter 2: Literature review 4

2.1. Importance of grapevine in agriculture **4**

2.2. Diseases affecting grapevine (*Vitis vinifera* L.) **5**

2.2.1 Bacterial blight 5

2.2.1.1 The causal agent 5

2.2.1.2 Symptoms, spread and distribution 6

2.2.1.3. Control measures 7

2.2.1.4 Bacterial detection and screening tests 8

2.3 Powdery mildew (PM) **9**

2.3.1 Causal agent 9

2.3.2 Symptoms 10

2.3.3 Control measures 11

2.4 Origin and characteristics of grape varieties **12**

2.4.1 Dauphine 12

2.4.2 Redglobe 12

2.4.3 C-3229 13

2.4.4 Kishmish Vatkana 14

2.5 Breeding for disease resistance **16**

2.6 Embryo Rescue **19**

2.7 Marker assisted selection **21**

2.8 Aims and objectives **22**

Chapter 3: Materials and Methods 23

3. 1 The establishment of an *in vitro* screening method for evaluating the resistance of grapevine cultivars to *Xylophilus ampelinus* **23**

3.1.1 Micropropagation of plants and growth conditions 23

3.1.2 Bacteria and growth conditions 24

3.1.3 Optimisation of in vitro plant inoculation method using Dauphine and Redglobe 24

3.1.3.1 Inoculation of plantlets with X. ampelinus using the scalpel method 25

3.1.3.2 Inoculation of plantlets with X. ampelinus using the needle pricking method..26
3.1.3.3 Disease scoring for bacterial blight symptoms on grapevine....26
3.1.3.4 Screening of C-3229 for resistance to Xylophilus ampelinus....27
3.1.4 Detection of Xylophilus ampelinus in inoculated plants....28
3.1.4.1 Re-isolation of Xylophilus ampelinus from inoculated plants....28
3.1.4.2 Detection of bacterial presence in plant material via nested PCR....29
3.1.4.3 Identification of candidate X. ampelinus colonies by specific PCR....31
3.1.4.4 Gel Electrophoresis....31
3.1.4.5 X. ampelinus genomic DNA isolation....31
3.1.5 Data analysis for disease phenotyping....32
3.2 Screening Kishmish Vatkana seedlings with molecular markers linked to powdery mildew resistance gene, *Ren1*....**32**
3.2.1 Origin of plant material....32
3.2.2 Embryo rescue and plant development....33
3.2.2.1 Transfer and acclimatisation of embryo rescue derived offspring to glasshouse conditions....35
3.2.3 Molecular techniques....36
3.2.3.1 Genomic DNA extraction from KV leaf tissue....36
3.2.3.2 Gel electrophoresis and DNA quantification....37
3.2.3.3 PCR conditions and amplification....37
3.2.3.4 Genotype data capturing....38
Chapter 4: Results....41
4.1 The establishment of an *in vitro* screening method for evaluating the resistance of grapevine cultivars to *Xylophilus ampelinus*....41
4.1.1 Micropropagation of plants and growth conditions....41
4.1.2 Bacterial inoculum preparation....41
4.1.3 Evaluation of in vitro plant inoculation methods: Comparing the scalpel and needle-pricking methods....43
4.1.4 Response of Dauphine and Redglobe to X. ampelinus infection....46
4.1.4.1 Detection of X. ampelinus DNA in plant extracts of Dauphine and Redglobe via nested PCR....53
4.1.4.2 Isolation of bacteria from macerated inoculated plants of Dauphine and Redglobe and confirmation of their identity....56
4.1.4.3 Evaluation of the phenotypic response of C-3229 to X. ampelinus infection..57
4.1.4.4 Plant phenotypic responses of Redglobe and C-3229....58
4.1.4.5 Detection of X. ampelinus in plant extracts of C-3229 and Redglobe via nPCR....61
4.1.4.6 Isolation of bacteria from Redglobe and C-3229 and confirmation of their identity....63

4.2 Screening Kishmish Vatkana seedlings with molecular markers linked to powdery mildew resistance gene, *Ren1* **........** 64

4.2.1. From laboratory to glasshouse: Acclimatisation of progeny from self-pollinated Kishmish Vatkana 64

4.2.2 Molecular characterisation to confirm zygosity of the progeny........ 65

4.2.2.1 DNA quantification 65

4.2.2.2 Classification of offspring........ 68

4.2.2.3 Marker performance 69

Chapter 5: Discussion 76

Chapter 6: Conclusion and recommendation 83

Reference 85

List of Figures

CHAPTER TWO

Figure 2.1 *Xylophilus ampelinus* infected grapevine ……………………..…………..….7

Figure 2.2 Powdery mildew symptoms …………………………………………………11

Figure 2.3 Table grapes included in this study ……………………………………...15

Figure 2.4 Molecular markers and their positions on chromosome 13 in a 7.4 centimorgan (cM) interval in Kishmish Vatkana ………………………………………...16

CHAPTER THREE

Figure 3.1. Scalpel inoculation method ....……………………………………………25

Figure 3.2. Inoculation of micropropagated plantlets using a modified-needle inoculation method ………………………………………………………………………...28

Figure 3.3 Sections used to confirm infection of *in vitro* inoculated plantlets ………29

Figure 3.4 Self-pollination of Kishmish Vatkana in the ARC Infruitec-Nietvoorbij vineyards ……………………………………………………………………………………33

Figure 3.5 Embryo rescue technique to develop offspring from self-pollinated Kishmish Vatkana ……………………………………………………………………………34

Figure 3.6 Acclimatisation of embryo rescued Kishmish Vatkana offspring ……......35

CHAPTER FOUR

Figure 4.1 Micropropagation of grapevine plantlets after four weeks in culture on WPM medium with no hormones …………………………………………………………….42

Figure 4.2 Micropropagation of grapevine plantlets after four weeks in culture showing improved growth on MS …………………………………………………………….42

Figure 4.3 Disease incidence in micropropagated Redglobe and Dauphine plants inoculated with suspensions of *Xylophilus* (10^8 cfu/ml) using different inoculation methods ……………………………………………………………………………..….45

Figure 4.4 Plant phenotypic responses of micropropagated Dauphine plantlets to *X ampelinus* inoculated using the needle method ……………………………………..49

Figure 4.5 Plant phenotypic responses of micropropagated Redglobe plantlets to *X. ampelinus* inoculated using the needle method ……………………………………….50

Figure 4.6 Amplified products from macerated samples of Redglobe inoculated by scalpel method four weeks post inoculation using nested-PCR ……………………...54

Figure 4.7 Amplified products from macerated samples of Redglobe inoculated by needle method four weeks post inoculation using nested PCR ……………………...55

Figure 4.8 Amplified products from macerated samples of Dauphine inoculated by scalpel method four weeks post inoculation using nested PCR ………………………55

Figure 4.9 Amplified products from macerated samples of Dauphine inoculated by needle method four weeks post inoculation using nested PCR ………………………56

Figure 4.10 Colony PCR amplification of bacteria isolated from Dauphine using *X. ampelinus*-specific primers S3 and S4 …………………………………………………57

Figure 4.11 Phenotypic response of Redglobe to *X. ampelinus* (10^8 cfu/ml) showing progressive bacterial necrosis four weeks post inoculation 59

Figure 4.12 Phenotypic response of C-3229 to *X. ampelinus* (10^8 cfu/ml) showing progressive bacterial necrosis ……………………………………………………………60

Figure 4.13 Disease progression in micropropagated C-3229 and Redglobe plants inoculated with suspensions of *X. ampelinus* (10^8 cfu/ml) using the modified needle method …………………………………………………………………………………...61

Figure 4.14 Amplified products from macerated samples of Redglobe inoculated by modified needle-pricking method and evaluated four weeks post inoculation. These results are representative of all nPCR results for Redglobe inoculated with *X. ampelinus* within this experiment …………………………………………………………62

Figure 4.15 Amplified products from macerated samples of C-3229 inoculated by modified needle-pricking method and evaluated four weeks post inoculation ………63

Figure 4.16 Colony PCR amplification of bacteria isolated from macerated samples of C-3229 four weeks post inoculation, using *X. ampelinus*-specific primers, S3 and S4. ………………………………………………………………………………………64

Figure 4.17 Offspring of self-pollinated Kishmish Vatkana …………………………..65

Figure 4.18 Genomic DNA (30ng/µl) quality check of Kishmish Vatkana offspring using agarose gel electrophoresis (0.8% agarose; 50 volts; 45 min) with 2µl DNA used per sample ………………………………………………………………………..66

Figure 4.19 Electropherograms for SSR marker UDV124 fluorescently labelled with a red dye (PET^{TM}) obtained after scoring of alleles in the individuals # KV1, KV4, KV6, KV26, and KV27 derived from self-pollination of Kishmish Vatkana …………………73

Figure 4.20 Electropherogram for SSR marker VMC9H4-2 fluorescently labelled with a red dye (PET^{TM}) obtained after scoring of alleles in the individuals # KV1, KV4, KV6, KV26, and KV27 derived from self-pollination of Kishmish Vatkana …………………74

Figure 4.21 Electropherogram for SSR marker VMCNG4E10-1Vic fluorescently labelled with a green dye (VIC^{TM}) obtained after scoring of alleles in the individuals # KV1, KV4, KV6, KV26, and KV27 derived from self-pollination of Kishmish Vatkana ……………………………………………………………………………………………..75

LIST OF TABLES

CHAPTER THREE

Table 3.1 The disease rating scale for bacterial blight of grapevine based on the visual symptoms ..……………………………………………………………………………...27

Table 3.2 Primers used for PCR detection of *Xylophilus ampelinus* DNA …..….……28

Table 3.3 PCR multiplex reaction protocol ……………………………………………...29

Table 3.4 SSR primers (René Veikondis, MSc. thesis, 2014) used in screening the progeny of self-pollinated Kishmish Vatkana …………………………………………40

CHAPTER FOUR

Table 4.1 Observations and calculated bacterial counts per ml of sample …………43

Table 4.2 Phenotypic responses of Dauphine inoculated with *X. ampelinus* using the needle inoculation method …………………………………………………………….51

Table 4.3 Phenotypic responses of Redglobe inoculated with *X. ampelinus* using the needle inoculation method …………………………………………………………….52

Table 4.4 Concentration and quality of genomic DNA isolated from Kishmish Vatkana offspring …………………………………………………………………………………67

Table 4.5 Allele comparison of population derived from self-pollinated Kishmish Vatkana using 5 SSR markers ………………………………………………………...71

Table 4.6 Progeny carrying a dominant allele (*Ren1*) for PM resistance derived from self-pollination of Kishmish Vatkana …………………………………………………..72

LIST OF ABREVIATIONS

μM	Micromolar
3’	Three prime
5’	Five prime
AFLP	Amplified Fragment Length Polymorphism
ARC	Agricultural Research Council
ANOVA	Analysis of variance
BA	6-benzylaminopurine
bp	base pair
CFU	Colony-Forming Unit
Cm	Centimetre
CTAB	Cetyltrimethylammonium Bromide
DAFF	Department of Agriculture, Forestry and Fisheries
EDTA	Ethylene Diamine Tetra-acetate
DNA	Deoxy-Ribonucleic Acid
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
g	Grams
IBA	Indole-3-Butyric acid (IBA)
Kb	Kilobases
MAS	Marker-Assisted Selection
$MgCl_2$	Magnesium Chloride
min	Minutes
ml	Millilitre
Mm	Millimetre
mM	Millimolar
MS	Murashige and Skoog
MW	Molecular weight
NA	nutrient agar
NBG	nutrient broth glycerol

NBS	nucleotide binding site
NCBI	National Center for Biotechnology Information
nm	nanometer
ng	Nanogram
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	Concentration of Hydrogen ions in a solution
Ren1	Resistance to *Erysiphe necator* 1
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SSR	Simple Sequence Repeats
Taq	T*hermus aquaticus* DNA polymerase
TE	Tris-Ethylenediamine
Tm	Melting temperature
Tris-HCL	Tris-aminomethane Hydrochloric Acid
TBE	Tris Borate EDTA
TBS	Tris-Buffered Saline
µL	microliter
µM	micromolar
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
WPM	woody plant medium
YPG	yeast peptone glucose
YPGA	yeast peptone glucose agar

Chapter 1: Introduction

1.1. Introduction

Grapevines are members of the family *Vitaceae* and belong to the genus *Vitis.* They are perennial, woody vines producing edible fruit native to Asia Minor, Europe, North America and other continents. There are over a dozen genera with over 50 species belonging to the genus *Vitis* with about 60 inter-fertile species occurring almost exclusively in the Northern Hemisphere. Members of this genus can be found in warm and temperate regions of the world, but there are also cold hardy species like *V. amurensis* and *V. riparia*. The genus *Vitis* consists of two subgenera: subgenus *Vitis* and subgenus Muscadinia (Planch.), with all species in subgenus *Vitis* being diploid (2n=38) (Mullins *et al*. 1992; Ma *et al*. 2018).

One of the most notable threats in agriculture is plant diseases. For this reason, it is crucial to establish crops with improved disease resistance to support the food demand of a growing population (Chavan and Smith, 2014). Crop yields in the field have been negatively impacted by diseases caused by plant pathogens. Therefore, identifying and making use of disease resistant plants can reduce yield loss. Many disease resistant cultivars are distinguished in plant species that form part of staple food, including wheat, rice, maize and sorghum, by inoculating the plant with a plant pathogen and selecting for resistant lines. In grapevine, there has been a great need for bacterial and fungal disease resistant lines as well as the need to have efficient inoculation methods that would be used to screen for resistance.

Grapevine (*V. vinifera* L) is susceptible to an array of diseases caused by bacteria, fungi, and viruses (Armijo *et al*. 2016). Both powdery mildew, a fungal disease, and bacterial blight have been known to affect grapevine cultivation by damaging fruit and reducing yield. Both diseases occur in South Africa and are the basis of this study. Bacterial blight, also known as bacterial necrosis, of grapevines is caused by the bacterium, *X. ampelinus* and is one of the important diseases in the major table grape production areas in the Western Cape province of South Africa (Du Plessis, 1940; Panagopoulos, 1987). Grapevines (*V. vinifera*) are the only known host or species affected (Panagopoulos, 1988). Severe infections can result in serious harvest losses

(Komutsu and Kondo, 2015). The disease is associated with warm, moist conditions and the bacteria survive in the vascular tissues of infected plants (Panagopoulos, 1987). The most characteristic symptom of the disease is the formation of cankers on canes, young shoots and leaf petioles. The extent of the development of symptoms and the type of symptoms vary and depend on different parameters including the grapevine genotype, environmental conditions and the infection period (Plantwise Knowledge Bank, 2013).

Chemicals have thus far failed to control the disease. Therefore, phytosanitary measures have been implemented to control the disease (Panagopoulos, 1987; EFSA PLH Panel, 2014;). To achieve this, tools used in pruning of grapevines should be thoroughly disinfected during use and overhead sprinkler irrigation should be avoided as this may promote the spread of the bacteria in the vineyards (Panagopoulos, 1987). The production of many important grape varieties is severely compromised by bacterial blight, so improvements in disease resistance are urgently needed by grapevine breeders. Successful breeding for disease resistance requires methodical screening techniques and often a need to screen large numbers of genotypes to find resistant lines (Agudelo-Romero *et al*., 2015; Peňázová *et al*., 2018). Plant disease resistance phenotyping can be done in the field, the greenhouse or *in vitro*. Among these three, the *in vitro* screening takes advantage of controlled environmental conditions but it has been argued previously that *in vitro* testing is not representative of what happens in nature, i.e. not considered to be as reliable as field and greenhouse tests. Although both of these screening tests are important, the benefit of controlled environmental conditions assist to speed up the process of resistance screening (Agudelo-Romero *et al*. 2015).

Grapevine powdery mildew (PM), on the other hand, caused by *Erysiphe necator*, is a major fungal disease that threatens grapevine-production in almost all grape growing countries worldwide (Katula-Debreceni, 2011). Fungal infection reduces yield, damages fruit and reduces wine quality. Breeding grapevine (*V. vinifera* L.) varieties resistant to PM is crucial to avoid excessive fungicide application, which comes at high cost with environmental and human health risks (Katula-Debreceni, 2011; Pap *et. al*. 2016). To help reduce breeding costs, DNA-based molecular markers linked to traits of interest are used. With this kind of marker-assisted selection (MAS), seedlings

containing genes of interest can be accurately identified shortly after germination and before any gene expression (Katula-Debreceni, 2011).

Kishmish Vatkna is a grapevine variety resistant to *E. necator*. KV is able to mount a post-penetration reaction against *Erysiphe necator*, a trait controlled by the dominant *Ren1* (Resistance to *E. necator* 1) gene positioned on chromosome 13. KV *Ren1* gene was the first PM resistance gene to be identified in *V. vinifera*. KV is, however, heterozygous for the *Ren1* gene (Hoffman e*t al*. 2008), resulting in only half the offspring from a cross with this cultivar possessing the trait. It would therefore be beneficial to use self-pollination to create offspring that are homozygous dominant for the *Ren1* gene, resulting in 100% *Ren1* positive hybrid offspring (Personal communication P. Burger, ARC-Nietvoorbij). KV is, however, a cultivar with soft seed remnants and a highly efficient embryo rescue technique will be needed to regenerate offspring from self-pollination (Spiegel-Roy *et al*. 1985).

Chapter 2: Literature review

2.1. Importance of grapevine in agriculture

Grapevine (*Vitis vinifera* L.) is one of South Africa's most widely grown fruit crops and is of major interest, mostly because of the global wine industry and due to the demand for fresh and dried fruit, juice, jams, jellies, raisins and other processed products (Diab *et al*. 2011; Abido *et al*. 2013). Grapes, as an ancient food in the life and history of humankind over several millennia, have increased in production, because the fresh fruit is nutritious for humans (Diab *et al*. 2011). As suggested by the archaeological records, grapevines were domesticated in the Mediterranean basin, in Western and Central Asia, and then cultivated in all temperate regions around the world (Sawler *et al*. 2013). The origin of domesticated grapes dates back 6000-8000 years (Sawler *et al*. 2013). However, today, countless of grape cultivars have been generated by natural or planned (by humans) crosses (Myles *et al*. 2011).

The genus, *Vitis*, is of major agronomic importance in the *Vitaceae* family (This *et al*. 2006). *V. vinifera* is the only species that is extensively used in the global wine industry within the genus (Zohary, 1995; Reisch *et al*. 2012). Thousands of *V. vinifera* cultivars exist with only a few cultivars dominating the global market for wine production due to how the wine is currently marketed, mainly because consumers associate good wine quality with well-known cultivars. Grapes commercially cultivated can usually be classified as either table or wine grapes based on their intended method of consumption; i.e., eaten raw (table grapes) or used to make wine (wine grapes) (This *et al*. 2006). South Africa produces a wide range of table grape cultivars that are harvested over a seven-month period between October and May. According to the Department of Agriculture, Forestry and Fisheries, the leading varieties produced in South Africa are Crimson Seedless at 20%, Prime seedless (8%), Thomson Seedless/ Sultana (7%), Flame Seedless (6%), Sugraone (5%) and the Redglobe (4%) (DAFF, 2017). However, it should be noted that these figures are not the same for the rest of the world. The four varieties used in this study are discussed in section 2.4.

2.2. Diseases affecting grapevine (*Vitis vinifera* L.)

Commercial cultivars of grapevines are threatened by a large number of pathogenic microorganisms that cause diseases during pre- and/or post-harvest periods, greatly affecting production, processing, export and fruit quality. The degree of susceptibility to disease differs depending on the cultivar, and damage can generally be severe. Bacteria, fungi and viruses cause the most common grapevine diseases, but the vast majority of plant pathogens are fungi (Armijo *et al*. 2016).

Some of the most important diseases affecting *V. vinifera* caused by fungi are downy mildew (*Plasmopara viticola*), PM (*Erysiphe necator*), grey mold (*Botryotinia fuckelina*), anthracnose (*Elsinoe ampelina*), and black rot (*Guignardia bidwellii*), while important disease caused by bacteria are crown gall (*Agrobacterium vitis*), Pierce's disease (*Xylella fastidiosa*), and bacterial blight (*Xylophilus ampelinus*) (Doman, 2015; Armijo *et al*. 2016).

Some bacterial diseases are characterised by relatively slow symptom progress compared to foliar and fruit cluster diseases caused by fungi. Bacterial pathogens causing grapevine diseases take place in the vascular system and intercellular spaces of their host plant and most can be cultivated on artificial media (Szegedi and Civerolo, 2011). In the sections to follow, more focus will be placed on PM and bacterial blight.

2.2.1 Bacterial blight

2.2.1.1 The causal agent

Bacterial blight of grapevine and its causal agent, *Xylophilus ampelinus*, were first described from Crete, Greece (Panagopoulos, 1969). Bacterial blight is known by different names in different countries. In Greece it is called 'tsilik marasi', 'vlamsiekte' in South Africa, 'mal nero' in Italy, and 'mal negro' in Portugal. The causal agent, *X. ampelinus*, is a Gram-negative bacterium that belongs to the family *Comamonadaceae* in the class beta-Proteobacteria (Willems *et al*., 1987). The bacterium was previously known as *Xanthomonas ampelina* (Panagopoulos, 1969), however, DNA-DNA and DNA-rRNA hybridisation showed that the bacterium belongs to the third rRNA superfamily where it forms a separate branch, now known as the genus *Xylophilus* (Willems *et al*. 1987). In culture at 28°C, growth is unhurried,

colonies are non-mucoid, smooth, yellow, round and whole (0.4-0.8 mm in diameter) and grow after 6-10 days on nutrient agar, which is a favourable growth medium (Panagopoulos, 1969).

2.2.1.2 Symptoms, spread and distribution

Bacterial blight of grapevine produced by *X. ampelinus,* is a disease that is particularly noticeable in European grapevines (*V. vinifera*) known to be susceptible (Panagopoulus, 1988). *X. ampelinus* has a restricted distribution, and does not take place in many parts of the world where grapes are grown. The disease has been reported to occur in Japan, the Mediterranean area and in remote sites in the Western Cape region of South Africa (Botha *et al*. 2001: Komutsu and Kondo, 2015). The bacterium is limited to vascular tissues within the plant. The pathogen is spread from infected vines to healthy ones via pruning tools, by wind and rain, and gain entry into the plant through open wounds, leaf scars and other affected sites. There are no known vectors of *X. ampelinus* (Bradbury, 1991; EFSA PLH Panel, 2014;). And there has been no reported bactericides that aid in disease control (Botha *et al*. 2001; Komutsu and Kondo, 2015).

Major infection occurs on one or two-year old shoots. The causative bacterium is transmitted with pruning tools and come in healthy tissues primarily through pruning wounds and by propagation material during grafting and harvesting (Panagopoulus, 1987; Komutsu and Kondo, 2015). The bacterium can also penetrate leaves through open stomata (Komutsu and Kondo, 2015). When infection arises through the stomata, *X. ampelinus* causes indigenous necrotic symptoms (Szegedi and Civerolo, 2011).

Symptoms include lesions on the stems and petioles, severe discolouration of the leaves and death of the infected canes and branches (Figure 2.1) (Botha *et al*. 2001; Komatsu and Kondo, 2015). Infection usually occurs on the lower two to three nodes of shoots that are 12-30 cm long, and spreads slowly upward. Very high bacterial concentrations (10^8 bacteria/g of plant tissue) can be found in the trunk that may or may not be showing symptoms (EFSA PLH Panel, 2014). Latent infections in 50% of symptomless canes from infected vineyards in Crete have been observed

(Panagopoulus, 1987). Infected shoots incline to be shorter, giving the vine an inhibited appearance. Leaves may be infiltrated by means of the petiole and then the veins, in which case the entire leaf dies. On the other hand, leaves are penetrated directly via the stomata, with development of angular, reddish-brown lesions (Figure 2.1b). As soon as infection occurs through the hydathodes, reddish-brown discolorations develop on the leaf tips. Light-yellow bacterial ooze may be observed on infected leaves when humidity is high. Flowers that have not reached maturity turn black and die back. Roots may also be infected, causing delay in shoot growth, whether the plant is grafted or on its own rootstock (Grall *et al*., 2005).

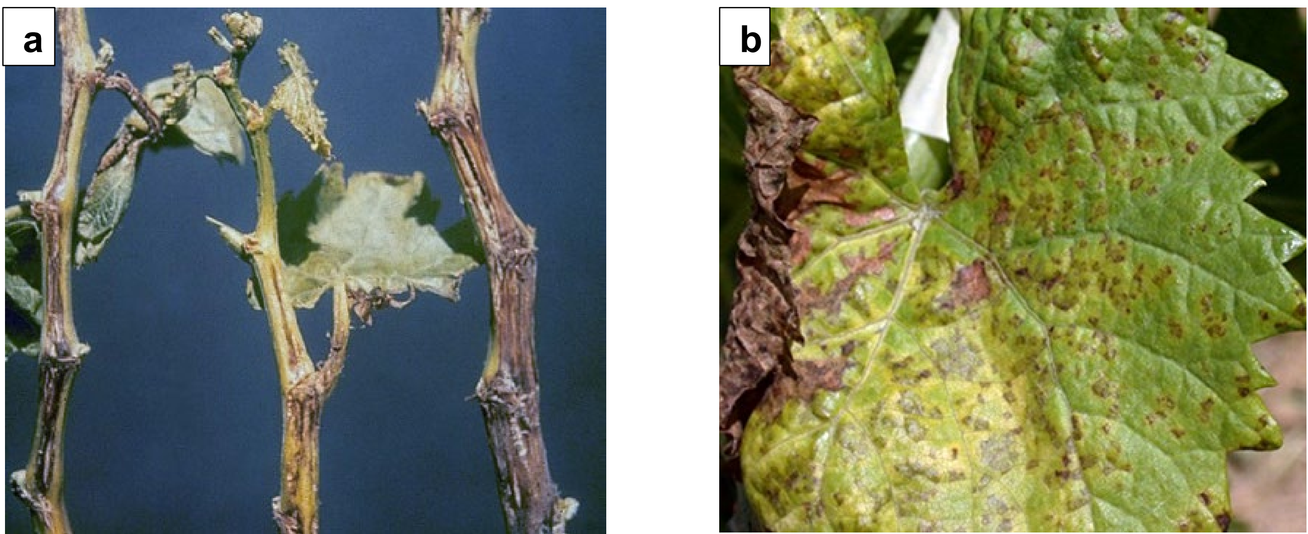


Figure 2.1: *Xylophilus ampelinus* infected grapevine showing: **a** - discolouration of plant tissue on the shoots/ canker formation. Necrosis appears on the stems and infection spreads along the branches **b** - Angular leaf spots seen on grape leaves. Images obtained from http://www.eppo.org/QUARANTINE/bacteria/Xylophilus_ ampelinus/XANTAM_images.htm

2.2.1.3. Control measures

Chemicals have failed to control bacterial blight of grapevine. Copper-containing agents were found to be the only chemicals that can be used for anti-bacterial treatment of grapevines. The bacterium did not colonise the xylem tissues but was found on the bottom surface of the bract and bud wool following overwintering. The copper wettable powder sulphate was effective in controlling the appearance of symptoms. However, this type of treatment will not work for grape plants where the bacterium has invaded the internal tissues. Therefore, these copper agents could only

be used to stop external contamination and the presence of symptoms (Komatsu and Kondo, 2015). As a result, viticulture practices have been implemented to control the disease (EFSA PLH Panel, 2014). In addition to developing resistant cultivars, control can be obtained through phytosanitary measures (Panagopoulos, 1987). To achieve this, tools used in pruning of grapevines should be carefully sterised throughout use. Overhead sprinkler irrigation should be evaded as this may promote the spread of the bacteria in the vineyards (Panagopoulos, 1987).

2.2.1.4 Bacterial detection and screening tests

Control of diseases produced by plant-pathogenic bacteria commonly requires correct detection and appropriate identification of the causal organism (Palacio-Bielsa, 2009). *X. ampelinus* is one of the bacterial pathogens that is spread through contaminated propagative materials, therefore exposure in infected plant products becomes of supreme importance (Alvarez, 2004). Detection necessitates field observation, analysis of plant tissues, isolation of the pathogen, description, and proof of Koch's postulates.

Symptoms in plants are not constantly precise and can be confused with those caused by other biotic or abiotic agents. Also, detection of bacteria in symptomless plant material for deterrent control is essential but can prove to be problematic, since low population levels and uneven distribution of the pathogen can occur. PCR has become an important tool in molecular biology and can be used to study minute quantities of DNA and its sensitivity has also led to applications in the diagnosis of pathogenic disease (Schaad *et al.* 2001). Primer sequences have already been defined that can be used with PCR protocols to recognise *X. ampelinus* (Botha *et al*. 2001; Alvarez, 2004; EFSA PLH Panel, 2014).

Many different methods for molecular recognition of *X. ampelinus* are available. A real-time PCR probe-based detection method offers a consistent and sensitive test for *X. ampelinus*, appropriate for a screening test. This method could be used to specifically identify isolated colonies and to relatively quantify *X. ampelinus* bacteria (Dreo *et al*. 2007). This method evades difficult post PCR handling and the related high risk of contamination. Nested PCR, another method for molecular detection of *X. ampelinus*,

has been used to overcome the problem of surplus non-target DNA template compared to target template. In grapevine cuttings, this method was able to detect low numbers of target cells when a surplus number of saprophytic bacteria were present (Botha *et al*. 2001). Nested PCR amplifies part of the 16S-23S rDNA intergenic spacer region (Botha *et al*. 2001).

With no effective chemical control measures and no true sources of disease resistance, it is of significant importance to produce bacterial blight resistant varieties. For this to be achieved, varieties need to be screened to identify those with desired traits and have a high potential breeding value and could be vital resources to plant breeders (Wang *et al*. 2015). In this work, three grape germplasm varieties were selected from the Nietvoorbij vineyards and investigated for tolerance/ susceptibility to bacterial blight. The three varieties used in this study are: Dauphine (a tolerant variety according to field studies), Redglobe (susceptible) and C-3229 (susceptibility to be confirmed) and will be discussed in more detail in the sections below.

2.3 Powdery mildew (PM)

2.3.1 Causal agent

One of the most vital diseases of cultivated grapevines universally is PM. *Erysiphe necator* is considered to be the only pm species adapted to *V. vinifera* (Agurto et al. 2017). The pathogen is an obligate biotrophic fungus that relies on the host cell, that is, on genera within the *Vitaceae*. This includes *Vitis, Cissus* and *Ampelopsis* (Gadoury *et al*. 2012; Qiu *et al*. 2015).

The disease is also identified as ‘oidium’ to grapevine producers. Powdery mildew is a severe problem for grape production worldwide. Due to the annual occurrence of the disease, precautions to control the disease have been ongoing (Halleen and Holz, 2001; Wang *et al*. 2014). In South Africa, PM has been reported to occur on South African vines since 1860 (Halleen and Holz, 2001). Hence, grape production is highly reliant on the recurrent use of fungicides. Hence, the integration of effective genetic resistance into cultivated grapevine would help lead to important financial and environmental benefits (Gadoury *et al*. 2012).

Knowledge of the pathogenicity of *E. necator* stems mostly from research conducted on European grapevine cultivars, the leaves and fruits of which are highly susceptible to this pathogen (Gadoury, 2012). The PM fungus infects green tissues of vines and can affect all phases of plant growth and infection reduces the winter hardiness of canes causing significant losses in yield and decrease in berry quality (Miazzi *et al.* 2010; Gadoury, 2012). Early infection of fruit with pm causes low wine quality due to reduced sugar content, and as a direct result of the fungus itself producing off-flavours (Pool *et al*. 1984).

2.3.2 Symptoms

Powdery mildew (*E. necator*) may be observed on the green parts of the plant including leaves (Fig.2.2a), immature berries (Fig.2.2b), branches, pedicels, inflorescences and shoots (Alimad *et al*. 2017). Chlorotic spots on the upper leaf surface are the initial symptoms that can be seen on leaves of grape plants. A short time later, signs of the pathogen appear as white, webby mycelium on the lower leaf surface. The leaves take on a white, powdery appearance as spores are formed. PM symptoms can be observed on foliage, fruit, flower parts and canes (British Columbia Ministry of Agriculture, 2015). Colonies are roughly circular and have a diameter size of between a few millimetres to a centimetre or more, occurring singly or in groups that coalesce to cover the leaf (Gadoury *et al*., 2012).

Distortion of leaves, drying, and premature drop is caused mainly by severe leaf infections. Fungus that look like powdery growth can cover infected berries and develop web-like blemishes (Figure 2.2a) (Halleen and Holz, 2001).

Figure 2.2 Powdery mildew symptoms. **a** - on leaves, **b** - severely infected berries are scarred, distorted and often split, and may not ripen properly. Images obtained from https://pnwhandbooks.org/plantdisease/host-disease/grape-vitis-spp-powdery-mildew.

2.3.3 Control measures

The primary control measure for PM is the application of chemicals and these are best used as protectants (Halleen and Holz, 2001; UC-Intergrated Pest Management Program, 2009). Timing of the first treatment is dependent on several factors in the treatment like the fungicide used, vine growth stage and the potential for disease infection. Moisture due to fog, dew or rain triggers ascospore release. After budbreak, ascospores begin to cause infections on green tissue when temperature exceeds 10°C. Soft chemistry products such as sulphur, biologicals and systemic acquired resistance products should be discontinued when disease pressure is high because they will not provide adequate control when used by themselves (UC-Intergrated Pest Management Program, 2009).

Organically acceptable methods are being used on organically certified grapes. These methods include Sulphur, Serenade Max, Sonata, Organic JMS stylet Oil and Purespray Green horticultural oil (UC-Intergrated Pest Management Program, 2009).

Sulphur, a protectant multi-site fungicide which acts as a general inhibitor of many fungal enzymes, was the first operative fungicide used for the control of PM on grapes. Today, the use of sulphur has become undesirable due to weather conditions like wind and rain, application has become difficult throughout the season (Halleen and Holz, 2001). The undesirable use of sulphur is also due to the danger of environmental pollution (affecting the biological activity of the soil) and allergic reaction to sulphur (Veikondis, 2014).

2.4 Origin and characteristics of grape varieties

2.4.1 Dauphine

Dauphine, a table grape cultivar, was developed by the Agricultural Research Council of South Africa and released in 1983. It was created from open pollinated progeny of Almeria, a late-producing white cultivar. Dauphine is a white seeded grape with large, oval berries, with compact bunches (Figure 2.3a). Dauphine has good storage ability after harvest and has a sweet to neutral taste (http://tulbaghnursery.co.za/fruit-trees-home/grapevines/). It is a late-ripening variety, ripening towards the end of March, thus making it one of the last grapes to be harvested in South Africa (Gütschow, 2001). It has good affinity with known rootstocks and thrives best on medium to heavy soils. It has reasonable resistance to berry splitting; however, neck cracking can take place (Gütschow, 2001).

Dauphine has been widely used as a parent for specific traits, like tolerance to powdery and downy mildew and have good fruit quality (Cain, 2010). Dauphine is also resistant to moderately resistant to *Botrytis cinerea,* causal agent of gray mold (Gütschow, 2001). Furthermore, field observations revealed that Dauphine is tolerant to bacterial blight. Therefore, this variety will allow a clear distinction or comparison between the other varieties that will be used in this study in their response to inoculation with *X. ampelinus*.

2.4.2 Redglobe

Redglobe is a complex hybrid bred by University of California-Davis in California, USA in 1958. It is a cross between Red Emperor, Hunisa and Nocera, and is a seeded red grape with huge berries (Figure 2.3b) (https://www.cooksinfo.com/red-globe-grapes). Red Emperor is a seeded table grape with large red to dark red sweet fruit. This variety remains domant in winter and can usually cope with temperature down to -15 °C (Glowinski, 1991; Cirami, 1996). Hunisa is a white seeded table grape variety native to Iran (*Vitis* International Variety Catalogue (VIVC), 2007). Nocera is a high-quality, red-wine grape variety from the north eastern region of Sicily and is now also grown in Calabria. It produces deeply coloured, high acidity wines and It has good vigor but poor disease resistance (D'Agata, 2014).

It is the world's second most-cultivated grape variety, harvested in late February in South Africa and is mostly planted in China, with a total of 91% vineyard area. Other countries where this variety is grown are United States, Spain, Portugal, Italy, Turkey, Chile, Argentina and South Africa (Focus OIV, 2017). Redglobe is an early-budding variety with a late maturity period (Focus OIV, 2017). It has shown to be susceptible to both downy mildew and PM (Van Heerden *et al.*, 2014). Redglobe has also been found to be susceptible to *Xylella fastidiosa*, which causes Pierce's disease (Krell *et al*. 2008).

For decades, infections by *X. ampelinus* have been occurring in South African vineyards due to shortage of disease resistant grape cultivars, effective chemical control measures, and favourable environmental conditions like high humidity in the growth season. Consequently, the removal of infected vines to replace with disease free material has been costly. It is therefore necessary to study the response of Redglobe to inoculation with *X. ampelinus* and breed for bacterial blight resistance in table grape cultivars.

2.4.3 C-3229

C-3229 is a white seedless selection with muscat flavour (Figure 2.3c). It is a cross between G4-682 (a breeding line from the ARC's table grape breeding programme) and Regal Seedless. Both parents are seedless and after cross-pollination, embryo rescue was employed to develop C-3229. G4-682 is early ripening with a slight muscat flavour, nice crispy berry texture, but is not very fertile. Regal Seedless, on the other hand, is very fertile, has large natural berry size and loose bunches. As in many situations in a breeding programme, crossing is done to either improve the quality of the fruit and/or to improve the cultivar's resistance against disease. In this case, the cross was made to get good muscat flavour with large natural berries with a crisp texture and good fertility (Personal communication P. Burger, ARC Infruitec-Nietvoorbij). Dauphine and Redglobe, mentioned above, have been described in the literature as tolerant and susceptible to bacterial blight respectively, while C-3229's response to bacterial blight is not known. This is the first screen of C-3229 for bacterial blight resistance and will determine whether this variety will be included in the breeding programmes.

2.4.4 Kishmish Vatkana

Kishmish Vatkana is a cultivated grapevine derived from Central Asia and belongs to *V. vinifera* L. subsp. *vinifera* (Hoffmann *et al*. 2008) (Figure 2.3d). The parentage is not clear but it has a parent-offspring relationship with the seedless table grape Sultanina (Hoffmann *et al*. 2008). KV yields large clusters with anthocyanin pigmented berries that contain the soft remains of aborted seeds. It has superior aroma and flavour characteristics (Qiu, 2015).

Kishmish Vatkana shows resistance to PM. It was shown that the resistance was linked to the Resistance to *E. necator* 1 (*Ren1*) gene, the first resistance gene found in *Vitis vinifera* (Hoffmann *et al*. 2008). *Ren1* confers single gene dominant resistance to powdery mildew and is heterozygous in KV. *Ren1* is located on linkage group 13 within a 7.4 centimorgan (cM) interval and with the closest markers located 0.9 cM away from the *Ren1* locus (Hoffmann *et al*. 2008) (Figure 2.4). Veikondis *et al*. (2018) selected the markers on linkage group 13 identified by Hoffmann *et al*. 2008 and verified that they were associated with the single dominant gene, *Ren1* in an offspring population from a Sunred Seedless x Kishmish Vatkana cross. Riaz *et al*. (2013) confirmed that KV is heterozygous at the *Ren1* locus.

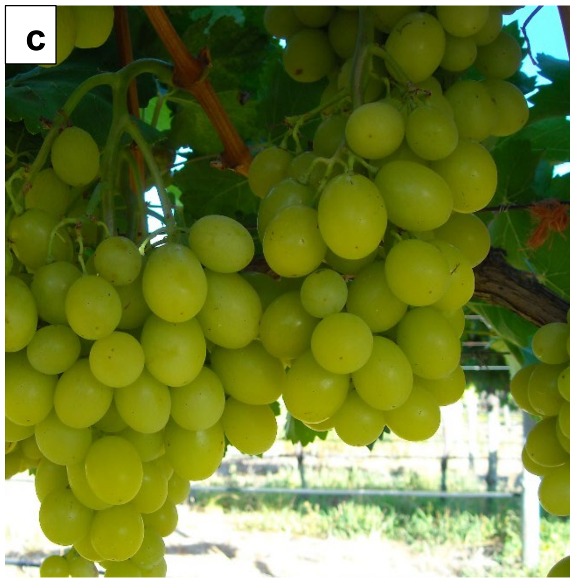


Photo: Phyllis Burger, ARC-Infruitec Stellenbosch **Photo:** www.tilia.zf.medelu.cz)

Figure 2.3 Table grapes included in this study: **a** - Dauphine, **b -** Red Globe, **c** – C-3229 **d** - Kishmish Vatkana,

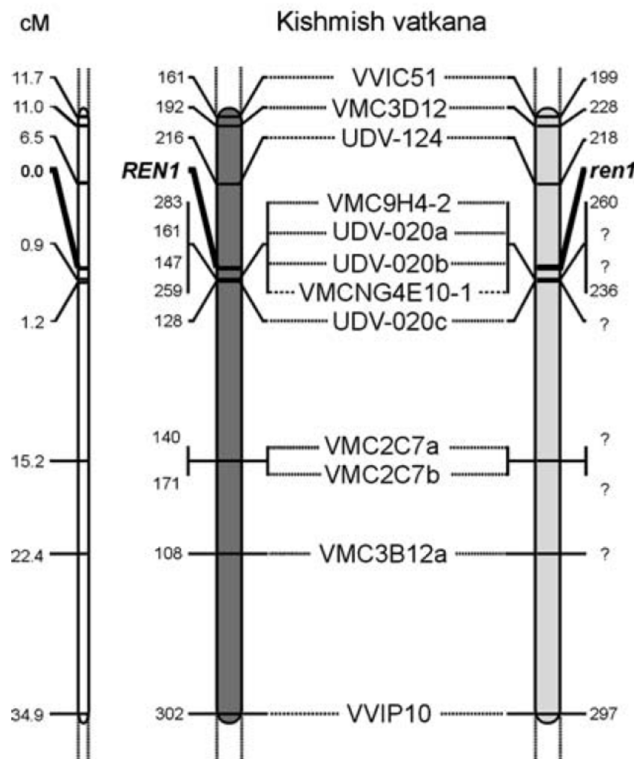


Figure 2.4: Molecular markers surrounding the Resistance to *Erysiphe necator* 1 (*Ren1*) gene and their positions on chromosome 13 in a 7.4 centimorgan (cM) interval in Kishmish Vatkana (Image from Hoffmann *et al*. 2008)

2.5 Breeding for disease resistance

Plant breeding is the genetic improvement of crops better suited for cultivation (Brown, 2011). Grapes (*V. vinifera* L.), as one of the most essential fruit crops in the world, are subject to intense breeding efforts in order to develop new disease resistant cultivars (Li *et al.* 2015). Breeding for disease resistance is one method of protecting crops from damage due to biotic factors. One of the valuable attributes to the grower is the inherited resistance of the crop as it is easy for the grower to manage and reduce the need for other methods of control. Growing disease resistant crop plants is also environmentally favourable (VIVC, 2007).

Bacterial diseases are of high importance in many crop species. Conventional breeding has been tested, and its efforts have proved to be successful especially in work done on bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo). Due to the importance of rice as a staple food, a substantial amount of research has

been done to combat bacterial blight caused by *Xanthomonas oryzae pv. oryzae (Xoo)* (Noroozi, *et al*. 2015).

In rice, at least 37 major disease resistance genes have been identified, seven of which have been isolated (Xa1, Xa3, Xa5, Xa21, Xa25, Xa26 and Xa27) (Noroozi *et al*. 2015). Bacterial races vary continually due to artificial and natural selection of genes coding for resistance to bacterial blight. It has therefore become critical to investigate and identify the new resistant resources to control the pathogen developing resistance (Noroozi *et al*. 2015)

Amongst grapevine diseases, those caused by various bacterial species are considerably important in most grape-growing countries. Chemical or biological control methods that could efficiently stop symptoms from developing are not obtainable for bacterial diseases, therefore breeding for resistance appears to be the best action. Both conventional plant breeding and genetic modification (GM) achieve crop improvement which are much needed tools to improve agricultural productivity (Gornal *et al.* 2010). Genetic modification in commercial varieties, especially in grapes is currently illegal. The South African Freeze Alliance on Genetic Engineering (SAFeAGE) has strong views against the use of GM. This group does not believe that South Africa needs GM foods. The reason they give include GM crops do not escalate yield potential, GM crops increase pesticide use, Integrated Pest Management, organic methods for controlling pests, and Marker Assisted Selection for plant breeding are farm technologies that have proven to be far more successful than using GM organisms (SAFeAGE, 2008, De Beer and Wynberg, 2018).

Pierce's disease (PD) is triggered by *Xyllela fastidiosa*, a bacterium that inhabits the xylem of a wide range of host plant species (Hickey *et al*. 2018). The disease spreads from plant to plant by sap feeding insects feeding on xylem fluid (UC Integrated Pest Management Program, 2009; Zhang *et al*. 2015). Powdery mildeww has caused major problems in a wide range of plant hosts and Californian vineyards. Much research is focussed on this disease and one of the research areas is developing resistant or tolerant varieties through identifying and using resistant germplasm in breeding and understanding the genetic control of the resistance. A source of PD resistance was

found in *Vitis arizonica* and a breeding programme to develop resistant cultivars was initiated at the University of California (Riaz *et al.* 2008).

It is obvious that bacterial diseases are of high importance and grapevine are highly affected by a large array of pathogens inducing disease in this plant (Compant, 2015). Selection of plants with improved resistance can aid in disease control of bacterial blight in grapevine. Due to limited knowledge of resistance to *X. ampelinus*, and the lack of effective chemical agents to aid in disease control, the initiative to breed for bacterial blight resistance in table grape cultivars was started at the ARC Institute. (Personal communication Y. Petersen, ARC-Infruitec).

Development of grapevine varieties resistant to powdery mildew is the most cost effective control method as opposed to heavy application of fungicides, which has proven to exert selection pressure on *E. necator* populations and advance the evolution of fungicide-resistant strains. In addition to this, increased concerns have been raised by consumers about the environmental impact of agrochemicals and the residues that remain in grape products (Li *et al*. 2015). Resistance to powdery mildew has been investigated in different species and cultivars of *Vitis* (Kozma, *et al*. 2009).

The resistance breeding to powdery mildew of grapevine was mostly based on *Vitis* species that are autochthonous in North America due to their natural resistance to powdery mildew and a number of resistance (R) loci have been identified and are used in different breeding programs for this disease including *RUN1*, *RUN2.1*, *RUN2.2*, *REN2*, *REN 3*, and *REN 5*. However, the wild germplasm in Asia, in the last decade, have become prime candidates for breeding due to the presence of high levels of resistance to powdery mildew (Pap *et al*. 2016). Some central Asian cultivars have been found with remarkable powdery mildew resistance (*Ren1*) with KV being the best among them (Hoffmann *et al*. 2008). The *Ren1* locus was also identified in other accessions of cultivated *V. vinifera* from central Asia (Riaz *et al*. 2013). New potential sources of PM resistance designated *REN6* and *REN7* were identified in the Chinese species, *V. piasezkii,* which is widely distributed in Northeast and Western China (Pap *et al*. 2016). Most commercial grapevine cultivars are *V. vinifera* and are susceptible to *E. necator*, but significant differences can be found in the grade of this attribute.

Kishmish Vatkana does not display symptoms after natural or artificial infection. This was attributed to the single dominant allele *Ren1*, a powdery mildew resistance gene which is heterozygous in KV (Hoffmann *et al.* 2008). This means that only half the off-spring from a cross with KV will inherit the trait. This study will investigate the inheritance of *Ren1* gene in offspring of self-pollinated KV to identify the individual plants homozygous for the *Ren1* locus. KV has only soft seed remnants and therefore off-spring from self-pollination were created and developed through embryo rescue (Personal communication P. Burger, ARC-Nietvoorbij)

2.6 Embryo Rescue

Embryo rescue techniques are among the oldest and successful procedures performed under sterile conditions. The term “embryo rescue” refers to a number of *in vitro* techniques to cultivate embryos on artificial media and to raise mature plants from them. For many years, embryo rescue has been widely used in many fruit crops, including peach, banana, apple, and watermelon (Anderson *et al*. 2002; Dantas *et al*. 2006; Uma *et al*. 2011; Li *et al*. 2015).

Depending on the organ cultured, embryo rescue is denoted as embryo, ovule, or ovary culture and culture must be begun before embryo abortion takes place. The most commonly used embryo rescue procedure is embryo culture, whereby embryos are excised and placed directly onto culture medium. Ovule and ovary culture are more appropriate for small-seeded species. Berries from controlled pollination of field-grown or greenhouse plants are collected prior to when embryo abortion is thought to occur. Since embryos are positioned in a sterile environment, disinfestation of the embryo itself is not necessary (Razi *et al.* 2013).

Overcoming embryo non-viability is the most common reason for the application of this technique (Geerts *at el*. 2011). Kishmish Vatkana is a cultivar with soft seed remnants (Hoffmann *et al*. 2008) and a highly efficient technique of embryo rescue is needed to develop offspring from self-pollination (Personal communication P. Burger, ARC Infruitec-Nietvoorbij). An embryo rescue technique involves three main steps: (1) Ovules are cultured *in vitro* (embryo development), (2) embryos are excised from the ovules and cultured (embryo germination and plantlet development), and (3) plantlet

roots are elongated, plants acclimated, and transplanted to soil (Reed, 2005). Several factors have been proposed to influence the outcome of embryo rescue in grape. These include the genotype of the grape cultivars used, the time of culture, media used, and culture method and condition (Li *et al.* 2015).

Embryo rescue has been used in many fruit crops like lime for different purposes such as seedless breeding (Kumari *et al*. 2018). Embryo development in seedless grapes is known to be strictly controlled by cultivar genotype (Ji *et al.* 2013a). Seedlessness in grapevine is induced via either stenospermocarpy or parthenocarpy. Grapevines known to be parthenocarpic, are able to fruit without pollination, fertilisation, and embryo formation. These are therefore not suitable as female parents for breeding by embryo rescue. Stenospermocarpic grapes on the other hand are widely used as female parents as pollination and fertilisation occur but embryo development is aborted in earlier stages and hard seeds do not develop (Ji *et al.* 2013a; Razi *et al.* 2013).

The successful use of embryo rescue of different grape genotypes can also vary with ripening seasons or seed trace sizes. Late maturing genotypes have been shown to result in fewer rescued embryos, germinated embryos, and transplantable plants as compared to early and mid- season ripening genotypes (Li *et al.* 2015). Additionally, genotypes with a larger ratio of seed trace weight to length normally provide larger numbers of ovules with embryos, more germinated embryos and more transplantable plants (Li *et al.* 2015).

It has been known that the type of medium needed for rescuing embryos is strongly dependent on the stage of embryo development (Reed, 2005). More than one type of medium formulation maybe required for cultures initiated using young embryos as the nutritional requirements differ (Agnihotri, 1993). In embryo rescue, the use of growth regulators has been common with the end results being quite inconsistent. In the research laboratories at the ARC, woody plant medium (WPM) without hormone (Lloyd and McCown, 1980) has been used as a standard operating procedure for the past few years. It has shown success in initiating and proliferating grapevine cultivars without changing concentration levels of sucrose and has been able to support growth of embryo development without hormones. Good response at all stages of embryo

and plant development are achieved (Personal communication Phyllis Burger, ARC Infruitec-Nietvoorbij).

2.7 Marker assisted selection

Molecular markers for plant breeding applications were developed and became popular in the early 1980s (Xu and Crouch, 2008). The establishment of marker assisted selection (MAS) as a new tool for grapevine breeders offers vast possibilities to increase breeding efficiency (Eibach and Töpfer, 2014). Breeding new and disease resistant grape varieties is time-consuming and resource-intensive due to grapes having a long generation cycle. MAS allows monitoring the segregation pattern of the resistance loci in the progeny and also identifies the genotypes with multiple resistance loci.

MAS has been used for major gene-controlled disease resistance in primary crops and crops of less interest to the private sector (Dwivedi *et al*. 2007). Using wheat as an example, William *et al.* (2007) reported the use of MAS in wheat breeding programs which have been developed for around 20 genes or chromosome regions used in cultivar development. Different kinds of molecular markers exist. The subsequent are examples of DNA based molecular markers: restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs) markers, amplified fragment length polymorphisms (AFLPs), single nucleotide polymorphisms (SNPs), simple sequence repeats (SSRs or also called microsatellites), sequence tagged microsatellite sites (STMS), and Sequence Tagged Sites (STS) (Tartarini, 2003; Merdinoglu *et al*. 2005). For the purpose of this review, I will focus on SSR markers since the research presented in this thesis involved the application of this type of marker.

Microsatellites or Simple Sequence Repeat (SSR) are DNA sections made of tandemly repeating units of mono-, di-, tri-, tetra- or penta-nucleotide, arranged throughout the genomes of prokaryotes and eukaryotes and are not randomly distributed along the genome (Kumari *et al*. 2009; Phumichai *et al*. 2015). In fact, in a study on *Arabidopsis thaliana* and rice (Lawson and Zhang, 2006), SSR distribution was found to be non-random and there were variations in different regions of the genes

(Flores-Renteria and Krohn, 2013). Over the past 20 years, SSRs have been the most widely used markers for genotyping plants as a result of being informative and transferable among related species (Mason, 2015). In cultivated plants, SSRs are used for constructing linkage maps, quantitative trait loci mapping (QTL), using marker assisted selection and defining DNA finger printing of cultivars (Jonah *et al*., 2011). Furthermore, the use of fluorescent primers in combination with automatic capillary or gel based DNA sequencers has become popular in most progressive laboratories and SSR are excellent markers for fluorescent techniques, multiplexing and high throughput analysis. Molecular markers tightly linked to resistance (R) genes can obviate the need for resistance testing to identify resistant individuals from early generations (Langridge and Chalmers, 2005). The published SSR markers for the *Ren1* gene were verified in offspring from a KV cross with a susceptible cultivar (Veikondis, 2014). These markers will be used to identify plants homozygous for *Ren1* in the offspring of self-pollinated KV.

2.8 Aims and objectives

The aims of the study were to:

1. develop an *in vitro* phenotypic screening method for resistance to bacterial blight of grapevine.
2. screen offspring of self-pollinated KV for individuals homozygous for the powdery mildew resistance gene, *Ren1*.

Objectives

1. Determine and optimise the parameters for *in vitro* screening of grapevine phenotypes for resistance to bacterial blight.
2. Employ *in vitro* embryo rescue to ensure development of offspring from self-pollination of KV
3. Identify individual offspring homozygous for *Ren1* by using molecular marker technology (published SSR markers).

This knowledge will be beneficial for the breeding of grapevines to improve bacterial blight and powdery mildew resistance in the future.

Chapter 3: Materials and Methods

3. 1 The establishment of an *in vitro* screening method for evaluating the resistance of grapevine cultivars to *Xylophilus ampelinus*

3.1.1 Micropropagation of plants and growth conditions

Chemicals used in this study were supplied by Kimix, (Cape Town, South Africa) and Separations (Johannesburg, South Africa). Three varieties namely, Dauphine, Redglobe and C-3229 (a cross between G4-682, a breeding line, and Regal Seedless), were used to achieve the objectives in this study. All plant material was obtained from the greenhouse. Plant material was chosen based on their response to bacterial blight. Redglobe is known to be susceptible to bacterial blight while Dauphine is tolerant, and C-3229 has an uncharacterised response to *X. ampelinus*. The use of Redglobe and Dauphine will allow for comparison between the two varieties with regard to their response to bacterial blight as well as facilitate application of the selected method to determine the resistance phenotype of C-3229.

Stems from each of the three varieties were collected and then soaked for 5 minutes in 3.5% sodium hypochlorite containing 0.1% Tween 80. Plant material was rinsed three times in sterile (autoclaved) tap water in a laminar flow cabinet. and then placed on woody plant medium (WPM) (Lloyd and McCown, 1980), supplemented with 3% sucrose, 1 g/L activated charcoal, 0.1 g/L myo-inositol and 7 g/L agar, and pH adjusted to 5.8 before autoclaving. Explants were Micropropagated at four week intervals, one per bottle, on WPM. Plant material was maintained in the growth room (24 °C under cool white fluorescent lights, 100 $µm^{-2}$ s^{-1}, with a photoperiod of 16 hours light/8 hours dark). Initially, all three varieties were propagated on WPM but showed different responses to the medium. Redglobe and C-3229 failed to grow in WPM, remaining stunted, while Dauphine continued to grow well. As a result, Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) amended with 1.1 mg/L 6-benzylaminopurine (BA) and 0.75 mg/L indole-3-butyric acid (IBA), was thus the medium of choice throughout this part of the study.

3.1.2 Bacteria and growth conditions

Chemicals used in this part of the study were supplied by Merck (Darmstadt, Germany) Oxoid, (Thermofisher Scientific, South Africa) and Kimix. The *X. ampelinus* strain VS20 (ARC-PPRI Culture Collection) used in this study was stored as a glycerol stock at -70 °C. The bacteria were grown on YPGA (7 g/L yeast extract, 7 g/L Bacto-peptone, 7 g/L glucose and 15 g/L Bacto-agar) as described by Grall and Manceau (2003). Plates were incubated at 28 °C for seven to ten days. Seven to ten-day old colonies were streaked onto fresh YPGA plates and incubated at 28 °C for four days. To prepare liquid inoculum, four-day old *X. ampelinus* cells were harvested by washing from the surface of the agar with sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) and calibrated to an optical density of 0.3 at a wavelength of 600nm, corresponding to approximately 1 x 10^8 colony forming units (CFU/ml). The bacterial concentration was confirmed by plating serial dilutions in triplicate onto YPGA and incubating the plates at 28 °C for seven days.

3.1.3 Optimisation of *in vitro* plant inoculation method using Dauphine and Redglobe

In the first year of study (2015-2016), a preliminary investigation was conducted to compare two inoculation methods - namely scalpel and needle inoculation methods - *in vitro* for their effectiveness in resolving susceptible and resistant reactions to bacterial blight of grapevine. In this experiment, cultivars Dauphine and Redglobe, tolerant and susceptible (based on observation in the field), respectively, were used. The selected method was then used to assess C-3229; a selection whose resistance reaction was unknown at the start of the study. All pathogenicity trials were repeated twice. All experiments involving inoculations were conducted inside a laminar-flow cabinet using standard sterile-technique protocols. The experimental unit consisted of a single plant in a 5 cm diameter culture jar.

For each method, twenty Dauphine plantlets were inoculated with *X. ampelinus* and six additional plantlets inoculated with PBS were used as controls. The reason for this was that as a tolerant cultivar, Dauphine was tested for the presence of the pathogen after both four and eight weeks following inoculation. Ten inoculated plantlets and

three control plantlets were used at each sampling for each method. For Redglobe, ten plantlets at the six-to-ten leaf stage were inoculated with an *X. ampelinus* bacterial suspension and PBS was used to inoculate three additional plantlets as controls.

3.1.3.1 Inoculation of plantlets with *X. ampelinus* using the scalpel method

For the scalpel inoculation method, plantlets at the six to ten leaf stage were inoculated with an *X. ampelinus* bacterial suspension of approximately 1 x 10^8 CFU/ml by cutting off the top of the plantlet at the second node using a scalpel blade [Swann-Morton® carbon steel surgical blade no. 11, Sheffield, England] that was previously dipped in a bacterial suspension of *X. ampelinus* (Figure 3.1). Control plantlets were inoculated by the same method with PBS solution.

Figure 3.1: Scalpel inoculation method: Dauphine plantlet cut at the second leaf node from the apex with a scalpel blade previously dipped in 1 x 10^8 CFU/ml suspension of *X. ampelinus*.

3.1.3.2 Inoculation of plantlets with *X. ampelinus* using the needle pricking method

Plantlets were inoculated using a sterile 26GX$^{5/8}$ gauge syringe needle connected to a 5 ml syringe. The needle was inserted along the stem of the plantlets at the second node from the apex and a drop of the inoculum (adjusted to approximately 1 x 10^8 CFU/ml) was placed on the injured stem. Control plantlets were treated the same using sterile PBS solution. After inoculation, both Dauphine and Redglobe plantlets were allowed to stand in the laminar flow cabinet for 2-3 minutes to allow the bacteria to be absorbed into the wound. Plantlets were then transferred to the growth room for incubation at 25 °C for four weeks for Redglobe and for up to eight weeks for Dauphine with a 16 h light/8h dark photoperiod. Across the inoculation methods, plantlets were monitored weekly for signs of necrosis at the inoculation points and to see whether the symptoms spread and the pathogen was able to translocate to other parts of the plantlet. The selected method was then used to assess C-3229, a selection whose resistance reaction was not known at the start of the study.

3.1.3.3 Disease scoring for bacterial blight symptoms on grapevine

The phenotypic response of all plants inoculated with *X. ampelinus* was recorded at 7, 14, 21 and 28 days post inoculation. For Dauphine, this was extended for another four weeks since it did not exhibit any symptoms in the first four weeks, extending the observation time to a total of eight weeks. Disease severity was visually assessed based on the appearance and progression of necrosis along the stem and through the whole plant, beginning at the inoculation point (IP) for both needle and scalpel inoculation methods. Based on the symptoms, disease severity was rated using the rating scale shown in Table 3.1. Disease incidence, defined here as the percentage of diseased plants was also recorded.

Table 3.1. The disease rating scale for bacterial blight of grapevine based on the visual symptoms

Disease severity score	Description
0	Absence of disease symptoms
1	Necrosis restricted to the inoculation point
2	Necrosis reaching the petiole adjacent IP
3	Necrosis expanding through the shoot above and /or below IP, leaves not affected
4	Necrosis spreading through the shoot, leaf stalk and reaching the leaves
5	Necrosis spreading through the shoot and causing thinning of the stem and petiole causing wilting of the plant

3.1.3.4 Screening of C-3229 for resistance to *Xylophilus ampelinus*

The purpose of method optimisation was to select the most effective method for screening cultivars and new selections for resistance to bacterial blight of grapevine. Based on the results obtained, it was decided to inoculate *in vitro* grown plants of the line C-3229 using a modified needle inoculation method to determine its reaction to infection by *X. ampelinus*. Dauphine and Redglobe were included in the study as controls to monitor the efficacy of the method. The pathogenicity trials were conducted three times to verify the plant phenotypes.

Using twenty plantlets of Dauphine, Redglobe and C-3229 at the six-to-ten leaf stage, a modified version of the needle inoculation method described in section 3.1.3.2 was performed using a sterile 26GX$^{5/8}$ gauge syringe needle connected to a 5ml syringe. Instead of directly inserting the needle attached to the syringe containing the inoculum into the plant, a small incision was made with the needle along the stem of the plantlets at the second node. A 3µl droplet of the bacterial suspension (inoculum) adjusted to approximately 1 x 10^8 CFU/ml of *X. ampelinus* was then pipetted onto the fresh wound (Figure 3.2). An additional three control plantlets of Redglobe and C-3229 and six plantlets of Dauphine were inoculated with the same procedure, but using sterile PBS solution. Four weeks post-inoculation, twenty inoculated plantlets and three control plantlets were assessed for the presence of the pathogen in Redglobe and C-3229. Dauphine, as a tolerant cultivar, was assessed twice, after four and eight weeks

following inoculation. Ten inoculated plantlets and three control plantlets were used at each sampling for Dauphine.

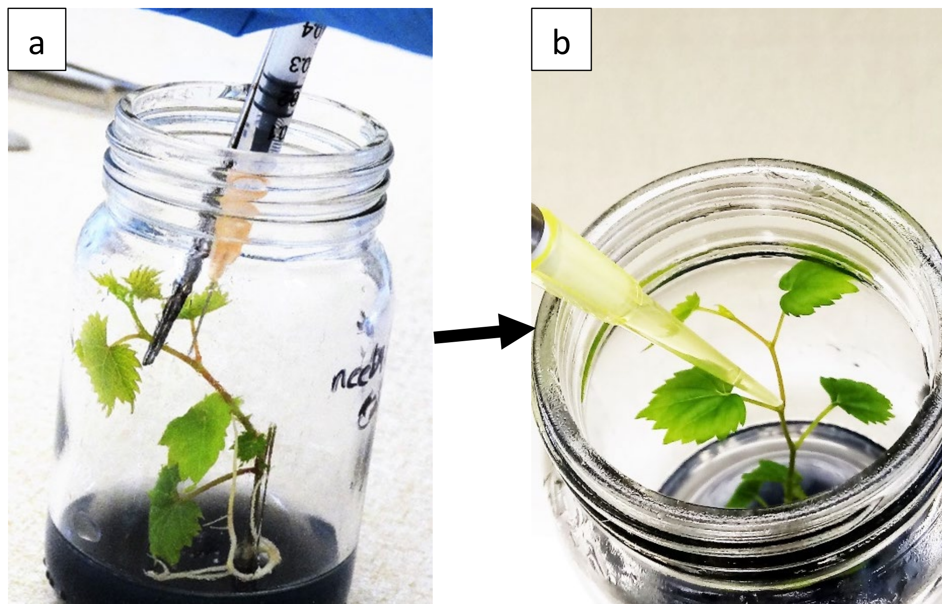


Figure 3.2: Inoculation of micropropagated plantlets using a modified needle inoculation method **a** - A small incision along the stem of the plantlets was made with the syringe needle at the second node from apical shoot while holding the plantlet with forceps. **b** - Using a micropipette, 3µl of the inoculum (approximately 10^8 CFU/ml) was placed onto the injured area.

3.1.4 Detection of *Xylophilus ampelinus* in inoculated plants

3.1.4.1 Re-isolation of *Xylophilus ampelinus* from inoculated plants

The Redglobe and C-3229 plantlets were tested for the presence of *X. ampelinus* four-weeks post-inoculation, and after four and eight weeks for Dauphine. For every experiment, three samples consisting of 0.3 cm segments of tissue from three different sections of the same inoculated plantlet of each variety were assessed, namely (a) the point of inoculation (IP), (b) 1.58 cm above the IP, and (c) 1.58 cm below the IP (Figure 3.3). Sections were surface-sterilised by rinsing in 70% ethanol, placed in 1.5 ml Eppendorf microcentrifuge tubes with 600 µl sterile Milli Q® water (Merck- Millipore, Darmstadt, Germany) and were thoroughly macerated using a sterile pestle. The macerates were left for one hour at room temperature (25 °C) to allow for diffusion of bacteria. Koch's postulate was confirmed by performing serial dilutions from the macerates, which were plated onto YPGA medium and incubated at 28 °C for seven

days for isolation and enumeration of *X. ampelinus* bacterial colonies to show conclusively that *X. ampelinus* was the cause of the observed disease symptoms. Plantlets inoculated with sterile PBS were used as controls.

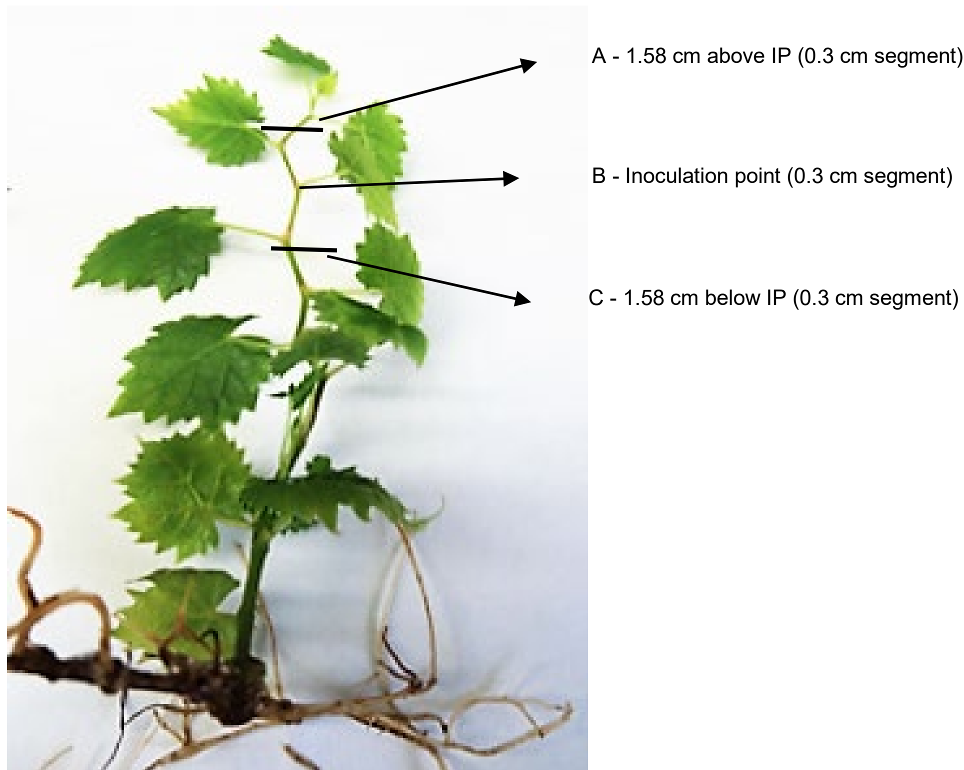


Figure 3.3: Sections used to confirm infection of *in vitro* inoculated plantlets (IP-Inoculation point).

3.1.4.2 Detection of bacterial presence in plant material via nested PCR

To confirm the presence of *Xylophilus ampelinus* DNA in infected micropropagated plantlets, nested-PCR was performed using primers listed in Table 3.2. The remaining macerate (section 3.2.5.1) was prepared for nested-PCR (nPCR) according to Botha *et al*. (2001). Briefly, samples were centrifuged at 10 000 rpm for 5 minutes and re-suspended in 1ml of wash buffer (100 mM Tris-HCl; 20 mM $NaCl_2$, pH 8.8). This step was repeated and the samples were then suspended in 50 µl of wash buffer plus a minute amount of polyvinylpyrrolidone (PVP). The samples were placed in boiling water for five-to-seven minutes before storage at -20 °C.

Each PCR amplification reaction was carried out in a 20 µl volume containing: 2 µl plant extract, the final concentration of each primer was 200nM, 1x final concentration

GoTaq Green master mix (10 µl) (Promega, Madison Wisconsin, USA), and 7.2 µl of sterile distilled water. The first PCR amplified a 742 base pair (bp) fragment using the primers A1 and B1 (Barry *et al*. 1991) under the following conditions: an initial denaturation step of 1 min at 94 °C followed by 30 cycles of 30 sec at 94 °C, 40 sec at 56 °C, 40 sec at 72 °C and a final extension at 72 °C for 2 min.

One microliter of the first PCR product was used as template for the second PCR. The second PCR made use of the *X. ampelinus*-specific primers, S3 and S4 (Botha *et al*., 2001), and amplified a 277 bp fragment. The PCR conditions were as follows: An initial denaturation step of 1 min at 94 °C followed by 30 cycles of 20 sec at 94 °C, 15 sec at 53 °C, 30 sec at 72 °C and a final extension at 72 °C for 2 min.

Table 3.2. Primers used for PCR detection of *X. ampelinus* DNA

Primer	Sequence	Description	Reference
A1	AGTCGTAACAAGGTAAGCCG	Forward primer for detection of *X. ampelinus,* Internal Transcribed Spacer (ITS) region; product is 742-bp	Barry *et al*. 1991
B1	CYRYTGCCAAGCATCCACT	Reverse primer for detection of *X. ampelinus* , ITS region; product is 742-bp	
S3	GGTGTTAGGCCGAGTAGTGAG	Forward primer for detection of *X. ampelinus* ITS region; product is 277-b	Botha *et al*. 2001
S4	GGTCTTTCACCTGACGCGTTA	Reverse primer for detection of *X. ampelinus* ITS region; product is 277-b	

3.1.4.3 Identification of candidate *X. ampelinus* colonies by specific PCR

Bacterial colonies resembling *X. ampelinus* isolated from the *in vitro* inoculated plants were transferred from YPGA plates into 30 µl sterile distilled water in microcentrifuge tubes. The samples were boiled for 5 min and 2 µl used as template in the PCR. To confirm the identity of *X. ampelinus*-like colonies recovered from the YPGA plates, PCR was performed, as described in section 2.2.5.2 with *X. ampelinus*-specific primer pair S3 and S4.

3.1.4.4 Gel Electrophoresis

The PCR products were resolved by electrophoresis through 1% agarose gels containing 2-3 µl of ethidium bromide in 1x TBE buffer (10.8 g/L Tris; 5.5 g/L Boric acid; 0.93 g/L EDTA). The samples were electrophoresed at 90 V/cm for 60 min. The PCR products were visualised and images recorded using a Syngene Ingenius LHR gel documentation system with GeneSnap™ version 7.12.06 (Syngene, Cambridge, United Kingdom).

3.1.4.5 *X. ampelinus* genomic DNA isolation

Total genomic DNA of *X. ampelinus* was extracted according to Mahuku (2004) to serve as a positive control every time PCR was performed. Bacteria were grown as described in section 3.1.2. A loopful of bacterial growth was transferred into sterile microcentrifuge tubes, and suspended in 1 ml of 1 M NaCl by vortexing. The suspension was centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was discarded and the cell pellets suspended a second time in 1M NaCl and centrifuged at 3000 rpm for 10 min to reduce and separate the cells from the polysaccharide. The pellets were suspended in 1ml of sterile distilled water and centrifuged at 4000 rpm for 10 min. This step was repeated. Pellets were resuspended in 500 µl of TES extraction buffer (0.2 M Tris-HCl pH 8, 10 mM EDTA pH 8, 0.5 M NaCl and 1% SDS) and 3 µl of 20 mg/ml proteinase K, mixed and incubated for 30 min at 65 °C. Ammonium acetate was added (250 µl of 7.5 M solution, pH 5.5) and the samples incubated on ice for 10 min, after which the samples were centrifuged at 13000 rpm and 4 °C for 15 min. The supernatant was transferred to a clean tube and DNA was precipitated by adding 0.6 volumes of cold isopropanol and incubated at -20

°C for 1-2 hours. The samples were then centrifuged for 10 min at 13000 rpm. Pellets were washed with cold 70% ethanol, air-dried and re-suspended in TE buffer (10 mM Tris-Cl (pH 8.0); 1 mM EDTA (pH 8.0)) when the pellet was dry. Two microliters of RNAse A was added for a final concentration of 1 µg/µl and incubated at 37 °C for 30-60 minutes. Isolated genomic DNA was quantified using the spectrophotometer ((Biodrop® Biochrom Ltd, Cambridge, UK). Measures of DNA purity was determined by the A260: A280 and A260: A230 ratios, which indicate protein, phenol, and carbohydrate contamination (Nazhad and Solouki, 2008). Isolated DNA was stored at -20 °C for future use.

3.1.5 Data analysis for disease phenotyping

Mean disease severity for each variety was calculated. Data were analysed for significant differences between inoculation methods and the varieties using Analysis of variance (ANOVA), and student's t-Least Significant Differences (LSD) test were performed in the case of significant effects ($P \leq 0.05$). Disease growth curve fitting on the data was done using Gompertz growth curve since this is a suitable and appropriate approach for this particular data analysis (Kaufman, 1981; Larsen, 2012).

3.2 Screening Kishmish Vatkana seedlings with molecular markers linked to powdery mildew resistance gene, *Ren1*

3.2.1 Origin of plant material

Kishmish Vatkana plants were self-pollinated in October of 2015. The population was created at the ARC Infruitec-Nietvoorbij, Stellenbosch. The aim was to create breeding lines homozygous for *Ren1* (the gene responsible for resistance to PM). Kishmish Vatkana is regarded as seedless, but small, soft, green rudimentary seeds are present. Since hard seeds do not develop, the standard seed stratification and germination protocols cannot be followed (Personal communication P. Burger, ARC Infruitec-Nietvoorbij). Embryos from seedless cultivars aborted a few weeks after flowers were pollinated and *in vitro* embryo rescue techniques were thus used to develop plants from the self-pollinated KV embryos.

Bunches were tagged and covered in paper bags (Figure 3.4) before flowers opened to prevent uncontrolled or accidental cross pollination from other cultivars. The anthers split open and released the pollen grains onto the stigmata for fertilisation to occur inside the paper bags. The bags were removed only after flowering was over and berries were well set (berry size was about 10 mm in diameter at the time). These immature berries were harvested in December 2015.

Figure 3.4: Self-pollination of Kishmish Vatkana in the ARC Infruitec-Nietvoorbij vineyards

3.2.2 Embryo rescue and plant development

Immature berries from self-pollinated KV lines were harvested within 6-8 weeks after fruit set and prior to embryo abortion. The berries were surface sterilised with 50% commercial bleach for 20 min followed by three washes of 5 min each in sterile (autoclaved) tap water in a laminar flow cabinet. Embryos were aseptically excised using a scalpel from the berries and placed in sterile Petri dishes (10 embryos per dish) containing woody plant culture medium (WPM) (Lloyd and McCown, 1980), supplemented with 0.1 g/L myo-inositol, 1 g/L activated charcoal, 30 g/L sucrose and 7 g/L agar. The pH of the medium was adjusted to 5.7 before autoclaving. The embryos were cultured in darkness at 25 °C.

After two and a half months, germinated embryos were removed and further cultured on fresh WPM, while those that had not already germinated were excised from the rudimentary seeds under a stereomicroscope (Figure 3.5a) and further cultured on fresh medium (Figure 3.5b). The excised embryos together with the germinated embryos were recorded and grown at 25 °C with 16/ 8h day/ night cycle of white fluorescent light (40 μmol.m^{-2}.s^{-1}) (Figure 3.5c). After one month (30 days) of culture, germination of all embryos was recorded and after another month, the number of well-rooted plantlets were recorded (Figure 3.5d).

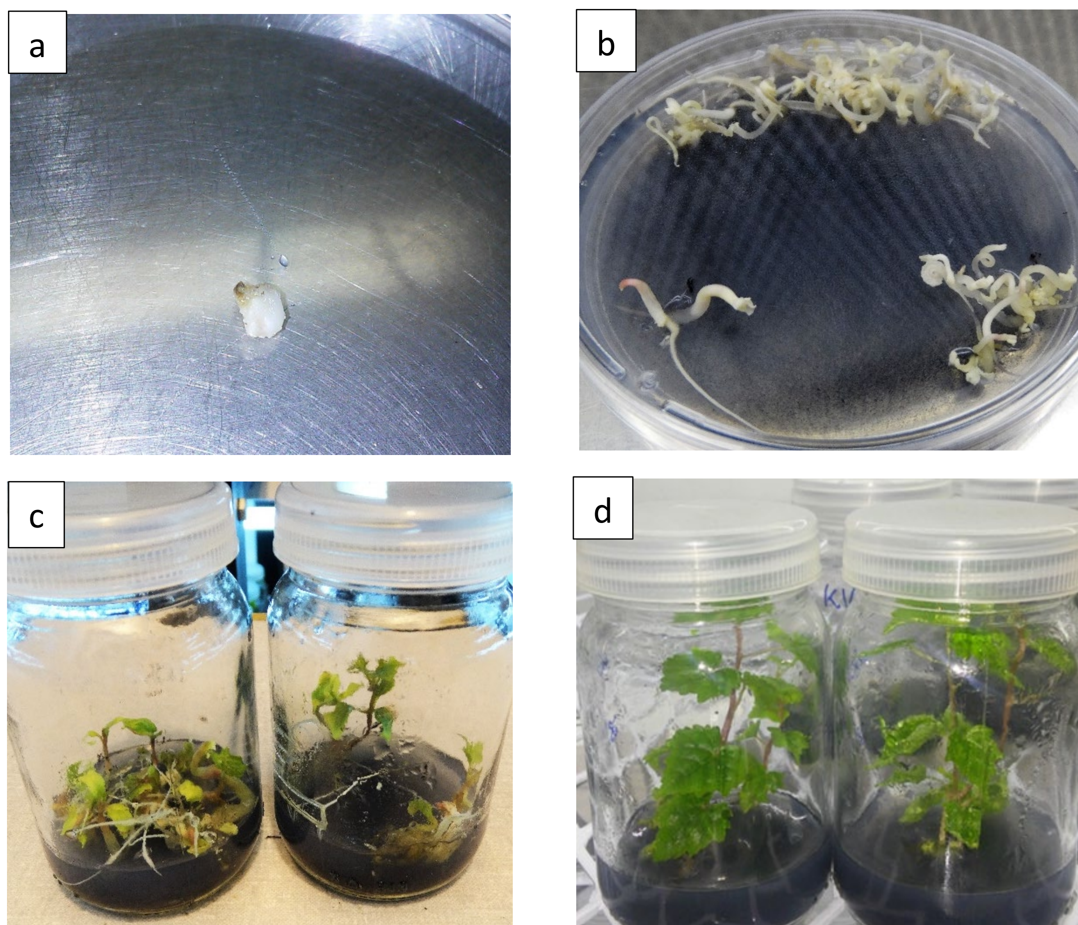


Figure 3.5: Embryo rescue technique to develop offspring from self-pollinated KV: **a** - Developed embryo from ovule as indicated by the arrow, **b** - Initial germination of embryos in WPM medium, **c** - Plantlets with true leaves, **d** - Whole plantlets developed from germinated embryos.

3.2.2.1 Transfer and acclimatisation of embryo rescue derived offspring to glasshouse conditions

When well-rooted plantlets reached a size at which they could easily be handled and further growth *in vitro* would be limited by the size of the culture bottles, they were transferred to a glasshouse. Seedlings were removed from the culture bottles, the agar-medium adhering to the roots rinsed off with distilled water, and planted in a commercial seedling growth mix (Hygromix) in plastic seedling trays. Each tray was covered in a plastic bag and placed in a glasshouse where it was protected from direct sunlight, but under growth lights at 25 °C with 16/ 8h day/ night cycle of white fluorescent light (40 $\mu mol.m^{-2}.s^{-1}$). To maximise relative humidity and prevent desiccation, plants were covered with plastic bags for several weeks and protected from direct sunlight (Figure 3.6a). The plastic bags were gradually opened to facilitate acclimatisation to conditions of lower humidity and temperature. The hardened-off seedlings were kept in the glasshouse under natural daylight and watered when required to ensure that the potting soil remained moist (Figure 3.6b). Six weeks after transfer to the glasshouse, the number of seedlings that had survived the hardening-off process was recorded.

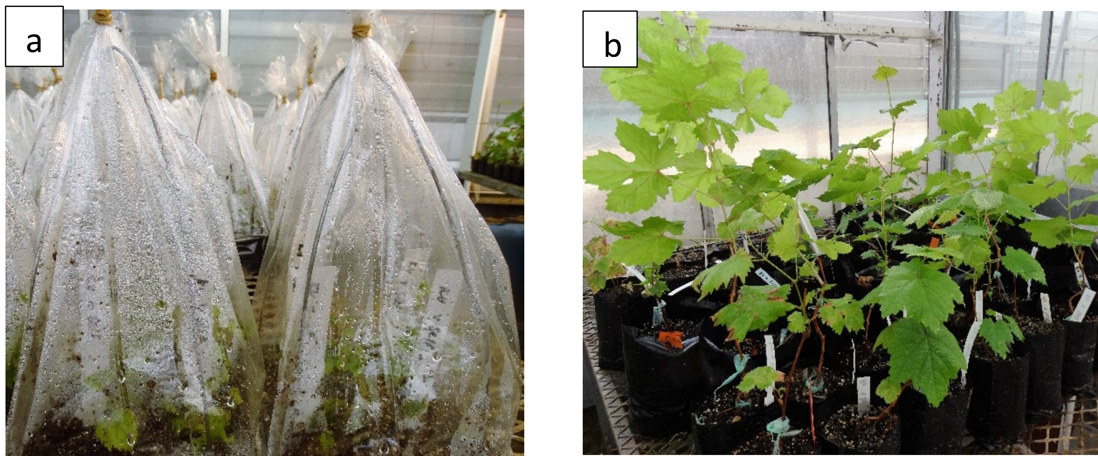


Figure 3.6: Acclimatisation of embryo rescued KV offspring: **a** - transplantation of plantlets into soil **b** - surviving seedlings in glasshouse.

3.2.3 Molecular techniques

3.2.3.1 Genomic DNA extraction from KV leaf tissue

It was difficult to get leaves from the population of 36 individuals derived from self-pollination of KV as the plants grew very poorly, most probably due to their failure to acclimatise to the outdoor environment from the *in vitro* conditions. As such, 21 offspring plants were lost. To ascertain zygosity status of the surviving 36 plantlets generated from the rescued embryos of self-pollinated KV plants, DNA was extracted from leaf material of glasshouse acclimated offspring, and from stems and leaves of micropropagated shoots using the cetyltrimethylammonium bromide (CTAB) DNA extraction method (Lodhi *et al*. 1994). Only fully open leaves as close as possible to the shoot tip were collected, placed into labelled plastic bags and transported in a cooler box to the laboratory for DNA extraction.

Leaf tissue (0.5 g fresh weight) was ground with carborundum at room temperature, using a sterile mortar and pestle and mixed with 5ml of DNA extraction buffer (20 mM Tris, 1 mM EDTA pH 8.0, 0.5 volumes of 5 M NaCl, 3% CTAB, 0.2% w/v 2-mercaptoethanol (added immediately prior to use)). The slurry was transferred to 15ml polypropylene centrifuge tubes, and 50 mg polyvinylpolypyrrolidine (PVP) added. Samples were incubated at 60 °C for 25 min after which 6 ml of 24:1 chloroform: isoamylalcohol was added. The extracts were gently mixed by inverting the tubes to form an emulsion, before centrifuging at 6000 rpm for 15 min at room temperature. The resulting aqueous solution was transferred to sterile 50ml centrifuge tubes using a wide-bore pipette tip without disturbing the pellets. A 50% v/v of 5 M NaCl was then added to the aqueous solution recovered from the previous step and mixed well. Two volumes of cold 95% ethanol (EtOH) was added to the aqueous solution and incubated at 4 °C for 20 min to precipitate genomic DNA. Samples were centrifuged at 6000 rpm, for 20 min, at 4 °C, after which the supernatant was discarded and the resulting pellets washed with cold 70% EtOH. DNA pellets were air dried at 37 °C for 30 min and resuspended in 200 µl TE buffer (10 mM Tris.HCl, 1 mM EDTA pH 8). Finally, one microliters of 20 mg/ ml RNase A was added per 100 µl of DNA and incubated at 37 °C for 15 min prior to storage at -20 °C.

3.2.3.2 Gel electrophoresis and DNA quantification

Integrity of the extracted genomic DNA was assessed by electrophoresis in a 1.0% agarose gel with 1 x Tris/ Borate/ EDTA (TBE) running buffer at 100 volts for one hour. Ten microliters of undiluted DNA were mixed with 3 µl of loading dye (Thermo Scientific, South Africa). The samples were electrophoresed at 90 V/cm for 45 minutes and visualised images recorded using a Syngene Ingenius LHR gel documentation system with GeneSnap™ version 7.12.06 (Syngene, Cambridge, United Kingdom).

Extracted genomic DNA was quantified using a spectrophotometer (Biodrop® Biochrom Ltd, Cambridge, CB40FJ UK). DNA purity was determined by the A260: A280 and A260: A230 ratios. These ratios indicate protein and polyphenol and carbohydrate contamination, respectively (Nazhad and Solouki, 2008). DNA samples from the extractions were diluted and adjusted to a final concentration of 30 ng/µl.

3.2.3.3 PCR conditions and amplification

All genotyping was performed at CenGen labs (Worcester, South Africa). A DNA quality check was done on 0.8% agarose gel at 50 volts for 45 minutes prior to PCR to confirm the results of PCR amplification. PCR conditions were optimised by CenGen laboratories (Worcester, South Africa) (Table 3.3). Amplifications with Simple Sequence Repeat (SSR) primers presented in Table 3.4 were performed in a final volume of 10 µl containing 1 µl of 30 ng/µl template DNA and the multiplex primer sequences for the *Ren1* linked markers on chromosome 13 (Veikondis, 2014). Samples were amplified using the GeneAmp PCR system 9700 and the Veriti 96-well thermocycler (Life Technologies, South Africa).

The PCR cycling parameters were an initial denaturation of 94 °C for 4 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing for 10 sec at and extension at 72 °C for 15 sec; and a final extension step at 72 °C for 30 min before cooling down to a 4 °C holding step to maintain product integrity. Markers used in this study had previously been optimised for marker combination per multiplex as well as annealing temperature (Veikondis, 2014). The annealing temperatures between 56 °C and 59 °C were used to test various marker combination for the optimised reaction set-up with all multiplex PCR reaction performed separately (Veikondis, 2014). All products were

pooled in equal volumes for multiplex one, two and three (Table 3.4) due to the differences in annealing temperatures, but the markers could be electrophoresed together to save cost.

SSR markers for linkage group 13 (LG13) were chosen based on the specific gene region (*Ren1* locus) targeted. We assayed VMC9h4.2, UDV020a, UDV124, VVIP10 and VMCNg4e10.1, which are all associated with the *Ren1* locus and previously verified in a KV cross (Veikondis, 2014). The *Ren1* locus is flanked by UDV124 on one side and the co-segregating VMC9h4.2 and VMCNg4e10.1 markers on the other side. The last two markers did not recombine and were determined by Hoffmann and co-workers (2008) to be located at a genetic distance of approximately 0.9 cM from the *Ren1* locus. The selected primer sets were commercially synthesized and fluorescently labelled at the 5' end with VIC^{TM} (green), NED^{TM} (yellow), PET^{TM} (red) or FAM^{TM} (blue) fluorophore dyes (Applied Biosystems, South Africa). The primer sequences have been reported in the following sources: UDV primer series is reported in Di Gaspero et al., 2007. The majority of the SSR markers used were from *Vitis* Microsatellite Consortium (VMC). Others were from the VVI and UDV marker series. These markers are available as NCBI uni-STS sequences (http://www.ncbi.nlm.nih.gov/). All these markers used in the study and their sources are referenced in table (Table 3.4).

3.2.3.4 Genotype data capturing

Once PCR amplification with fluorescently labelled primers was achieved, PCR products were electrophoresed at the Central Analytical Facilities (CAF) at the University of Stellenbosch using the ABI 3130 Prism® Genetic Analyzer (Applied Biosystems). Genemapper® software version 4.8.1 (Life Technologies, RSA) was used to label peaks and create appropriate bins. Genotypes showing a single amplified fragment or a prominent peak were considered as an allele with two peaks expected for a heterozygous and one peak for a homozygote for that particular locus. The labelled alleles were checked to ensure that data was correct. Allelic scores were verified by a competent technician and data was captured in excel Microsoft office spreadsheets for further reference and usage. Data was independently validated by the Cengen laboratory in Worcester and collated in Microsoft excel office. The

software summarised the data for each KV offspring plant. Segregation analysis was performed by referring to the different fragments obtained when screening each offspring with a specific marker, thus allowing allelic distribution in the progeny to be determined. The electropherograms of each individual from the progeny was compared.

Table 3.3: PCR multiplex reaction protocol

Reaction components	Stock concentration	Final concentration	Volume per reaction
Buffer (Separations)	10X	1X	1 µl
dNTP (Kapa Biosystems, RSA)	5 mM	0.1 mM	0.4 µl
25 mM $MgCl_2$ (Separations)	25 mM	1.8 mM	0.72 µl
Supertherm Taq (Separations)	5 U/µl	0.75 U	0.15 µl
Multiplex appropriate primers	10 pmol/µl	0.3 pmol/µl	0.2 µl of each forward and reverse primer
DNA	100 ng/µl	30 ng/µl	1 µl
ddH_20			Add to total volume of 10 µl

Table 3.4: SSR primers (René Veikondis, PhD thesis, 2014) used in screening the progeny of self-pollinated KV

Reaction	SSR Marker Name	Forward primer sequence(5'-3')	Reverse primer sequence(3'-5')	Source	LG[2]	Label[3] colour	TM
1	UDV020	tgttggtgtgtgtttgtacgtg	tgttggcctgatgttgagag	NCBI	13	FAM	57°C
2[1]	UDV124	gcatcttcttcttcccaacc	gagtgcatttgtcaaagtcgtg	NCBI	13	PET	56°C
	VMC9H4-2	cacatcattcattgatgaggct	gcagttgatgcaaaacaacagt	*Vitis* Microsatellite Consortium	13	PET	
	VVIP10	tgccttgacattgttttcatcc	gaaactgggctgttattgttga	Merdinoglu et al., 2005		FAM	
3	VMCNG4e10-1	aatgcagcagcgccagatg	gcaggctgctgctgtttg	*Vitis* Microsatellite Consortium	13	VIC	59°C

TM – Annealing temperature

[1] Multiplex PCR reaction

[2] Linkage group or chromosome number that the SSR marker is positioned on

[3] Fluorescent label colour

Chapter 4: Results

4.1 The establishment of an *in vitro* screening method for evaluating the resistance of grapevine cultivars to *Xylophilus ampelinus*

4.1.1 Micropropagation of plants and growth conditions

Three varieties namely, Dauphine, Redglobe and C-3229 were established on WPM medium with no hormones for propagation. However, it was observed that C-3229 and Redglobe showed abnormal growth. The shoots of these varieties were not able to elongate, only to form dwarf shoots and a few green leaves, as a type of vitrification phenomenon (Figure 4.1a and 4.1b). However, Dauphine grew well on the same medium showing elongation of stems (Figure 4.1c). Since the stem elongation and leaf appearance of Redglobe and C-3229 cultured in WPM medium were considered to be not as desirable as shoots propagated on MS (Figure 4.2), it was decided that MS with 6-benzylaminopurine (BA) and indole-3-butyric acid (IBA) hormones would be used for shoot multiplication and root initiation.

4.1.2 Bacterial inoculum preparation

An optical density of 0.3 at 600 nm was used in determining the colony forming units in the prepared bacterial suspension and plate count serial dilutions were done to verify that the inoculum to be used was at 10^8 cfu/ml. Table 4.1 shows the bacterial count after serial dilution.

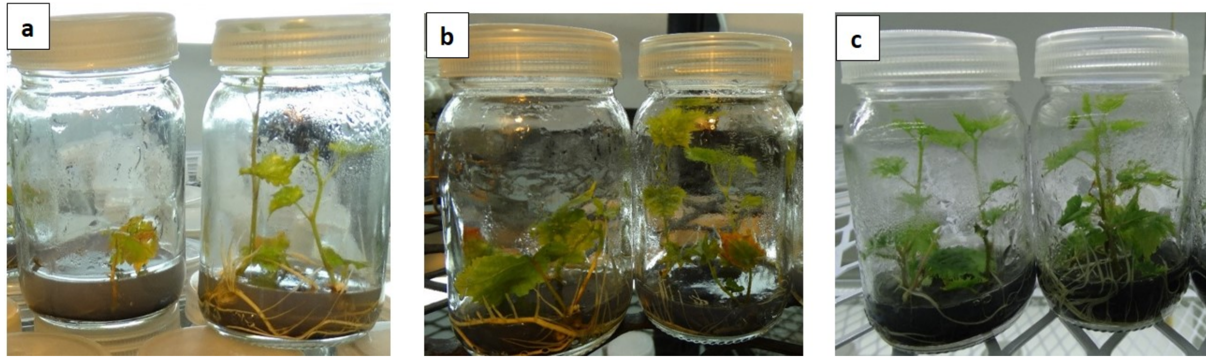


Figure .4.1: Micropropagation of grapevine plantlets after four weeks in culture on WPM medium with no hormones **a** – Redglobe, **b** - C-3229, **c** - Dauphine. Redglobe and C-3229 exhibited poor growth on the medium while Dauphine showed good growth.

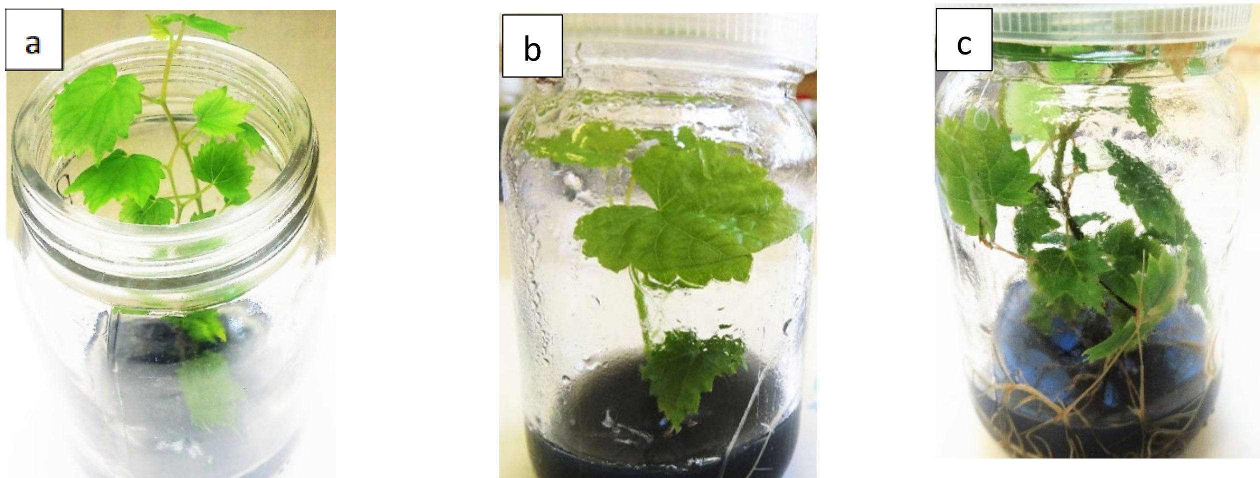


Figure 4.2: Micropropagation of grapevine plantlets after four weeks in culture showing improved growth on MS medium supplemented with 1.1 g/L BA and 0.75 g/L. **a** - Redglobe, **b** - C-3229 and **c** - Dauphine.

Table 4.1: Observations and calculated bacterial counts per ml of plant sample

Dilution	ml of dilution plated	Number of colonies	Bacterial count per ml of sample	Average count per ml of sample
10^2	0.1 ml	TNTC[1]	NA[2]	NA
10^2	0.1 ml	TNTC	NA	NA
10^2	0.1 ml	TNTC	NA	NA
10^3	0.1 ml	TNTC	NA	NA
10^3	0.1 ml	TNTC	NA	NA
10^3	0.1 ml	TNTC	NA	NA
10^4	0.1 ml	700	7×10^7	4.19×10^7
10^4	0.1 ml	499	4.99×10^7	
10^4	0.1 ml	460	4.6×10^7	
10^5	0.1 ml	187	1.87×10^8	1.59×10^8
10^5	0.1 ml	157	1.57×10^8	
10^5	0.1 ml	132	1.32×10^8	
10^6	0.1 ml	23		3.5×10^8
10^6	0.1 ml	38	3.8×10^8	
10^6	0.1 ml	43	4.3×10^8	

[1] Too numerous to count

[2] Non applicable

4.1.3 Evaluation of *in vitro* plant inoculation methods: Comparing the scalpel and needle-pricking methods

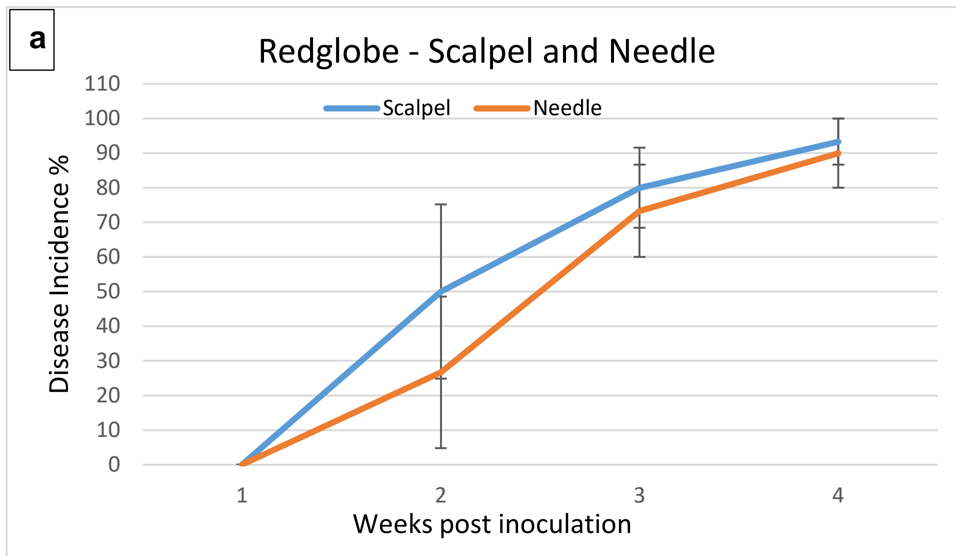
In order to compare the susceptibility/ tolerance level of the two grape varieties that were used in this study (Redglobe and Dauphine) two inoculation methods were applied to discriminate between resistant and susceptible phenotypes. To verify that the phenotype observed for the experiment plants was the result of the inoculated bacteria, the phenotypes of the experiment plants were compared to control plants inoculated with PBS solution. The results of three independent experiments showed symptom development on the stem for both susceptible and tolerant plants, with symptoms appearing later on the tolerant Dauphine plants than on susceptible Redglobe plants. The control plants remained healthy, with the exception of callus formation at the inoculation point due to injury. Thus indicating that symptom

development on the experiment plants was due to inoculation with *X. ampelinus*. Mean disease incidence (the number of plants exhibiting symptoms) was calculated as a percentage for both methods.

Disease incidence values were fitted as a function of time using the modified Gompertz model (Figure 4.3) in order to analyse the effect of the inoculation method on the incubation period, that is, the initial appearance of the symptoms. Symptoms on Redglobe appeared after one week in plants inoculated with both methods. The average disease incidence for the scalpel method was 50%, 80% and 93% at week two, three and four, respectively (Figure 4.3a). The same pattern was observed for the needle method where disease incidence was 46%, 76% and 90% at week two, three and four, respectively (Figure 4.3a).

In contrast, Dauphine plants inoculated with either method did not show any symptom for the first five weeks and symptoms only developed between the sixth and eighth week after inoculation. These symptoms consisted of discolouration of tissue on the shoots at the point of inoculation, brown discolouration that moved to the neighbouring tissues, including the stem and petioles. This resulted in necrosis on the tissue and in some cases wilting of the plant due to thinning of the stems and petioles. The average disease incidence for the scalpel method was 88%, at both six and seven weeks, post inoculation; and 100% at eight weeks post inoculation for the variety, Dauphine (Figure 4.3b). For the needle method, the average values of disease incidence at six, seven and eight weeks' inoculation was 66%, 82% and 94%, respectively (Figure 4.3.b).

Data were analysed for significant differences among varieties and methods. The interaction between inoculation method and variety was not significant ($P \leq 0.05$). However, there is a significant difference between the two varieties in disease incidence four weeks, post inoculation ($P \leq 0.05$; Figure 4.3). By this time, the severity of disease symptom expression for Redglobe was high, while Dauphine had to be kept in the same medium for longer to further observe its response to the pathogen. It was only at week six that Dauphine started to show any symptoms. By this time, it had been in the medium longer than Redglobe, which may have then had an influence on the observed symptoms.



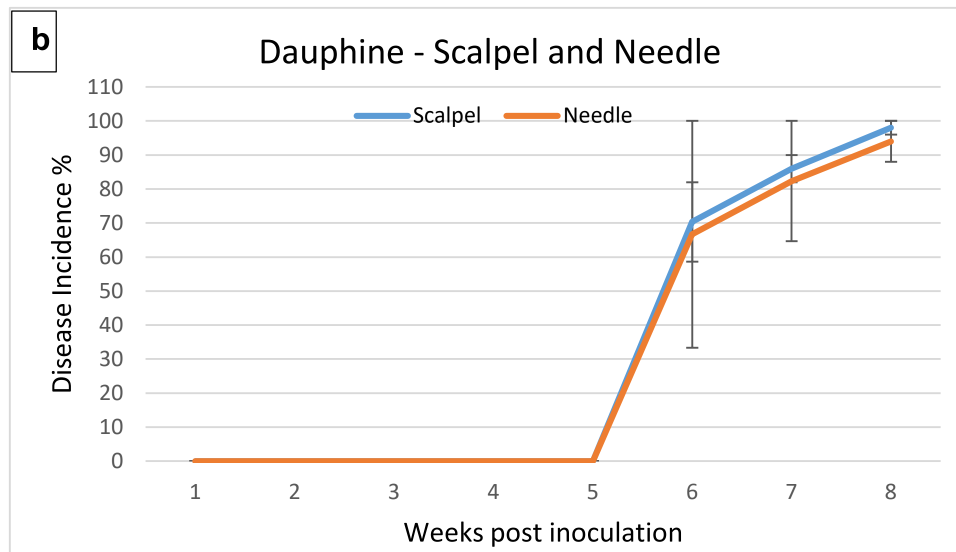


Figure 4.3: Disease incidence in micropropagated grape plantlets inoculated with suspensions of *X. ampelinus* (10^8 cfu/ml) using different inoculation methods: **a** – Redglobe inoculated with scalpel and needle inoculation methods; **b** – Dauphine inoculated with scalpel and needle inoculation methods. Values are the mean of the three replicated trials, error bars correspond to the standard error of the mean. Redglobe (n=20) at 1–4 weeks post-inoculation and Dauphine (n=20) at 1-8 weeks post-inoculation.

4.1.4 Response of Dauphine and Redglobe to *X. ampelinus* infection

Both control and inoculated plants of each variety were visually assessed on a weekly basis for four to eight weeks, depending on the variety. The development of disease symptoms was observed within one week of inoculation for Redglobe and after five weeks for Dauphine. While evaluating disease resistance under *in vitro* conditions, it was deemed necessary to record the phenotypic characters, which would correlate with the disease severity. Through this approach, the phenotype of a given variety can be quantified according to the symptoms. Hence, six phenotypic parameters were recorded to correlate with the disease severity (Table 3.1, section 3.1.3.3).

The control inoculation, an injection with sterile PBS solution, caused no disease symptoms (Figure 4.4a, c, e). Dauphine plantlets did not show any symptoms in the first five weeks after inoculation (Figure 4.4b), they continued to grow normally, and similar to plantlets inoculated with sterile PBS. This was observed for both scalpel and needle-inoculation methods. However, from week six to week eight the symptom progression increased quickly reaching a disease severity score of three. This pattern in behaviour was observed for the scalpel method as well as the needle method, although none of the plantlets collapsed. At six weeks, control plants remained disease free (Figure 4.4c) while some plantlets inoculated with bacteria using both methods were observed to develop necrosis beyond the point of inoculation margin above and below the stem and also at the adjacent petioles (Figure 4.4d). Healthy looking control plantlets were observed after eight weeks of inoculation. At eight weeks post inoculation, additional plantlets started to show symptoms, while the symptoms on already infected plantlets became more pronounced and included stunting of plants and brittle leaves (Figure 4.4f). Collapse in plants was observed after eight weeks post inoculation with the needle method (Figure 4.4f). It must be noted that the reason for this phenotypic response might relate to the fact that Dauphine had been in the same medium for eight weeks, which may have affected the health status of the plants. Especially since, at eight weeks, plantlets inoculated with sterile PBS started to wither with leaves starting to brown and fall off, but to a lesser extent than those inoculated with *X. ampelinus*.

As previously mentioned, symptoms on Redglobe appeared earlier than on Dauphine plantlets. Control plantlets inoculated with sterile PBS using the scalpel method did not exhibit any symptoms throughout the duration of the experiment (symptom rating scale 0) (Figure 4.5a). At week two, plants inoculated with bacterial suspension of 10^8 cfm/ml started showing symptoms restricted to the inoculation point for scalpel, correlating to a disease severity score of one (Figure 4.5b). At week two, control plantlets inoculated with sterile PBS using the needle method showed no symptoms (Figure 4.5c), while plantlets inoculated with bacteria showed symptoms restricted to the point of inoculation correlating to a disease severity score of one (Figure 4.5d). When comparing the two methods for each variety, we found that at the beginning of the experiments the disease development was uniform, whether symptoms started showing at week two (as was the case with Redglobe) or at week six (as was the case with Dauphine). However, as time progressed, we observed a difference at how quickly the disease symptoms progressed and the final disease severity but not necessarily in the final resistance ranking of the varieties tested.

Most of the plants inoculated with the scalpel method did not show the spread of discolouration to neighbouring tissues and symptoms were limited to the inoculation point. After a further two weeks, plants inoculated via the needle method showed necrotic lesions formed beyond the margins of the inoculation point and spreading through the shoot upwards and downwards, resulting in a disease severity score of three (Figure 4.5e). At week four, most plantlets were showing necrosis spreading through the shoot and causing thinning of the stem and petioles. This led to the plant losing its strength and collapsing, symptom scale of five (Figure 4.5f). Most of the plants inoculated using the scalpel method did not experience plant collapse. Therefore, tables 4.2 and 4.3 provide a summary of the disease severity scores and incidence observed per week using needle inoculation method for Dauphine and Redglobe, respectively, over the three trials conducted. The needle method gives a clear picture of the different levels of the appearance of disease symptoms and how it progressed leading to the final resistance ranking of the two varieties.

For Dauphine, at week seven, up to 62% of plants exhibited symptoms only restricted to the inoculation point, a disease grade of one. At the end of week eight, 94% plants were showing a disease grade of three with plants showing necrosis expanding

through the shoot above and/or below IP and withering of leaves. Overall, a disease grade ranging from 0-3 was observed after five weeks post-inoculation for Dauphine. For Redglobe, on the other hand, at week one, a disease grade of 0 was observed with 0% symptom development. By week two disease incidence reached up to 47% with 20% of the plants showing a disease severity score of two and 27% a disease severity score of three. (Table 4.3). By end of week four, up to 53% of the plants were wilting and 37 % showed necrosis that had spread to the adjacent petioles from the inoculation point, a disease grade of five and two, respectively (Table 4.3).

Overall, these data confirmed the phenotypes of Dauphine and Redglobe and demonstrated that they were useful varieties for our purpose of evaluating the efficacy of different inoculation methods for in *in vitro* assessment of disease resistance.

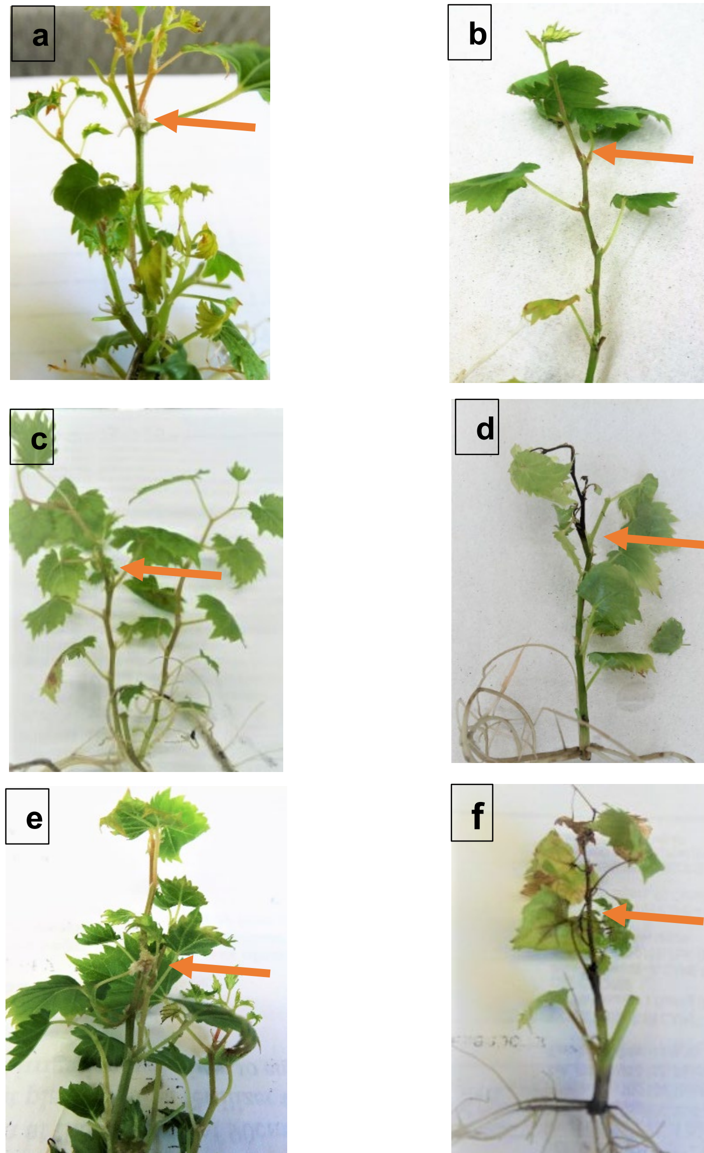


Figure 4.4: Plant phenotypic responses of micropropagated Dauphine plantlets to *X. ampelinus* inoculated using the needle method. Errows pointing at the IP point, **a** – plantlet inoculated with sterile PBS buffer (control) at four weeks showing no symptoms. **b** - plantlet inoculated with bacterial suspension (10^8 cfu/ml) at four weeks post-inoculation, showing callus formation; **c** - plantlet inoculated with sterile PBS buffer (control) at six weeks showing no symptoms; **d** - plantlet inoculated with bacterial suspension (10^8 cfu/ml) at six weeks post-inoculation showing necrosis both above and below beyond the point of inoculation margin; **e -** plantlet inoculated with sterile PBS buffer (control) at eight weeks showing no symptoms; **f** - plantlet inoculated with bacterial suspension (10^8 cfu/ml) at eight weeks post-inoculation showing withering of leaves.

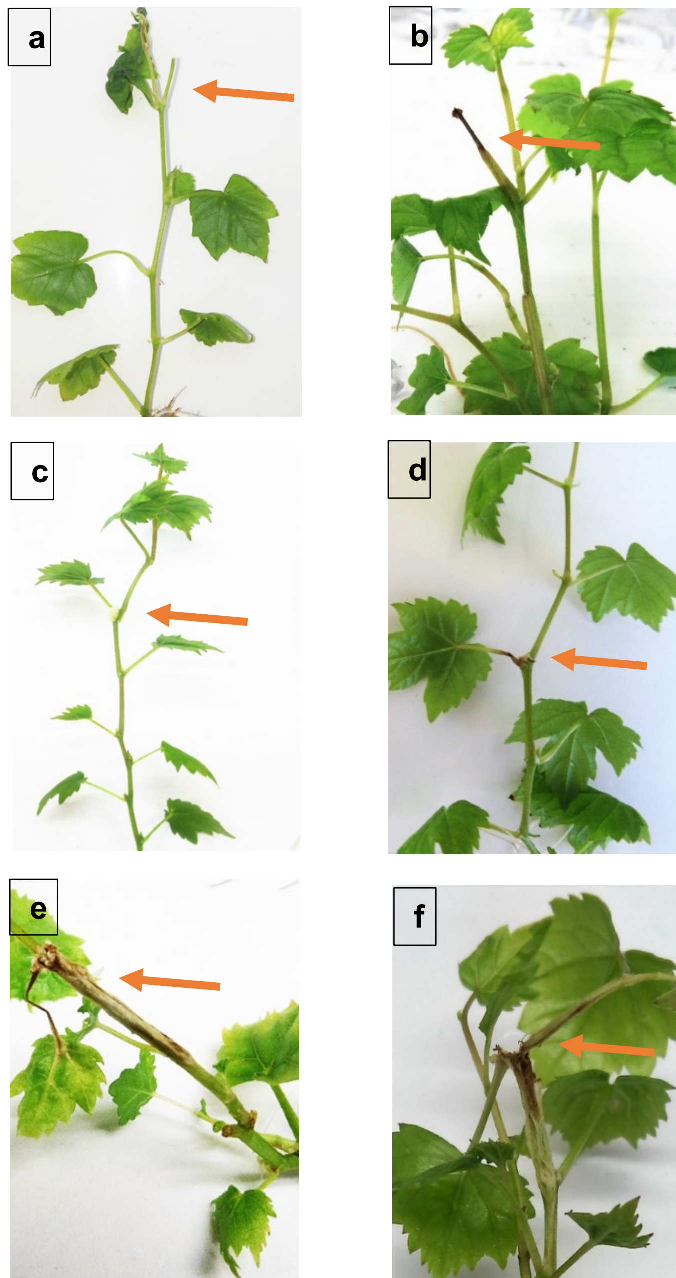


Figure 4.5: Plant phenotypic responses of micropropagated Redglobe plantlets to *X. ampelinus* inoculated using the scalpel and needle methods. Arrows pointing at the IP point, **a** - plantlet inoculated with sterile PBS buffer (control) at two weeks showing no symptoms using scalpel method. **b** - plantlet inoculated with bacterial suspension (10^8 cfu/ml) at two weeks post inoculation, showing symptoms restricted to the inoculation point, representing a disease severity score of 1; **c** - plantlet inoculated with sterile PBS buffer (control) at two weeks showing no symptoms using needle method, **d** - plantlet inoculated with bacterial suspension (10^8 cfu/ml) at two weeks post needle inoculation, showing symptoms restricted to the inoculation point, representing a disease severity score of 1, **e** - plantlet inoculated with bacterial suspension (10^8 cfu/ml) at four weeks post needle inoculation representing a disease severity score of 3; **f** - plantlet at four weeks post needle inoculation representing a disease severity score of 5.

Table 4.2: Phenotypic responses of Dauphine inoculated with *X. ampelinus* using the needle inoculation method

Week post-inoculation	Disease score	Symptom description	% plants [a]
5	0	Absence of disease symptoms	100%
6	1	Necrosis restricted to the inoculation point	47%
	3	Necrosis expanding through the shoot above and/or below IP, leaves not affected	20%
	0	Absence of disease symptoms	33%
7	1	Necrosis restricted to the inoculation point	62%
	3	Necrosis expanding through the shoot above and/or below IP, leaves not affected	20%
	0	Absence of disease symptoms	18%
8	3	Necrosis expanding through the shoot above and/or below IP, withering of leaves	94%
	0	Absence of disease symptoms	6%

[a] Classification of data is the average of 3 trials

Table 4.3: Phenotypic responses of Redglobe inoculated with *X. ampelinus* using the needle inoculation method

Weeks	Disease grade	Symptom description	% plants [a]
1	0	Absence of disease symptoms	0%
2	1	Necrosis restricted to the inoculation point	20%
	3	Necrosis expanding through the shoot above and/or below IP, leaves not affected	27%
	0	Absence of disease symptoms	53%
3	2	Necrosis reaching the petiole adjacent IP	53%
	5	Necrosis spreading through the shoot and causing thinning of the stem and petiole causing wilting of the plant	20%
	0	Absence of disease symptoms	27%
4	2	Necrosis reaching the petiole adjacent IP	37%
	5	Necrosis spreading through the shoot and causing thinning of the stem and petiole causing wilting of the plant	53%
	0	Absence of disease symptoms	10%

[a] Classification of data is the average of 3 trials

4.1.4.1 Detection of *X. ampelinus* DNA in plant extracts of Dauphine and Redglobe via nested PCR

It is important to confirm whether the observed symptoms on the plants were actually caused by *X. ampelinus* and whether the symptomless plants were infected with the pathogen at all. DNA from the macerated plants inoculated with *X. ampelinus* bacterial suspension was amplified using nested PCR (nPCR) as developed by Botha *et al*. (2001). As anticipated, no amplification was obtained from the negative controls while a bacterial-specific fragment with an expected length of 277 bp was detected in segments taken from the point of inoculation and also 1.58cm below the IP (in case of the scalpel method and 1.58cm both above and below IP (in the case of the needle method), of all tested plants four weeks after inoculation for Redglobe. (Figures 4.6 4.7). It should be noted that in Figure 4.7b, no amplification was obtained for the points of inoculation. This could be because all bacteria in that area have moved to other parts of the plant.

Similar results were obtained for Dauphine at four weeks post inoculation using the scalpel method (Figure 4.8) and at four weeks post inoculation using the needle method (Figure 4.9). These results confirm the efficacy of the conducted inoculations, as well as confirmation of Dauphine as a tolerant variety. PCR results show that in the first four weeks following inoculation, plants were colonised by the bacteria but showed no visual symptoms. The use of nested PCR also helped evaluate the spreading of the bacteria through plant tissues. Bacteria were found to be present in stem sections taken from the point of inoculation as well as 1.58 cm above and below the IP, in the case of the needle method, and from the point of inoculation and 1.58cm below the IP in the case of scalpel method, proving that the bacteria have the capacity to enter and translocate within the plant. *X. ampelinus* was not detected in control plants inoculated with sterile PBS. Despite this movement of bacteria beyond the point of inoculation as shown by PCR analysis, no symptoms were observed on the plant surface when using the scalpel method.

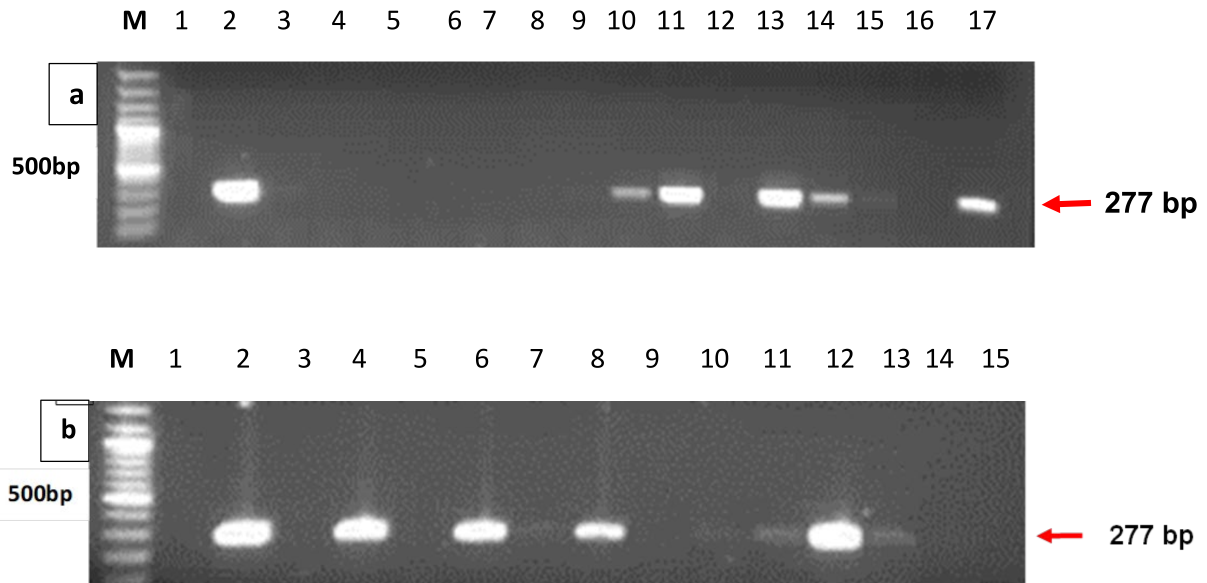
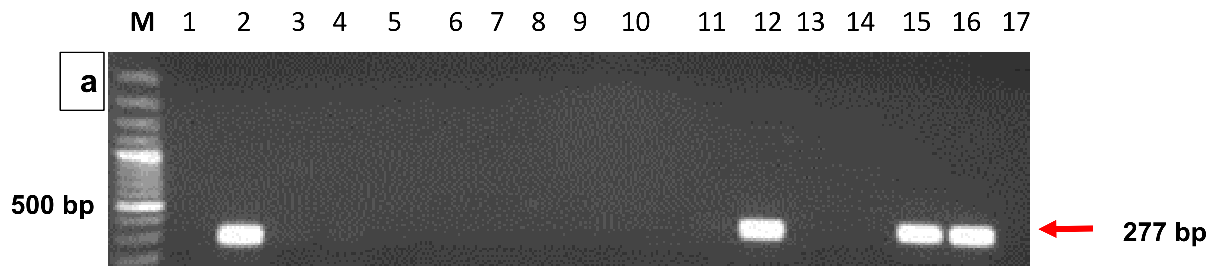


Figure 4.6: Amplified products from macerated samples of Redglobe inoculated by scalpel method four weeks post inoculation using nested-PCR: **a** - Lane M-100 bp Plus marker, lane1-negative control, lane 2-positive control, lane 3 to 8-control plants inoculated with sterile PBS, 9-IP, 10-below IP, 11-IP, 12-below IP, 13IP, 14-below IP, 15-IP, 16-below IP and 17-IP. **b** - Lane M-100 bp Plus marker, lane1-negative control, lane 2-positive control, lane 3-below IP, 4-IP, 5-below IP, 6-IP, 7-below IP, 8-IP, 9-below IP, 10-IP, 11-below IP, 12-IP, 13-IP.



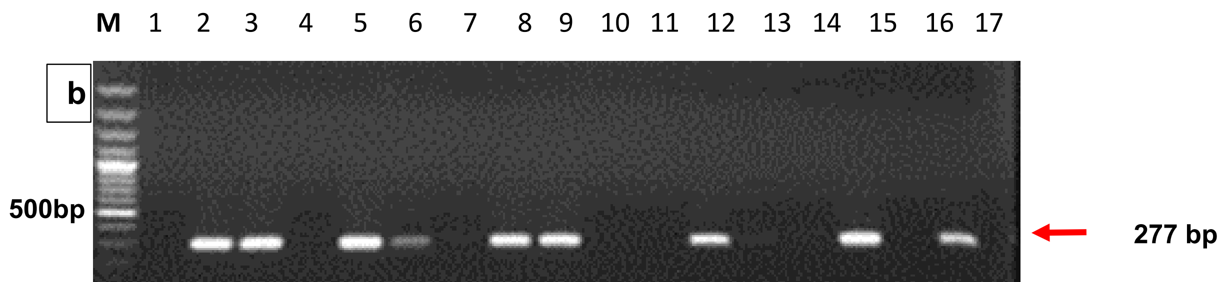


Figure 4.7: Amplified products from macerated samples of Redglobe inoculated by needle method four weeks post inoculation using nested-PCR: **a** - Lane M-100 bp Plus marker, lane 1-negative control, lane 2-positive control, lane 3 to 11-control plants inoculated with sterile PBS, lanes 12-above IP, 13-IP, 14-below IP, 15-above IP, 16-IP, 17-below IP. **b** - Lane M-100 bp Plus marker, 1-negative control, 2-positive control, 3-above IP, 4-IP, 5-below IP, 6-above IP, 7-IP, 8-below IP, 9-above IP, 10-IP, 11-below IP, 12-above IP, 13-IP, 14-below IP, 15-above IP, 16-IP and 17-below IP.

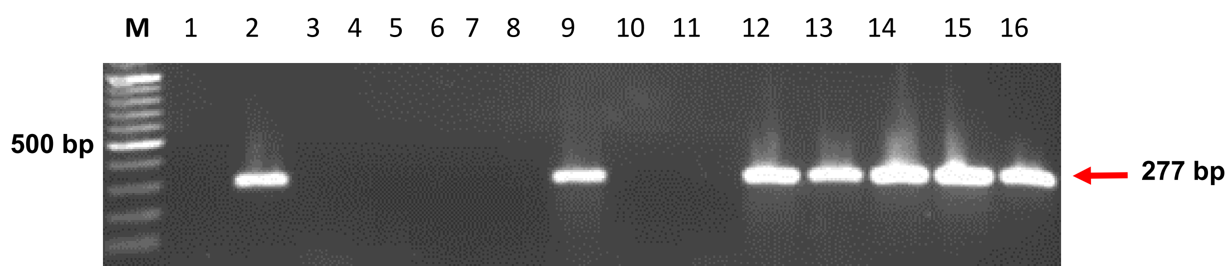


Figure 4.8: Amplified products from macerated samples of Dauphine inoculated by scalpel method four weeks post inoculation using nested-PCR**.** Lane M-100 bp Plus marker, 1-negative control, 2-positive control, 3 to 8-control plants inoculated with sterile PBS, 9-IP, 10-below IP, 11-IP, 12-below IP, 13-IP, 14-below IP, 15-IP and 16-below IP.

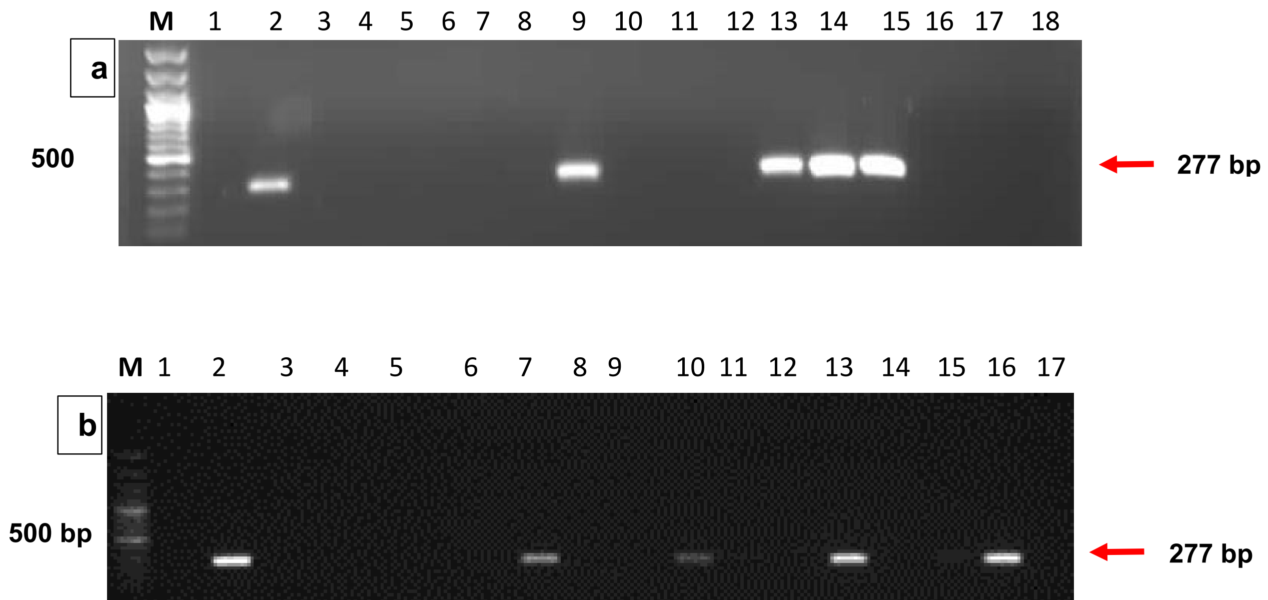


Figure 4.9: Amplified products from macerated samples of Dauphine inoculated by needle method four weeks post inoculation using nested-PCR: **a** - Lane M-100 bp Plus marker, 1 - negative control, 2 - positive control, 3 - 8 control plants inoculated with sterile PBS (above IP, IP and below IP), 9 - above IP, 10 - IP, 11 - below IP, 12 - above IP, 13 - IP, 14 - below IP, 15 - above IP, 16 - IP and 17 - below IP. **b** - Lane M-100 bp Plus marker, lane 1 - negative control, lane 2 - positive control, lane 3 - 5 control plants inoculated with sterile PBS (above IP, IP and below IP), 6 - above IP, 7 - IP, 8 - below IP, 9 - above IP, 10 - IP, below IP, below IP, 11 - below IP, 12 - above IP, 13 - IP, 14 - below IP, 15 - above IP, 16 – IP and 17 - below IP.

4.1.4.2 Isolation of bacteria from macerated inoculated plants of Dauphine and Redglobe and confirmation of their identity

As mentioned in section 3.1.4.2, detection of the bacterium by nPCR of plant material and isolation on YPGA were done in parallel. When isolation of bacteria from inoculated plantlets was done, yellow, slow-growing colonies were obtained. These colonies, obtained for both Dauphine and Redglobe, were subjected to colony PCR. In most cases, saprophytic bacteria overgrew the plates, making isolation difficult. *Xylophilus ampelinus* colonies were re-isolated from Dauphine at four and eight weeks, respectively (Figure 4.10), but not from Redglobe due to overgrowth of saprophytic bacteria on plates.

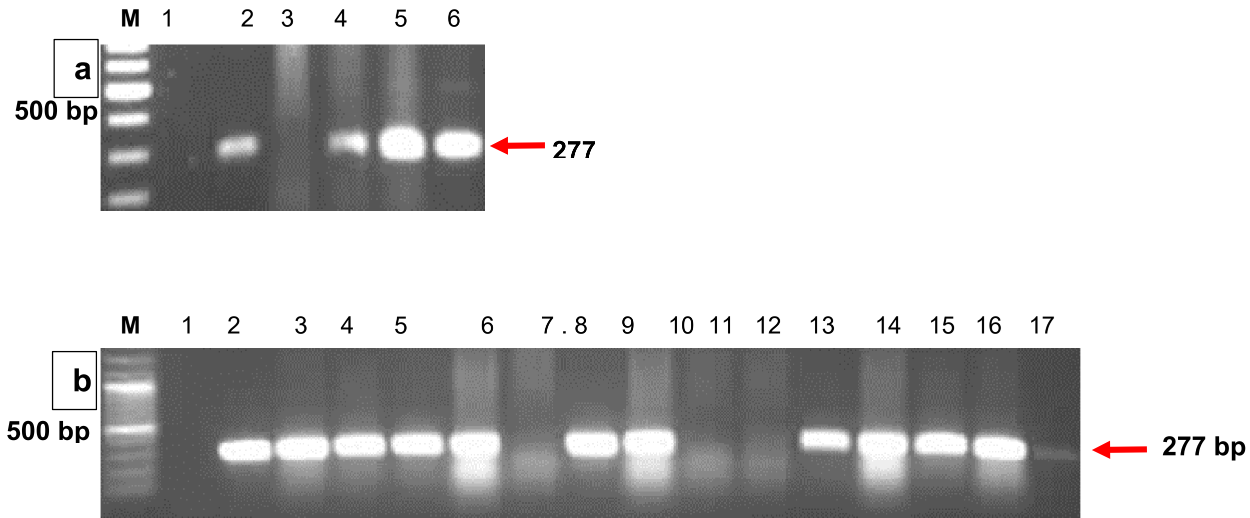


Figure 4.10: Colony PCR amplification of bacteria isolated from Dauphine using *X. ampelinus*-specific primers S3 and S4. **a** - Dauphine at four weeks post-inoculation. M lane: 100 bp Plus marker; 1: negative control; 2: positive control, 3, 4, 5 and 6 bands showing positive results for *X. ampelinus*. **b** - Dauphine at eight weeks post-inoculation: M lane - 100 bp Plus marker; 1 - negative control; 2 - positive control, lanes 3-17 candidate colonies tested.

4.1.4.3 Evaluation of the phenotypic response of C-3229 to *X. ampelinus* infection

In sections 3.1.3.1 and 3.1.3.2, two inoculation methods were evaluated based on their capacity to induce *X. ampelinus* infections in Dauphine and Redglobe based on their previously reported susceptibility, leading to the development of symptoms under laboratory conditions. Although both methods gave relatively fast symptom expression, progression of disease symptoms in plants was more enhanced when using the needle method. Necrosis was visually observed even 1.58 cm from the inoculation point and beyond. However, plants inoculated using the scalpel method showed no disease progression to the neighbouring parts of the plants. Based on these results, it was decided that *X. ampelinus* pathogenicity studies are best carried out through the inoculation of bacteria using a modified needle-inoculation method. It was also noted that disease symptoms corresponded to those observed in natural infections, including disease progression into petioles and shoots.

In the following sections, only the responses of Redglobe and C-3229 are discussed. The reason for this is that during the course of the experiment it was observed that

Dauphine plantlets were negatively affected by long-term micropropagation. Plants were propagated for longer than two years in culture media. The expected response of Dauphine to infection by *X. ampelinus* changed from that of a tolerant cultivar to a susceptible one due to continuous subculture, which exposes the plants to oxidative stress leading to mutations (Carvalho *et al*. 2015).

4.1.4.4 Plant phenotypic responses of Redglobe and C-3229

The majority of the inoculated plantlets of Redglobe were susceptible to *X. ampelinus* infection and these results are similar to those of the previous experiments, which evaluated the efficiency of two inoculation methods. Both Redglobe and C-3229 reacted similarly towards the bacteria, reaching average disease incidence of 88% and 90%, respectively, at four weeks post-inoculation. Redglobe and C-3229 plants inoculated with sterile PBS, did not show any symptoms four weeks post-inoculation (Figure 4.11a). At two weeks post-inoculation, the susceptible plantlets of Redglobe showed discolouration of tissue restricted to the point of inoculation, a disease severity scale of two (Figure 4.11b). Severe disease expression was demonstrated by stem and shoot discolouration at three weeks post-inoculation, which later led to necrosis/canker formation and eventually the collapse of the plantlets at week four, which is a disease severity rating of five (Figure 4.11c). Figure 4.11d shows Redglobe plant with dying leaves.

C-3229 followed the same pattern of symptom development as Redglobe. PBS-inoculated plants did not show any symptoms throughout the experiment (Figure 4.12a). At week two, the symptoms exhibited by most plantlets rated three on the disease incidence scale, meaning that discolouration of tissues in most plantlets occurred, which progressed to necrosis on shoots moving upwards towards the shoot apex (Figure 4.12b). At week four, a disease severity rating of five was observed as plantlets collapsed due to thinning of petioles (Figure 4.12c). Disease incidence values were fitted as a function of time using the Gompertz model (Figure 4.13) in order to analyse the effect of the inoculation with *X. ampelinus* on the rate of disease progression between C-3229 and Redglobe. The analyses of variance for all estimated parameters showed no significant difference between the response of Redglobe and

C-3229 to *X. ampelinus* infection (P≤0.5), indicating that C-3229 is as susceptible to bacterial blight as Redglobe.

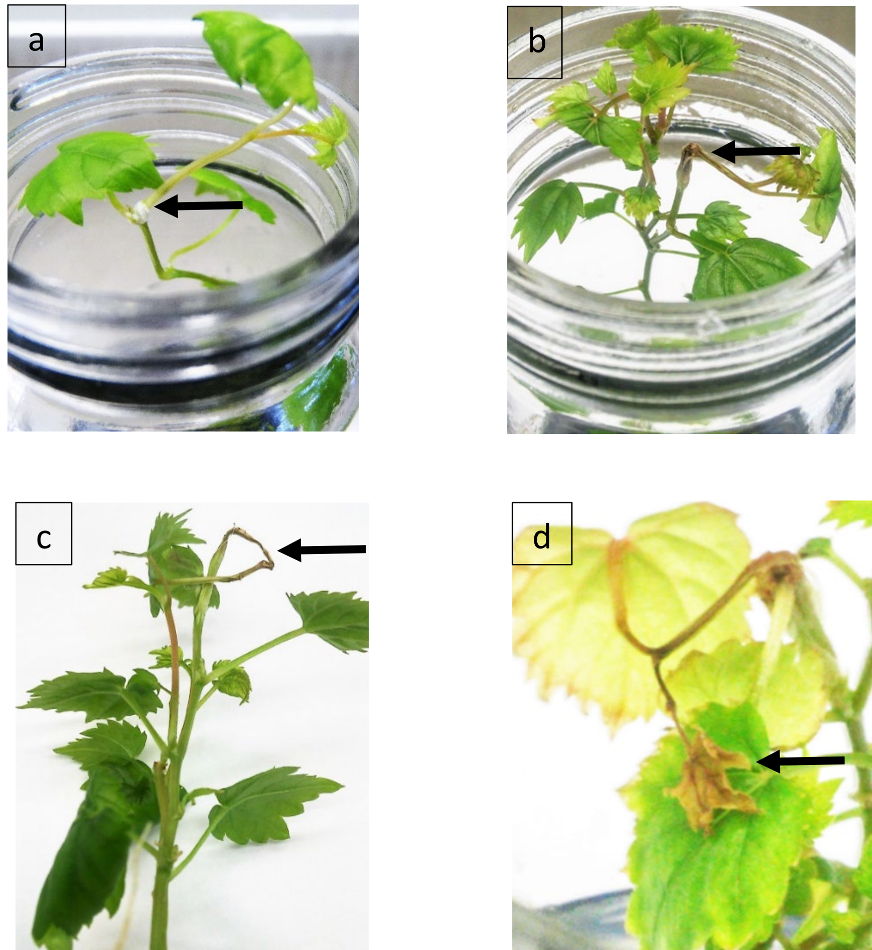


Figure 4.11: Phenotypic response of Redglobe to *X. ampelinus* (10^8 cfu/ml) showing progressive bacterial necrosis four weeks post inoculation. Arrows pointing at the IP point**, a** - PBS-inoculated control plant, the arrow indicates callus formation at the IP; **b** - the arrow indicates necrosis restricted to the IP, and **c** - the arrow indicates collapse of the plantlet at four weeks, **d** - the arrow indicates dying leaves.

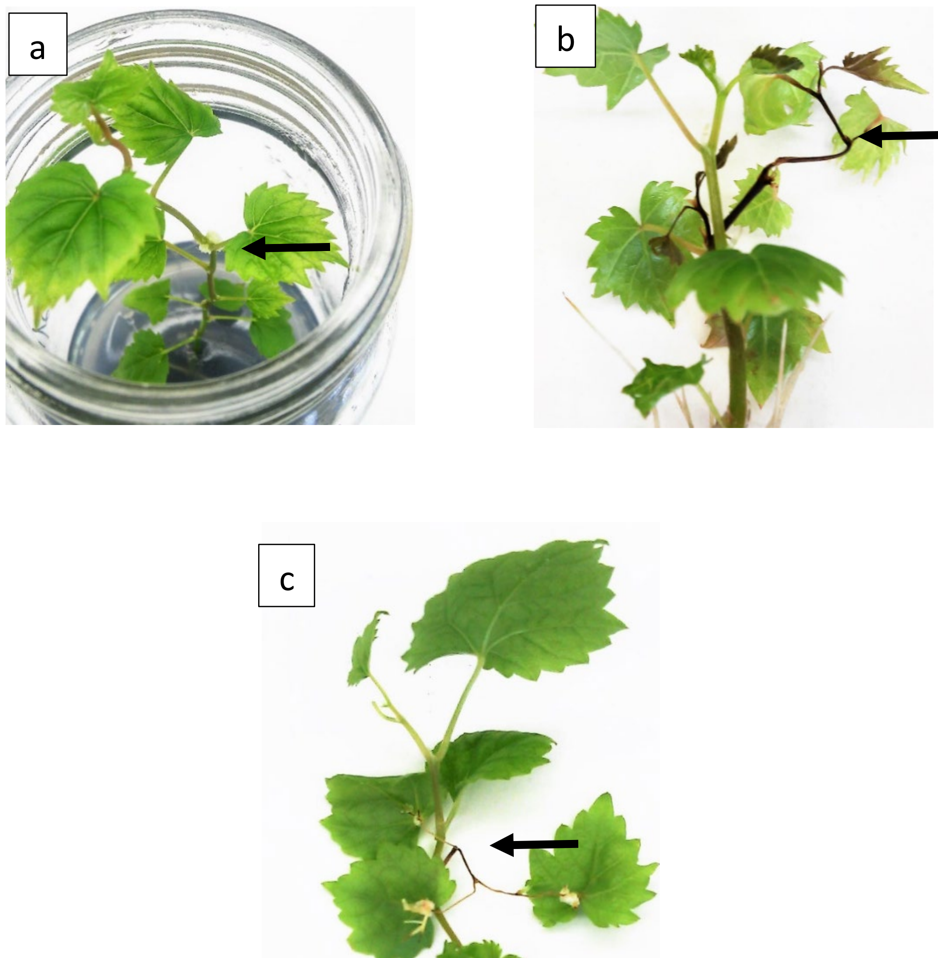


Figure 4.12: Phenotypic response of C-3229 to *X. ampelinus* (10^8 cfu/ml) showing progressive bacterial necrosis. Arrows pointing at the IP point, **a** - PBS-inoculated control plant with no symptoms, disease severity rating 0, **b** - the arrow indicates necrosis extending from the IP towards the shoot apex, necrosis spreading through the shoot, leaf stalk and reaching the leaves three weeks post inoculation and **c** - the arrow indicates necrosis extending through the shoot into the petioles and leaves, resulting in wilting and eventually the collapse of the plant at four weeks post-inoculation.

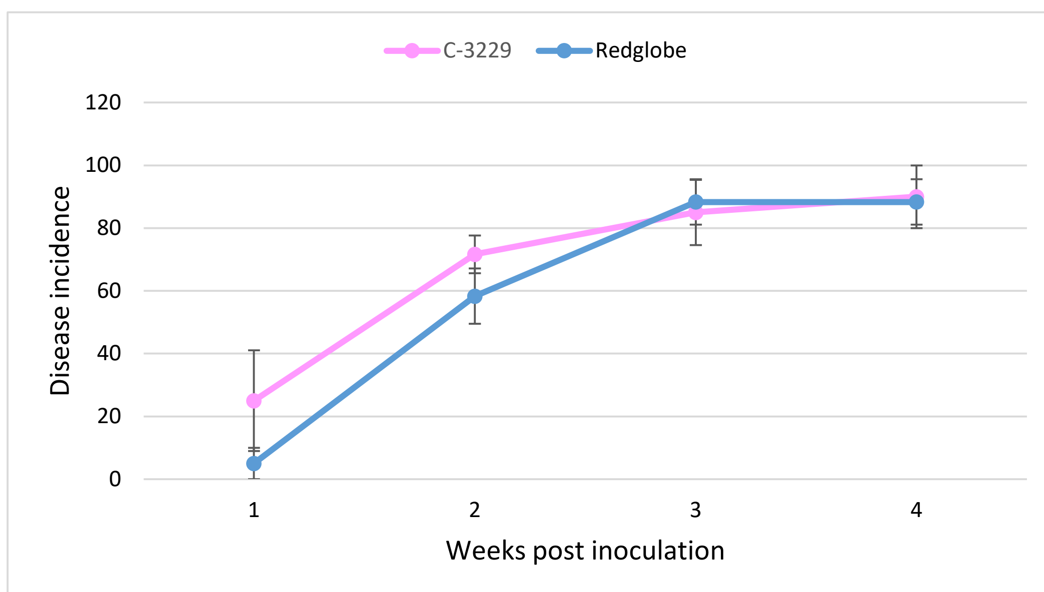


Figure 4.13: Disease progression in micropropagated C-3229 and Redglobe plants inoculated with suspensions of *X. ampelinus* (10^8 cfu/ml) using the modified needle-pricking method. Values are the mean of the three replicated trials, error bars correspond to the standard error of the mean. Redglobe and C-3229 (n=20) at 1–4 weeks post-inoculation.

4.1.4.5 Detection of *X. ampelinus* in plant extracts of C-3229 and Redglobe via nPCR

As was the case in the evaluation of the two inoculation methods, the primers used were able to amplify the expected PCR fragment of 277 bp from the plant extracts, thus confirming the presence of bacterial DNA in inoculated plant samples at the point of inoculation and also 1.58 cm above and below the inoculation point for both Redglobe and C-3229 (Figures 4.14 and 4.15). The results presented here suggest that *X. ampelinus* can enter and translocate within the plants, and that the bacteria probably move via the vascular system.

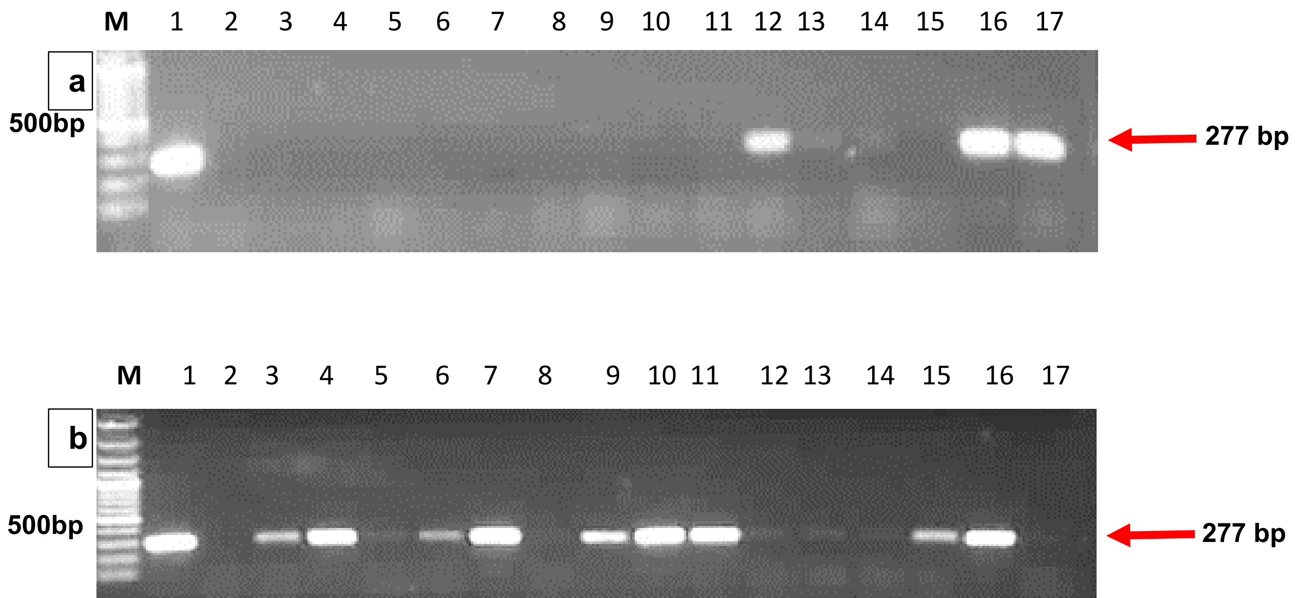


Figure 4.14: Amplified products from macerated samples of Redglobe inoculated by modified needle-pricking method and evaluated four weeks post inoculation. These results are representative of all nPCR results for Redglobe inoculated with *X. ampelinus* within this experiment: **a** - Lane M-100 bp Plus marker, 1 - positive control, 2 - negative control, 3 to 11 - control plants inoculated with sterile PBS, 12 - above IP, 13 - IP, 14 - below IP, 15 - above IP, 16 - IP, 17 - below IP. **b** - Lane M-100 bp Plus marker, 1 - positive control, 2 - negative control, 3 - above IP, 4 - IP, 5 - below IP, 6 - above IP, 7 - IP, 8 - below IP, 9 - above IP, 10 - IP, 11 - below IP, 12 - above IP, 13 - IP, 14 - below IP, 15 - above IP, 16 - IP, 17 - below IP.

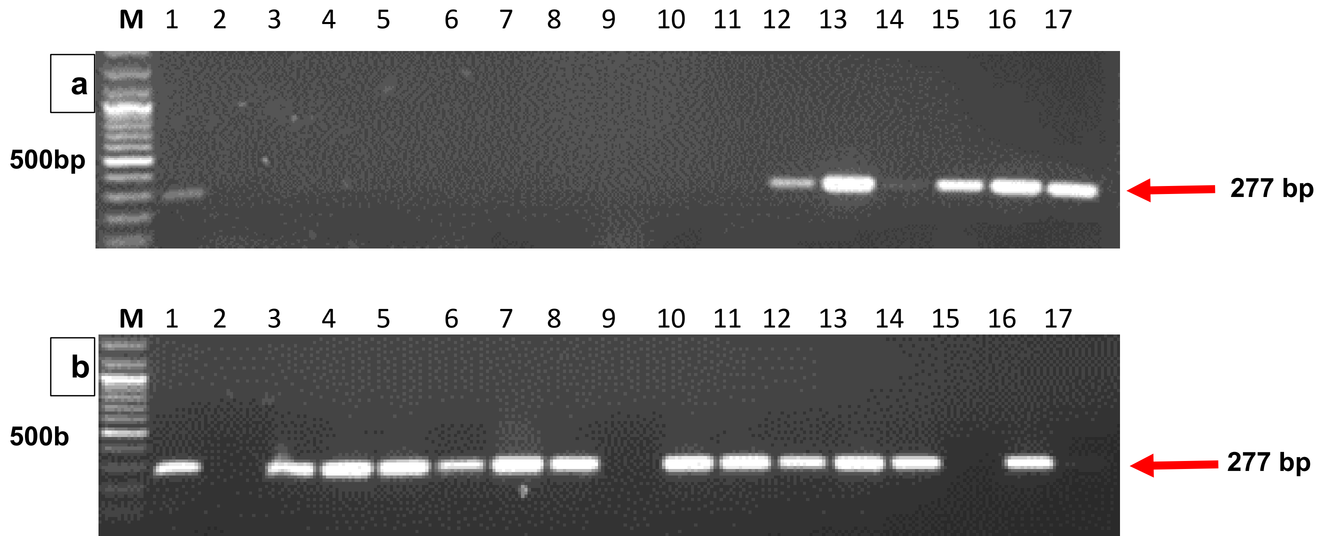


Figure 4.15: Amplified products from macerated samples of C-3229 inoculated by modified needle-pricking method and evaluated four weeks post inoculation. These results are representative of all nPCR reactions conducted for C-3229 inoculated with *X. ampelinus*: **a** - Lane M-100 bp Plus marker, 1 - positive control, 2 - negative control, 3 to 11 - control plants inoculated with sterile PBS, 12 - above IP, 13 - IP, 14 - below IP, 15 - above IP, 16 - IP, 17 - below IP. **b** - Lane M-100 bp Plus marker, 1 - positive control, 2 - negative control, 3 - above IP, 4 - IP, 5 - below IP, 6 - above IP, 7 - IP, 8 - below IP, 9 - above IP, 10 - IP, 11 - below IP, 12 - above IP, 13 - IP, 14 - below IP, 15 - above IP, 16 - IP, 17 - below IP.

4.1.4.6 Isolation of bacteria from Redglobe and C-3229 and confirmation of their identity

All tested colonies obtained from serial dilutions of macerated samples of C-3229 and Redglobe were confirmed as *X. ampelinus*, based on amplification of the 277 bp DNA fragment. Taken together, these results suggest that *X. ampelinus* is the causative microbe causing the disease symptoms that can be visually seen and imply that the bacteria have the capacity to translocate in the direction of both the shoot apex and roots (Figure 4.16). As was the case during the method optimisation experiments, difficulty was experienced in isolating bacteria from the infected Redglobe plant material for which the presence of the organism's DNA was confirmed by PCR.

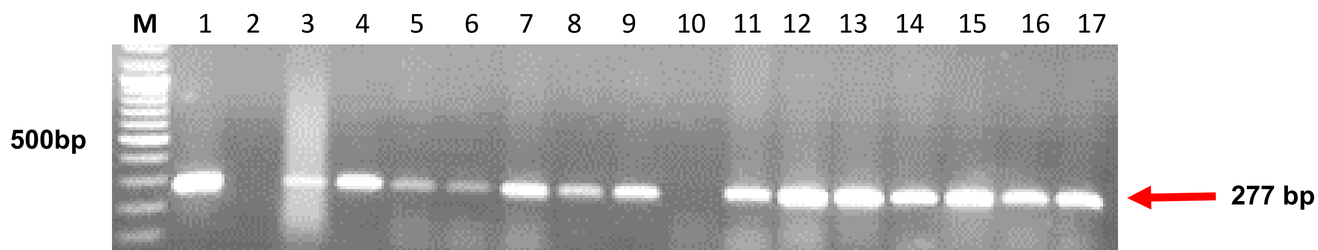


Figure 4.16: Colony PCR amplification of bacteria isolated from macerated samples of C-3229 four weeks post inoculation, using *X. ampelinus*-specific primers, S3 and S4. (M) 100 bp Plus marker; 1 - (+ve), 2 - (-ve) control; 3 - above IP, 4 - IP, 5 - IP, 6 - IP, 7 - IP, 8 - IP, 9 - IP, 10 - IP, 11 to17 - below-IP.

4.2 Screening Kishmish Vatkana seedlings with molecular markers linked to powdery mildew resistance gene, *Ren1*

4.2.1. From laboratory to glasshouse: Acclimatisation of progeny from self-pollinated Kishmish Vatkana

Self-pollination of KV was performed to generate grape genotypes resistant to PM that are homozygous for the *Ren1* gene. KV was self-pollinated at the ARC – Nietvoorbij vineyards in October 2015. Embryo rescue was deemed an effective way to obtain viable offspring from self-pollinated plants.

A total number of 444 rudimentary seeds were obtained from self-pollinated KV and cultured on WPM. A total of 35 (7.8%) embryos germinated from the seeds, while 30 (6.7%) embryos were excised. Most rudimentary seeds were empty when dissected indicating that the embryo had already aborted. A total of 65 (14.6%) embryos were recovered from the rudimentary seeds. Germinated embryos that developed into plantlets were transferred to soil and only 36 plantlets were recovered and extraction of DNA was done on these plantlets. Figure 4.17a shows healthy offspring whose leaves were used for DNA extraction. Some of the offspring grew poorly and it was difficult to get the leaves for DNA extraction (Figures 4.17b and c).

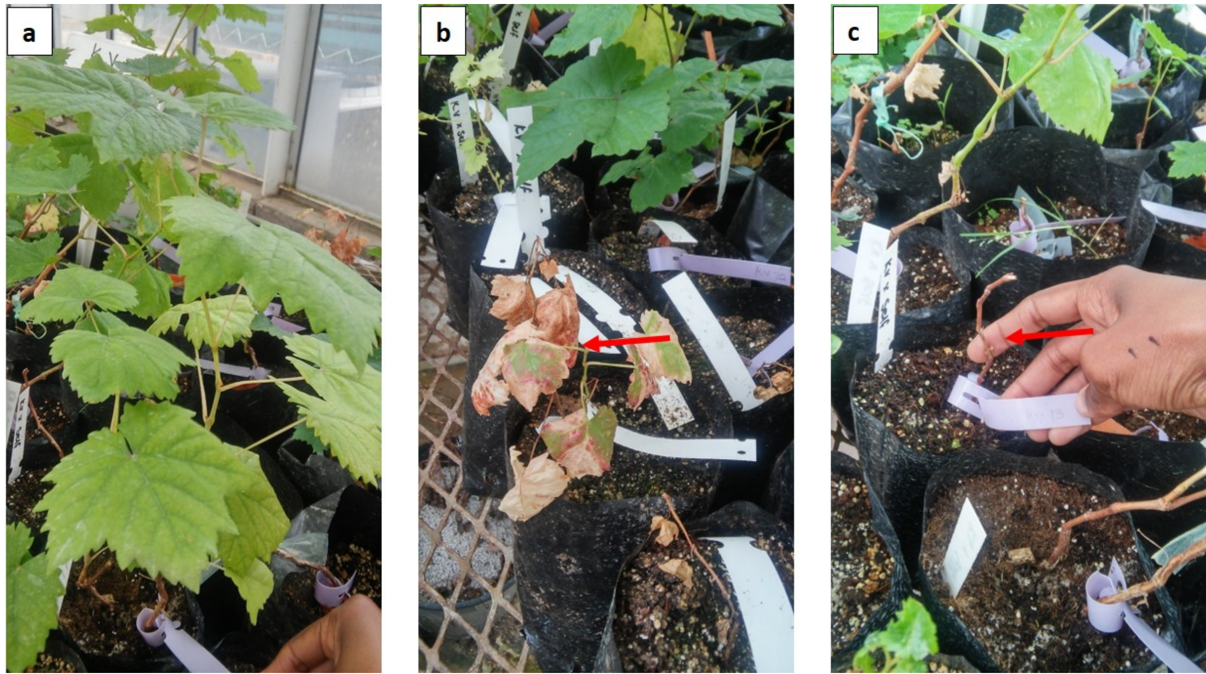


Figure 4.17 Offspring of self-pollinated KV: **a** - Healthy growing offspring, **b** - Slow growing offspring, **c** - Dead offspring.

4.2.2 Molecular characterisation to confirm zygosity of the progeny

4.2.2.1 DNA quantification

Total genomic DNA was successfully isolated from the self-pollinated KV individuals using CTAB method. DNA recovery varied from 5.41 ng/µl to 311.86 ng/µl. The ratio of DNA to protein ranged from 1.28 to 1.95 (Table 4.4). This can be attributed to the variation of carbohydrate content and polyphenols from plant to plant which led to the wide variation in DNA yields. Expected A260: A280 values are commonly in the range 1.5 - 2.0 ng/µl. Undiluted DNA samples (30 ng/µl) were checked for quality on the 0.8% agarose gel as depicted in Figure 4.18. Because of the high DNA concentrations, 10x dilutions were prepared and from this diluted DNA, 15 ng/µl stock solutions were prepared for use in PCR

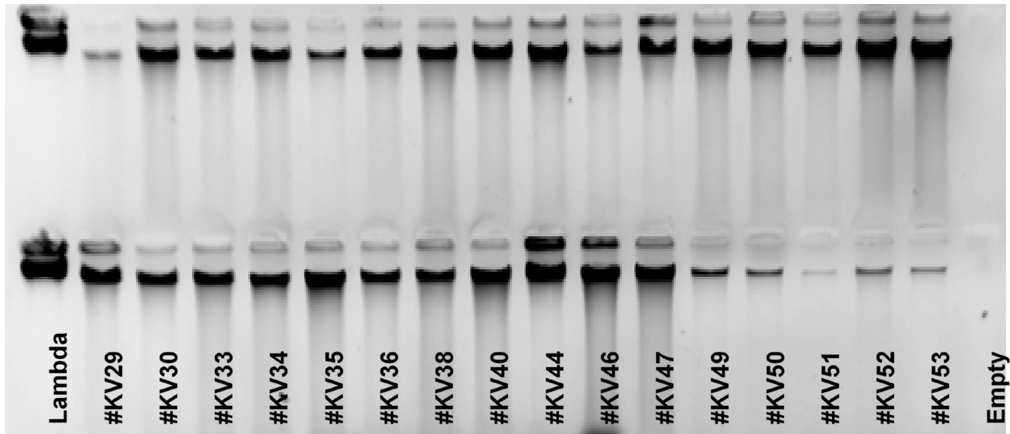


Figure 4.18 Undiluted genomic DNA (30 ng/µl) quality check of KV offspring using agarose gel electrophoresis (0.8% agarose; 50 volts; 45 min) with 2µl DNA used per sample.

Table 4.4: Concentration and quality of genomic DNA isolated from Kishmish Vatkana offspring

Sample ID	ng/µl	260/280	260/230	DNA (15 ng/µl)	H_2O 30 µl
KV1: 10 x	**117,67**	1,9	1,7	3,8	26,2
KV2: 10 x	**165,33**	1,88	1,65	2,7	27,3
KV3: 10 x	**256,36**	1,92	1,92	1,8	28,2
KV4: 10 x	**215,89**	1,92	1,71	2,1	27,9
KV6: 10 x	**114,51**	1,87	1,36	3,9	26,1
KV7: 10 x	**162,32**	1,87	1,36	2,8	27,2
KV11: 10 x	**186,8**	1,87	1,58	2,4	27,6
KV12: 10 x	**234,55**	1,89	1,74	1,9	28,1
KV13: 10 x	**123,52**	1,82	1,53	3,6	26,4
KV14: 10 x	**302,81**	1,92	1,68	1,5	28,5
KV15: 10 x	**201,21**	1,94	1,73	2,2	27,8
KV16: 10 x	**218,39**	1,89	1,43	2,1	27,9
KV21: 10 x	**208,73**	1,92	1,79	2,2	27,8
KV26: 10 x	**216,08**	1,9	1,67	2,1	27,9
KV27: 10 x	**294,18**	1,92	1,89	1,5	28,5
KV28: 10 x	**274,02**	1,93	1,97	1,6	28,4
KV29: 10 x	**323,87**	1,93	2,08	1,4	28,6
KV30: 10 x	**275,46**	1,9	1,73	1,6	28,4
KV33: 10 x	**310,08**	1,9	1,82	1,5	28,5
KV34: 10 x	**228,79**	1,92	1,72	2,0	28,0
KV35: 10 x	**274,74**	1,89	1,67	1,6	28,4
KV36: 10 x	**173,36**	1,86	1,54	2,6	27,4
KV38: 10 x	**296,74**	1,95	1,82	1,5	28,5
KV40: 10 x	**311,86**	1,91	1,89	1,4	28,6
KV44: 10 x	**272,58**	1,94	1,85	1,7	28,3
KV46: 10 x	**193,84**	1,89	1,73	2,3	27,7
KV47: 10 x	**285,1**	1,91	1,88	1,6	28,4
KV49: 10 x	**44,61**	1,95	1,64	10,1	19,9
KV50: 10 x	**57,56**	1,75	1,78	7,8	22,2
KV51: 10 x	**14,34**	1,92	1,14	*Use as is*	
KV52: 10 x	**58,53**	1,88	1,68	7,7	22,3
KV53: 10 x	**30,06**	2	1,52	15,0	15,0
KV54: 10 x	**60,68**	1,85	1,72	7,4	22,6
KV55: 10 x	**108,85**	1,82	1,96	4,1	25,9
KV56: 10 x	**5,41**	1,28	0,68	*Use as is*	
KV57: 10 x	**74**	1,84	1,95	6,1	23,9
Parent 52: 10 x	**592,42**	1,93	2,1	0,8	29,2

4.2.2.2 Classification of offspring

In this study, we looked at the inheritance of the Ren1 gene conferring resistance to powdery mildew (PM) in 36 offspring from self-pollinated KV to identify offspring homozygous for the *Ren1* gene. The following SSR markers were used for the screening, VMC9H4-2 and VMCNG4E10-1 (which are the most tightly linked markers to *Ren1*), UDV124 and UDV020, that flank *Ren1* and lastly VVIP10, which is located furthest from the *Ren1* locus. Allele sizes for all mentioned SSR markers for all genotypes are shown in Tables 4.5 and 4.6.

1. The off types: An error in parentage was assumed for KV1, KV33, KV34 and KV35 as allele sizes for each marker did not support self-pollination of KV. They were therefore excluded from this study. (Table 4.5).

2. Individuals with homozygous dominant alleles: Ten individuals (KV2, KV4, KV6, KV11, KV12, KV16, KV40, KV46, KV47 and KV52) were found to be homozygous, carrying the dominant alleles for two markers (VMC9H4-2 and VMCNG4E10-1), which co-segregate and are tightly linked to *REN*1. These individuals were identified as a source of PM resistance that could be used in breeding programs.

3. Individuals with homozygous recessive alleles: Some individuals, namely KV26, KV27, KV28, KV30 and KV44 were found to be homozygous but carrying the recessive alleles for markers UDV124, VMC9H4-2, VMCNG4E10-1 and VVIP10. Even though individual KV44 carries the 300 bp allele for marker VVIP10, associated with *Ren1* it carries only recessive alleles for the other markers. These individuals mentioned above, although homozygous, could not be considered for the breeding program.

4. The last group of individuals were found to be heterozygous. These individuals, even though they are resistant, will not be used for breeding purposes.

KV56 could not be amplified due to low concentration of DNA in the sample. Therefore, we could not determine which class this individual belongs to.

4.2.2.3 Marker performance

Screening genotypes from the self-pollinated KV homozygous for the *Ren1* locus was undertaken using SSR markers UDV124, VMC9H4-2, VMCNG4E10-1, UDV020 and VVIP10 shown in Table 4.5. The locus UDV020 was the most polymorphic with up to four alleles. UDV020 presented challenges when scoring alleles. The allele sizes 125, 134, 145 and 160 bp were recorded for UDV020, and the 125 bp and 145 bp alleles were always present when the 160bp allele was inherited. All the other markers had two alleles, i.e., homozygous for the susceptible allele. In this study, markers VMC9H4-2 and VMCNG4E10-1 were homozygous for certain individuals mentioned in section 4.2.2.2 above and were used to determine the homozygous individuals with the dominant alleles. For marker UDV124 none of the individuals were homozygous for the dominant allele. Most of the individuals were heterozygous, presenting a 217 bp allele size associated with resistance. A few of the individuals were homozygous with recessive alleles of 219 bp (in other words, homozygous for susceptibility to PM). Marker VVIP10 is furthest from the *Ren1* locus and thus recombination may occur more often. Amplicon size differences allowed for unambiguous distinction of *Ren1* and its homologous alleles. Allele sizes for all of the markers mentioned above are shown in Table 4.4. The individuals shared one or both of the parental alleles at most loci. In KV, which is heterozygous for the *Ren1* locus, allele 217 bp, 296 bp, 259 bp and 300 bp are the markers for resistance (*Ren1*) (with markers UDV124, VMC9H4-2, VMCNG4E10-1 and VVIP10, respectively) (personal communication with Veikondis and Cengen). The SSR screening in this study confirmed that KV (parent) was heterozygous at the *Ren1* locus and that it carried the dominant allele for powdery mildew resistance.

Markers, UDV124, VMC9h4.2 and VMCNg4e10.1, produced SSR profiles that were easily scorable and were therefore included in the construction of the electropherograms (Figures 4.19 - 4.21). SSR marker UDV124 exhibited stutter peaks, but they didn't impair the recognition or differentiation between heterozygotes and homozygotes (Figure 4.19). All of the alleles of different loci exhibited good peak resolutions and were clearly identified for SSR marker VMC9h4.2 and VMCNg4e10.1 (Figures 4.20 and 4.21 respectively). The majority of the 36 individuals tested showed amplification of two peaks for each locus. Individuals showing one peak were

considered to be homozygous for that locus, whereas individuals with two peaks were heterozygous.

Marker UDV020 gave multiple alleles and these allele sizes were difficult to score, while allele scores for marker UDV124 identified that individuals were either heterozygous (217/219) for *Ren1* or homozygous (219/219) for susceptibility (*ren1*) Marker VVIP10 is furthest from the *Ren1* locus and thus recombinations may occur more often. To identify the individuals homozygous for *Ren1* only the two markers (VMC9H4-2 and VMCNG4E10-1) which are known to be tightly linked to the *Ren1* locus were included (Table 4.6).

Table 4.5: Allele comparison of population derived from self-pollinated Kishmish Vatkana using 5 SSR markers.

Individuals	*Ren 1* - selected markers				
	UDV020	UDV124	VMC9H4-2	VMCNG4E10-1	VVIP10
Parent	125/134/145/160	217/219	271/296	236/259	294/300
KV1	145/160[1]	195/230	287/327	250/288	275/294
KV2	125/145/160	217/219	296/296[2]	259/259	300/300
KV3	125/134/160	219/219	271/296	236/259	294/300
KV4	125/145/160	217/219	296/296	259/259	300/300
KV6	125/145/160	217/219	296/296	259/259	300/300
KV7	125/134/160	217/219	271/296	236/259	294/300
KV11	125/145//160	217/219	296/296	259/259	300/300
KV12	125/145/160	217/219	296/296	259/259	300/300
KV13	125/145/160	217/219	271/296	236/259	294/300
KV14	125/134/145/160	217/219	271/296	236/259	294/300
KV15	125134/145/160	217/219	271/296	236/259	294/300
KV16	125/145/160	217/219	296/296	259/259	300/300
KV17	125/134/145/160	219/219	271/296	236/259	294/300
KV26	134/134	219/219	271/271	236/236	294/294
KV27	134/134	219/219	271/271	236/236	294/294
KV28	134/134	219/219	271/271	236/236	294/294
KV29	125/134/145/160	217/219	271/296	236/259	300/300
KV30	134/134	219/219	271/271	236/236	294/294
KV33	134/146//158	195/209	271/274	236/239	294/294
KV34	134/134	209/209	271/271	236/236	294/294
KV35	134/136/158	195/209	271/274	236/239	294/294
KV36	125/134/145/160	217/219	271/296	236/259	294/300
KV38	125/134/145/160	217/219	271/296	236/259	294/300
KV40	125/145/160	217/219	296/296	259/259	294/300
KV44	125/134/145/160	219/219	271/271	236/236	300/300
KV46	125/145/160	217/219	296/296	259/259	300/300
KV47	125/145/160	217/219	296/296	259/259	294/300
KV49	125/134/145/160	217/219	271/296	236/259	294/300
KV50	125/134/145/160	217/219	271/296	236/259	294/300
KV51_10x	125/134/145/160	217/219	271/296	236/259	294/300 very low
KV52	125/145/160	217/219	296/296	259/259	300/300
KV53	125/134/145/160	217/219	271/296	236/259	294/300
KV54	125/134/145/160	217219	271/296	236/259	294/300
KV55	125/134/145/160	217/219	271/296	236/259	294/300
KV56	Poor Amp[3]				Poor DNA
KV57	125/134/145/160	217/219	271/296	236/259	294/300

[1] Alleles present in off types indicated in blue

[2]Allele sizes associated with resistance markers as shown in red

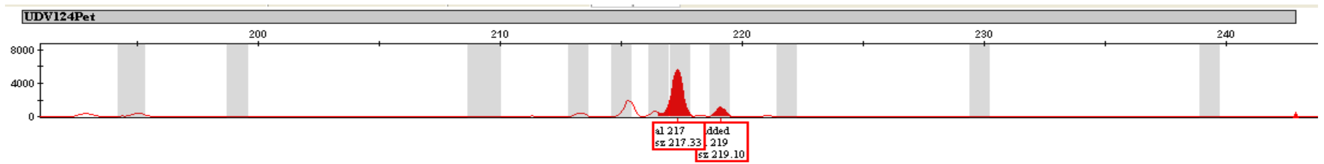
[3]Allele size could not be determined due to poor DNA quality

Table 4.6: Progeny carrying homozygous alleles for the two co-segregating markers (VMC9H4-2 and VMCNG4E10-1) most closely linked to *Ren1* for PM resistance derived from self-pollination of KV.

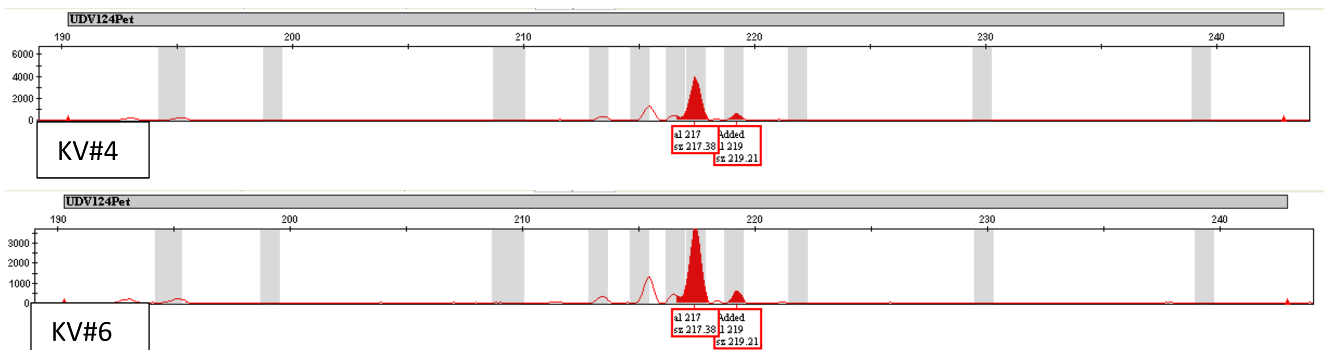
Individuals	***Ren 1* - selected markers**	
	VMC9H4-2	**VMCNG4E10-1**
Parent	**271/ 296**	**236/ 259**
KV2	**296**	**259**
KV4	**296**	**259**
KV6	**296**	**259**
KV11	**296**	**259**
KV12	**296**	**259**
KV16	**296**	**259**
KV40	**296**	**259**
KV46	**296**	**259**
KV47	**296**	**259**
KV52	**296**	**259**

UDV124Pet

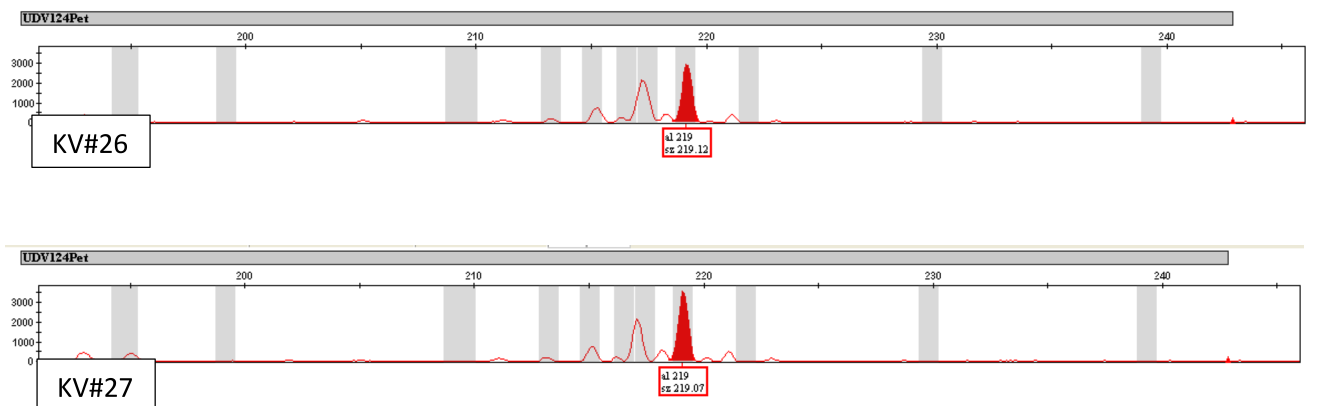
Parent – Heterozygous (217 bp/ 219 bp)



Heterozygous offspring (217 bp/ 219 bp)



Homozygous offspring with recessive alleles (219 bp)



Off type plant

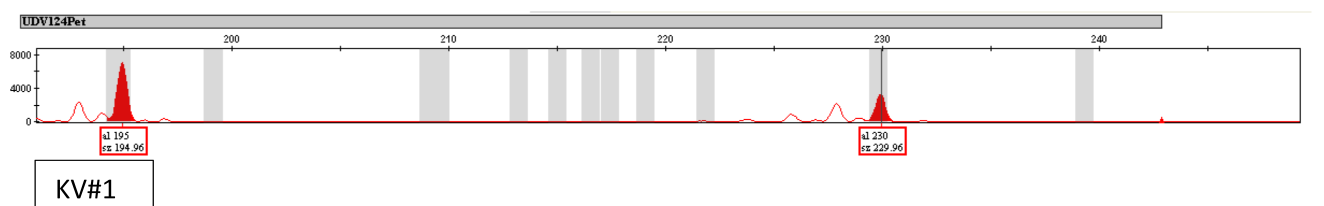
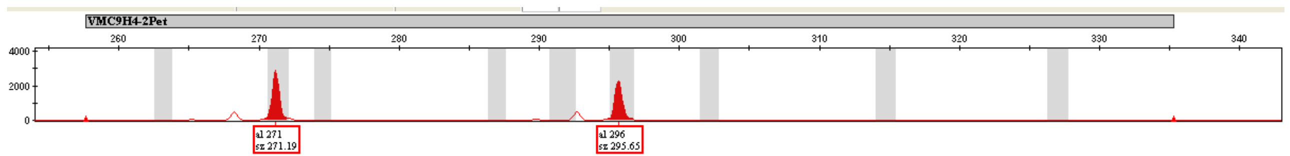


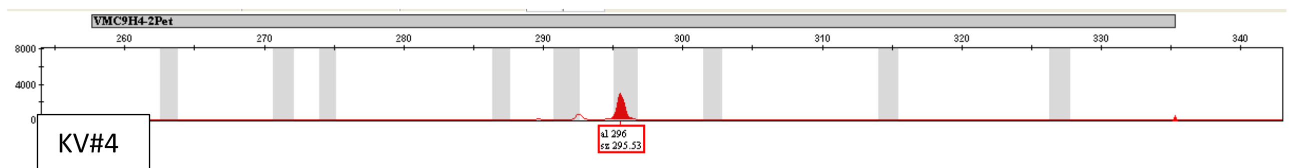
Figure 4.19: Electropherograms for SSR marker UDV124 fluorescently labelled with a red dye (PET™) obtained after scoring of alleles in the individuals # KV1, KV4, KV6, KV26, and KV27 derived from self-pollination of KV.

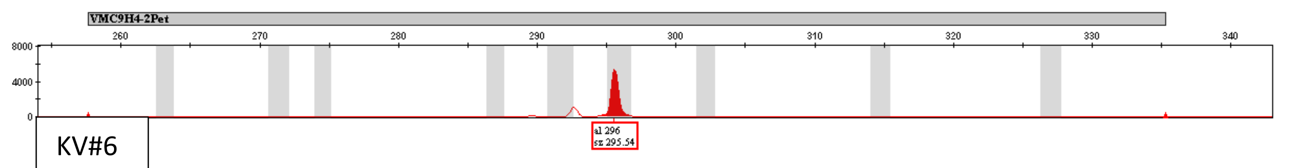
VMC9H4-2Pet

Parent (271 bp/ 296 bp)

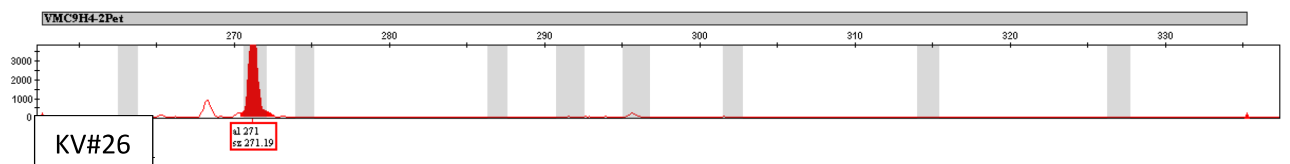


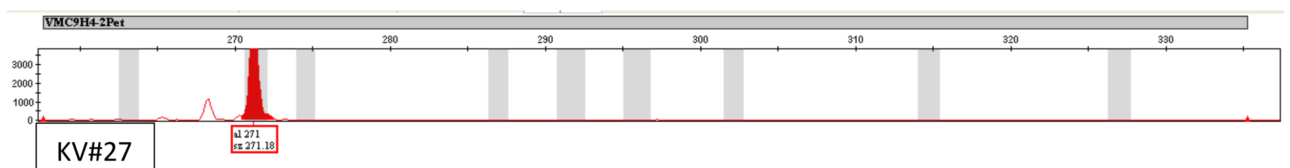
Homozygous offspring with resistant alleles (296 bp)





Homozygous offspring with recessive alleles (271 bp)





Off type plant

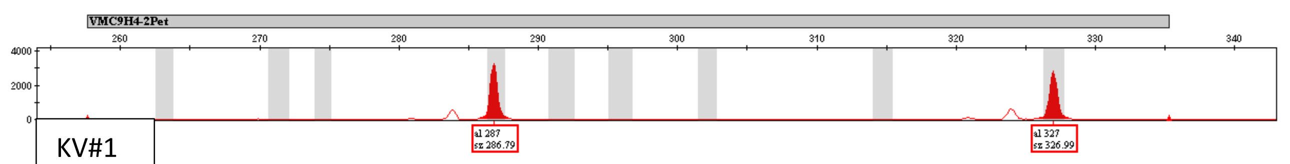
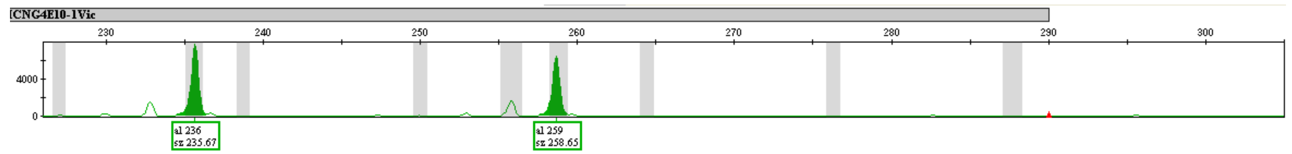


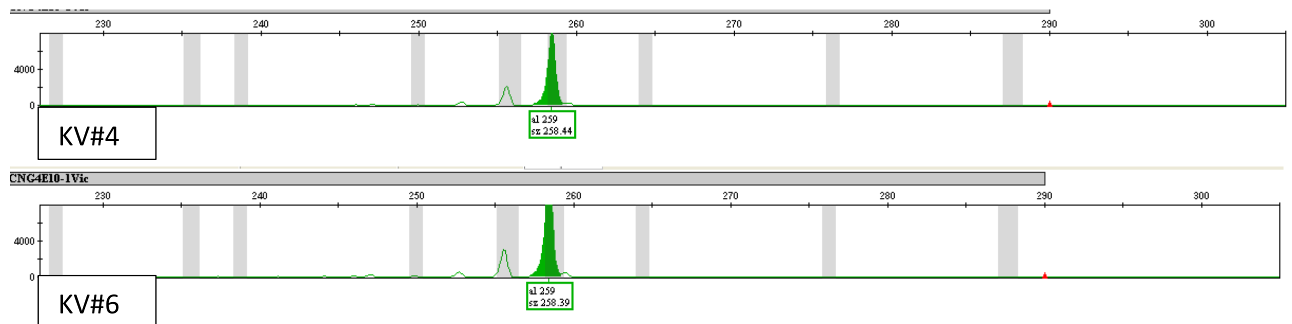
Figure 4.20: Electropherogram for SSR marker VMC9H4-2 fluorescently labelled with a red dye (PET^{TM}) obtained after scoring of alleles in the individuals # KV1, KV4, KV6, KV26, and KV27 derived from self-pollination of KV.

VMCNG4E10-1Vic

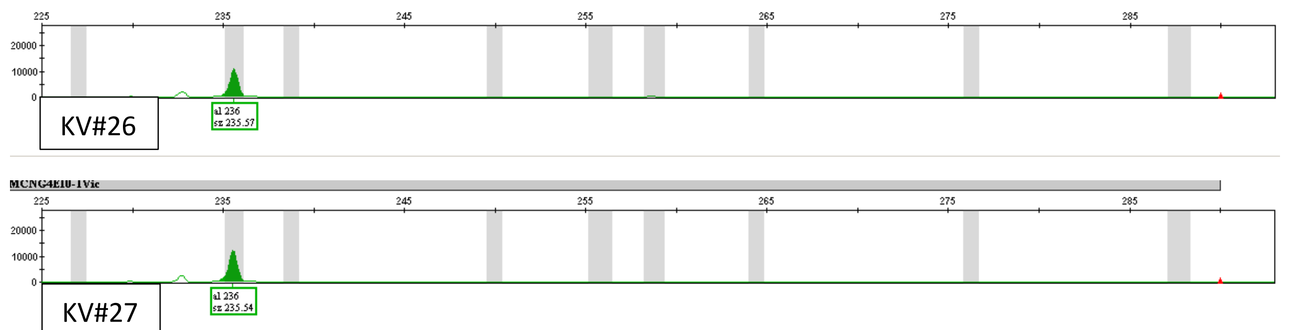
Parent (236 bp/ <u>259 bp</u>)



Homozygous offspring with resistant alleles (259 bp)



Homozygous offspring with recessive alleles (236 bp)



Off type plant

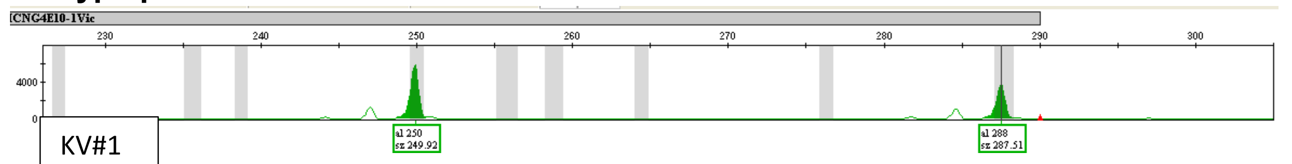


Figure 4.21: Electropherogram for SSR marker VMCNG4E10-1Vic fluorescently labelled with a green dye (VIC^{TM}) obtained after scoring of alleles in the individuals # KV1, KV4, KV6, KV26, and KV27 derived from self-pollination of KV.

Chapter 5: Discussion

Control of bacterial and fungal diseases is a concern for breeders and growers of grapevine. Therefore, efforts are made to develop varieties that have disease resistance genes from multiple sources. In this way, durable resistance can be obtained that is beneficial to breeders and growers. The research presented in this thesis aimed to contribute to the search for bacterial blight resistant grapevine varieties as well as identification of individuals homozygous for the powdery mildew resistance gene, *Ren1*, that can be used in breeding programmes.

The first part of this research was conducted to determine an *in vitro* inoculation method that could be used to consistently identify grapevine genotypes with resistance to *X. ampelinus,* whether screening tests would be faster than greenhouse screening, and to evaluate spread of the pathogen within the plant. In the current study, an efficient *in vitro*-inoculation method for assessment of resistance to grapevine bacterial blight was developed. This method is suitable for screening different grapevine genotypes for their responses to *X. ampelinus.* As a result, it was possible to confirm the phenotypic response of two different *Vitis vinifera* varieties namely Redglobe and Dauphine and establish the response of a new selection C-3229 (a cross between G4-682, a breeding line, and Regal Seedless) to *X. ampelinus*, the causal agent of grapevine bacterial blight.

Establishment of a visual resistance reaction rating scale is important to consistently and accurately identify plants tolerant or resistant to the pathogen. It is also important that the same resistance phenotypes be observed in at least two separate trials. In this study, three trials of each variety, Dauphine, Redglobe and C-3229, were consistent. In the optimisation section of this study where Dauphine and Redglobe were used as controls to compare the needle and scalpel inoculation method, it was observed that, when comparing these genotypes using visual rating, there were significant differences in disease severity.

Redglobe, at the end of week four, already expressed disease symptoms, while Dauphine exhibited no symptoms. It was only at six weeks post inoculation that symptoms were observed in Dauphine. It should be noted that 30% of Redglobe plants

at three weeks post inoculation, had reached the maximum disease rating of five (Table 4.3). For Dauphine, at eight weeks post inoculation, 94% of plants had only reached a disease severity rating of three. This does not only suggest that necrosis is an early response to pathogen invasion, but also that visual symptoms vary significantly between host genotypes. This was an important indicator of host susceptibility and is an essential component in assessing the extent of disease symptom expression in relation to colonisation of host tissue.

Both scalpel and needle inoculation methods used, resulted in the successful inoculation of *X. ampelinus* suspensions into plantlets of the genotypes under study and the development of disease symptoms in plantlets maintained in a growth room for four to eight weeks. Discolouration of tissue on the shoots were the earliest symptoms of bacterial blight. As expected, Dauphine exhibited tolerance, with plants exhibiting necrosis around the inoculation site only after six weeks and the necrosis extending away from the inoculation point at eight weeks, post-inoculation. It should be taken into consideration that Dauphine had been in the same medium for eight weeks, which could have affected the health of the plants. Depletion of minerals may occur in the medium after a long period of time and this may cause stress on the plants; leaving the plants with a greater tendency to wilt (Nuffield Foundation, 2011). On the other hand, Redglobe and C-3229 were both susceptible to *X. ampelinus* infection and exhibited symptoms of necrosis two weeks post-inoculation, and these symptoms became progressively more severe with time, with wilting developing between the third and fourth week post inoculation.

Wilting in plants is a conspicuous symptom of extremely susceptible genotypes, and both Redglobe and C-3229 exhibited these symptoms with some part of the plantlets appearing flaccid, proving that these two varieties are indeed susceptible. Wilting of plantlets and their collapse may have been a result of a decrease in water availability in *X. ampelinus*-infected plantlets, which leads to a more pronounced reduction in stomatal conductance causing water limitation in the plant. A study done by McElrone *et al*. 2003, on xylem-limited bacterial infection on the water relations using *Xylella fastidiosa* produced similar symptoms. They found that the water-stress experienced by the plants due to bacterial invasion caused the plants to lose their turgidity leading to plant wilting. The recovery / PCR detection of *X. ampelinus* from stem sections

taken 1.58 cm above and below the inoculation point, four weeks (in the case of Redglobe and C-3229) and eight weeks (in the case of Dauphine) after inoculation, established that the bacteria can be transported via the vascular system. This allowed migration along the stem and the petioles in some instances, resulting in more plantlets showing collapse from the inoculation point. This comes as no surprise since *X. ampelinus* is known as a bacterium that infects xylem, but also inhabits other tissues (Bové and Garnier, 2002).

Chatelet *et al*. (2011) did a study on xylem structure of grape varieties, both tolerant and susceptible to the xylem-limited bacterium *Xylella fastidious*. The study showed that the xylem of susceptible grapevine varieties could have greater inter vessel pitting which allowed *X. fastidiosa* more access to adjacent vessels via pit membranes. On the other hand, the xylem tissue of tolerant grapevines had more isolated vessels, preventing bacterial movement to other vessels. A similar logic could be applied to the movement of *X. ampelinus* in the xylem of grapevine genotypes under study. This could explain why susceptible Redglobe and C-3229 exhibited disease symptoms as early as one week, while tolerant Dauphine had delayed symptom appearance. However, the type of inter-vessel pitting of these varieties would have to be determined to confirm this.

The age of plantlets used in this study emerged as an important factor. Plant material that has been continuously transplanted for over two years should not be used. In the second round of trials in this study, results for Dauphine had to be excluded, since the resistance phenotype of Dauphine against *X. ampelinus* was compromised after many cycles of sub-culturing in tissue culture media. Continuous sub-culturing exposes the plants to oxidative stress, which may result in mutations (Krishna *et al*. 2016). Smulders and De Klerk (2011) found that extreme procedures such as protoplast culture and callus formation impose stress. Varieties, like Dauphine, that go through a callus phase can promote a higher mutation rate (Zayova *et al.* 2010). Furthermore, the genetic stability of a tissue type may be affected by rapid multiplication of that tissue (Khan *et al*. 2011). Bairu *et al*. (2011) and Currais *et al.* (2013) established that genetic variations occur in undifferentiated cells, calli, tissues and morphological traits of *in vitro* raised plants.

Necrosis and wilting symptoms are simpler and faster to observe, but this needs to be accompanied by molecular analysis. It should be noted that a particular set of symptoms could result from a number of causes other than the pathogen under study. There are other slow growing bacteria that are regularly isolated from grapevine material, which can easily be confused with *X. ampelinus* (Serfontein *et al*. 1997). Therefore, bacterial isolation and PCR was performed to prove that *X. ampelinus* was the cause of the symptoms observed in the inoculated plantlets and also to detect the movement of the bacteria to other parts of the plant that did not necessarily show symptoms. The recovery of bacteria in sections taken 1.58 cm away from the point of inoculation confirms that the bacteria were transported within the stem in both methods. Such behaviour has already been described for grapevine plants inoculated with *X*. *ampelinus* (Grall and Manceau, 2003; Langenhoven and Petersen, 2007). It was interesting to see that PCR results showed positive results for *X. ampelinus* in Dauphine samples after four weeks post inoculation, even though plants did not show symptoms at this stage for both methods. This suggests that the methods provided a good entry point for the pathogen to colonise the host plants. However, the pathogen was not able to cause symptoms until after six weeks, proving Dauphine's tolerance levels to the bacteria. PCR showed that *X. ampelinus* efficiently colonised grapevine tissues regardless of the inoculation method used. However, the mode of entry through wounds caused by scalpel method shows it might not be as successful as using the needle method. Symptoms in plants inoculated using the scalpel method did not spread as far as was observed when using the needle method. One logical reason would be that the needle inoculation method allows colonisation of bacteria in both directions: up and down perhaps due to delivery of the inoculums deeper into the vascular bundles of the host species through the needle method. This then allows the bacteria to multiply more in the plants and bacterial invasion increases. Such behaviour has been reported for grapevine plants inoculated with *X. ampelinus* (Grall and Manceau, 2003; Grall et al. 2005).

Isolation of *X. ampelinus* on YPGA media was difficult due to faster growth of saprophytes, especially when it came to Redglobe. It should be taken into consideration that *X. ampelinus* grows very slowly on artificial media and colonies become visible only after ten days of culture. This makes it easy for the saprophytic bacteria to overgrow the pathogen (Komatsu and Kondo, 2015).

The results from this study showed that evaluation of grapevine varieties for their response to *X. ampelinus* infection using micropropagated plants could be accomplished within two to six weeks compared to the three months required for screening plants in the greenhouse. In addition, the results on host tolerance and susceptibility, determined in the present work, agree with nursery and field observations for Redglobe and Dauphine (Langenhoven and Petersen, 2007). This is in contrast to observations for other crops and pathogens, e.g. screening of cassava genotypes for resistance to bacterial blight caused by *Xanthomonas axonopodis* pv. *manihotis,* which showed that not all host-pathogen interactions yielded the same result for both glasshouse and field trials (Banito *et al*. 2010)

Plants in this study were inoculated with *X. ampelinus* strain VS20. In a study done by Petersen *et al*. (2019), 20 isolates from South Africa, as well as from Europe, were tested and analysis showed that all isolates moved beyond the point of inoculation and induced symptoms, thus revealing that there is not much variation virulent among the isolates. It therefore stands to reason that we need only use VS20, as it is representative of isolates available. This is not always the case, as was shown by the responses of two cabbage (*Brassica oleracea*) cultivars inoculated with four different isolates of *Xanthomonas campestris* pv *campestris*, causal agent of black rot, which indicated differences in the virulence of the isolates (Peňázová *et al*. 2018).

The results of the present study confirm that grape variety Dauphine is tolerant to *X. ampelinus*. Therefore, this variety may be useful as donor parent in breeding programs for developing improved grape varieties with tolerance to bacterial blight. Furthermore, these results indicate that offspring from a Dauphine x C-3229 cross can be used as a mapping population for bacterial blight resistance analysis.

Using conventional breeding procedures, it was possible to obtain progeny from self-pollinated KV resistant to PM. The main goal is to combine the high quality traits from *Vitis vinifera* with resistance characteristics typical of wild species from America and Asia (Zini *et al*. 2015). The discovery of the PM resistance loci, *Ren1*, in both KV and Dzhandzhal kara became an important contribution to PM mildew resistance breeding, which led to the screening of additional germplasm in attempts to find other homologs possibly carrying the PM resistance genes. Riaz *et al*. (2013) investigated these

possible additional homologs as sources of resistance to PM and found six varieties with resistant alleles using SSR markers, UDV124 and VMCNg4e10.1. These varieties are all *V. vinifera* and included Husseine, Late Vavilov, Sochal, Baidh-ul-Haman and the two already mentioned above. With the identification of these varieties, parents could be selected to be used in new crosses to improve resistance to PM in breeding programs.

With two unique loci to restrict powdery mildew infection and their lack of negative fruit quality attributes, Chinese *Vitis* species have attracted attention from grape breeders. In a study done by Pap *et al*. 2016, quantitative trait locus (QTL) analyses identified two major powdery mildew resistance loci on chromosome 9 (*REN6*) and on chromosome 19 (*REN7*) in the Chinese grape species *Vitis piasezkii.* Their location on different chromosomes offers the potential for grape breeders to combine these resistance genes with the existing powdery mildew resistance loci (*Ren1*) to produce grape germplasm with more durable resistance against powdery mildew.

Research done by Kozma *et al*. 2009, combined two independently evolved resistance genes, *RUN1* (from *Muscadinia rotundifolia*) and *Ren1* from (*V. vinifera* cv. KV) for a secure and durable resistance against new races of *E. necator.* They concluded that such combination of various resistance genes made it possible to breed new cultivars with high level complex resistance. Based on the relationships among and characteristics of varieties, scientists can better protect genetic resources and conduct breeding programs.

Based on the method used in this study, it was possible to select valuable, homozygous dominant KV lines at an early stage that would make beneficial breeding resources. In this study, five SSR markers were used in screening 36 individuals derived from self-pollination of KV. Of the 36 individuals screened with the SSR markers, VMC9h4.2, UDV020a, UDV124, VVIP10 and VMCNg4e10.1, ten were homozygous for the *Ren1* gene. The primer sets in the study amplified successfully. VMC9h4.2 and VMCNg4e10.1 markers successfully validated the presence of the single dominant *Ren1* powdery mildew resistance locus. In the current study, allele sizes differ slightly from that of Hoffman *et al*. (2008), but were verified locally by Veikondis (2014). VMC9H4-2 and VMCNG4E10-1 are the markers most closely linked

to and reliably co-segregated with the *Ren1* locus (Hoffmann *et al*. 2008, Coleman *et al.* 2009, Kozma *et al*. 2009). However, screening progeny of self-pollinated KV with *Ren1* linked gene marker, UDV-020, produced multiple alleles. Veikondis, 2014, while studying the resistance genes in grapevine using molecular marker technology, also observed that this marker resulted in multiple alleles in the KV parent due to the primer having multiple binding sites. Coleman (2009) explained the phenomenon behind this by observing that the area around the *Ren1* gene is highly repetitive. However, based on the results obtained with the Nimrang x KV cross where progeny is genotyped with the UD020 marker – it was concluded that this marker is reliable in MAS (Katula-Debreceni *et al*. 2010).).

Multiplexing SSR markers from the PCR analysis to generate genotypic data were used, to make the process cost effective. This is important as it will help reduce the financial impact on breeders who incorporate this approach into breeding programs. In multiplex PCR, more than one pair of primers with different fluorescent labels were amplified in a single reaction. Care was taken to make sure that markers labelled in the same colour i.e. UDV124 and VMC9H4-2, were non-overlapping in their allele ranges and as long as the size range of alleles for the markers were compatible, the amplification product identity could be readily determined (Flores-Renteria and Krohn, 2013). In this study, lines homozygous for *Ren1* gene, namely KV2, KV4, KV6, KV11, KV12, KV16, KV40, KV46, KV47 and KV52 were identified. The benefit will be that whenever crosses are made with a homozygous dominant gene, all the offspring should inherit the trait.

Chapter 6: Conclusion and recommendation

Currently there are no published reports available concerning resistance of grapevine germplasm to bacterial blight caused by *X. ampelinus*. The lack of resistance in germplasm is a serious issue that needs to be addressed urgently as the disease can cause heavy yield losses. In this study, an efficient method, using a syringe needle to wound the stem, suitable for *in vitro* inoculation of grape plants with *X. ampelinus* was developed. Inoculation of plants with the needle method was both rapid and produce sufficiently high levels of infection to identify susceptible and tolerant grapevine genotypes. This method proved to be feasible and can now be applied to a more detailed study involving a large number of grapevine varieties to identify grapevine genotypes with varying levels of resistance to bacterial blight and to better select which grape genotypes should be integrated into a breeding program.

The current study verified that the SSR markers used to assay the genotypes from the self-pollinated Kishmish Vatkana were able to identify the *Ren1* gene that is homozygous in the offspring at an early stage. Plants not carrying the desired gene can now be discarded thus reducing the resources spent in the breeding program. Marker assisted selection will help to accelerate future breeding programmes for disease resistance and save breeders the time and expenses involved in developing and maintaining populations to maturity for the identification of traits.

With *E. necator* being a rapidly evolving pathogen as a result of strong selection pressure due to extensive use of synthetic fungicides, there is a desperate need to breed for durable field resistance. With major powdery mildew disease resistance loci that have been identified in Chinese and American *Vitis* species, combining their resistance genes (pyramiding) with that of KV can slow the evolution of virulent isolates and achieve durable resistance in the field. This is possibly a primary objective of grape breeders worldwide and something to look into for future studies.

The individuals that were identified in this study which are homozygous for the dominant gene *Ren1* are intended for use as parents in future breeding programmes. This will help ensure that whenever crosses are made with these cultivars, all the offspring should inherit the trait. This makes these breeding lines very useful in crosses

to combine powdery mildew resistance from more than one source (pyramiding of genes).

It is evident that Dauphine with tolerance to bacterial blight and the KV offspring homozygous for the *Ren1* gene resistant to powdery mildew, can be used as pollen parents in breeding programs. It was evident during this study that molecular studies can help breeders save time and spare costs in developing populations that are without the desired traits without having to wait until the plant have reached maturity.

Reference

Abido A., Aly M., Hassanen S., Rayan G. 2013. *In vitro* propagation of grapevine (*Vitis vinifera* L.) Muscat of Alexandria cv. For conservation of endangerment. Middle East Journal of Scientific Research 13: 328-337.

Agnihotri A. 1993. Hybrid embryo rescue. In: Lindsey K. (eds) Plant Tissue Culture Manual. Springer, Dordrecht. Doi.org/10.1007/978-94-009-0103-2_46

Agudelo-Romero P., Erban A, Rego C., Carbonell-Bejerano P., Nascimento T., Sousa L., Martínez-Zapater J.M., Kopka J., Fortes A.M. 2015. Transcriptome and metabolome reprogramming in *Vitis vinifera* cv. Trincadeira berries upon infection with *Botrytis cinerea*. Journal of Experimental Botany 66(7): 1769-1785.

Agurto M., Schlechter R. O., Armijo G., Solano E., Serrano C., Contreras R.A., Zúñiga G.E., Arce-Johnson P. 2017. RUN1 and REN1 pyramiding in grapevine (*Vitis vinifera* cv. Crimson Seedless) displays an improved defense response leading to enhanced resistance to powdery mildew (*Erysiphe necator*). Frontiers in Plant Science 8:758. doi: 10.3389/fpls.2017.00758.

Alimad N., Naff W., Azmeh F. 2017. Overwintering form of *Erysiphe necator*, the causal agent of grapevine powdery mildew in Southern Syria. Journal of Pant Protection Research 57(2): 129- 135.

Alvarez A. 2004. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. Annual Review of Phytopathology Journal 42: 339-366.

Anderson N., Byrne D., Sinclair J., Burrell A. 2002. Cooler temperature during germination improves the survival of embryo cultured peach seed. HortScience Journals 37: 402-403.

Armijo G., Schlechter R., Agurto M., Munoz D., Nunerz C., Arce-Johnson P. 2016. Grapevine Pathogenic Microorganisms: Understanding infection strategies and host response scenarios. Frontiers in Plant Science 7: 1-18.

Bairu M.W., Aremu A.O., Staden J.V. 2011. Soma clonal variation in plants: causes and detection methods. Plant Growth Regulation 63(2):147-173.

Banito A., Kpémoua K. E., Wydra K. 2010. Screening of Cassava genotypes for resistance to bacterial blight using strain x genotype interactions. Journal of Plant Pathology 92(1): 181-186.

Barry T. Colleran G., Glennon M., Duncan L., Gannon F. 1991. The 16s/23s ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods and Applications 1: 51-61.

Botha W., Serfontein S., Greyling M., Berger D. 2001. Detection of *Xylophilus ampelinus* in grapevine cuttings using a nested polymerase chain reaction. Plant Pathology 50: 515-526.

Bové J.M., Garnier M., 2002. Phloem and xylem-restricted plant pathogenic bacteria. Plant Science 163: 1083-1098.

Bradbury J. 1991. *Xylophilus ampelinus*. CMI Descriptions of Pathogenic Fungi and Bacteria No. 1050. *CAB International* formerly Centre for Agriculture and Biosciences International (CABI)*, Wallingford, UK*.

British Columbia Ministry of Agriculture. 2015. Grape Powdery mildew., [online] Available at: https://www2.gov.bc.ca/assets/gov/farming-natural-resources-and-industry/agriculture-and-seafood/animal-and-crops/plant-health/grape_powdery_mildew.pdf.

Brown J. 2011. Breeding for resistance as a component of integrated crop disease control and sustainable food production. [online] Available at: http://www./ [Accessed 6 September 2017].

Cain D. 2010. New diversity in table grapes: A commercial perspective. Journal of the American Pomological society 64(2): 83-85.

Carvalho L.C., Vidigal P., Amânclo S. 2015. Oxidative stress homeostasis in grapevine (*Vitis vinifera* L.). Environmental Toxicology, Frontiers in Environmental Sciences, doi.org/10.3389/fenvs.2015.00020.

Chatelet D. S., Wistrom C. M, Purcell A. H., Rost T. L., Matthews M. A. 2011. Xylem structure of four grape varieties and 12 alternative hosts to the xylem-limited bacterium *Xylella fastodia*. Ann Bot. 2011 Jul;108(1): 73-85.doi:10.1093/aob/mcr106.Epub 2011 May 5.

Cirami R. 1996. Tablegrapes for the home garden: A practical guide to growing tablegrapes in your garden. Winetitles, Marleston, SA.

Chavan S., Smith S. M. 2014. A rapid and efficient method for assessing pathogenicity of *Ustilago maydis* on Maize and Teosinte lines. Journal of Visualised Experiments, (83), e50712, doi: 10.3791/50712.

Coleman C, Copetti D, Cipriani G, Hoffmann S, Kozma P, Kovács L, Morgante M, Testolin R, Di Gaspero G. 2009. The powdery resistance gene *Ren1* co-segregates with an NBSLRR gene cluster in two Central Asian grapevines. BioMed Central Genetics 10:89. doi: 10.1186/1471-2156-10-89.

Compant S., Brader G., Muzammil S., Sessitsch A., Lebrihi A., Mathieu F. 2015. Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. BioControl 58 (4): 435-455. Springer Verlag.

Currais L., Loureiro J., Santos C., Canhoto J. M. 2013. Ploidy stability in embryogenic cultures and regenerated plantlets of tamarillo. Plant Cell Tissue Organ Culture 114(2): 149-159.

DAFF. 2017. A profile of the South African table grapes market value chain. [Online]. Available at: https://www.nda.agric.za/doaDev/sideMenu/Marketing/Annual%20Publications/Commodity%20Profiles/field%20crops/Table%20Grape%20Market%20Value%20Chain%20Profile%202017.pdf
D'Agata, I. 2014. Native Wine Grapes of Italy. University of California Press. p. 374-5. ISBN 978-0-520-27226-2.

Dantas A., Boneti J., Nodari R., Guerra M. 2006. Embryo rescue from interspecific crosses in apple rootstock. Pesquisa Agropecuária Brasileira 41: 969-973.

De Beer T. and Wynberg R. 2018. Developing and implementing policy for the mandatory labelling of genetically modified food in South Africa. South African Journal of Science 114(7/8), Art. #2017-0137, 7 pages. http://dx.doi.org/10.17159/sajs.2018/20170137.

Diab A., Khalil S., Ismail R. 2011. Regeneration and micropropagation of grapevine (*Vitis vinifera* L.) through shoot tips. International Journal of Advanced Biotechnology and Research 2(4): 484-491.

Di Gaspero G, Cipriani G, Adam-Blondon A-F, Testolin R. 2007. Linkage maps of grapevine displaying the chromosomal locations of 420 microsatellite markers and 82 markers for *R*-gene candidates. Theoretical Applied Genetics 114:1249-1263.

Doman E. 2015. Seven common grapevine diseases. [online] Extension. Available at: URL https://learn.winecoolerdirect.com/common-grapevine-diseases. Accessed 2 October 2018.

Dreo T, Gruden K, Manceau C, Janse J., Ravnikar M. 2007. Development of a real-time PCR-based method for detection of *Xylophilus ampelinus*. Plant Pathology 56: 9-16.

Du Plessis S.J. 1940. Bacterial blight of vines (vlamsiekte) in South Africa caused by *Erwinia vitivora* (Bacc.) DuP. Union of South Africa, Department of Agriculture and Forestry, Science Bulletin 214: 1-105.

Dwivedi S., Crouch J., Mackill D., Xu Y., Blait M., Ragot M., Upadhyaya H., Ortiz R. 2007. The molecularization of public sector crop breeding: progress, problems, and prospects. Advance Agronomy 95: 163-318.

EFSA PLH Panel (EFSA Panel on Plant Health) 2014. Scientific Opinion on the pest categorisation of *Xylophilus ampelinus.* European Food Safety Authority (EFSA) Journal 12*(*12): 2903-3921.

Eibach R., Töpfer R. 2014. Progress in grapevine breeding. Acta Horticulturae Journal (ISHS,) 1046: 197-209.

Flores-Renteria L., Krohn A. 2013. Scoring Microsatellite loci. In: Kantartzi S. (eds) Microsatelites. Methods in Molecular biology, Human Press, Totowa, New Jersey.

Focus International Organization of Vine and wine OIV. 2017. Distribution of the world's grapevine varieties. International organization of vine and wine18 rued'AguesseauF-75008, Paris–France. [online] Available at: http://www.oiv.int/public/medias/5888/en-distribution-of-the-worlds-grapevine-varieties.pdf.

Gadaury D. M, Cadle-David L., Wilcox W. F., Dry I. B., Seem R. C., Milgroom M. G. 2012. Grapevine powdery mildew (*Erysiphe necator*): A fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. Molecular Plant Pathology 13(1): 1-16.

Geerts P., Toussaint A., Mergeai G., Baudoin J. 2011. Phaseolus immature embryo rescue technology. In: Thorpe T., Yeung E. (eds) Plant Embryo Culture. Methods in molecular biology (Methods and Protocols), 710, pp.117-129 Humana Press.

https://www.nda.agric.za/doaDev/sideMenu/Marketing/Annual%20Publications/Commodity%20Profiles/field%20crops/Table%20Grape%20Market%20Value%20Chain%20Profile%202017.pdf.

Glowinski, L. 1991. The complete book of fruit growing in Australia. Lothian Publishing Company, Port Melbourne.
Gornal J., Betts R., Burker E., Clark R., Camp J., Willett K., Wiltshire A. 2010. Implications of climate change for agricultural productivity in the early twenty-first century. Philosophical Transactions of the Royal Society, 365: 2973-2989.

Grall S., Manceau C. 2003. Colonization of Vitis vinifera by a Green Fluorescence Protein-labeled, gfp-marked strain of *Xylophilus ampelinus*, the causal agent of bacterial necrosis of grapevine. Applied and Environmental Microbiology 69(4): 1904-1912.

Grall S, Roulland C, Guillaumes J., Manceau C. 2005. Bleeding sap and old wood are the two main sources of contamination of merging organs of vine plants by *Xylophilus ampelinus*, the causal agent of bacterial necrosis. Applied and Environmental Microbiology 71: 8292–8300.

Gütschow, M., 2001. Resistance to *Botrytis cinerea* in parts of leaves and bunches of grapevine. MSc. Thesis. Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Halleen F., Holz G. 2001. A South African perspective of powdery mildew in grapevines. Viticulture Research, Winetech technical. [Online] Available at: https://www.wineland.co.za/a-south-african-perspective-of-powdery-mildew-in-grapevines/.

Hickey C. C., Blaauw B., Brannen P., Pfeifer D., Nita M., Hoffmann M. 2018. Viticulture Management. University of Georgia Extension Circular 1151. Retrieved from https://extension.uga.edu/publications/detail. html?number=C1151

Hoffmann S., Di Gaspero G., Kovács L., Howard S., Kiss E., Galbács Z., Testolin R., Kozma P. 2008. Resistance to *Erysiphe necator* in the grapevine ‘Kishmish vatkana’ is controlled by a single locus through restriction of hyphal growth. Theoretical and Applied Genetics 116: 427-438.

Ji W., Li Z., Yao W., Gong P., Wang Y. 2013. Abnormal seedlings emerged during embryo rescue and its remedy for seedless grape breeding. Korean Journal of Horticultural Science and Technology 31: 483-489.

Jonah P., Bello L., Lucky O., Midau A. Moruppa S. 2011. Review: The importance of molecular markers in plant breeding programmes. Global Journal of Science Frontier Research 11(5): 5 -12.

Katula-Debreceni D. 2011. Molecular identification of fungi resistant grape genotypes. PhD thesis. Szent István University, Hungary.

Katula-Debreceni D., Lencsés A. K., Szöke, A. Veres A, Hoffmann S., Kozma P., Kovacs L. G., Heszky L., Kiss E. 2010. Marker-assisted selection for two dominant powdery mildew resistance genes introgressed into a hybrid grape population. Scientia Horticulturae 126 (2010) 448-453.

Kaufman, K. W. 1981. Fitting and using growth curves Oecologia 49:293. https://doi.org/10.1007/BF00347588

Khan S., Kauser N, Saeed B. 2011. Establishment of genetic fidelity of *in-vitro* raised banana plantlets. Paktanis. Journal of Botany 43: 233-242.

Komutsu T., Kondo N. 2015. Winter habitat of *Xylophilus ampelinus*, the cause of bacterial blight of grapevine in Japan. Journal of General Plant Pathology 81: 237-242.

Kozma P, Kiss, E., Hoffmann S., Galbács Z., Dula T. 2009. Using the powdery mildew resistant *Muscadinia rotundifolia* and *Vitis vinifera* 'Kishmish vatkana' for breeding new cultivars. In: Peterlunger, E. et al (eds). Proceedings of the IXth International Conference on Grape Genetics and Breeding. Acta Horticulturae. 827559-564.

Krell R., Perring T., Hashim-Buckey J., Pinckard T. 2008. Susceptibility of *Vitis vinifera* L. cv. Redglobe and Thompson Seedless to Pierce's disease. American Journal of Enology and Viticulture 59: 61-66.

Krishna H., Alizadeh M., Singh D., Singh U., Chauhan N., Eftekhari M. Sadh R. K. 2016. Somaclonal variations and their applications in horticultural crops improvements. 3 Biotech 3(54): 1-8.

Kumari P., Gupta V. K., Misra A. K., Modi D. R., Pandey B. K. 2009. Potential of molecular markers in plant biotechnology. Plant Omics Journal 2(4): 141-162.

Kumari P., Thaneshwari, Rahul. 2018. Embryo rescue in Horticultural Crops. International Journal of Current Microbiology and Applied Science 7(6): 3350-3358.

Langenhoven W. E., Petersen Y. 2007. Detection of *Xylophilus ampelinus* in greenhouse and field infected samples. Pages 147-148 in: Extended Abstracts of 5th International Table Grape Symposium.

Larsen R.J., Marx M.L. 2012. An Introduction to mathematical statistics and its applications. 5th ed., International ed. Boston, Mass.; London: Prentice Hall.

Lloyd G. McCown B. 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. Combined Proceedings of International Plant Propagators' Society 30: 421-427.

Langridge P., Chalmers K. 2005. The Principle: Identification and Application of Molecular Markers. In: Molecular Marker Systems in Plant Breeding and Crop Improvement, pp. 3-22. Lörz, H. & Wenzel, G. (eds). Springer-Verlag, Berlin, Heidelberg.

Lawson M. J., Zhang L. 2006. Distinct patterns of SSR distribution in the Arabidopsis thaliana and rice genomes. Genome Biology 7(2): 1-11.

Li J., Wang X., Wang X., Wang Y. 2015. Embryo rescue technique and its applications for seedless breeding in grape. Plant Cell Tissue Organ culture 120: 861-880.

Lloyd G., McCown B. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Combined Proceedings of the International Plant Propagators Society 30: 421–427.

Lodhi M., Guang-Ning Y., Norman F. W., Reisch B. I. 1994. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant Molecular Biology Reporter 12(1): 6-13. 10.1007/BF02668658.

Ma Z., Wen J., Tian J., Jamal A., Chen L., Liu X. 2018. Testing reticulate evolution of four Vitis species from East Asia using restriction-site associated DNA sequencing. Journal of Systematics and Evolution 56(4): 331–339. Doi: 10.1111/jse.12444

Mahuku G. 2004. A simple extraction method suitable for PCR based analysis of plant, fungal, and bacterial DNA. Plant Molecular Biology Reporter (22): 71-81.

Mason A.S. 2015. SSR Genotyping. In: Plant Genotyping. Methods in Molecular Biology (Methods and Protocols): 77-89. Batley J. (eds). Humana Press, New York, NY.

McElrone A. J., Sherald J. L. and Forseth I. N. 2003. Interactive effects of water stress and xylem-limited bacterial infection on the water relations of a host vine. Journal of Experimental Botany 54(381): 419–430.

Merdinoglu D, Butterlin G, Bevilacqua L, Chiquet V, Adam-Blondon A., Decroocq S. 2005. Development and characterization of a large set of microsatellite markers in grapevine (*Vitis vinifera*) suitable for multiplex PCR. Molecular Breeding 15: 349-366'.

Miazzi M., Ramadan H., Faretra F. 2010. An *In vitro* method to evaluate grapevine cultivars for *Erysiphe necator* susceptibility. *In vitro* Cellular and Developmental Biology-Plant 46(4): 363-367.

Mullins M., Bouquet A., Wilson L. 1992. Biology of the grapevines. Cambridge University Press., New York.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with 1tobacco tissue cultures. Physiology Plant 15: 473-497.

Myles S., Boyko A., Owens C., Brown P., Grassi Aradhya M., Prins B., Reynolds A., Chia J., Ware D., Bustamante C. Buckler E. S. 2011. Genetic structure and domestication history of grape. Proceedings of the National Academy of Sciences 108(9): 3530-3535.

Nazhad N., Solouki M. 2008. Seperation of DNA for molecular markers Analysis from leaves of the *Vitis vinifera*. Pakistan Journal of Biological Sciences 11:1436-1442.

Noroozi M., Beheshtizadeh H., Sattari A. 2015. Bacterial blight in rice: A review. International Journal of Scientific Research in Science and Technology 1(2): 20-23.

Nuffield Foundation. 2001. Investigating the effect of minerals on plant growth. Practical Biology in partnership with Society of Biology [online]. Available at: https://www.nuffieldfoundation.org/practical-biology/investigating-effect-minerals-plant-growth.

Palacio-Bielsa A., Cambra M. López M. 2009. PCR detection and identification of plant-pathogen bacteria. Journal of Plant Pathology 91(2): 249-297.
Panagopoulos C.1987. Recent research progress on *Xanthomonas ampelina*. Bulletin OEPP 17: 225-230.

Panagopoulos C.G. 1969. The disease "tsilik marasi" of grapevine: its description and identification of the causal agent (*Xanthomonas ampelina* sp. nov.). Annales de l'Institut Phytopathologique Benaki 9: 59-81.

Panagopoulus C.1987. Recent research progress on *Xanthomonas ampelina*. Bulletin OEPP 17, pp. 225-230.

Panagopoulos C.G. 1988. Xanthomonas ampelina Panagopoulos. In: European Handbook of Plant Diseases [ed. By Smith, I. M. \Dunez, J. \Lelliot, R. A. \Philips, D. H. \Archer, S. A.].Oxford, London, Edinburgh, Boston, Palo Alto, Melbourne, UK & USA & Australia: Blackwell Scientific Publications, 157–158.

Pap D., Riaz S., Dry I. B., Jermakow A., Tenscher A. C., Cantu D., Oláh R. and Walker M. A. 2016. Identification of two novel powdery mildew resistance loci, REN6 and REN7, from the wild Chinese grape species *Vitis piasezkii*. BMC Plant Biology 16(170): 1-19.

Peňázová E., Kopta T., Jurica M., Pečenka J., Eichmeier, Pokluda R. 2018. Testing of inoculation methods and susceptibility testing of perspective cabbages breeding lines (*Brassica Oleracea Convar. Capitata*) to the black rot disease caused by *Xanthomonas campestris* pv. *campestris.* Acta Universitatis Agriculture ET Silviculturae Mendelianae Brunensis 66(16): 139-148.

Petersen Y., Burger P., Langenhoven W.E. 2019. Genotypic diversity and virulence of *Xylophilus ampelinus* isolates. (Abstract) 51st Congress of the Southern African Society for Plant Pathology. 21-23 January 2019. Mykonos, Western Cape.

Phumichai C., Phumichai T. Wongkaew A. 2015. Novel chloroplast microsatellite (cpSSR) markers for genetic diversity assessment of cultivated and wild *Hevea* rubber. Plant Molecular Biology Report 33: 1486-1498.

Plantwise Knowledge Bank. 2013. Cancer of grapevine (*Xylophilus ampelinus*). DPI NSW factsheets, New South Wales Government, [online]. Available at: www.plantwise.org/KnowledgeBank.

Pool R., Pearson R., Welser M., Lakso A., Seem R. 1984. Influence of powdery mildew on yield and growth of Rosette grapevines. Plant diseases 68: 590-593.

Qiu W., Feechan A., Dry I. 2015. Current understanding of grapevine defence mechanisms against the bio trophic fungus (*Erysiphe necator*), the causal agent of

powdery mildew disease. Horticulture Research, 2, 15020; doi:10.1038/hortres.2015.20.

Razi M., Marandi R., Baneh H., Hosseini B. Darvishzadeha R. 2013. Effect of Paternal Genotypes Sprays with BA and IAA Concentration on Embryo Rescue of F1 Progenies from 'Askari' (*Vitis vinifera* L.) Cultivar. Journal of Agricultural Science and Technology, 15: 1023-1032.

Reed S. 2005. Embryo Rescue. In: Plant Development and Biotechnology. Trigiano R.N. and Gray D.J. (ed). CRC Press Boca Raton, FL pp. 235-239. Rosewood, Denver.

Reisch B. I., Owens C.L., Cousins P.S. 2012. Grape. In: Badenes M. L. and Byrne D. H. (ed.) Fruit Breeding, Handbook of Plant Breeding, pp. 225-262. Springer, New York USA.

Riaz S., Boursquot J., Dangl G. S., Lacombe T., Laucou V., Tenscher A. C., Walker M. A. 2013. Identification of mildew resistance in wild and cultivated Central Asian grape germplasm. BMC Plant Biology 13(149): 1-21.

Riaz S., Tenscher A., Rubin J., Graziani R., Pao S., Walker M. 2008. Fine-scale genetic mapping of two Pierce's disease resistance loci and a major segregation distortion region on chromosome 14 of grape. Theoretical and Applied Genetics 117(5): 671-68.

Sawler J., Reisch B., Aradhya M., Prins B., Zhong G., Schwaninger H., Myles S. 2013. Genomics Assisted Ancestry Deconvolution in Grape. *PLoS ONE*, *8*(11), e80791. http://doi.org/10.1371/journal.pone.0080791.

Schaad N., Jones J., Chun W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3rd. Ed. APS Press, St. Paul, Minnesota, USA.

Sefc K.M., Lefort F., Grando M.S., Scott K.D., Steinkellner H. and Thomas M.R. 2001. Molecular Biology and Biotechnology of Grapevine. First edition, The Netherlands.

Serfontein S., Serfontein J. J., Botha W. J. Staphorst J.L. 1997. The isolation and characterisation of Xylophilus ampelinus. Vitis 36(4): 209-210.

Smulders M. J. M., Klerk de G. J. M. 2011. Epigenetics in plant tissue culture. Plant Growth Regulation 63(2): 137-46.

South African Freeze Alliance on Genetic Engineering (SAFeAge). 2008. Consumer rights recognised: GM foods will be labelled in South Africa [press release]. 2008 September 17 [cited 2017 Dec 08]. Available from: http://db.zs-intern.de/uploads/1221732671-Safeage-GELabelling.pdf 9. Gosling M. GM labelling clause reinstated in Bill. Cape.

Spiegel-Roy P., Sahar N., Baron J., Lavi V. 1985. *In vitro* culture and plant formation from grape cultivars with abortive Ovules and seeds. Journal of the American Society for Horticultural Science 110: 109-112.

Szegedi E., Civerolo E. 2011. Bacterial diseases of grapevines. International Journal of Horticultural Science 17(3): 45-49.

Tartarini S. 2003. Marker-assisted selection in pome fruit breeding. In *Proc. Int.* Workshop on Marker-Assisted Selection: A Fast Track to Increase Genetic Gain in Plant and Animal Breeding. [Online] available at: www.fao.org/biotech/docs/Tartarini.pdf).

This P., Lacombe T., Thomas M. 2006. Historical origins and genetic diversity of wine grapes. Trends in Genetics 22(9): 511-519.

UC-Intergrated Pest Management Program. 2009. Powdery mildew on ornamentals. [Online] Available at:
http://ipm.ucanr.edu/PMG/r280101011.

Uma S., Lakshmi S., Saraswathi M., Akbar A., Mustaffa M. 2011. Embryo rescue and plant regenation in banana (*Musa* spp.). Plant Cell Tissue Organ 105: 105-111.
University of Californai, Intergrated Pest magement Program. 2017. Grape Pierce's disease pathogen: *Xylella fastidiosa*. [online] Available at: www. http://ipm.ucanr.edu/PMG/r302101211.html.

Van Heerden C. J., Burger P., Vermeulen A., Prins R. 2014. Detection of downy and powdery mildew resistance QTL in a 'Regent' x 'Redglobe' population. Euphytica 200: 281-295.

Veikondis R. 2014. Genetic characteristic of fungal disease resistance genes in grapevine using molecular marker technology. MSc. Thesis. Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa. http://hdl.handle.net/10019.96090.

Veikondis R., Burger P., Vermeulen A., Van Heerden C. J., Prins R. 2018. Confirmation of the effectiveness and genetic positions of disease resistance Loci in 'Kishmish Vatkana' (*Ren1*) and 'Villard Blanc' (*REN3* and *Rpv3*). South African Journal of Enology and Viticulture 39(2): 185-195.

Vitis International Variety Catalogue (VIVC). 2007. Federal Centre for Breeding Research on Cultivated Plants (BAZ). Institute for Grapevine Breeding Geilweilerhof (IRZ).

Wang L., Zhang J., Liu L., Zhang L., Wei L., Hu D. 2015. Genetic diversity of grape germplasm as revealed by microsatellite (SSR) markers. African Journal of Biotechnology 14(12): 990-998.

Weng K., Li Z., Liu R., Wang L., Xu Y. 2014. Transcriptome of *Erysiphe necator*-infected *Vitis pseudoreticulata* leaves provides insight into grapevine resistance to powdery mildew. Horticulture Research 14049; doi:10.1038/hortres.2014.49.

Willems A., Gillis M., Kersters K., Van Den Broecke L., De Ley J. 1987. The taxonomic position of *Xanthomonas ampelina*. Bulletin OEPP, 17(2), pp. 237-24031.

William H., Trethowan, R., Crosby-Galvan, E. 2007. Wheat breeding assisted by markers: CIMMYT's experience. Euphytica 157: 307-319.

Xu Y., Crouch J. 2008. Marker assisted selection in plant breeding: From publication to practice. Crop Science 483: 91-407.

Zayova E., Vassilevska-Ivanova R., Kraptchev B., Stoeva D. 2010. Somaclonal variations through indirect organogenesis in eggplant (*Solanum melongena* L.). Biological Diversity and Conversation 3(3): 1-5.

Zhang X., Harvey P.R., Stummer B. E., Warren R. A., Zhang G., Guo., Li J., Yang H. 2015. Antibiosis functions during interactions of *Trichoderma afroharzianum* and *Trichoderma gamsii* with plant pathogenic *Rhizoctonia* and *Pythium*. Functional and Intergrative. Genomics 15(5): 599-610.

Zini E., Raffeiner M., Di Gaspero G., Eibach r., Grando M. S., Letschka T. 2015. Applying a defined set of molecular markers to improve selection of resistant grapevine accessions. Acta Horticulture 1082: 73-78 DOI: 10.17660/ActaHortic.2015.1082.9.

Zohary D. 1995. Domestication of the grapevine *Vitis vinifera* L. in the Near East. In: McGovern P.E., Fleming S.J. and Katz S.H., eds. The origins and ancient history of wine. New York: Gordon and Breach, 23–30.