Anomalous PCR results to Grapevine leafroll associated closterovirus type 3 in South Africa

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I declare that the thesis/dissertation, which I hereby submit for the degree MSc. Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution

SIGNATURE:…….

DATE:……..

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INDEX

INDEX (Continue)

INDEX (Continue)

LIST OF FIGURES

LIST OF FIGURES (CONTINUE)

LIST OF TABLES

ABSTRACT

Grapevine leafroll-associated virus type 3 (GLRaV-3) is the major causative agent of grapevine leafroll disease. The disease has a major negative impact on grape production for wineries, and can cause up to 62.8% loss in production. Despite the negative impact of GLRaV-3 on the grapevine industry worldwide, knowledge on the variability of the virus, which is essential for developing effective control measure of the virus in vineyards, is surprisingly scarce. To test this, six primers sets used in a one tube, one step polymerase chain reaction (PCR) protocol, together with ELISA were used to detect GLRaV-3 virus in 135 plant samples collected from a single vineyard. As expected the more sensitive PCR detected more infected samples than ELISA. However, some samples yielded positive results with the ELISA, but negative results using PCR. This might suggest that strain variants exist. Amongst PCR results of the different primer sets, anomalous results occurred, as often a plant will yield an amplicon with one primer set, but not with another primer set. Using the entire set of 7 PCR results per sample, each plant was assigned a PCR 'fingerprint'. This yielded 24 different fingerprints in the vineyard. Mapping the spatial distribution of given fingerprints supported the possibility that strain variants exist. However, sequencing areas incorporating the primer binding sites showed no nucleotide sequence differences, indicating that the anomalous PCR results were not due to variants, but rather to protocol error.

The PCR protocol used initially was adapted to obtain more optimal detection of virus. Different extraction methods and PCR protocols were tested. It was found that using the two step RT-PCR and using a less dilute plant macerate in ELISA extraction buffer (1:5), yielded amplicon of the expected size from all known infected plant samples. The protocol was further optimized with regards the RT step and subsequent PCR. Since the modified protocol did detect all known infected plant samples it can be concluded that in the 135 plant samples tested no significant sequence variation in strains at the primer binding sites occurred and that the anomalous PCR results initially obtained were due to a sub-optimal extraction method.

CHAPTER 1

INTRODUCTION

1.1. Introduction

The South African wine industry encompasses wine (natural, fortified and sparkling); rebate wine; distilling wine; brandy and other spirits distilled from wine, grape juice and grape juice concentrate (Sawis, 2005). The wine industry in South Africa (RSA) produced a total of 1 015.697 million liters of beverages in 2004, earning an income of 2 790.540 million rand (Sawis, 2005). In 2004 RSA produced 351 746 825 vines, which were distributed over 124 749 hectares of land (Sawis, 2005).

Grapevine is naturally infected by 20 different disease causing viruses (Sutic, 1999). Viral diseases are among the most economically damaging of all grapevine diseases since in contrast to most fungal and bacterial diseases, once infected, the canes remain systemically infected with no cure (Sutic, 1999). Grapevine is susceptible to several graft transmissible diseases caused by viruses and virus-like pathogenic agents (Credi & Giunchedi, 1996). Amongst these, Grapevine leafroll disease (GLD) has a high incidence in all the viticultural regions of the world (Credi & Giunchedi, 1996) and is one of the most important viral diseases of grapevine.

Grapevine leafroll disease was first detected in California in 1958 where the virus was reported that to affect fruit coloration, maturation and yield in the red table grape, Emperor (Rowhani, 1997). GLD is also known as Rougeau, Flavescence or Bruinsure in France (1853), Rolkrankheit in Germany (Goheen, 1958) and red-leaf in California (Charles *et al.*, 2006). Scheu (1936) was the first in Europe to prove that a virus caused leafroll disease (Weber *et al*., 1993). The disease has a major negative impact on grape production for wineries and can cause up to 62.8% loss in production (Fajardo *et al.*, 2002). Nine different members of the *Closteroviridae* are associated with grapevine leafroll disease (Alkowni *et al.*, 2004). Grapevine leafroll-associated virus type 3 (GLRaV-3) is the major causative agent of grapevine leafroll disease, not only in RSA, but worldwide (Turturo *et al.*, 2005).

Commercial wine and rootstock cultivars in the Western Cape were tested in 1970 for a variety of viruses (Nel & Engelbrecht, 1972). In 1970 a survey was undertaken on the incidence of leafroll in the vineyards of the Western Cape (Nel & Engelbrecht, 1972). An extraordinary number of vines were found to be infected, with 68,4% of these infections represented by leafroll virus (Nel & Engelbrecht, 1972). The perception of an increase in GLD infection in RSA vineyards may be due to the fact that more red wine vines, displaying more visible symptoms, are now cultivated (Carstens, 2002). A survey performed by Pietersen (2006) to monitor the spread of GLD is RSA

showed that the disease is a serious problem in the RSA wine industry, due to its rapid spread and infection of certified planting material.

The only way to control the disease is by planting healthy plant material and to prevent subsequent re-infection. Spatio-temporal analysis of GLD spread in grapevine motherblocks (Pietersen, 2006) showed that control strategies have to incorporate a) planting of only certified planting material, followed by b) monitoring vines for infections with grapevine leafroll viruses which may have been latent at the time of establishment and c) removal of such infected material (rouging) (Spreeth *et al.*, 2006). The development of certified plant material is regulated by the certification schemes of a country (Rowhani, 1997). KWV-Vititec (a plant improvement organization) is such an organisation in South Africa maintaining the certified planting material. KWV-Vititec tests plants for the virus using techniques such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), however for these techniques to be effective, they must be able to detect all the different strains of the virus.

Apart from control strategies such as rouging, resistant plant material may be used to control the spread of a virus (Aaziz & Tepher, 1999). Developing resistance in plants using molecular approaches would require the characterisation of different strains to obtain resistance to all the strains and not just a selected few (Aaziz & Tepher, 1999). The expression of nucleotide sequences derived from plant viruses in transgenic plants can induce protection against viral diseases, a concept referred to as pathogen-derived resistance (Bendahmane *et al.*, 1997). Pathogen-derived resistance (PDR) was first reported by Powell-Abel *et al.* (1986) for the tobacco mosaic tobamovirus (TMV) coat protein (CP). The molecular events that mediate PDR, including resistance provided by coat protein molecules, remains unclear, and it is likely to involve a variety of cellular and molecular mechanisms (Bendahmane *et al.*, 1997). In the case of coat protein-mediated resistance (CP-MR) against TMV, the accumulation of CP in the transgenic plants is indispensable for protection against TMV. Furthermore, CP-MR against TMV is somewhat specific, and there is a correlation between resistance and the degree of amino acid sequence similarity between the CP in the transgenic plants and the CP of the challenging virus (Bendahmane *et al.*, 1997). Freeborough & Burger (2006) are performing studies on producing transgenic plants that will show resistance to GLRaV-3 using the heat shock protein 70 kDa homolog (HSP70h). If strains of GLRaV-3 exist in a plant it will therefore only show total resistance to GLRaV-3 if resistance is generated against all occurring strains.

It is apparent that because of the wide occurrence and the negative impact of the disease on the industry in RSA and its association with GLRaV-3 that it is necessary to know whether different GLRaV-3 strains are present in RSA; this in return will give a general idea on how to control the disease. Should different strains occur, and each has a unique marker, it would be possible to determine which of the strains the vector distributes preferentially. The markers of each strain would also give the opportunity to follow the distribution and determine if long distance spread is a common phenomenon. If the vector were dispersed over long distances, one control measure that can be implemented would be planting rows of trees, which can survive as a wind barrier.

The aim of this study was to confirm the possibility that different strains of GLRaV-3 exist in a selected area of a chosen vineyard, and to characterise these with regards molecular markers. If strains do occur the data can be used to try and determine the best control strategy to stop the spread of the virus.

1.2. Summary of aims

In this study we wish to:

1) Determine whether anomalous PCR results (plant sample showing positive ELISA reaction, but no amplicon when amplified with PCR (personal communication G. Pietersen) were due to strain variation by:

   (a) Characterizing variability in a selected 9 row by 15 plant matrix within a selected vineyard using 6 PCR systems and assigning a PCR fingerprint to each plant sample.

   (b) Obtain supporting evidence that different strains do occur through spatial analysis of the distribution of plants of a given PCR fingerprint.

   (c) Conforming the presence of variants by obtaining sequences of a number of plant samples representing different PCR fingerprints and hence possibly different strains.

Or; falling the above

2) To determine whether the anomalous PCR results (one primer set amplifying a plant sample, indicating that virus is present, while another primer set yields no amplicon on the same plant sample (personal communication G. Pietersen) were due to sub-optimal extraction or inefficient PCR protocols by;

   a) Determining whether the problem is due to the extraction buffer, by testing if any of the following extraction methods can give consistent results in the PCR:

      - Total RNA extraction with Promega kit (Promega, Madison, USA)
      - Extracting RNA using the Phenol-chloroform extraction method (Mallory *et al.,* 2001)
      - Concentrating the virus using antibody (Immunocapture) or adhesion to PCR tube (Simple-direct tube method) (Suehiro *et al*., 2005) before PCR was performed

- ❖ Extracting double stranded RNA from the plant sample (Valverde, 1990)
- ❖ Grinding the sample in ELISA extraction buffer (according to manufactures, ARC-PPRI, Pretoria)
- ❖ Spotting plant sample on nitrocellulose transfer membrane (Osman & Rowhani, 2006).

b) Determining the most effective amplification protocol, which will give accurate consistent amplification

- ❖ Two-step PCR. RT according to enzyme manufacture's protocol (Sigma protocol)
- ❖ One tube, one step RT-PCR followed by nested PCR (G. Pietersen, personal communication).

c) Determining the titer range in which detection of the virus will still be successful using plants with known high and low virus titer.

2.4) Determining the role, if any, that RNA degradation of extracted plant sample plays in the consistency of PCR results.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Grapevine has been an ancient food plant in the life of man for several millennia (Sutic *et al.*, 1999) and production is constantly increasing because the fresh fruit is nutritious and is increasingly becoming useful as an industrial raw material for developing new food products, juices, and beverages (Sutic *et al.*, 1999). Sustained efforts to ensure high, reliable yields are continuously threatened with damage to production caused by numerous diseases (Sutic *et al.*, 1999). In Brazil, grapevine leafroll virus stands out as an economically important disease. An incidence of 78% has been reported in Sao Paulo, and 15.6–98% in Rio Grande do Dul (Dianese *et al.*, 2005). Severely affected plants have been observed to suffer a reduction of 42.4% in number of clusters, 62.8% in production and 65.2% in vigor (Fajardo *et al.*, 2002). The consistent association of Grapevine leafroll-associated virus type 3 (GLRaV-3) with leafroll disease of grapevine prompted research to identify GLRaV-3 as a genuine agent of this economically most important disease (Boscia *et al.*, 1995). Despite the negative impact of GLRaV-3 on the grapevine industry worldwide, knowledge on variability of the virus, which is essential for developing effective control of the virus in vineyards, is surprisingly scarce (Jooste *et al.*, 2005).

2.2 Characterization of the family and the virus

2.2 a) Characterization of the Family: *Closteroviridae*

The plant virus family *Closteroviridae* is comprised of viruses with flexuous rod-shaped virions of 1 250 to 2 200 nm in length (Alkowni *et al.*, 2004). These viruses all contains a positive sense single-stranded RNA genome of 15.5 to 19.3 kb (Alkowni *et al.*, 2004). Initially, when established in 1998 the family consisted of just two genera, Closterovirus and Crinivirus. The major differentiating trait of the two genera was the possession of a monopartite and bipartite genome respectively (Martelli *et al.*, 2002). Karasev (2000) argued that closteroviruses should be classified by the type of insect vector rather than by the number of genomic RNAs. He proposed a genus named Vinivirus, but the International Committee on Taxonomy of Viruses (ICTV) study group on Closteroviruses and Allied viruses changed it to Ampelovirus (from ampelos, Greek for grapevine) to prevent confusion with the genus Vitivirus (Martelli *et al.*, 2002). The revised version of the family was approved by the ICTV in July 2002. The family consists of 3 genera: Closterovirus, Crinivirus and Ampelovirus, with the differentiating trait being the family of the insect vector (Figure 2.1) (Martelli *et al.*, 2002).

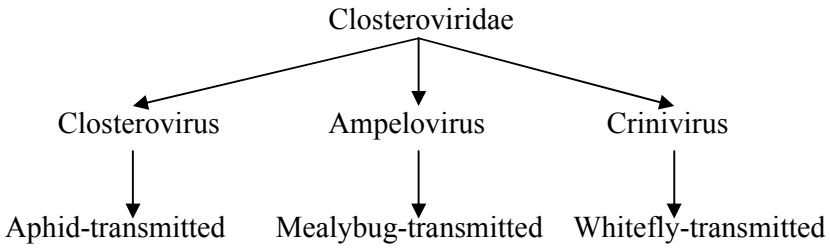

Figure 2.1: The three genera of the family *Closteroviridae* with its associated insect vector

The ancestral virus in the evolution of the closteroviruses was most likely a monopartite virus. This suggestion is based on the phylogenetic clustering of all the whitefly transmitted closteroviruses and the great uniformity in their genome organization. This also indicates a recent origin of the bipartite genome that has not yet had enough time to significantly evolve (Karasev, 2000). The genome of the ancestor had 7 open reading frames (ORF) that were organized into a replication-associated gene block and one closterovirus-specific gene block (Figure 2.2). These 7 ORF's consisted of ORF 1a (methyltransferase (MET) and helicase (HEL)) and ORF 1b (RNA dependent RNA (RdRp) polymerase) coding for the replication associated gene block, while the remaining 5 ORF's represented the closterovirus-specific gene block that included the genes for the heat-shock protein (HSP 70), the two structural proteins: the coat protein (CP) and the diverged coat protein (CPd) and two small proteins, p21 and p60 (Figure 2.2) (Karasev, 2000). The other closteroviral features that the ancestral virus had was the +1 translational frameshift and the expression of the 3′-terminal genes via sub-genomic RNA (sgRNA) (Karasev, 2000).

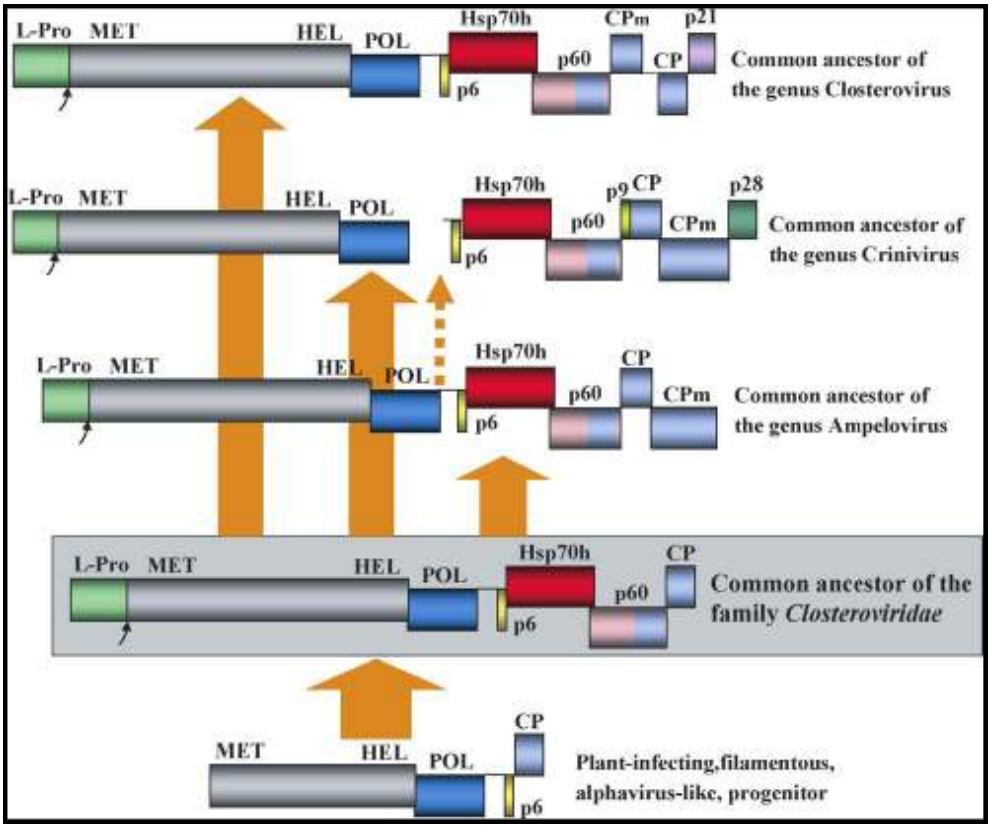

Figure 2.2: The hypothetical ancestor of the Family Closteroviridae. Image reproduced from Dolja *et al.* (2006)

Phylogenies inferred from the amino acid sequences of the closterovirus HEL, RdRp and the HSP 70 suggests that the closterovirus co-evolved with their insect vectors. This co-evolution probably took place over a considerable period of time for the great diversity to arise. Thus evolution within the family *Closteroviridae* most probably followed the three Families of insects: aphids, mealybugs and whiteflies (Karasev, 2000).

2.2 b) Characterization of Grapevine-leafroll type 3

Grapevine leafroll-associated virus type 3 (GLRaV-3) is the type species of the genus Ampelovirus (Ling *et al.*, 1997) and is the major causative agent of the disease, grapevine leafroll in grapevines. The virus is 1800-2000 nm in length and consists of a single-stranded RNA genome (Figure 2.3). The genome is positive sense and non-adenylated (Ling *et al.*, 1997). The size of the RNA genome is 17 919 nucleotides which is organized into 13 ORFs (Ling *et al.*, 1997). The virus genome is protected by the virions. Another principle function of the virion is to facilitate the transmission of the virus from host to host or within the host (Satyanarayana *et al.*, 2004).

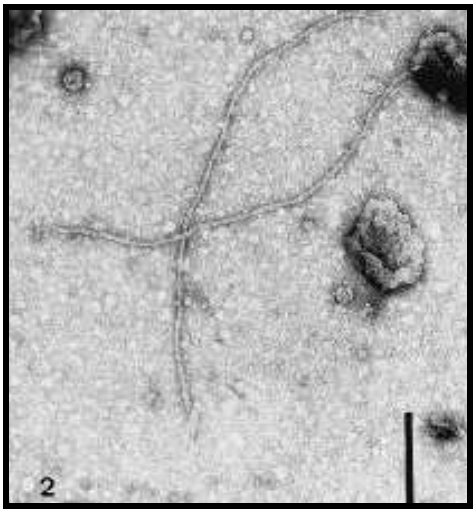

Figure 2.3: Electron micrograph of grapevine-leafroll associated type 3 virus (Scagliusi *et al.*, 2002.).

Most plant viruses have virions consisting of helical nucleocapsids, which are open structures, usually consisting of a single-coat protein with regular stacking around a nucleic acid in a constant relationship of amplitude and pitch. The long flexuous virions of *citrus tristeza virus* (CTV) and other viruses of the *Closteroviridae* family have a helical architecture consisting of two coat proteins (Satyanarayana *et al.*, 2004). The major coat protein encapsulates most (>95%) of the virion (the "body"), but the second (minor) coat protein (CPm) encapsulates the other end (the "tail") resulting in a "rattlesnake-like" virion (Figure 2.4) (Satyanarayana *et al.*, 2004).

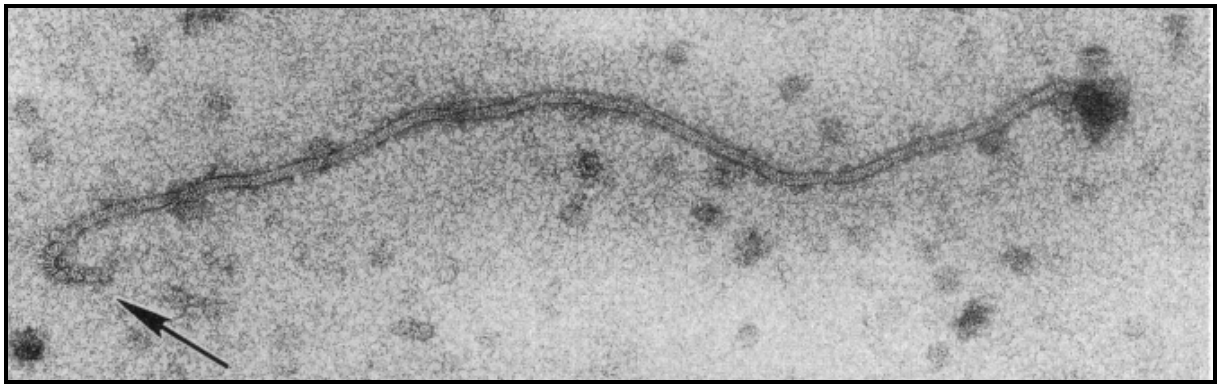

Figure 2.4: The "rattlesnake-like" virion structure of grapevine-leafroll associated type 3 (Agranovsky *et al.*, 1995)

2.3. Molecular characterization of Grapevine-leafroll type 3

2.3.1) Genome organization of Grapevine-leafroll type 3

In a study done by Ling *et al.* (1998) it showed that the genome of GLRaV-3 encompassed 13 ORF's. These ORF's were designated ORF 1a, ORF 1b and ORF 2-12 following the conventional closterovirus designation. Major genetic components such as HEL, RdRp, HSP 70, a putative HSP 90 homologue, CP and a CPm were identified. In general, the genome organization of GLRaV-3 is consistent with that expected for a typical monopartite closterovirus (Figure 2.5) (Ling *et al.*, 1998).

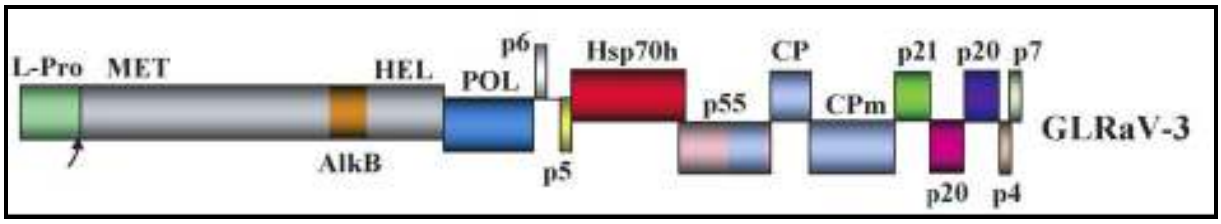

Figure 2.5: Schematic genome organization of Grapevine-leafroll associated type 3 (Martelli, 2002).

GLRaV-3 contains a 5′ untranslated region (UTR) that consists of 158 nucleotides. GLRaV-3 has the longest 5′ UTR among closteroviruses sequenced to date, followed by BYV and CTV (107nt), LIYV (97 nt) and LChV-1 (76 nt). The 5′ UTR has a low (30%) G + C content. The resulting low degree of secondary structure would facilitate ribosome binding in the 5′ UTR and initiate an efficient translational process (Ling *et al.*, 2004).

ORF 1a encodes a polypeptide with a size of 245 kDa. This ORF starts from the first ATG at the positions 159-161 and terminates at nucleotide 6870-6872 (Ling *et al.*, 2004). The start position is a favourable context for a translational initiation with a G at the + 4 and – 3 positions. In a multi-sequence alignment of GLRaV-3 with another Ampelovirus (Pineapple mealybug wilt associated virus, PMWAV-2), using 92 amino acid residues located from amino acid position 2801 to 371, Ling *et al*. (2004) demonstrated that ORF 1a contains a characteristic papain-like protease signature similar to the catalytic cysteine and histidine residues. The GLRaV-3 p-protease was predicted to cleave the ORF 1a polypeptide between residues Gly^{371} and Gly^{372}, similar to the Beet yellow virus (BYV) papain-like protease. This cleavage generates a N-terminal peptide of 371 amino acids and 42 kDa, following the p-protease processing site of the leader protein. ORF 1a also contained the

newly identified methyltransferase-like domain (MT) and the previously characterized helicase domain (Ling *et al.*, 2004).

Upstream of the helicase domain, an alkylated DNA repair protein (AlkB) domain is located within the viral replicase domain. The function of the AlkB in viral infection is unknown, but it seems likely that, similarly to certain cellular AlkBs, it might be involved in RNA repair via methylation reversal (Dolja *et al.*, 2006).

ORF 1b overlaps with the last 113 nucleotides of ORF 1a and terminates at the UAA codon at positions 3711-3713 on the genome. As first suggested for BYV, a +1 frameshift mechanism may also be used for the expression of ORF 1a/1b fusion protein in GLRaV-3. However the so-called "slippery" GGGUUU sequence and stem-loop structure that were proposed to be involved in BYV frameshift were absent from the GLRaV-3 ORF1a/1b overlap. Additional experiments on *in vitro* expression of GLRaV-3 genomic RNA are needed in order to determine whether a large fusion protein is actually produced (Ling *et al.*, 1998). ORF 1b is necessary to encode the RdRp, which is a protein that consists of 553 amino acids with an overall molecular mass of 60 678 Da (Ling *et al.*, 1998).

ORF 2 is responsible for encoding a small protein with a molecular mass of 5 927 Da. The open reading frame is followed by a long intergenic region of 1 067 nucleotides. The sequence showed no homologue when compared with the BLAST function (Ling *et al.*, 1998). It is thought that ORF 3 encodes a small hydrophobic transmembrane protein. The peptide consists of 45 amino acids that has a molecular mass of 5 090 Da (Ling *et al.*, 1998).

ORF 4 transcribes and translates the HSP 70 protein, which is 549 amino acids in length and has a molecular mass of 59 113 Da (Ling *et al.*, 1998). The HSP 70 protein constitutes a highly conserved family of proteins required for cell viability in all organisms and protein folding, protein translation, and thermal tolerance (Wilbanks *et al.*, 1994). Ling *et al.* (1998) showed that a multiple amino acid sequence alignment of GLRaV-3 p 59 with HSP 70 homologues of closteroviruses showed a sequence similarity among eight conserved motifs. Functionally important motifs, characteristic of all proteins containing the ATPase domain of the HSP 70 type, were also preserved in GLRaV-3 p 59. These results suggested that this HSP 70 chaperone-like protein might also possess ATPase activity on its N-terminal domain and protein-protein interaction on its C-terminal domain.

Viruses generally don't encode HSP 70s, although many have been demonstrated to recruit cellular HSP70s to aid virion assembly or genome replication (Peremyslov *et al.*, 1999). Several animal and plant viruses specifically regulate the expression of cellular HSPs 70 (Peremyslov *et al.*, 1999). Plant closteroviruses represent a unique family of large positive strand RNA viruses that encode homologues for HSP 70s (Peremyslov *et al.*, 1999). It seems likely that a common ancestor of closteroviruses acquired the HSP 70 gene by recombination with a host messenger RNA (mRNA) coding for HSP 70 (Peremyslov *et al.*, 1999). Inspection of a tentative phylogenetic relationship among closterovirus HSP 70s and a cellular HSP 70 shows that closterovirus HSP 70 are grouped together and separated from the plant HSP 70 (Ling *et al.*, 1998).

As closteroviruses do not encode a movement protein (MP), it has been suggested that the HSP 70 functions in closteroviral transport similarly to the participation of cellular HSP 70s in various translocation processes (Peremyslov *et al.*, 1999). Although closteroviral HSP 70 is structurally unrelated to the MPs of other plant viruses, it shares several features with diverse MP families. Similar to the p30 MP of tobacco mosaic virus (TMV), BYV HSP 70 is expressed early in infection and is found in the plasmodesmata, the structures that interconnect adjacent cells and provide a route for viral trafficking. Moreover HSP 70 seems to posses *in vitro* microtubule-binding ability and has been demonstrated that TMV MP bind microtubules *in vivo* (Peremyslov *et al.*, 1999). While the transport mechanism of the HSP 70 action is not known, apparent association of HSP 70 with the virions and plasmodesmata suggests that it may bind virions; chaperone them towards the plasmodesmata; and pull them into plasmodesmatal channels (Peremyslov *et al.*, 1999). It is hypothesized that the virion translocation through plasmodesmata occurs in a directional, tail-first manner (Prokhnevsky, 2002).

ORF 5 potentially encodes a protein of 483 amino acids. The molecular mass of the protein encoded by the ORF is 54 852 Da (Ling *et al.*, 1998).

ORF 6 was identified as the reading frame that transcribes the coat protein (CP) of GLRaV-3 and contains four conserved amino acids (N, R, G and D) that are identified in all the closterovirus coat proteins (Ling *et al.*, 1998). The coat protein has a molecular weight of 43 kDa and is larger that the 22-28 kDa coat proteins of closteroviruses that affect other crops (Ling *et al.*, 1997). The CP forms a long, helical body of the flexuous filamentous virions and encapsidates ~95% of the viral RNA (Dolja *et al.*, 2006).

The ORF that is responsible for encoding the diverged coat protein (CPm) follows the coat protein. This is confirmed by the presence of four conserved closterovirus coat protein residues in the C-terminal portion of this protein. The molecular mass of the diverged coat protein is 53 104 Da (Ling *et al.*, 1998). Analysis of the phylogenetic relationship with closteroviruses using the CPs and diverged copies again placed GLRaV-3 in an independent lineage from aphid-transmissible and whitefly-transmissible closteroviruses (Ling *et al.*, 1998). This CPm is responsible for the formation of the virion tail, while the tail encapsidates the 5′ terminal ~700 nt-long RNA-region (Dolja *et al.*, 2006).

The remaining five ORF's downstream of CPm (ORF 8-12) potentially encode proteins of 185, 177, 179, 36 and 60 amino acids with molecular mass values of 21 248, 19 588, 19 652, 3 933 and 6 768 Da, respectively (Ling *et al.*, 1998). Searches of databases using the BLAST program did not reveal statistically significant homologies for the proteins in the study performed by Ling *et al.* (1998).

ORF 8 was shown to have suppressor-silencing functions (p21). Interestingly, most of the dedicated viral suppressors represent small protein families without detectable homologous relationship to any other viral or host proteins (Dolja *et al.*, 2006). It has been found that p21 binds to double-stranded forms of the small interfering RNA (siRNA) or microRNA (miRNA). Thus, the molecular mechanism of p21 action is similar to that of another well-studied suppressor, p19 of *Tomato stunt virus.* Both suppressors sequester small RNA effectors of silencing and prevent their loading into the RNA-induced Silencing Complex (RISC) (Dolja *et al.*, 2006). However these suppressors cannot discriminate between siRNA that target viral genomes and endogenous siRNAs and miRNAs that are involved in regulation of plant development. Accumulation of p21 and p19 in plants induce developmental abnormalities many of which are identical to symptoms of viral infection (Dolja *et al.*, 2006). ORF 9 and ORF 10 encode p20, which represent a p21-like suppressor of RNA-silencing (Dolja *et al.*, 2006).

The 3′ UTR consists of 277 nt, for which database searches found no significant match except to the p20 sequence identified in another isolate of GLRaV-3. The extensive secondary structure in the 3′ UTR suggests that this region of the GRLaV-3 RNA may play a role in the viral replication cycle (Ling *et al.*, 1998).

2.3.2) Factors contributing to the diversity in Grapevine-leafroll type 3

Plant viruses utilize several mechanisms to generate the large amount of genetic diversity found both between and within species (Roossinck, 1997). Evidence for genetic variation of plant viruses was reported as early as 1926 (Garcia-Arenal, 2001). Three major forces drive the evolution of viruses: mutation, recombination and reassortment. These are essentially the same forces that drive all evolution; recombination in viruses is analogues to recombination in meiosis, and reassortment in viruses is analogues to chromosomal reassortment during sexual reproduction (Roossinck, 1997).

Several studies have estimated the error rates of the RdRp of animal RNA viruses; these rates have an average about 10^{-4} or one error per 10 kb genome (Roossinck, 1997). This is generally accepted as the error rate of all RdRps, but this finding needs to be confirmed for plant viruses, and it also should be examined in the context of a whole organism. In addition to misincorporation, RdRps can probably introduce variation by replication slippage, which results in short repeats. Although no direct evidence for this slippage exists, the presence of short repeats in some viral genomes is indicative of the presence of slippage from the RdRps (Roossinck, 1997).

Recombination is one of the main factors in the evolution of positive-stranded RNA viruses including those that infect plants. They involve either homologous recombination between two nearly identical RNAs, or non-homologous recombination between two RNAs that have a short anti-parallel stretch of complementarity. Homologous recombination can be either precise or imprecise and the sequence context appears to control crossover precision (Roossinck, 1997).

Homologous recombination can allow two viral genomes with different deleterious mutations to regenerate a functional genome. It can also allow related viruses to exchange genes in mixed infections, potentially generating even more fit variants. Phylogenetic analyses have yielded different estimations for the evolutionary relationships of RNA viruses when different genes are used. This strongly suggests that recombination has played a very important role in their evolution. Non-homologous recombination results in the deletions, insertions and repetitions of viral sequences (Roossinck, 1997). RNA recombination can generate different types of diversity that selection can act upon (Roossinck, 1997). In 1995 two lines of evidence suggested that recombination is quite common in the course of closterovirus infection (Karasev, 2000). The most direct evidence is the presence of defective RNAs in infected plants. Other, indirect evidence stems from the findings of different types of chimeric genomes in closteroviruses (Karasev, 2000).

In a study done on sequence variation in *Grapevine virus B* (GVB) a large amount of variation was obtained in the four regions (intergenic region, ORF 1, ORF 4, ORF 5) of the genome studied (Shi *et al.*, 2004). A possible explanation for the presence of extensive sequence variation in the GVB RNA may be due to the extensive vegetative propagation of the grapevine, its only natural host, over millennia (Shi *et al.*, 2004). This, together with grafting practices has resulted in the mixing of GVB isolates from various sources (Shi *et al.*, 2004). This not only may evoke RNA recombination but also may force the virus to induce changes to its sequence in order to be able to adapt to the new environment (Shi *et al.*, 2004).

Although defective RNAs (D-RNA) are more extensively found and studied in *Citrus tristeza* virus, D-RNA's are probably characteristic of all closteroviruses. In 1995, a small subgenomic (sg) dsRNA species was cloned from GLRaV-3 infected grapevines and used as a specific probe for GLRaV-3 detection. Only after nearly the complete GLRaV-3 genome was cloned and sequenced was the sgRNA species found to represent a GLRaV-3 specific D-RNA (Karasev, 2000).

Duplication of a gene, followed by rapid divergence, usually to provide a new or modified function, is a common theme in the evolution of organisms with large genomes, but it has rarely been seen in plant viruses. One instance was described: the closteroviruses have a duplication of their coat protein gene, which has since diverged, and encodes a protein in the unique tail-like structure at one end of the virion (Roossinck, 1997). Since the CP-like domain containing genes are present in all of the closteroviruses whose genomes have been sequenced so far, it appears that the duplication occurred prior to the divergence of these viruses from their common ancestor (Napuli *et al.*, 2003).

2.4. Host and symptoms of the disease

No alternative hosts are known to GLRaV-3 and leafroll-associated closteroviruses have not been identified in any wild or cultivated plant species other than *Vitis* species (Pathirana & McKenzie, 2005). When the vine is infected the fruit maturation is delayed and the yield is decreased by 20-40% (Alkowni *et al.*, 2004). Other symptoms include interveinal reddening (red grape varieties) or yellowing (white grape varieties) in late summer and fall leaves, down rolling of leaves and phloem disruption (Alkowni *et al.*, 2004) (Figure 2.6).

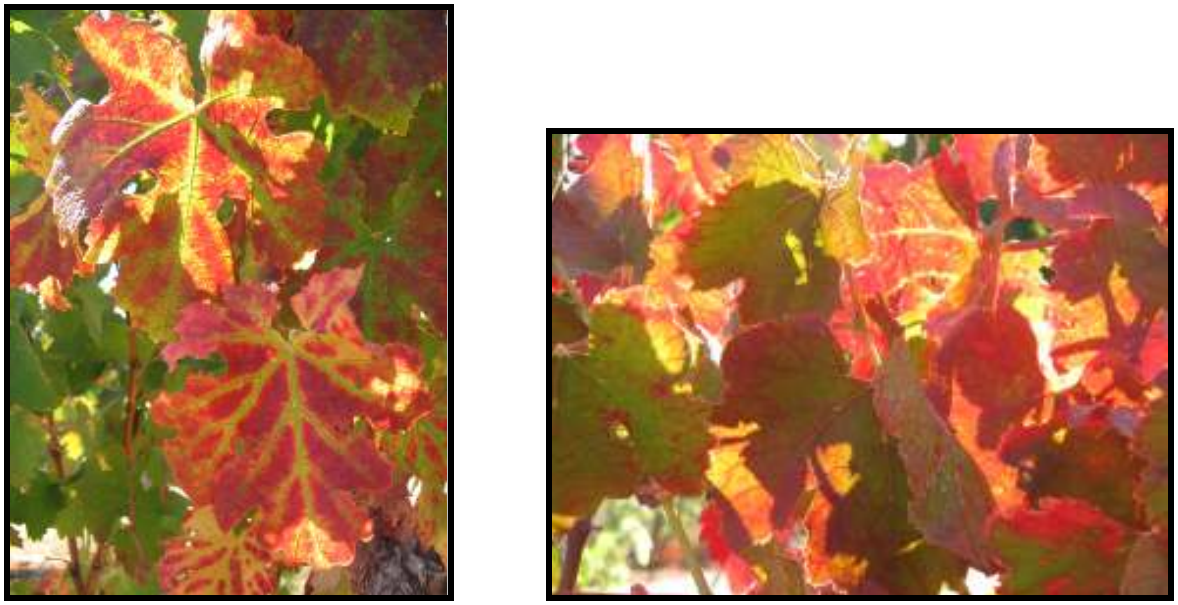

Figure 2.6: Symptoms caused by the Grapevine leafroll-associated virus type 3 on a red cultivar (Picture O. Koch)

Disease symptoms are dependent upon the virus isolate, grape variety and/or environmental conditions; because of this many infected vines may be asymptomatic making visual diagnosis difficult (Rowhani, 1997). Symptoms of grapevine-leafroll disease can resemble those caused by mechanical damage to the trunk and canes; by other diseases of the phloem tissue and by arthropod damage. This also complicates visual diagnosis (Alkowni *et al.*, 2004). The altered pigmentation can also be caused by magnesium and iron deficiencies in white-berried varieties (Pathirana & McKenzie, 2005). The symptoms of the disease are ideally expressed at temperatures between 22 and 25°C (Pathirana & McKenzie, 2005).

2.5. Economic losses caused by the disease

Virus-induced diseases are the most economically damaging diseases of the grapevine industry, because unlike fungal and bacterial diseases, once the grapevine is infected, the canes will remain infected for life with no prospect of a cure. Grapevine-leafroll disease is responsible for lowering the quality of the grapes by delaying the accumulation of sugar and lowering the production of anthocyanin in red wine cultivars. Wine made from these grapes is lower in alcohol, colour and tannins (Martelli *et al.*, 2002).

The disease is also responsible for lowering crop quality, because the vector, *Planococcus ficus*, excretes honeydew. The honeydew acts as a substrate for sooty mold, which will infect the grape bunches that are covered with the excreted honeydew (Daane *et al.*, 2004). Both the honeydew and the sooty mold lower the crop quality of the grapes.

2.6. Control of the disease

To control virus diseases in perennials, the most efficient method is to prevent infection of the plant. This is achieved mainly with the use of measures that are based on two principles: firstly the planting of healthy stock and then protection of the stock against subsequent infection. These protocols are usually performed with certification schemes (Rowhani *et al.*, 2004). Certification schemes are generally regulated by governments and often share the same objectives: to identify healthy sources for propagation through the application of time-tested indexing procedures as well as more recently developed molecular assays (Figure 2.7) (Rowhani *et al.*, 2004).

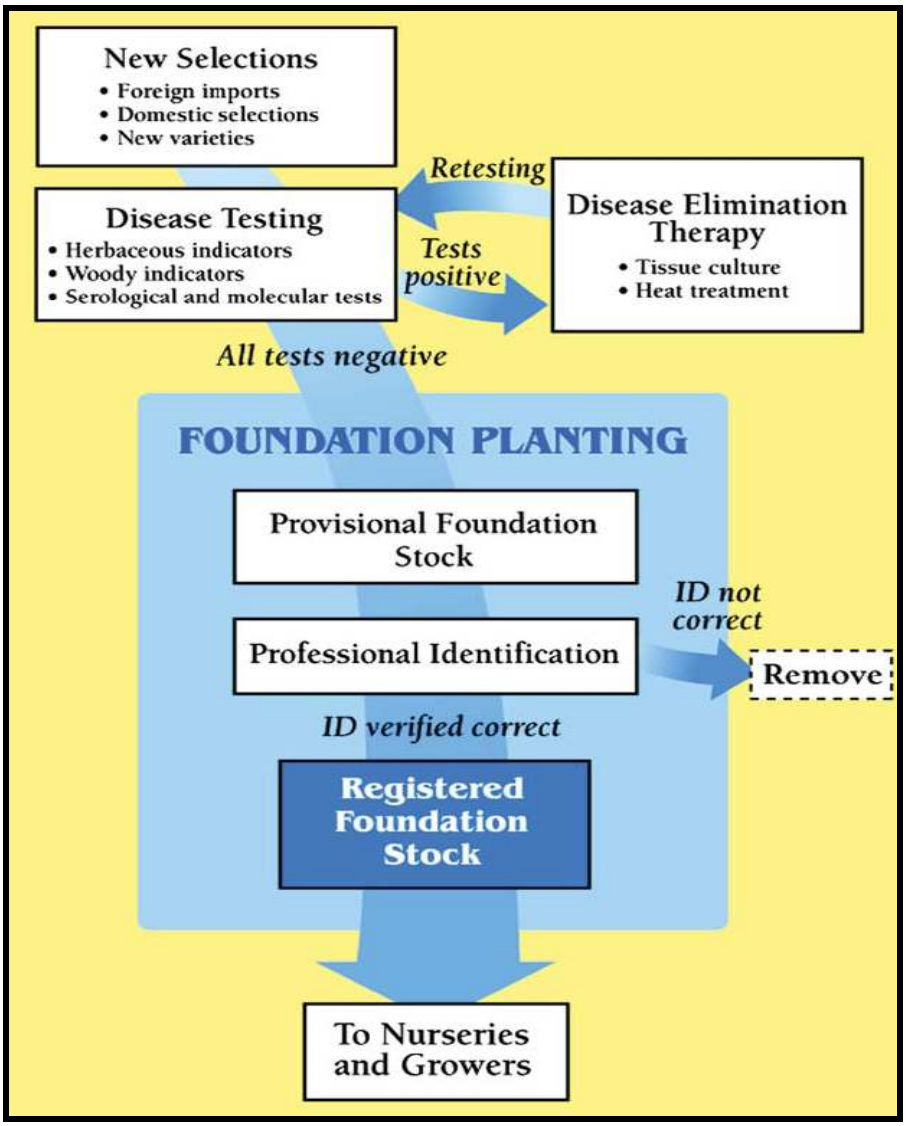

Figure 2.7: Scheme for testing and establishing foundation plantings (Rowhani *et al.*, 2004).

2.6.1) Disease testing

2.6.1 a) Biological indexing

There are two major types of indicator plants. Firstly there is the herbaceous group, which is used for sap-transmitted viruses, and the assay is performed over a period of weeks. Secondly there are the woody plants, which need a longer incubation period and the assays may take years before completion (Rowhani *et al.*, 2004).

To detect leafroll, woody indicators are used. The plant used as indicator for leafroll is *Vitis vinifera* cv. Cabernet Franc. The symptoms on Cabernet Franc are interveinal reddening of the leaf blade with the primary veins being prominently green. The leaf margins may roll downward, causing curling of the leaf and often the internodes are shortened (Rowhani *et al.*, 2004).

2.6.1 b) Serological and molecular testing

Serology describes the techniques where antibodies are used to detect the antigen. Enzyme-linked immunosorbent assay (ELISA) is a technique that utilizes the ability of antibodies, which are produced in animals, to recognize proteins, usually the coat protein of the virus of interest (Webster *et al.*, 2004). Antibodies are fixed to the surface of a well within a microtitre plate, and a sap extract from the plant is added to the well. If the virus of interest is present in the plant, it will bind to the anti-bodies fixed on the surface. Any unbound extract is washed-off before a secondary antibody that recognize that recognizes the first antibody is added. The secondary antibody allows for indirect detection of the virus because it has a reporter molecule attached to it, usually an enzyme that acts on a substrate that changes colour, which is detected visually by a calibrated microtitre plate spectrophotometer (Webster *et al.*, 2004). This method can be used for testing multiple plants for a single virus using one well per plant sample, or alternatively a single plant can be simultaneously tested for many viruses on a single plate with different antibodies coated to each well in duplicate or triplicate for reproducibility. Two types of ELISA protocols are used for surveillance namely double antibody sandwich (DAS) ELISA or indirect DAS ELISA. Direct methods such as DAS-ELISA involve enzyme attachment to the antibody probe. In the indirect method the antibody probe remains unlabeled. Instead, the enzyme is attached to a second antibody or Protein A reactive specifically to the probe antibody (Rowhani *et al*, 2004). Indirect DAS ELISA is preferred over the DAS-ELISA because of its greater sensitivity, broader reactivity and convenience (Rowhani *et al*, 2004). ELISA is performed annually on the foundation vineyards for pathogens known to spread naturally, this includes GLRaV-3 and it allows qualitative and quantitative analysis, high through-put, and high sensitivity (Rowhani *et al.*, 2004).

Although ELISA is the most useful technique for routine plant virus diagnosis, the technique is not sensitive enough for detection of viral RNA targets in some woody plant tissues and vectors due to low viral titre (Olmos *et al.*, 1999). More sensitive techniques have been assayed to overcome the diagnosis problem (Olmos *et al.*, 1999). The polymerase chain reaction (PCR) is an example of a more sensitive technique (Olmos *et al.*, 1999).

The PCR is a molecular assay that is sensitive, reliable, fast and highly versatile. In the case where the virus contains a RNA genome, reverse transcriptase-PCR (RT-PCR) is used (Rowhani *et al.*, 2004). In a PCR reaction the oligonucleotide primers flanking part of the genome, extends the flanked region by a thermostable DNA polymerase in a series of denaturation and extension steps that exponentially increases the target DNA. High sensitivity can easily lead to false positive results from contamination. The risk of false positives makes adequate controls essential. There are a number of variations on the basic technique, designed to increase sensitivity, alter specificity or allow automation of detection (Webster, 2004).

2.6.1 c) Comparison between serological and biological assays in the detection of grapevine-leafroll associated virus.

In theory ELISA is more specific and more sensitive, the test can also be completed in one or two days, which is an advantage over the lengthier incubation period needed for biological indexing on Cabernet franc (Rowhani, 1997). Rowhani (1997) did a survey containing a total of 221 grapevines. All the vines that tested positive by ELISA for GLRaV–1, GLRaV–2 and GLRaV–4 were symptomatic for leafroll disease, however, 8 of the ELISA GLRaV-3 positives tested negative with indexing. To confirm that the ELISA positives did not represent ELISA false-positives an Immunocapture reverse transcription PCR (IC/RT-PCR) was performed. Test results remained positive for the 8 samples. This discrepancy can indicate that GLRaV-3 was not uniformly distributed in the vines and that the use of an occasional healthy bud chip from infected grapevines, or failure of an infected bud chip to survive contributes to the negative results of the bioassay (Rowhani, 1997).

Rowhani (1997) suggests that biological indexing is still needed for the establishment of the foundation stocks or to clear selections through quarantine, but that ELISA as a valuable test is used for both early screening and subsequent testing to verify the continued health status of vines in registration and certification schemes (Rowhani, 1997).

2.7. Transmission of the virus

Closteroviruses are transmitted in a semi-persistent manner, with a minimum acquisition period of 0.5 to 1 hour, and may retain infectivity in an insect for up to 9 days, depending on the virus-insect combination (Karasev, 2000). Infection could not be transmitted vertically, and infectivity is

normally lost after molting. So far strict specialization has been observed between a virus and an insect vector at the family level, i.e. aphid-transmitted closteroviruses could not be transmitted by whiteflies or mealybugs, and vice versa (Karasev, 2000).

Four common distribution patterns were observed when research was done on the most important means of spread of leafroll disease, and the dispersal of viruliferous mealybugs. This was achieved by determining the temporal and spatial changes in distribution of grapevine leafroll disease infected vines in 70 mother block vineyards in the Western Cape, South Africa (Pietersen, 2006). Changes in the number of leafroll infected vine in vineyards in which leafroll had occurred and where rouging was not applied (57 vineyards) were monitored from 2001 to 2005.

The most prominent spatial distribution pattern of the infected vines was significant runs of adjacent infected vines. This pattern was mainly along rows and the number of infected plants in the row increased form season to season. These results indicated secondary spread within the vineyards after establishment (Pietersen, 2006). Secondary spread is the most important pattern of leafroll spread in local vineyards.

Circumstantial evidence of spread of leafroll by infected planting material was gathered on two mother blocks that was newly established on soil that was not previously planted with vines. These two motherblocks contained randomly occurring leafroll infections within the first or second season of establishment. These infected vines were also associated with specific rootstocks or scion clones only (Pietersen, 2006).

Evidence of leafroll spread from a preceding vineyard was presented based on the association of greater numbers of infected vines in one half of a mother block. This infected vines correlated spatially with a severely leafroll infected Cabernet franc vineyard previously planted in that half (Pietersen, 2006).

Gradients of leafroll infected vines; often associated with proximal leafroll infected vineyards were also observed (Pietersen, 2006) and are known as primary spread.

2.8. Vector

Insect vectors transmitting closteroviruses all belong to the order Homoptera, suborder Sternorrhyncha, and have been found in three famlies: Aphididae (aphids), Aleyrodidae (whiteflies)

and Pseudococcidae (mealybugs). These insects prefer feeding on the phloem tissue and their piercing-sucking mouthparts generally cause little damage to the plant (Karasev, 2000).

Until the 1980s, the spread of grapevine-leafroll disease was assumed to occur only through infected plant material, mainly asymptomatic American grapevine rootstock. In 1983, however it was found that some mealybugs are vectors of grapevine-leafroll associated viruses (Cabaleiro, 1997). Mealybugs transmit Grapevine leafroll-associated virus type 3. The mealybugs that are responsible for the spread of GLRaV-3 are *Pseudococcus longispinus*, *Planococcus ficus, Planococcus citri and Pseudococcus affinis* (Cabaleira & Segura, 1997). The classification of the mealybugs is as follows: Order: Homoptera, Sub-order: Sternorrhyncha, family: Pseudococcidae (Karasev, 2000). The virus is also transferred by a scale insect, *Pulvinaria vitis* (Cabaleira & Segura, 1997).

Planococcus ficus, the vine mealybug, is a key pest in South African vineyards (Figure 2.8) (Walton *et al*, 2004). It feeds on the vine roots, trunk, cordon, canes, leaves and fruit. *Planococcus ficus* has between 3 to 4 generations per year (Walton *et al*., 2004). The mature female is sessile, while the males have wings with which they can fly (Walton *et al.*, 2004). The mealybugs have seven development stages (Figure 2.9). In the first stage the mealybug is in an egg, from which they emerge as bright orange to yellow crawlers also known as 1st instars. In their third life stage the 1st instars begins to secrete a wax that will give the body of the instar a white appearance. The 2nd and 3rd instar mealybug will develop distinctive lateral and caudal spines and their body size will increase. From the 3rd instar they will develop into immature females and then to adults. The male mealybugs develop the same as the females from the egg to the 3rd instar stage. After the 3rd instar stage the male will develop into a non-feeding pupil stage. From the pupa a winged adult male will emerge. There are no over wintering stages in the lifecycle, all life stages can be found throughout the year.

Figure 2.8: The vine mealybug, *Planococcus ficus* (Vaughn Walton)

The mealybug excretes an abundant amount of honeydew. The honeydew provides a good food source for sooty mold and this can reduce the quality of the harvested grapes (Walton *et al*, 2004). In moderate to high numbers *Planococcus ficus* can lead to defoliated and infested grape bunches, both which result in reduced crop quality and yield. Less commonly, high *Planococcus ficus* population densities can lead to weak vines and, with repeated annual infestations, vine death (Walton *et al.*, 2004).

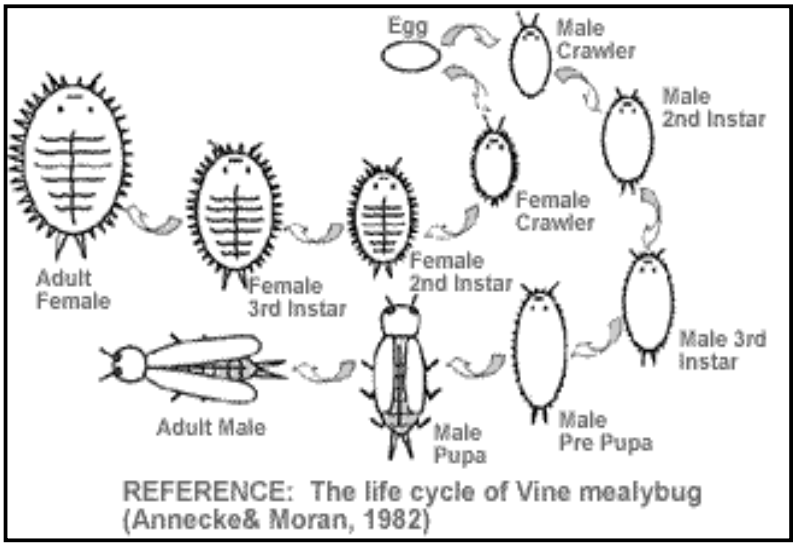

Figure 2.9: The life cycle of the vine mealybug, *Planococcus ficus* (Annecke & Moran, 1982)

2.9. Monitoring and Control of the vector

2.9.1.Monitoring *Planococcus ficus*

To determine the presence and density of mealybugs in a vineyard, visual sampling is done most of the time. Visually sampling vineyard for mealybugs is often a labour-intensive process that consists of time-consuming examination of many individual vines. Visual monitoring methods are most effective in late summer when the mealybugs are located on exposed locations and have high densities. Regrettably, this period and the conditions are often encountered after crop damage has already occurred (Walton *et al.*, 2004). An alternative method is the use of pheromone traps.

The use of a synthetic pheromone was developed and tested as a monitoring tool for *Planococcus ficus* in Californian vineyards (Walton *et al*., 2004). The pheromone is a monoterpene ester known as lavandulyl senecioate (Walton *et al.*, 2004). The pheromone is inserted into a plastic holder and placed in the field. The pheromones will attract the male mealybugs. It is shown that the amount of mealybugs caught in these traps is equivalently related to the amount of meaylybugs spotted in a field and that the pheromone traps are successful in catching *Planococcus ficus* males at extremely low pest population infestation levels (Walton *et al*., 2004). Based on studies in South Africa and those completed in California these trapping programs will be too cumbersome and expensive (Walton *et al*., 2004). Even if the monitoring is done, the mealybugs if present in the field must be controlled to prevent huge amounts of damage to the vineyards.

2.9.2 Controlling *Planococcus ficus*

Planococcus ficus was first recorded as a pest in the Western Cape Province (South Africa) during 1930 (Walton & Pringle, 2004). In South Africa the most predominant *Planococcus ficus* insecticide program includes one or more application(s) of long-residual organophospates (chlorpyrifos and/or tolouthion), applied just before bud break, followed by one or more application(s) of a short-residual organophosphate (mevinphos), applied during the growing season (Walton *et al.*, 2004).

This control method is incomplete due to the poor coverage of the insecticide when the mealybugs reside under dense leafs or under bark. The mealybugs also have a waxy excretion that covers its

body and can cause the applied insecticide to run off. There is also the issue of the negative impact insecticides have on the natural enemies of *Planococcus ficus* (Walton *et al.,* 2004).

Anagyrus pseudococci were first introduced in 1934 in California from South America to control citrus mealybug, but currently it is used to suppress vine mealybugs, *Planococcus ficus* as well (Daane *et al*., 2004). *Anagyrus pseudococci* (Hymenoptera, Encrytidae) is a polyphagous parasitoid of the citrus mealybug, *Planococcus citri* (Daane *et al*., 2004). The parasitoid also attacks distantly related species such as *Pseudococcus comstocki, Phenacoccus herreni, Dismicoccus brevipes* and *Maconellicoccus hersutus* (Daane *et al*., 2004). Due to the wide host and geographical range *Anagyrus pseudococci* is often used for biological control of the pseudococcids (Daane *et al*., 2004).

A range of natural enemies, many of which commonly occur in the Western Cape province, attack *Planococcus ficus* populations (Walton & Pringle, 2004). The most common natural enemies in this area include, in descending order of abundance:

Parasitoids: *Anagyrus* spp, *Cooccidoxenoides perminutus*, *Leptomastix dactylopii* (Walton & Pringle, 2004),

Predatory beetles: *Nephus bineavatus*, *N. angustus* and N. *quadrivittatus* (Walton & Pringle, 2004).

Biological control was severely hampered by the presence of a variety of ant species in vineyards in the Western Cape Province (Walton & Pringle, 2004). Ant control has been achieved using chemical stem-barrier treatments. Walton (2003) did a two-year field study of mass releases of *C. perminutus*, a parasitoid of the first, second and third instars of *Planococcus ficus*. This method of control was at least as effective as the currently used chemical control programme (Walton & Pringle, 2004).

Presently, integrated production of wine is encouraged by the wine industry in South Africa (Walton & Pringle, 2004). This system includes sound integrated pest management strategies for suppressing pests such as *Planococcus ficus.* Strategies include monitoring pest activity, pest-control practices such as trunk barriers, optimised use of biological control and limited use of chemicals during the growing season (Walton & Pringle, 2004).

CHAPTER 3

Characterisation of GLRaV-3 variation within a vineyard

3.1 INTRODUCTION

Ribonucleic acid (RNA) viruses are the most abundant molecular parasites infecting humans, animals and plants (Domingo & Holland, 1997). Among RNA plant viruses, genetic diversity arises from error-prone replication mechanisms that result in high mutation rates, as well from recombination and reassortment (Power, 2000). Evolution of viruses that rely on a vector to move between hosts is constrained not only by adaption to hosts and host defenses, but also by the requirements of vector compatibility (Power, 2000). Studying the variability of plant virus populations is an important aspect of plant pathology and may be relevant to diagnostic and control procedures (Alioto *et al.*, 2003).

Different types of variability occur and may be identified with different techniques. Three types of variability can be distinguished namely serological, biological and molecular variability. The existence of variation should be considered in the selection of methods for diagnosis during surveys for viruses (Theilman *et al.*, 2004). Serological variability is distinguished by the use of either polyclonal antisera or monoclonal antibodies (Hauser *et al.*, 2000), since the variable region will be the coat protein.

Molecular variability occurs when the strains differ in nucleotide sequence (Hauser *et al.*, 2000). Cloning of complementary DNA (cDNA) followed by polymerase chain reaction (PCR) and with restriction fragment length polymorphism (RLFP) or sequencing provides data for the determination of variability between strains (Candresse & Cambra, 2006).

The family *Closteroviridae* is divided into 3 genera: Ampelovirus, Closterovirus and Crinivirus (Martelli *et al.*, 2002). Grapevine leafroll-associated virus type 3 (GLRaV-3) belongs to the genus Ampelovirus. Significant variation in different members of the *Closteroviridae* is observed, but little is known about the variability that exists in GLRaV-3 (Turturo *et al.*, 2005).

The Closterovirus, *Citrus tristeza* virus (CTV), is the causal agent of the most economically important disease of Citrus spp. in the world (Lbida *et al*., 2005). Lbida *et al.* (2005) studied the genomic variability of the coat protein from three CTV virus isolates by a single-strand conformation polymorphism (SSCP) assay. The assay was applied directly to extracts from infected plants (Lbida *et al*., 2005). Comparison of the genomic variability of CTV, the intensity of

symptoms induced on indicator plants and the reaction to specific monoclonal antibodies showed clear differences between the Moroccan isolate (P1) and Spanish isolates (P2 and R1).

Comparison of cDNA sequences of the Florida CTV isolates T3 and T30 and the genome of the Israeli VT isolate showed a relatively consistent or symmetrical distribution of nucleotide sequence identity in both the 5′ and 3′ regions of the 19.2 kb genome (Hilf *et al.*, 1999). By contrast, when these sequences were compared to the sequence of isolate T36, they showed a dramatic decrease in sequence identity in the 5′ proximal 11 kb of the genome (Hilf *et al.*, 1999). The data obtained splits the CTV group into two major groups based on sequence differences (Hilf *et al.*, 1999).

Variation in natural CTV populations in Eastern Spain was studied by comparing the SSCP patterns of two genomic RNA (gRNA) regions in randomly selected trees at various locations (d'Urso *et al*., 2003). The gRNA regions selected in this study included the p20 gene and segment A (located in ORF1a) (d'Urso *et al*., 2003). The p20 gene is located within the conserved region of the CTV genome, and is involved in the formation of inclusion bodies (d'Urso *et al*., 2003). Segment A is located in a highly variable region between the protease and methyltransferase domains of ORF1a (d'Urso *et al*., 2003). The data presented suggested that even in neighboring trees within the same grove the predominant sequence variants of the pathogenicity-involved genomic regions may be different, thus explaining in part the variations in symptom expression observed (d'Urso *et al*., 2003).

Data analyses in a study performed by Rubio *et al.* (2001) showed that most isolates studied contained a population of CTV sequence variants, with one variant being predominant. Four of the isolates showed two major sequence variants in some genomic regions (Rubio *et al.,* 2001). d'Urso *et al.* (2000) characterised the population of genomic RNA sequence variants between CTV isolates by SSCP analysis using cDNA of the p18 and p20 genes. Comparison of field and aphid-transmitted isolates showed that aphid transmission frequently altered the SSCP pattern of both genes, indicating changes in the population of genomic RNA variants (d'Urso *et al.*, 2000).

Sixteen double-stranded RNA (dsRNA) profiles differing by the number and/or position of subgenomic bands were detected when a study on the variability among Spanish CTV virus isolates were conducted (Guerri *et al.*, 1991). One of these profiles was detected in more than half the trees analysed (Guerri *et al.*, 1991). The most diversity was found in the oldest groves and where the highest CTV incidences occurred (Guerri *et al.*, 1991).

The analysis of the variability observed in the CTV 5′ untranslated region (UTR) allowed classification of the sequences into three groups (Lopez *et al.*, 1998). In the same study it was established that the spectrum of sequence variation in the 5′ UTR is not continuous, but concentrated in peaks and separated by valleys, a general feature of viral quasispecies (Lopez *et al.*, 1998).

A survey for viruses was carried out in 2003 in the main olive-growing areas of Lebanon (Fadel *et al.*, 2005). Reverse transcriptase PCR was used to test for the presence of Olive leaf yellowing associated virus (OLYaV) (Fadel *et al.*, 2005). This Closterovirus was detected in 23.7% the samples (Fadel *et al.*, 2005). High variability was detected in the heat shock protein 70 (HSP70) gene of the Lebanese and Italian OLYaV isolates (Fadel *et al.*, 2005). At least nine different patterns were obtained when this genomic region was subjected to SSCP (Fadel *et al.*, 2005).

Another Closterovirus where a high degree of variability was detected was Grapevine leafroll associated virus 1 (GLRaV-1) (Little & Rezaian, 2006). Analysis of the nucleotide sequences of 10 open reading frames (ORFs) from GLRaV-1 revealed the presence of an unusually high degree of sequence variation in ORFs 3, 6 and 7 (Little *et al.*, 2001). Open reading frames 3, 6 and 7 encode the homologue of heat shock protein 70 and the two divergent copies of the coat protein, respectively (Little *et al.*, 2001). None of the changes in the genes resulted in a frame shift or stop codon and there was a trend for the conservation of amino acids or change to amino acids having similar physiochemical properties (Little *et al.*, 2001). These observations suggested that GLRaV-1 may exist in the form of a heterogeneous population, possibly resulting from the lack of selective pressure and mixing of virus strains due to viticulture practices of vegetative propagation and grafting over the centuries (Little *et al.*, 2001).

Cucurbit yellowing stunting disorder virus (CYSDV) is a virus that shows considerable genetic variation in the genus Crinivirus (Aguilar *et al.*, 2003). In a study by Marco & Aranda (2005) they observed that variability is not distributed evenly among the different regions of the viral genome, with the coat protein gene showing more diversity compared to the other coding regions (p25, p22, HSP70). Little cherry virus (LChV) is a representative of the Ampelovirus genus that contains sequence variability (Theilmann *et al.*, 2004). In a study performed by Theilmann *et al.* (2004) on LChV a high level of sequence variability was found. Nucleotide sequence divergence between one

isolate (LChV-LC5) and the other sequenced isolates ranged for 0 to 19.7% and the amino acid divergence range from 0 to 9.1% (Theilmann *et al.*, 2004).

Eight diverse GLRaV-2 were identified among the infected vines tested from Brazil, France, Greece and Italy (Angelini *et al.*, 2004). The technique heteroduplex mobility assay (HMA) was used to detect the variability (Angelini *et al.*, 2004). Heteroduplex mobility assay is based on the fact that structural deformations in double-stranded DNA fragments, which result from nucleotide mismatches or gaps, reduce their electrophoretic mobility in a polyacrylamide gel (Angelini *et al*., 2004).

Based on the variability of other members in the family *Closteroviridae*, it is reasonable to expect a high level of variability in GLRaV-3. However, few researchers have tested for the variability of GLRaV-3. Dianese *et al.* (2005) performed a study where the objective of the work was to compare the variability of a fragment of the viral polymerase gene of GLRaV-3 of three isolates from grapevines grown in: (a) the Vale da Sao Francisco, Pernambuco, Brazil, (b) North America (AF037268) and (c) Southern Brazil (AF438411). Three sequences were compared using the programs BLAST (Dianese *et al.*, 2005) and Bioedit (Dianese *et al.*, 2005). The three samples showed nucleotide substitutions when compared to each other. This indicated a variability that could be explained by natural variation of GLRaV-3 isolates within grapevine (Dianese *et al.*, 2005). Variability could also be due to vegetative propagation and the long life cycle of the plant associated with error prone RNA-dependent RNA polymerase (Little *et al.*, 2001).

In a paper presented by Turturo *et al*. (2005) the population structure and genetic variability of 45 GLRaV-3 isolates from 14 countries were investigated by SSCP and sequence analysis of 3 different genomic regions. The three regions, covering 9.2% of the entire genome, encode the RNA dependent RNA polymerase (RdRP), HSP 70 and the coat protein (CP) (Turturo *et al*., 2005). Analysis of the three genomic regions of the 45 isolates examined showed the existence of six, five and nine different SSCP patterns in the RdRp, HSP 70 and CP genes, respectively. Differences in the genetic diversity and constraints existing in the three regions analysed indicated a higher variability in the CP gene (Turturo *et al*., 2005).

In a study performed by Jooste and Goszczynski (2005) on the variability of GLRaV-3 using SSCP and sequencing they discovered that the 5′ terminal parts were clearly more divergent. This was achieved by sequencing fragments of the 5′ UTR + ORF 1a, 3′ terminal part of ORF 1a, ORF 4,

ORF 5, ORF 6 and ORF 7 of three isolates (Jooste & Goszczynski, 2005). Using sequencing it was determined that the most and least divergent fragments were of ORF 5 (91.8% - 92.3%) and ORF 7 (96.0% - 96.2%), respectively. Results further revealed that although the 3′ terminal half of the genome and the sequence located in the 3′ terminal part of ORF 1a were relatively similar among isolates (91.3% - 96.2% nt identity), their 5′ terminal parts were divergent (Jooste & Goszczynski, 2005).

The potential impact of virus variability in long-lived hosts is unclear (Turturo *et al*., 2005). Another reason for studying the closterovirus variability is the size and complexity of the genome, which uses combined strategies of polyprotein processing and subgenomic RNA for expression and evolution (Karasev, 2000).

In the present study, the genetic variability of GLRaV-3 obtained from 135 vine canes was investigated utilising PCR with 6 primer sets targeting the virus genome. Canes were collected from a vineyard belonging to Laborie vine estate at La Concorde. The specific vineyard was chosen as it had been used in a study to determine whether grapevine leafroll virus infected plants can be detected on a large scale with remote sensing (Pietersen, 2006b). Remote sensing exploits the difference in refraction spectrum of infected and healthy plants captured by aerial photography (Pietersen, 2006b). The above-mentioned study was done to minimise the need for plant-for-plant monitoring of vines for symptoms (Pietersen, 2006b). Plant-for-plant monitoring is labour intensive and can only be performed in a short period of the year between the onset of autumn and the fall of vine leaves (Pietersen, 2006a). In order to establish a “ground-truth” an enzyme-linked immunosorbent assay (ELISA) was performed to identify infected plants (Pietersen, 2006b). In addition to ELISA, the more sensitive PCR technique was performed to identify infected plants. As expected due to the greater sensitivity of PCR some ELISA negative samples, where positive by PCR (G. Pietersen, personal communication). However sometimes the plant material tested positive for the leafroll virus in ELISA but was negative using PCR (Pietersen, 2006b). It was speculated that these apparently counter intuitive results may be due to the presence of grapevine leafroll type 3 variants (G. Pietersen, personal communication). As *Citrus tristeza* virus has greater divergence in the 5′ terminal parts of the genome (Albiach-marti *et al.*, 2000), and GLRaV-3 belongs to the same family, it was considered reasonable to assume that with GLRaV-3 the variance will most probably exist in the 5′ region of the genome too. Six primers sets were therefore designed targeting regions in the 5′ half of the genome (Pietersen, unpublished). The primers were designed using the only GLRaV-3 genome sequence available on Genbank (AF032768) in the

initial stages of that study (G. Pietersen, personal communication) with no prior knowledge of variation.

The aim of the project was to characterise the perceived variability within 135 plants, collected from the same vineyard in which Prof. Pietersen performed his studies, and to identify molecular markers. These molecular markers may be exploited in strain-specific PCR's to monitor the spread of different variants in epidemiological studies.

3.2 MATERIALS & METHODS

3.2.1 Sample origin and preparation for ELISA and PCR

Grapevine canes were collected in the late autumn/early winter of 2004, from a vineyard belonging to Laborie wine estate at La Concorde, Paarl, Western Cape, South Africa (Figure 3.1). The vineyard has been monitored for leafroll spread since 2001 (www.sawis.co.za). The block was originally planted with *Vitis Vinifera* cv. Merlot clone 3 on rootstock 101-14 in 1992. Canes were collected from 135 vines in a 9 row by 15 plant matrix (n=135) within a selected area of the vineyard irrespective of the symptoms. The bark was removed from the canes, and shavings were prepared of the phloem. These shavings were macerated in ELISA extraction buffer (0.01 M Tris, 0.01 M $MgSO_4.7H_2O$, pH 7.6, 0.2% 2-Mercaptoethanol, 2% Triton X-100, 0.36 M Polyvinylpolipyrrolidone) at a ratio of 1:10 (1 g/10 ml) within plastic maceration bags and strained through a fine mesh, using a Homex 6 macerator (Bioreba AG, Switzerland).

Figure 3.1: Aerial image of the site within the vineyard where canes were collected (Upper vineyard, Block 49 Paarl, Upper vineyard Left bottom corner = S 33°45'36.85", E 18°57'48.22", Upper vineyard Top right corner = S 33°45'44.81", E 18°57'52.32")

3.2.2 Indirect enzyme-linked immunosorbent assay (ELISA)

A mixture of polyclonal Rabbit-anti GLRaV-1, Rabbit-anti GLRaV-2 and Rabbit-anti GLRaV-3 (1:1:1) sera (ARC-PPRI, Pretoria) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 7 mM NaN_3, pH 9.6) was used to coat the wells (100 µl) of standard 96 well microtiter plates (Nunc, Maxisorb, Denmark). The edge rows of plates were not used to avoid edge effects. Edge effects occur when liquid from the edge rows evaporate due to the temperature difference when compared with the wells in the middle of the plate. To avoid the edge effect, the edge wells were filled with water for all the incubation steps. The plate was incubated for 2 hours at 37ºC after coating. The plates were then washed 5 times with PBS-Tween (0.14 M NaCl, 8.33 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, pH 7.4, 0.05% Tween) to remove unbound antibodies. Cane tissue was used and the extraction was performed as described previously (section 3.2.1). Phloem from plants

was extracted in 1:5 ratio (1g/5ml) in ELISA extraction buffer and 100 µl of this extraction was added to the wells. The ELISA test was performed in triplicate for all samples. The plates contained a healthy control, a buffer control and a positive control (known infected vine). The plates containing the plant samples were incubated overnight at 4ºC, followed by washing 5 times with PBS-Tween. Following the washing step, 100 µl of Rabbit anti-GLRaV-3 (ARC-PPRI, Pretoria), diluted 1:10 000 in antibody buffer (PBS, pH 7.4, 0.02 g/ml polyvinylpyrrolidone and 0.002 g/ml egg albumen powder, pH 7.4), was added per well. The plate was incubated for two hours at 37ºC, thereafter it was washed 5 times with PBS-Tween. Goat anti-rabbit globulin/alkaline phosphatase conjugate (GAR-AP) (SIGMA, Missouri, USA) was diluted 1:2000 in antibody buffer and 100 µl of the dilution added per well, followed by a two-hour incubation step at 37ºC. After washing the plate 5 times, 100 µl substrate (ρ-nitrophenyl phosphate) (1mg/ml) was added per well. The absorbance at 405 nm (A_{405}) was measured after 30 minutes incubation at 25ºC, followed by further readings every 30 minutes. The positive/negative threshold selected at the average value of the healthy control plus 2 X the Standard deviation (SD).

3.2.3 One tube, one step RT-PCR

The following PCR protocol was used with the primer pairs described in Table 3.1 for the positive identification of GLRaV-3 in macerates of vine samples prepared as previously described (section 3.2.1). The reaction mixture consisted of 1.25 µl of 2% Triton X-100, 2.50 µl of 10x NH_4 reaction buffer (Bioline, Boston, USA), 1.25 µl of 3.5 mM deoxyribonucleotide triphosphate mix (dNTPs), 1.25 µl of 0.5 µM of each primer (Table 3.1), 1.25 µl of 0.2 M Dithiotreitol (DTT), 1.50 µl of 0.05 M $MgCl_2$, 18 units of Human placenta ribonuclease inhibitor (HPRI RNAse) (Amersham Bioscience, London), 40 units of moloney-murine leukemia virus (M-MLV) reverse transcriptase (SIGMA, Missouri, USA), 0.5 µl sample as template and 0.5 units of Gotaq Flexi® DNA polymerase (Promega, Madison, USA). The reaction volume was made up to 25 µl with molecular grade water (SIGMA, Missouri, USA).

The thermal cycling programme was conducted on the ABI GeneAmp 2700 (Applied biosystems, Warrington, United Kingdom). The cycle programme consisted of an incubation step at 37°C for 45 min for cDNA synthesis, followed by the amplification cycles consisting of a denaturation step at 94°C for 2 minutes followed by 35 cycles of denaturation (94°C, 1 minute), annealing (50°C, 1 minute) and extension (72°C, 2 minutes). The cycle programme concluded with a final extension

step at 72ºC for 10 minutes. When using primer set H420/C693 the annealing temperature used was 60ºC (McKenzie, 1997).

The genebank sequence AF037268 was used as template for primer design. Figure 3.2 illustrates the primer binding sites on the GLRaV-3 (AF037268) genome.

Table 3.1: The primers used in the one tube, one step RT-PCR reactions

Primers	Sequence	Amplicon size
1392 (Pietersen, unpublished data)	5′AGGTCGTTAACAAAGCTCG3’	356 bp
1393 (Pietersen, unpublished data)	5′CAGTGTCCATCCCGTG3′	
1401 (Pietersen, unpublished data)	5′CGAGGTAGGATAGGGGC3′	149 bp
1402 (Pietersen, unpublished data)	5′CGGTCAACTCGTTCAACC3′	
1399 (Pietersen, unpublished data)	5′GGTGGCTTGTGGGGCTA3′	152 bp
1400 (Pietersen, unpublished data)	5′TATGAACAGGGTATTGGACT3′	
93-25 (1253) (Ling *et al.*, 2001)	5′ATTTAGAAAGTACGATCGTGC3′	199 bp
93-40 (1254) (Ling *et al.*, 2001)	5′CAGTCAGGGGTAACATCTTA3′	
H420 (McKenzie, 1997)	5′GATTTAAGCGCGTTTTTCAGGAC3′	209 bp
C629 (McKenzie, 1997)	5′CGGCACGATCGTACTTTCTAA3′	
1397 (Pietersen, unpublished data)	5′CAATTGGAAGACGCTGAGTT3′	162 bp
1398 (Pietersen, unpublished data)	5′GTGTGGTAGAGTAGTTCCGGT3′	
1379 (Pietersen, unpublished data)	5′TCGCTATAGCGCAAGCCATTG3′	490 bp
1396 (Pietersen, unpublished data)	5′GGATATCTACGATGAGCGG3′	

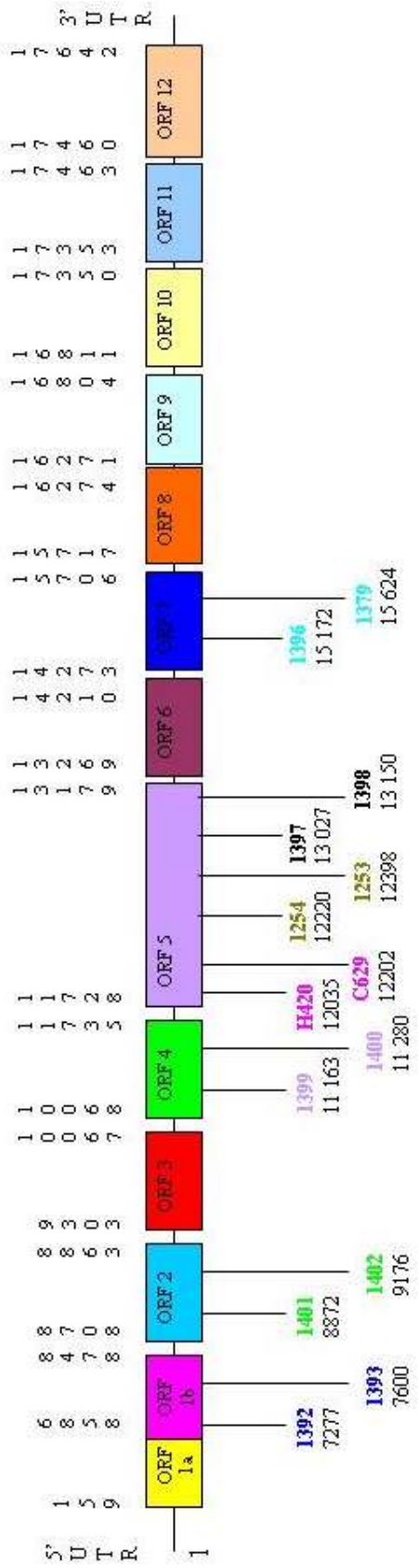

Figure 3.2: Schematic representation of the GLRaV-3 genome (AF037268) annotated with ORFs and target sites of primers utilised in this study. Primer name and nucleotide position indicated on the genome

The PCR products were visualised by gel electrophoresis using 1% agarose gels. The size of the amplicon was determined with the aid of a molecular DNA marker (Hyperladder IV) (Bioline, Boston, USA), 5 µl (600 ng) of the marker was loaded on the gel. From the RT-PCR products, 15 µl were mixed with 3 µl of loading buffer (0.01 M sucrose and 0.02 mM Bromophenol blue). Gels were electrophoresed for 35 minutes at 100 V in 1x Sodium boric acid (SB) eletrophoreses buffer (0.2 M NaOH and 0.73 M boric acid, pH 8). After electrophoresis, the gel was stained with ethidium bromide (EtBr) (1 mg/ml) for 10 minutes and washed twice with water. The gel was visualised on an UV transilluminator (UVP, Model M-15) and photographed using a digital camera mounted on a hood covering the transilluminator (Vilber Lourmat, France).

The results from different primer sets were combined to assign a so-called PCR fingerprint to each sample. The RT-PCR was repeated three times on 5 chosen samples, each with a different PCR fingerprint, with all 6 primers to test the consistency of the RT-PCR fingerprint profile.

Spatial analysis of the plants with a given PCR fingerprint was determined by plotting aerial distribution charts (Figure 3.8). Morisita's index of dispersal (Morisita, 1959) was used to determine whether significant clumping of the different fingerprints occurred or whether the distribution was random. All the calculations were performed with the program PATCHY (Maixner, 1993).

3.2.4 Two-step PCR

The two-step PCR was performed using primers flanking the primers used in the one tube, one step RT-PCR (section 3.2.3) to obtain amplicons for sequencing (section 3.2.6).

Reverse transcription (RT) was performed according to the manufacturer's protocol (SIGMA, Missouri, USA), with a few modifications. Briefly the modifications were as follows: 5 µl of total RNA and 2.5 ul of 0.5 mM of each of the reverse and forward primers (Table 3.2) were heat denatured in 35 µl (total volume) at 90°C for 2 minutes. After heat denaturation the samples were snap chilled on ice for 2 minutes and incubated for 20 minutes at room temperature. Reverse transcription reaction mixtures were assembled in 15 µl (total volume) and included: 5 µl RT buffer (SIGMA, Missouri, USA), 2.5 µl of 3.5 mM dNTPs, 2.5 µl of 0.5 µM of each primer, 2.5 µl of 0.2 M DTT, 18 units of HPRI RNAse inhibitor (Amersham Bioscience, London) and 40 units of

M-MLV (SIGMA, Missouri, USA). The samples were incubated at 37°C for 60 minutes to allow cDNA synthesis. One µl of product was used as template in the PCR reaction described below (section 3.2.4.2). The primers used were tabulated (Table 3.2) and the binding sites of the primers on the GLRaV-3 genome (AF0327368) are illustrated in figure 3.3.

The reaction mixture used for the two-step PCR contained: 1.25 µl of 2% Triton X-100, 2.5 µl of 10x NH_4 reaction buffer (Bioline, Boston, USA), 1.25 µl of 3.5 mM dNTPs, 1.25 µl of 0.5 µM of each primer (Table 3.2), 1.25 µl of 0.2 M DTT, 1.5 µl of 0.05 M $MgCl_2$ and 0.5 units of Gotaq Flexi® DNA polymerase (Promega, Madison, USA) in a total volume of 25 µl.

The thermal cycling programme consisted of an incubation step at 94°C for 2 minutes followed by 35 cycles of denaturation (94°C, 15 seconds), annealing (50°C, 30 seconds) and extension (72°C, 1 minute adding 5 sec per cycle). The final extension cycle was set at 72°C for 7 minutes. 15 µl of the PCR products were electrophoresed and visualised as described earlier (section 3.2.3).

Table 3.2: The primers used in the two-step PCR

Primer	Primer sequence	Amplicon size
93-25 (1253) (Ling *et al.*, 2001)	5′ATTTAGAAAGTACGATCGTGC3′	477 bp
92-98 (1256) (Ling *et al.,* 2001)	5′ACCAACTTCTCGGCGATCTC3′	
1386 (Pietersen, unpublished data)	5′TCTTAGGCGTGCCATCGTG3′	750 bp
1387 (Pietersen, unpublished data)	5′GGAGATGTTTCCCGACCCCG3′	
1388 (Pietersen, unpublished data)	5′CGCGGTTGTAACGTGGGTTG3′	661 bp
1389 (Pietersen, unpublished data)	5′CGCCTCTGAGAGCCGACAGA3′	

3.2.5 Sequencing

To determine if variation at the primer binding site (primers used in the one tube, one step RT-PCR) was present, the two-step PCR amplicons (using primers flanking the first binding sites) (section 3.2.5) were sequenced. Bands of the expected size (Table 3.2) were cut out of the gel and purified with the Wizard SV gel and PCR clean-up system (Promega, Madison, USA) according to the manufacturer's protocol. The purified products were electrophoresed to determine the concentration of the amplicon for sequencing.

The reaction mixture used for sequencing contained: 2 µl Big Dye v3.1 (Applied biosystems, Warrington, United Kingdom), 1 µl sequencing buffer (Applied biosystems, Warrington, United Kingdom), 1 µl of 3.2 pmol primer, 100 ng template and molecular grade water (Sigma, Missouri, USA) up to 10 µl. The thermal cycling programme consisted of an incubation step at 94°C for 1 minute followed by 25 cycles of denaturation (94°C, 10 seconds), annealing (50°C, 5 seconds) and extension (60°C, 4 minutes). Sequencing was done in the forward and the reverse orientations with the primers used for amplification.

Precipitation of the sequencing reactions was performed with the Ethylenediaminetetraacetic acid disodium salt/Sodium acetate/ethanol (EDTA/NaOAc/EtOH) method (BigDye Terminator V3.1 kit cycle sequence protocol). Briefly the method involves: adding 1 µl of 125 mM EDTA, 1 µl of 3 mM NaOAc, 25 µl of 100% EtOH to the sample and centrifuging the sample at 4°C for 30 minutes. After centrifugation the supernatant was removed and 100 µl of 70% EtOH added to wash the pellet. The samples were centrifuged for 15 minutes (at room temperature) after which the supernatant was removed. The wash step was performed twice and finally air-dried for 20 minutes. Sequence analysis was conducted at the sequencing facility of the University of Pretoria on an ABI PRISM® 3100/3130 genetic analyser. Sequence trace files obtained were analysed utilising BLAST and DNAMAN (Lynnon Biosoft, Quebec, Canada). The consensus sequences obtained were based on the forward and the reverse sequences data.

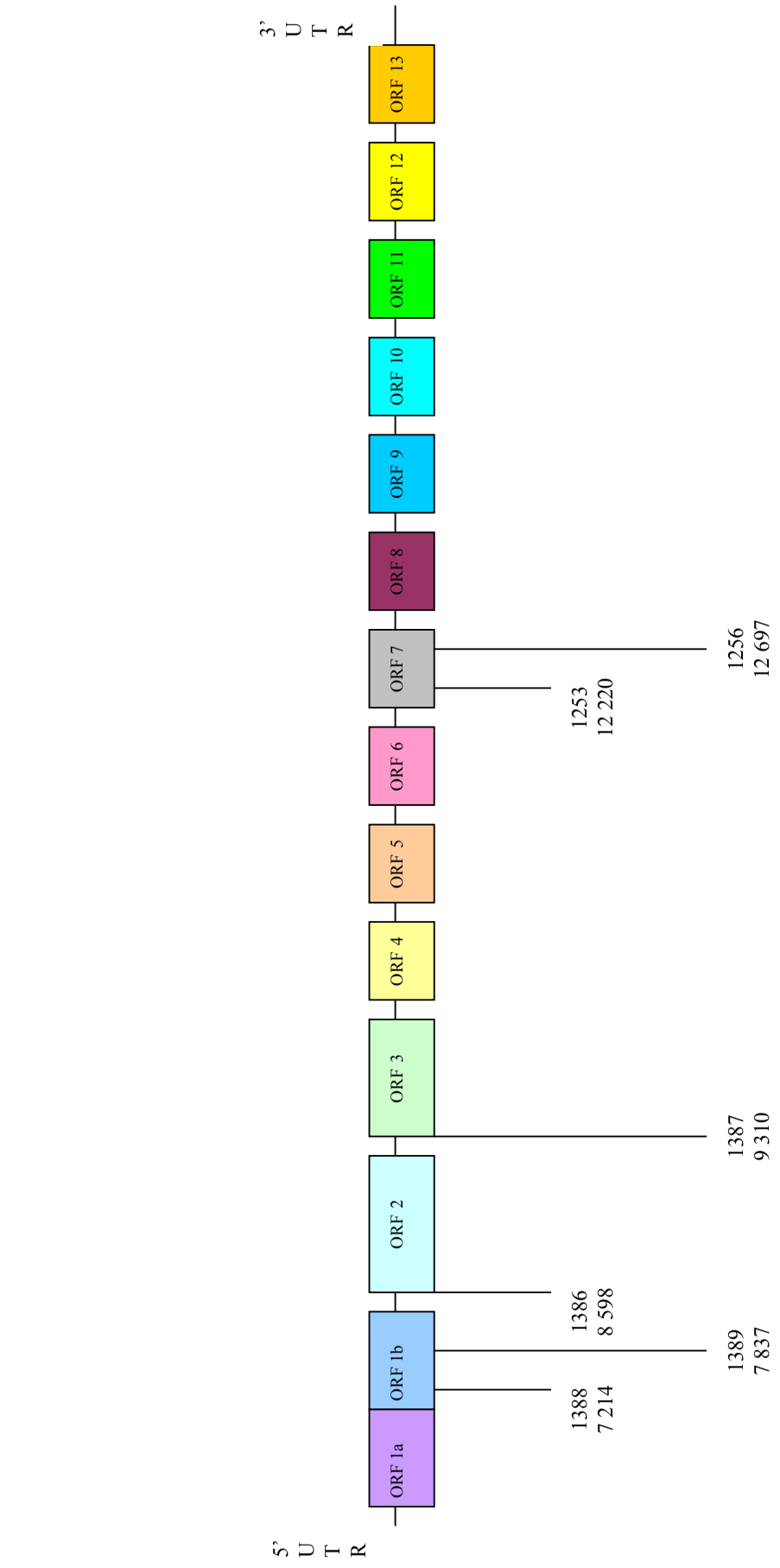

Figure 3.3: Schematic diagram of GLRaV-3 genome organisation showing the binding positions of primers used for sequencing (AF037268)

3.3 RESULTS

The 135 samples were each subjected to PCR with each of the 6 primer sets. All primer sets appeared to work efficiently in the one tube, one step RT-PCR and amplicons of the expected sizes were obtained. Specific plant samples yielding amplicon with one primer set did not necessarily yield amplicon with other primer sets (Figure 3.4). This PCR differentiation pattern could indicate possible variation in the primer-binding site in instances where no amplification was obtained.

PCR fingerprints were assigned to each of the 135 samples based on the results of the amplification with the 6 primer sets (Appendix B). If the primer set gave positive PCR results, the primer set for that specific plant was scored with a "+", while if the primer set yielded a negative PCR result the primer set for that specific plant was scored with a "-". For example, the PCR results for plant 49/13/02 (vineyard number, row number, plant position in row) illustrated in figure 3.4 were designated to a PCR fingerprint as illustrated in table 3.3. Completing the PCRs on all 135 plants, 24 different PCR fingerprints were obtained.

When the consistency of the one tube, one step RT-PCR was tested using 5 selected plant samples, 4 of the plant samples gave the exact fingerprint in three repetitions as originally obtained. Only one plant gave a different fingerprint in one of the repetitions when one primer set did not yield amplicon, whereas in the initial PCR it did.

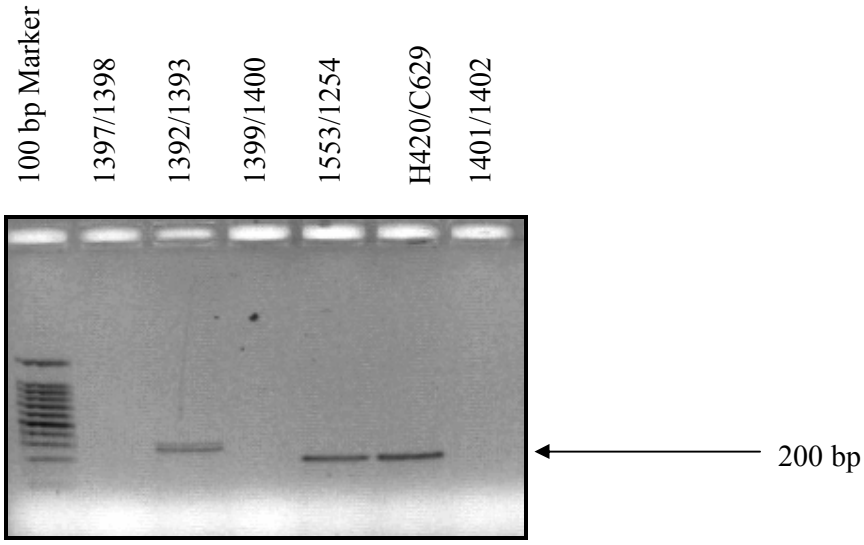

Figure 3.4: Example of an Agarose gel showing the PCR amplicons yielded from six primer sets on plant sample 49/13/02. This combination referred to as a "PCR fingerprint" in this study

Table 3.3 PCR fingerprint for plant sample 49/13/02

1397/1398	1401/1402	H420/C629	1379/1396	1392/1393	1399/1400	1253/1254
-	+	-	-	+	-	+

When vine symptoms were compared to the PCR data obtained from the 6 primer sets, it was noted that as high as 45% of plants were infected with GLRaV-3 without showing symptoms (Figure 3.5). Plants yielding a positive reaction on any one of the PCR systems were considered infected (Figure 3.5).

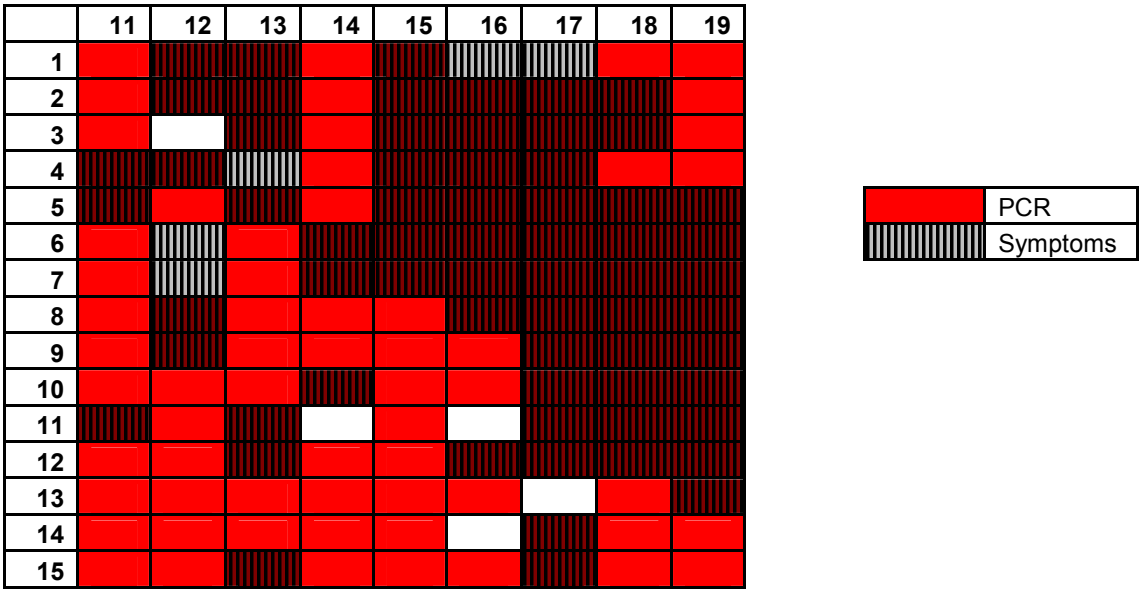

Figure 3.5: An aerial representation of the 9 row by 15 plant matrix correlating symptoms monitored in 2006 with positive PCR results (all primer combinations)

While comparing the PCR data with the ELISA data it became evident that a number of plant samples showed positive results with the PCR but negative results with the ELISA (e.g. plant 3 of row 14, Figure 3.6). A possible explanation for this phenomenon may be a lower sensitivity of the ELISA assay (Acheche *et al.*, 1999, Klerks *et al.*, 2001). Conducting only ELISA assay to screen for GLRaV-3 is therefore not recommended. Additional PCR assays with specific primer sets may prove to be more useful. In other instances plants yielded a positive signal with ELISA, but the same plant sample tested negative in at least one of the PCR reactions or sometimes in all of them (e.g. plant 3 of row 12, Figure 3.6). As PCR is generally more sensitive than ELISA this suggests that variants possibly exist.

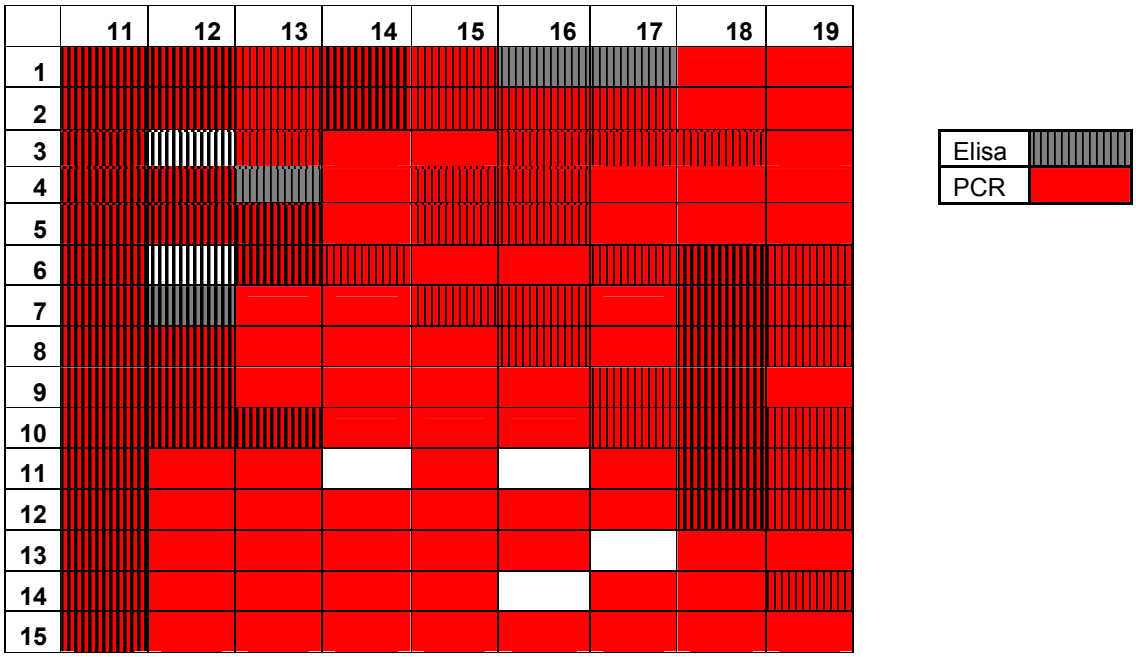

Figure 3.6: An aerial representation of the 9 row by 15 plant matrix correlating ELISA and the PCR system results

To determine the distribution pattern of the 24 PCR fingerprints, aerial distribution charts were drawn (Figure 3.7; Figure 3.7 and Appendix C: Figure C1 – Figure C20. Morisita's index (Morisita, 1959) was used to determine whether the clumping of plants of a given fingerprint was significant. The estimated mean focus size varied between 2 X 2 (quadrant size of 2 plants X 2 plants) and 4 X 3 (quadrant size of 4 plants X 3 plants). Utilising the Morisita index 10 of the 24 fingerprints (Fingerprint 2, 3, 7, 9, 13, 14, 16, 17 and 22) showed significant clumping results ($\delta > 1$).

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	-	+	-	-	-

	11	12	13	14	15	16	17	18	19
1		2005							
2						2001			
3									
4		2004							
5									
6									
7					2004				
8									
9									
10									
11									
12							2002		
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.591	0.451	0.571
3	0.813	0.613	0.595
4	0.398	-	-
5	1.437	-	-
6	-	-	-
7	-	-	-

Figure 3.7: The figure illustrates PCR fingerprint 1, spatial distribution (9 row by 15 plant matrix), year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index analysis of vines in a given fingerprint

Two examples of a PCR fingerprint, its spatial distribution and determination on Morisita index are presented here. In the first instance (Figure 3.7) the Morisita's index showed no significant clumping. This means the vines of the given fingerprint are distributed randomly over the block monitored. The clumping is not significant when analysed with quadrants ranging from 2 X 2 to 4 X 7. PCR fingerprints 4-6 (Appendix C: Figure C2; C3; C4), PCR fingerprint 8 (Appendix C: Figure C6), PCR fingerprints 10-12 (Appendix C: Figures C8; C9; C10), PCR fingerprint 15 (Appendix C: Figure C13) and PCR fingerprint 18-21 (Appendix C: Figure C16; C17; C18 and C19) and also showed no significant clumping.

In the second instance, fingerprint 2 (Figure 3.8) showed significant clumping when the mean focus size is small. This significant ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$) clumping of plants with GLRaV-3 of a single PCR fingerprint further supports the hypothesis for presence of virus strains spreading amongst spatially closely distributed plants. PCR fingerprint 3, 7, 9, 13, 14, 16, 17 and 22 (Appendix C: Figures C1; C5; C7; C11; C12; C14; C15; C20 also showed significant clumping.

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
-	+	+	-	+	-

	11	12	13	14	15	16	17	18	19
1									
2									
3			2001						
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	30	0.95	16
3	0.947	0.929	0.889
4	14	-	-
5	14	-	-
6	-	-	-
7	-	-	-

Figure 3.8: The figure illustrates PCR fingerprint 2, spatial distribution (9 row by 15 plant matrix), year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index analysis of vines in a given fingerprint

Thus far, results suggested the presence of GLRaV-3 sequence variants. Deoxyribonucleic acid sequencing was therefore performed for 5 randomly selected samples (performed on amplicon from 2-step PCR), each assigned to a group with a different PCR fingerprint, to verify that the diverse patterns seen were due to different strains. These sequencing reactions were performed with primers 1253 and 1256. Initially, these specific primers were chosen as they flank the primers that yielded low PCR efficiency scores in the one step, one tube RT-PCR (Table 3.4), possibly due to binding of the primers in a region with increase nucleotide variability. The following formulas (Gunnarsson & Lanke, 2002) were used to calculate the PCR sensitivity and specificity for the different primers used:

$$\% \text{ Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}}$$

$$\% \text{ Specificity} = \frac{\text{True negative}}{\text{False positive} + \text{True negative}}$$

The calculations were given in Appendix A and the result tabulated in table 3.4.

In the formulae a true positive plant sample would be a sample that tested positive in both the ELISA and the PCR, while the false negative would be a plant that yielded no amplicon in the PCR reactions but gave a positive colour reaction with ELISA. A true negative plant sample will represent a sample that gave a negative colour reaction with ELISA and yielded no amplicon in the PCR reaction. A false positive plant sample will represent a plant sample that gave a negative colour reaction with ELISA, but yielded amplicon in the PCR reaction.

Table 3.4: PCR sensitivity and specificity

Primers	Sensitivity	Specificity
93-25/93-40	83.82 %	23.89 %
1399/1400	44.12 %	23.89 %
1397/1398	57.35 %	47.76 %
1401/1402	25 %	83.85 %
H420/C629	26.47 %	65.67 %
1379/1396	16.18 %	92.54 %
1392/1393	17.64 %	94.03 %

The sequence generated from sample 49/12/02 was used as reference to align the other samples with because it amplified with most of the primers; it was also used as a positive control for the sequence systems. This could be an indication that no variation in this viral population occurred due to the fact that all the primers bind to the genome. The multiple alignments of four samples (the fifth sequence was not usable) with primer 1253 were illustrated in Figure 3.9. The sequences were 100% similar.

Further sequencing reactions (performed on amplicon from 2-step PCR) were conducted with primer sets 1386/1387 and 1388/1389. These sequences were presented in Appendix D. Comparison of these sequences revealed high homology. The sequences were 100% similar when primers 1386, 1387, 1388 and 1389 were used.

Sample	Sequence	Position
Plant49/12/02	GATGCTTTACCGAAGGTGGTATCTGCATACGTAGAtcTTT	40
Plant49/13/09	GATGCTTTACCGAAGGTGGtATCTGCATACGTAGAtcTTT	40
Plant49/15/03	GATGCTTTACCGAAGGTGGtATCTGCATACGTAGAtcTTT	40
Plant49/17/06	GATGCTTTACCGAAGGTGGTATCTGCATACGTAGAtcTTT	40
Consensus	gatgctttaccgaaggtggtatctgcatacgtagatcttt	
Plant49/12/02	ATACGAACTTGCAGGAGTTGCTGTCAGATGAAGTAACTAA	80
Plant49/13/09	ATACGAACTTGCAGGAGTTGCTGTCAGATGAAGTAaCTAA	80
Plant49/15/03	ATACGAACTTGCAGGAGTTGCTGTCAGATGAAGTAaCTAA	80
Plant49/17/06	ATACGAACTTGCAGGAGTTGCTGTCAGATGAAGtAaCTAA	80
Consensus	atacgaacttgcaggagttgctgtcagatgaagtaactaa	
Plant49/12/02	GGCCAGAACCGATACAGTTTCTGCCTACGCTACTGACTCT	120
Plant49/13/09	GGCCAGAACCGATACAGTTTCTGCCTACGCTACTGACTCT	120
Plant49/15/03	GGCCAGAACCGATACAGTTTCTGCCTACGCTACTGACTCT	120
Plant49/17/06	GGCCAGAACCGATACAGTTTCTGCCTACGCTACTGACTCT	120
Consensus	ggccagaaccgatacagtttctgcctacgctactgactct	
Plant49/12/02	ATGGCTTTCTTAGTTAAGATGTTGCCCCTGACAGCCCGTG	160
Plant49/13/09	ATGGCTTTCTTAGTTAAGATGTTGCCCCTGACAGCCCGTG	160
Plant49/15/03	ATGGCTTTCTTAGTTAAGATGTTGCCCCTGACAGCCCGTG	160
Plant49/17/06	ATGGCTTTCTTAGTTAAGATGTTGCCCCTGACAGCCCGTG	160
Consensus	atggctttcttagttaagatgttgcccctgacagcccgtg	
Plant49/12/02	AGCAGTGGTTGAGAGACATGTTAGGATATCTGCTAGTACG	200
Plant49/13/05	AGCAGTGGTTGAGAGACATGTTAGGATATCTGCTAGTACG	200
Plant49/15/03	AGCAGTGGTTGAGAGACATGTTAGGATATCTGCTAGTACG	200
Plant49/17/06	AGCAGTGGTTGAGAGACATGTTAGGATATCTGCTAGTACG	200
Consensus	agcagtggttgagagacatgttaggatatctgctagtacg	
Plant49/12/02	AAGACGACCGGCAAATTTTTCCTACGACATAAGAGTAGCT	240
Plant49/13/09	AAGACGACCGGCAAATTTTTCCTACGACATAAGAGTAGCT	240
Plant49/15/03	AAGACGACCGGCAAATTTTTCCTACGACATAAGAGTAGCT	240
Plant49/17/06	AAGACGACCGGCAAATTTTTCCTACGACATAAGAGTAGCT	240
Consensus	aagacgaccggcaaatttttcctacgacataagagtagct	
Plant49/12/02	TGGGTATATGACGTGATCGCTACGCTCAAGCTGGTCATAA	280
Plant49/13/09	TGGGTATATGACGTGATCGCTACGCTCAAGCTGGTCATAA	280
Plant49/15/03	TGGGTATATGACGTGATCGCTACGCTCAAGCTGGTCATAA	280
Plant49/17/06	TGGGTATATGACGTGATCGCTACGCTCAAGCTGGTCATAA	280
Consensus	tgggtatatgacgtgatcgctacgctcaagctggtcataa	
Plant49/12/02	AATCGTTTTTCAACAAGGACACACCCGGGGGTATTAAAGA	320
Plant49/13/09	AATCGTTTTTCAACAAGGACACACCCGGGGGTATTAAAGA	320
Plant49/15/03	AATCGTTTTTCAACAAGGACACACCCGGGGGTATTAAAGA	320
Plant49/17/06	AATCGTTTTTCAACAAGGACACACCCGGGgGTATTAAAGA	320
Consensus	aatcgtttttcaacaaggacacacccgggggtattaaaga	
Plant49/12/02	CTTAAAACCATATGTGCCTATAGAGTCCTTCGACCCCTTT	360
Plant49/13/09	CTTAAAACCATATGTGCCTATAGAGTCCTTCGACCCCTTT	360
Plant49/15/03	CTTAAAACCATATGTGCCTATAGAGTCCTTCGACCCCTTT	360
Plant49/17/06	CTTAAAACCATATGTGCCTATAgAGTCCTTCGACCCCTTT	360
Consensus	cttaaaaccatatgtgcctatagagtccttcgacccctttt	
Plant49/12/02	CACGAGCTTTCATCCTATTTCTCTAGGTTGAGTTATGAGA	400
Plant49/13/09	CACGAGCTTTCATCCTATTTCTCTAGGTTGAGTTATGAGA	400
Plant49/15/03	CACGAGCTTTCATCCTATTTCTCTAGGTTGAGTTATGAGA	400
Plant49/17/06	CACGAGCTTTCATCCTATTTCTCTAGGTTGAGTTATGAGA	400
Consensus	cacgagctttcatcctatttctctaggttgagttatgaga	

Figure 3.9: Multiple alignment of 4 sequences generated from 4 different samples (49/12/02; 49/13/09; 49/15/03; 49/1706) for using primer 1253 to the sequencing. Consensus sequence present below the aligned sequences of the plant samples

3.4 DISCUSSION & CONCLUSION

Based on the initial experiments conducted, where different PCR systems were employed to detect GLRaV-3, it appears that different strains of GLRaV-3 exist within the samples analysed. It was found that not all primer sets yielded amplicon with some plant samples, shown to be positive with another primer set. This phenomenon may indicate variation in the nucleotide sequence of the viruses where the primers bind. It is known that false negative PCR results may arise when there is sequence variability in the primer-binding region of the amplicon (Barlow *et al.*, 1997).

In the present study, each sample was assigned a PCR fingerprint based on the amplification patterns obtained from the one step, one tube RT-PCR using 6 primer sets. Based on the PCR fingerprints assigned to the plant samples in the present study it was possible that more than one variant of GLRaV-3 exist in the 9 row by 15 plant matrix. The PCR results yielded 24 combinations of different PCR fingerprints, possibly suggesting the existence of 24 different strains. Dianese *et al.* (2005) showed with the aid of sequencing that different strains for GLRaV-3 do exist. When compared to the results obtained by Jooste & Goszczynski (2005) and Turturo *et al.* (2005) the results of the current study suggest that there are more variants than previously found. The results of Jooste & Goszczynski (2005) for example found only two variants for GLRaV-3 from 36 isolates screened in their study.

Reliability of the PCR system utilized (one step, one tube RT-PCR) was tested by repeating PCR using the same primer sets on plants samples 49/12/12, 49/13/09, 49/15/03, 49/16/11 and 49/17/16 with 3 replicates. Plant 49/16/11 was used as the healthy control. The 5 plant samples were selected to represent five different PCR fingerprints. 49/12/02 was selected as it amplifies in almost all the PCRs using different primer sets. Four out of the five yielded the same PCR fingerprint as in the first trail. This supports the suggestion that more than one variant does exist in the block tested.

Any given PCR fingerprint however may represent the result of a mixed infection of specific strains. In a study done by Turturo *et al*. (2005) to determine the variability of GLRaV-3, analysis of population structures of those isolates having a pattern composed of several bands revealed the existence of mixed infections, which was confirmed by the high values of inter-isolate genetic diversity and the inconsistent clustering in the phylogenetic tree of these isolates. Within the same family, *Closteroviridae*, CTV is usually present in field trees as a mixture or complex of isolates (Roy & Briansky, 2004). A mixed infection is very probable, as GLRaV-3 hosts are long-living perennial plants, allowing the possibility of repeated inoculation of GLRaV-3 by viruliferous

mealybugs (Rubio *et al.*, 2001). The existence of mixed infections can be invoked to explain why one of the 5 plants, used to test consistency of the RT-PCR, gave a different PCR fingerprint in one of the repeats. One of the primers gave no amplicon in one of the repeats, where it was originally found to yield amplicons. This could be due to the fact that different petioles were tested in each repetition and the original RT-PCR. Testing different petioles may result in a difference in the dominant strain when amplified, resulting in two different PCR fingerprints. Furthermore one of the problems that can be encountered in the detection of GLRaV-3 is the erratic distribution of the virus in grapevine tissue (Acheche *et al.*, 1999). A study done by d'Urso *et al.* (2000) showed that different SSCP patterns for CTV were obtained when RNA was extracted from small pieces of tissue sampled at different sites of the same plant, indicating uneven distribution of the genomic RNA variants within the infected plant. If mixed infections were present in the current study material, the number of PCR fingerprints and strains may be lower than 24, but it will most probably still be higher than the two as proposed by Jooste & Goszczynski (2005). Dianese *et al.* (2005) reported in a study on variation in the 3′ terminal of the polymerase gene that a plant source was infected with two virus populations.

However, the fact that one of the PCR fingerprints differed at one PCR primer set when repeatability was tested, suggests that false negative PCR results may occur. In the study done by Pico *et al.* (1999) erratic amplification was obtained with fast sap extraction procedures. False negative PCR results may however be due to one or a combination of the following factors: the presence of inhibitors of Taq polymerase or the other components of the reaction; a low number of copies of the starting target sequence; or sequence variability in the primer binding region of the amplicon (Barlow *et al.*, 1997). In the current study it is unlikely as two or three primer sets were run in parallel on the same plant sap extract. While amplification did not occur with all primer sets, in such instances it did for some, indicating that sufficient intact viral RNA was present for amplification. It was considered most unlikely that the 'false' negatives were due to low virus copy number as the same extract often worked in other primer combinations.

To gain more evidence that putative GLRaV-3 strains exist and to assess the usefulness of using the current PCR fingerprint system as a molecular marker in spatial distribution studies, the spatial analysis of the positions of the plants with the same PCR fingerprint was performed. Aerial distribution charts were drawn to determine if vines with virus populations yielding similar fingerprints cluster together. The distribution charts show strong clustering of a number of vines with a specific PCR fingerprint, which supports the possibility of different strains. Morisita's index

was also performed to determine whether clustering is significant and random. The Morisita's index of dispersion compares the ratio of the observed chance of drawing two individuals at random without replacement from the same quadrant over a number of quadrants, to the expected chance of the same event for individuals randomly dispersed over the quadrants (Morisita, 1959). The estimated mean focus size analysed varied between 2 X 2 (quadrant size of 2 plants X 2 plants) and 4 X 3 (quadrant size of 4 plants X 3 plants). The index is unity (one) when individuals are randomly dispersed, irrespective of quadrant size or mean density of individuals per quadrant. Values greater than unity indicates clumping and those falling between 0 and 1 indicate uniformity (Morisita, 1959). Ten (PCR fingerprints 2, 3, 7, 9, 13, 14, 16, 17 and 22) out of the 24 clustering patterns had significant clumping (P values being $p \leq 0.05$ and/or $p \leq 0.01$ and/or $p \leq 0.001$). This is further evidence that different strains of GLRaV-3 can exist in the infected plant material tested, and that the spread of the strains from occurred one plant (plant that was initially infected) to the neighbouring plants.

As a final definitive confirmation as to the possibility that nucleotide sequence variation of GLRaV-3 exists, sequencing with primers flanking three primer sets (1392 and 1393, 1401 and 1402, 1253 and 1254), used to determine the distribution patterns, were done to verify if there are different strains. Based on the sequences obtained no variation exists and therefore no strains were detected. As this represents the most stringent test of possibility of presence of strains these results must be accepted and must be concluded that the amplification patterns were due to an error in the protocol: either the extraction or the amplification step. The high genetic stability found could be attributed to negative or purifying selection to maintain the functional integrity of the viral genome (Marco & Aranda, 2005). The sequence data accumulated during the past ten years shows that genetic stability is the rule, rather than the exception, in natural populations of plant viruses (Garcia-Arenal *et al*., 2001).

Plant RNA viruses such as GLRaV-3, in particular those infecting perennial crops, have a great potential for genetic variation (Kominek *et al*., 2005). The mechanisms of inducing RNA virus variation include mutation, homologous and non-homologous recombination and genome segment reassortment. Different viral families exploit the above-mentioned mechanisms to different extents. Molecular recombination is prominent in plant and animal positive strand RNA viruses (Domingo & Holland, 1997). Molecular recombination in conjunction with short replication times and a high multiplicity, leads towards a dynamic mutant population, a unique feature for RNA viruses termed virus quasi-species and corresponding to a swarm of sequence variants (Aaziz & Tepfer, 1999). A

study done by Little *et al*. (2001) showed that the high intra-isolate genetic diversity of GLRaV-1 was probably related to the absence of a 'genetic bottleneck', due to a perennial character of the virus host and virus transmission via vegetative propagation and grafting. A reason why the block of vines in the current study showed no variation could be due to the fact that tissue culture is used in the certification schemes to develop virus-free material. When virus-free material is planted these plants may be infected in the vineyard by virus-carrying vectors. For recombination to occur the plant needs to be re-infected with another stain of the virus. Since in practice infected plants and neighbouring plants are immediately removed after showing symptoms, the formation and spread of strains is highly unlikely. This practice may lead up to the formation of a genetic bottleneck.

Another possibility why no strains were detected in this study could be explained by the small area in which the study was conducted. If in future one should want to identify different strains for GLRaV-3, the study should be conducted over a widespread area, to increase the probability of identifying different strains.

In this study sequence variability at the primer binding sites was probably not the causative element for anomalous PCR results obtained. Further research should be directed towards establishing sensitive and consistent assays for detecting GLRaV-3.

CHAPTER 4

OPTIMISATION OF EXTRACTION AND PCR PROTOCOLS

4.1 INTRODUCTION

Since its first description by Saiki in 1958, the polymerase chain reaction (PCR) has revolutionised the methodology of gene manipulation (Sellner *et al.*, 1992). This effect has been most pronounced in the years since a thermostable DNA polymerase, isolated from a thermophillic bacterium, *Thermus aquaticus,* was applied to PCR (Saiki *et al.*, 1988). Oligonucleotide primers, flanking part of the genome of the virus, are extended by a thermostable DNA polymerase in a series of denaturation and extension steps that exponentially increases the target DNA (Saiki *et al.*, 1988). The procedure is extremely sensitive (Arnheim & Erlich, 1992). One area where PCR has shown great value is the detection of viral pathogens, particularly those for which culturing is difficult or where serologically based detection systems are inadequate (Chandler *et al.*, 1998). Polymerase chain reaction has been used successfully to detect a range of viruses including both DNA and RNA viruses (Chandler *et al.*, 1998). Detection of viral RNA by PCR requires reverse transcription (RT) of viral RNA prior to the reaction (Lopez *et al.*, 2003.).

Several variations of RT-PCR have been developed, including nested- (Mullis *et. al.*, 1986), one step- (Powell *et al.*, 1987), multiplex- and real-time RT-PCR (Higuchi *et al*., 1992). Nested-PCR is designed for highly specific detection of templates present in very low amounts (Rowhani *et al.*, 2004). In this method, an external primer is used for initial amplification (Rowhani *et al.*, 2004). Then, a second primer pair, designed to hybridise within the initial amplified product, is used to prime a second round of PCR to further amplify the targeted segment (Rowhani *et al.*, 2004).

In a one-step RT-PCR all reagents required for reverse transcription and amplification are combined in a single thermocycler tube (Powell *et al.*, 1987). The thermal cycling programme accomplishes reverse transcription first followed by multiple cycles of PCR (Powell *et al.*, 1987).

Preparation of plant extracts is a critical aspect of RT-PCR (Rowhani *et al.*, 2004). Nucleic acid extraction from plant tissues is the most laborious and time-consuming step in the detection of a virus (Rowhani *et al.*, 2004). In addition, it is sometimes difficult to extract relatively pure nucleic acids from some plants (Nakahara *et al.*, 1999). The presence of contaminants such as polyphenols and polysaccharides in the plant extracts often decreases the detection sensitivity of PCR (Nakaune & Nakano, 2006). Polyphenolics are secondary plant metabolites (Friedman, 1997) that occur at different concentrations in the leaves, bark and fruit of higher plants (Koonjul *et al.*, 1999). The concentration of inhibitory substances varies with age, cultivar and the degree of pathogen resistance of the cultivar (Singh *et al.*, 1998). The oxidation products of phenolic compounds

appear to be involved in the defence of plants against invading pathogens, including bacteria, fungi, and viruses (Friedman, 1997). An important characteristic of many polyphenolics is their tendency to form complexes with nucleic acids, resulting in inhibition of molecular manipulation (Koonjul *et al*., 1999). Nucleic acid extracts from plant tissue should be purified with resins or columns before the nucleic acid extract is used for PCR amplification (Koonjul *et al*., 1999).

To overcome the inhibitory aspect of some plant extracts and to avoid organic solvents, various approaches involving nucleic acid and protein immobilisation on solid supports have been used (Rowhani *et al.,* 1995). These include: Immunocapture (IC) with specific antibodies (Rowhani *et al.,* 1995; James, 1999); immobilisation onto polypropylene or polystyrene surfaces; use of charged nylon membranes (La Notte *et al.*, 1997; Myslik & Nassuth, 2001; Dovas & Katis, 2003); immobilisation of plant sap on filter paper, nylon membranes and untreated nitrocellulose membranes (Olmos *et al.*, 1996, Osman & Rowhani, 2005). The inhibition of the PCR reaction can also be reversed by the addition of polyvinylpyrrolidone (PVP) to the PCR reaction (Koonjul *et al*., 1999). PVP has been reported to form complexes through hydrogen bonding with polyphenolics, effectively removing them from the homogenate (Su & Gibor, 1988).

The use of different detection techniques may lead to non-comparable and contradictory results (Pico *et al.*, 1999). In the paper presented by Pico *et al.* (1999) four extraction procedures were tested and compared to establish which procedure could be used to improve the detection of tomato yellow leaf curl virus in tomatoes. In a study done by Van der Merwe *et al*. (2007) on *Vitis* material they compared different extraction buffers for grinding the plant material (winter wood cuttings of cultivar Black Spanish, bark scrapings were used) to obtain optimal amplification using IC-RT-nested PCR. The buffers used were IC-RT-nested PCR buffer (0.02 M PBS, pH 7, 0.5 ml Tween, 2% PVP, 0.2% ovalbumin), immunosorbent electron microscopy (ISEM) buffer (0.1 M PO_4, pH 7, 2% PVP) and Grapevine ELISA buffer (0.1 M Tris-HCl, pH 7.6, 4% PVP-P (insoluble), 0.2% β-mercapto-ethanol, 2% Triton X-100) (Van der Merwe *et al*., 2007). In the study van der Merwe *et al*. (2007) found that the Grapevine ELISA buffer improved the detection of GLRaV-3 in PCR by 8-fold and in ELISA by 4-fold when compared to the other two buffers, making this the preferred buffer.

Sellner *et al*. (1992) established that inhibition of the PCR reaction is not just attributed to plant compounds or polyphenols, but inhibition could also occur from the enzymes in the PCR reaction mixture. In order to minimise the number of manual manipulations required for processing large numbers of samples, Sellner *et al.* (1992) attempted to design a system whereby all the reagents

required for both reverse transcription and amplification could be added to one tube and a single, non-interrupted thermal cycling programme performed (Sellner *et al.*, 1992). The results initially showed that reverse transcription followed by PCR (RT-PCR) in one tube was only successful if a relatively large amount of viral RNA was present (Sellner *et al.*, 1992). When Sellner *et al.* (1992) investigated this phenomenon it was revealed that the reverse transcriptase (RT) directly interfered with the Taq polymerase (Sellner *et al.*, 1992). Other researchers may have unwittingly avoided this problem by either using a lower RT:Taq polymerase ratio in RT-PCR, or performing the cDNA synthesis separately and diluting this before adding it to the PCR reaction (Sellner *et al.*, 199).

Research done by Chandler *et al.* (1998) suggested that the inhibitory effect of RT on PCR is mediated through the RT interactions with the specific messenger RNA (mRNA) or complementary DNA (cDNA) and that the inhibitory effect is dependent upon template concentration (or copy number). One of the problems encountered in the detection of GLRaV-3 is the low concentration of virus in grapevine tissue (Acheche *et al.*, 1999), which will increase the probability of inhibition from the RT enzyme on the PCR (Chandler *et al.*, 1998).

In previous experiments (Chapter 3) it was found that some plant samples tested positive with ELISA, but yielded inconsistent results with 6 primer sets used in PCR (Chapter 3). Furthermore it was shown that the PCR negative results were not due to sequence variation affecting primer binding (Chapter 3). In the current chapter different extraction techniques and PCR protocols were tested to determine whether false negative PCR results obtained with known infected sources was a protocol error, rather than variability of the pathogen. This may be due to inhibition from sap extracts, PCR inhibition by RT enzyme or suboptimal PCR conditions.

4.2 MATERIALS & METHODS

4.2.1 PCR template preparation

Seven different preparation methods to obtain template for PCR were assessed: 1) grinding in extraction buffer (as done in Chapter 3); 2) total RNA extraction using the Promega kit (Promega, Madison, USA); 3) Phenol-chloroform extraction (Mallory *et al.*, 2001); 4) Spotting of plant sap onto nitrocellulose transfer membrane (Osman & Rowhani, 2003); 5) double stranded RNA (dsRNA) extraction (Valverde, 1990); 6) Imumunocapture and 7) the simple-direct tube method (Suehiro *et al.*, 2005).

4.2.1.1 Grinding plant sample in extraction buffer

The petioles were ground in an enzyme-linked immunosorbent assay (ELISA) extraction buffer (0.01 M Tris, 0.01 M $MgSO_4.7H_2O$, pH 7.6, 0.2% 2-Mercaptoethanol, 2% Triton X-100, 0.36 M PVP) at a ratio of 1:5 (1g/5ml) with a Homex 6 macerator (Bioreba AG, Switzerland). Typically 0.5 µl of this sap was used in the PCR protocol.

4.2.1.2 Total RNA extraction

Petioles (300 mg) were prepared for total RNA extraction by maceration in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the SV Total RNA isolation System (Promega, Madison, USA) according to the protocol of the manufacturers.

4.2.1.3 Phenol-Chloroform extraction

A phenol-chloroform extraction was performed to extract viral RNA from infected grapevine petioles as described by Mallory *et al.* (2001) with minor modifications. Briefly the protocol involves: 100 mg of plant sample was ground with a mortar and pestle in liquid nitrogen. The ground sample was prepared for maceration by the addition of extraction buffer (0.1g/0.1ml) (100 mM NaCl, 2% SDS, 50 mM Tris/HCl, pH 9, 10 mM Ethylenediaminetetraacetic acid disodium salt (EDTA), 20 mM β-mercaptoethanol) and macerated for 1 minute. Following maceration, 100 µl aqueous phenol (Fluka, Switzerland) and 100 µl chloroform (SIGMA, Missouri, USA) was added to the sample. The samples were then mixed and centrifuged (8000g) for 10 minutes at 4°C. After centrifugation the aqueous phase was removed and 100 µl aqueous phenol (Fluka, Switzerland) and 100 µl chloroform (SIGMA, Missouri, USA) was added. The samples were then mixed and centrifuged (8000g) for 10 minutes at 4°C. Following the second centrifugation step, 200 µl chloroform (SIGMA, Missouri, USA) was added to the supernatant and the sample centrifuged at 8000g for 10 minutes at 4°C. The aqueous phase was then removed and the nucleic acids precipitated with sodium acetate (3 M NaOAc) and 100% ethanol at -80°C for 1 hour (Mallory *et al.,* 2001). The pellet was washed with 70% ethanol and re-suspended in 100 µl molecular grade water (SIGMA, Missouri, USA).

4.2.1.4 Spotting sample preparation

The plant sap was spotted on a nitrocellulose transfer membrane to prevent inhibition of the plant polysaccharides in the PCR. The spotting sample preparation was performed as described by Osman & Rowhani (2006). Briefly the protocol involved the following: The petioles were ground in ELISA extraction buffer (0.01 M Tris, 0.01 M $MgSO_4.7H_2O$, pH 7.6, 0.2% 2-Mercaptoethanol, 2% Triton X-100, 0.36 M Polyvinylpolipyrrolidone) in a ratio of 1:10 (1g/10ml). Ten microliters of this extract was spotted onto nitrocellulose transfer membrane (Micron Separations, Inc., Westboro, MA). The membrane was left at room temperature for 30 minutes to dry. To release the nucleic acid, the membranes were boiled in 100 µl GES buffer (0.1 M Glycine-NaOH, pH 9, 0.05 M NaCl, 1 mM EDTA, containing 0.5% Triton X-100) in micro-centrifuge tubes at 95°C for 10 minutes, vortexed and placed on ice. In the subsequent PCR reaction 1 µl of the sample was used as template.

4.2.1.5 Double-stranded RNA extraction

A double-stranded RNA extraction (dsRNA) extraction was performed to extract viral RNA from infected grapevine petioles as described by Valverde (1990) with minor modifications**.** Briefly the protocol involved: Tissue (3.5 g) was ground in 50 ml 1 x Saline/Tris/EDTA (STE) buffer (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 6.8), added 1 ml 10% SDS, 0.5 ml bentonite and 9 ml 1x STE-saturated phenol afterwards. The aqueous phase was removed and 2.1 ml 95% ethanol added and mixed. Cellulose CF-11 (Whatman Chemicals, Hillsboro, OR) (1 g) was placed in a syringe and 25 ml 1x STE buffer (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 6.8) containing ethanol (16% v/v) added on the cellulose to wash the column. The sap (obtained from macerated tissue) was placed in the column to filter the solution; this solution was discarded and the column flushed with 40 ml 1x STE buffer containing ethanol. The nucleic acid was eluted from the cellulose with 6 ml 1x STE buffer. Following elution 20 ml 95% ethanol and 0.5 ml 3 M sodium acetate (Calbiochem, Germany) was added and the samples incubated at –20°C for at least 2 hours to precipitate dsRNA. The samples were centrifuged (25 minutes, 8000g) to collect the dsRNA in pellet format. The ethanol was poured off and the pellet re-suspended in 20 µl TE buffer (0.2 M Tris, 0.05 M EDTA, pH 8). In the subsequent PCR reaction 0.5 µl of the dsRNA extraction was used as template.

4.2.1.6 Immunocapture

Polymerase chain reaction (PCR) tubes (0.2 ml, polypropylene, Whitehead Scientific Ltd, Brackenfell) were incubated with 50 µl of Goat anti-GLRaV-3 globulin (ARC-PPRI, Pretoria) diluted 500 times with coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 7 mM NaN_3, pH 9.6) at 4°C overnight. After washing the tubes three times for 3 minutes each with PBST (phosphate buffered saline containing 0.05% Tween-20), 50 µl of sap (obtained by maceration of plant sample in ELISA extraction buffer described in section 3.2.1) was added to each pre-coated PCR tube. The tubes containing the plant material were incubated overnight at 4°C. Tubes were washed three times with PBST as described above and 25 µl of PCR master mix was added thereafter.

4.2.1.7 Simple-direct-tube method

The simple-direct-tube method was performed as described by Suehiro *et al*. (2005). Briefly the protocol involved: Dividing the petioles into two halves. One half was ground in a 1:1 ratio in PBST, while the other half was ground in ELISA extraction buffer (1:1 ratio). The resulting crude sap (200 µl) was pipetted into a PCR tube (0.2 µl, polypropylene, Whitehead Scientific Ltd, Brackenfell). After incubation at room temperature for 15 minutes, the sap was removed and the tube washed three times with 200 µl of PBST to remove residual tissue. Thirty microlitres of molecular water (SIGMA, Missouri, USA) containing 15 units of RNAse inhibitor (Amersham bioscience, London) was added to the tube and immediately incubated at 95°C for 1 minute (denaturation) and cooled on ice for 1 minute. In the subsequent PCR reaction 0.5 µl of the sample was used as template.

4.2.2 PCR protocols

Different PCR protocols ((1) two step PCR, (2) One step, one tube RT-PCR followed by nested PCR) were tested to obtain optimal amplification.

4.2.2.1 Two-step PCR

Reverse transcription was performed according to the protocol of the manufacturer of the reverse transcriptase (SIGMA, Missouri, USA), with modifications to the protocol. Briefly the protocol involved a reaction mix of 35 µl molecular grade H_2O (SIGMA, Missouri, USA), 5 µl of total RNA and 2.5 ul of 0.5 mM of each reverse and forward primers were added and heat denatured at 90°C for 2 minutes. After heat denaturation, the samples were snap cooled on ice for 2 minutes and incubated for 20 minutes at room temperature. Reverse transcription reaction mixtures were assembled in 10 µl and included: 5 µl RT buffer (SIGMA, Missouri, USA), 2.5 µl of 3.5 mM deoxyribonucleotide triphosphate mix (dNTPs), 2.5 µl of 0.5 µM of each primer, 2.5 µl of 0.2 M Dithiotreitol (DTT), 18 units of Human placenta ribonuclease inhibitor (HPRI RNAse)(Amersham Bioscience, London) and 40 units of moloney-murine leukemia virus (M-MLV) reverse transcriptase (SIGMA, Missouri, USA). The samples were incubated at 37°C for 60 minutes. 0.5 µl of the product was used as template for PCR as described below. The primers used were 93-25 and 93-40 (Table 4.2), while the expected size for the amplification product was 200 bp.

The reaction mixture used for the amplification contained: 1.25 µl of 2% Triton X-100, 2.5 µl of 10x NH_4 Reaction buffer (Bioline, Boston, USA), 1.25 µl of 3.5 mM dNTPs, 1.25 µl of 0.5 µM of each primer (93-25/93-40), 1.25 µl of 0.2 M DTT, 1.5 µl of 0.05 M $MgCl_2$ and 0.5 units of Gotaq Flexi® DNA polymerase (Promega, Madison, USA) in a total volume of 25 µl.

The thermal cycling programme consisted of a precycle at 94°C for 2 minutes followed by 35 cycles of denaturation (94°C, 15 seconds), annealing (50°C, 30 seconds) and extension (94°C, 1 minute adding 5 sec per cycle). The final extension cycle was set at 72°C for 7 minutes. The PCR was performed using the GeneAmp 2700 thermocycler (Applied Biosystems, Warrington, United Kingdom).

4.2.2.2 One tube, one step RT-PCR (first round amplification)

The reaction mixture contained: 0.5 µl template, 2.5 µl of 2% Triton X-100, 10 µl of 10x NH_4 Reaction buffer (Bioline, Boston, USA), 2.5 µl of 3.5 mM dNTPs, 2.5 µl of 0.5 µM of each primer, 2.5 µl of 0.2 M DTT, 3 µl of 0.025 M $MgCl_2$, 18 units of HPRI RNAse inhibitor (Amersham Bioscience, London), 40 units of M-MLV reverse transcriptase (SIGMA, Missouri, USA) and 0.75 units of Gotaq Flexi® DNA polymerase (Promega, Madison, USA). The primers used were 93-110 and 92-98 (Table 4.1). The final volume of the reaction was 25 µl.

The thermal cycling programme involved one incubation step for cDNA synthesis at 37°C for 45 minutes, followed by the PCR parameters consisting of a precycle at 94°C for 2 minutes followed by 35 cycles of denaturation (94°C, 1 minute), annealing (50°C, 1 minute) and extension (94°C, 2 minutes). The final extension cycle was set at 72°C for 10 minutes. The PCR was performed using the GeneAmp 2700 thermocycler (Applied biosystems, Warrington, United Kingdom). 0.5 µl product was used as the template in the nested PCR (section 4.2.2.3).

Table 4.1: Primers used in the one tube, one step RT-PCR reactions

Primers	Sequence	Amplicon size
93-110 (Ling *et al*., 2001)	5′TGTGGACAGCAATCTTCCAA3′	628 bp
92-98 (Ling *et al.,* 2001)	5′ACCAACTTCTCGGCGATCTC3′	

4.2.2.3 Nested PCR

0.5 µl of the first round amplification product (section 4.2.2.2) was used in a reaction mixture containing: 10 µl of 10x NH_4 Reaction buffer (Bioline, Boston, USA), 2.5 µl of 3.5 mM dNTPs, 2.5 µl of 0.5 µM of each primer, 3 µl of 0.025 M $MgCl_2$ and 0.75 units of Gotaq Flexi® DNA polymerase (Promega, Madison, USA). The primers used were 93-25 and 93-40 (Table 4.2). The final volume of the reaction was 25 µl.

Table 4.2: The primers used in the nested PCR

Primers	Sequence	Amplicon size
93-25 (Ling *et al.*, 2001)	5′ATTTAGAAAGTACGATCGTGC3′	199 bp
93-40 (Ling *et al.*, 2001)	5′CAGTCAGGGGTAACATCTTA3′	

The thermal cycling programme consisted of a pre-cycle step at 94°C for 2 minutes followed by 33 cycles of denaturation (94°C, 1 minute), annealing (50°C, 1 minute) and extension (94°C, 2 minutes). The final extension cycle was set at 72°C for 7 min. The PCR was performed on the GeneAmp 2700 thermocycler (Applied biosystems, Warrington, United Kingdom).

15 µl of the RT-PCR products were electrophoresed in a 1% agarose gel at 100 V for 35 minutes in 1x Sodium boric acid (SB) electrophoresis buffer (0.2 M NaOH and 0.73 M boric acid, pH 8). The size of the amplicon was determined with the aid of a molecular DNA marker (Hyperladder IV, Bioline, Boston, USA) with 5 µl (600 ng) of the marker loaded on the gel. After electrophoresis the agarose gels were post-stained with EtBr-SB buffer (1 mg/ml) for 10 min, washed twice with water and viewed on an UV transilluminator (UVP, Model M-15) The DNA fragments were photographed using a digital camera mounted on a hood over the transilluminator (Vilber Lourmat, France).

4.3 RESULTS

4.3.1 Protocol change

To assess the possibility that either the reverse transcriptase or the PCR step were not reliable, leading to inconsistent results, cDNA synthesis by reverse transcriptase (RT) was performed on two occasions on three samples, previously positive in PCR (Chapter 3), followed by three replicates of separate PCR reactions using each of the two cDNA's as template (Figure 4.2). To avoid the inhibition of the RT on the Taq enzyme, the reverse transcription was performed separately to the PCR in a two step, two tube protocol. A total RNA extraction was done to purify and concentrate the RNA of the samples and to eliminate the presence of contaminants, which may inhibit the PCR reaction. The primers used were 93-25 and 93-40 (Table 4.2) and the three plant samples are

expected to yield amplicon in all the PCR reactions, as these plant samples did amplify previously using these primers (Chapter 3) and they were ELISA positive.

Because of the replicates in this experiment it could also be determined if the false negatives were due to human error (e.g. inconsistent sample addition). In figure 4.1 the top gel represents the amplicons obtained on cDNA templates from the first RT on the plant samples, while the bottom gel represents the amplicon obtained on the cDNA templates from the second RT. All three plants samples yielded amplicon on all the replicates, while the negative control showed no amplification. The expected bands size was obtained.

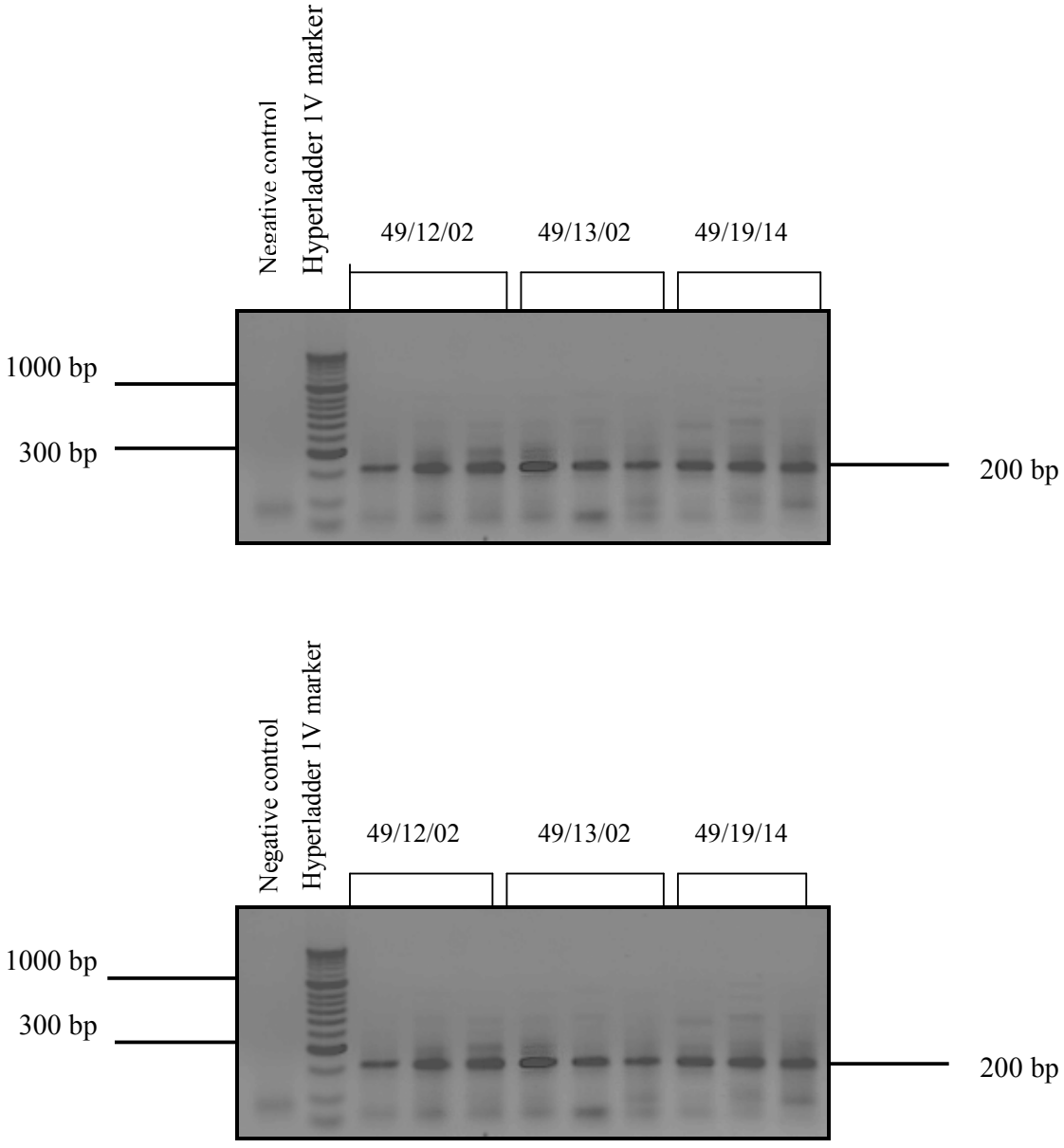

Figure 4.1: Testing the reliability of RT and PCR. Top picture illustrates the amplicons obtained on cDNA templates from the first RT reaction, while the bottom picture is the amplicons obtained from a second RT reaction. The two RT's were performed using the same plant samples

The results obtained, suggest that both the RT step and the PCR are reliable, and that the unexpected negative PCR results previously obtained on some of the plant samples were not due to inconsistent amplification. To determine if the negative PCR results previously obtained was not due to RT enzyme inhibition on Taq enzyme, three plant samples (49/12/02; 49/13/09 and 49/15/03) were used to perform a total RNA extraction and a two step PCR.

The three plant samples (49/12/02; 49/13/09 and 49/15/03) were used in the PCR and were expected to yield amplicons, as they had produced an amplicon with at least one of the six primer sets in Chapter 3 (Sample 49/13/09 yielded amplicon with primer set 1392/1392 but not with primer set 93-25/93-40). Sample 49/15/03 had also tested positive in ELISA. The results obtained showed that some of these known infected samples gave negative results in the PCR reaction (Figure 4.2). The expected 200 bp band was obtained from only 49/12/02 when amplified with primer set 93-25/93-40, indicating that the PCR performed does work, but no amplification was obtained from plant samples 49/13/09 and 49/15/03. The negative control showed no amplicon, indicating that there was no master mix reagent contamination.

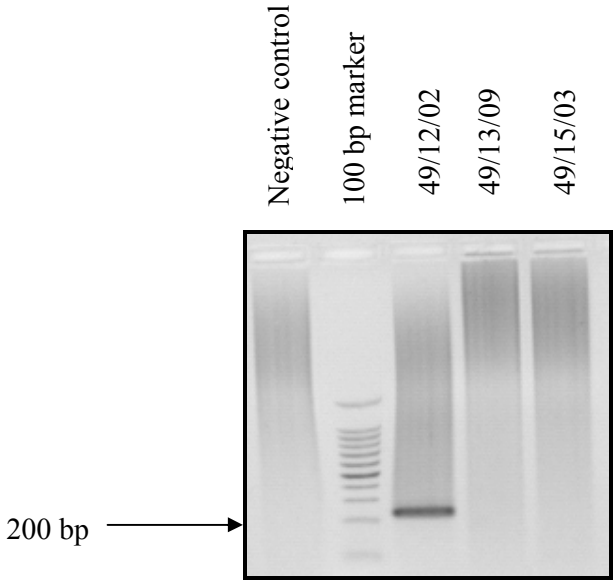

Figure 4.2: Amplification of three plant samples using primer set 93-25/93-40 performed with a two-step PCR system

Negative results obtained previously (Chapter 3), are unlikely to be due to RT enzyme inhibition on the PCR, since some plant samples still yielded no amplicon even after the RT step and the PCR were separated from each other. Different extraction methods were tested to determine which method yielded the best template for amplification.

4.3.2 Template preparation

The phenol-chloroform extraction was chosen as an alternative extraction method to the total RNA extraction and was done based on parallel studies conducted by N. Douglas (unpublished data). N. Douglas is a MSc. student at the entomology department of the University of Pretoria. The aim of her study was to determine how efficient the vector, *Planococcus ficus,* was in spreading the virus, GLRaV-3 and the distance over which the vector could spread the virus. But first she needed to know whether the vector was carrying the virus. She used PCR to detect the virus within the vector. The primers that she used were also 93-10/92-98 (Ling *et al.*, 2001), followed with primers 93-25/93-40 (Ling *et al.*, 2001) in the nested PCR. To achieve the most accurate and consistent results in the PCR she also tested a few extraction methods and kits to determine which yielded optimal and consistent amplification. The methods and kits tested were: RNeasy Mini Kit from QIAGEN (Valencia, CA, USA), Promega SV total RNA isolation kit (Promega, Madison, USA), Gentra Purescript RNA Isolation kit (Gentra Systems Inc, Minneapolis, MN, USA), Qiagen QIAzol (Valencia, CA, USA) and Chloroform-phenol extraction (Mallory *et al.,* 2001). Her studies indicated that with the phenol-chloroform extractions all expected positive samples yielded positive PCR results, whereas the other extraction methods did not consistently give positive PCR results on the same samples.

4.3.3 Phenol-chloroform extraction

Initially PCR on the phenol-chloroform extraction was done with reverse transcription separate from the PCR reaction, to ensure that if no amplification took place it was not due to RT-Taq inhibition. The two-step RT-PCR was done to verify if the extractions were successful and if previously positive reactions will still give positive results Primers 93-25 and 93-40 were used (Table 4.2). The known positive plant sample yielded the correct size band of 199 bp in all three replicates (Figure 4.3). The negative control did not yield any amplificons. The results suggest that the extraction was successful, since samples that previously yielded amplicon (Chapter 3) still yielded amplicon using the chloroform-phenol extracts.

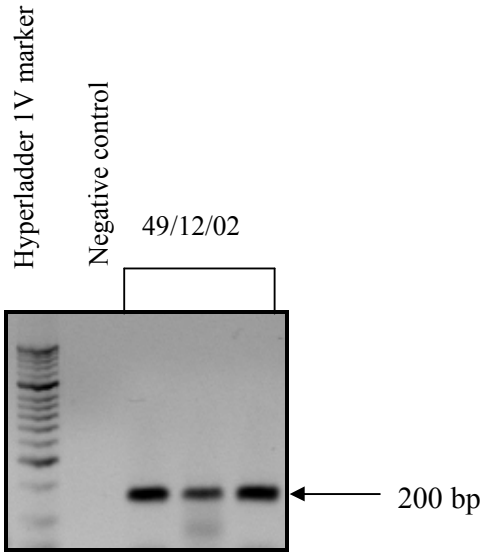

Figure 4.3: Verifying the phenol-chloroform extractions worked by doing a two-step PCR

Since the extractions were successful, samples (known to be infected) but not yielding amplicon previously (Chapter 3), were amplified using the exact protocol N. Douglas used in her studies. The protocol consisted of a one-step, one-tube RT-PCR, followed by a nested PCR. As the RT-PCR protocol of N. Douglas, using primer set 93-110/92-98 followed by 93-25/93-40 in the nested PCR, successfully amplified all the known infected samples in her study, this specific RT-PCR protocol was used on the phenol-chloroform extractions done in the current study. Primers 93-110 and 92-98 were designed (Ling *et al.*, 2001) to bind to the region of the genome flanking the regions where primers 93-25 and 93-40 bind. In the RT-PCR the expected band size of 199 bp was only observed in one of the plant samples, 49/12/02, but not 49/15/03 (Figure 4.4), which is known to be GLRaV-3 infected. The negative control did not yield any amplicons.

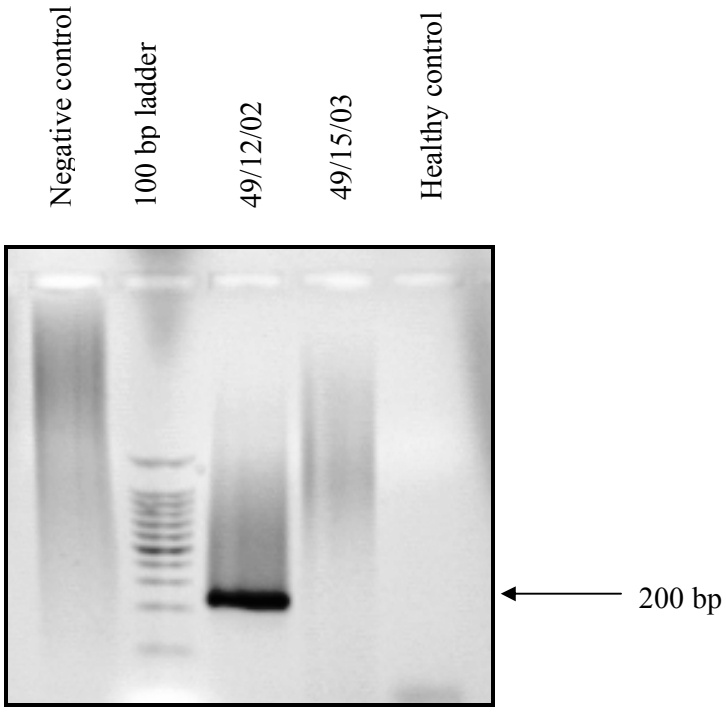

Figure 4.4: PCR results on phenol-chloroform extracts using the PCR protocol of N. Douglas

4.3.3 Template preparation: Immunocapture, dsRNA extraction and sap extraction

Figure 4.5 illustrates the PCR results of the immunocapture, dsRNA extraction and sap extraction. Three plants were tested and a healthy control (49/17/13) was included making sure that the amplicon obtained was not non-specific amplification of DNA from the plant genome. Polymerase chain reactions were done in triplicate for each extraction method. The PCR protocol followed was exactly the same as the protocol N. Douglas used (described in section 4.3.2). The primers used were 93-110 and 92-98, followed by primers 93-25 and 93-40 in the nested PCR reaction. The primers were used because of the success N. Douglas obtained when using them in her studies and to be able to compare the results with the phenol-chloroform extraction results. Using the same conditions in different experiments one can establish which extraction method is the best without the complication of PCR variations, for example the efficiency and sensitivity of primers in detecting the virus.

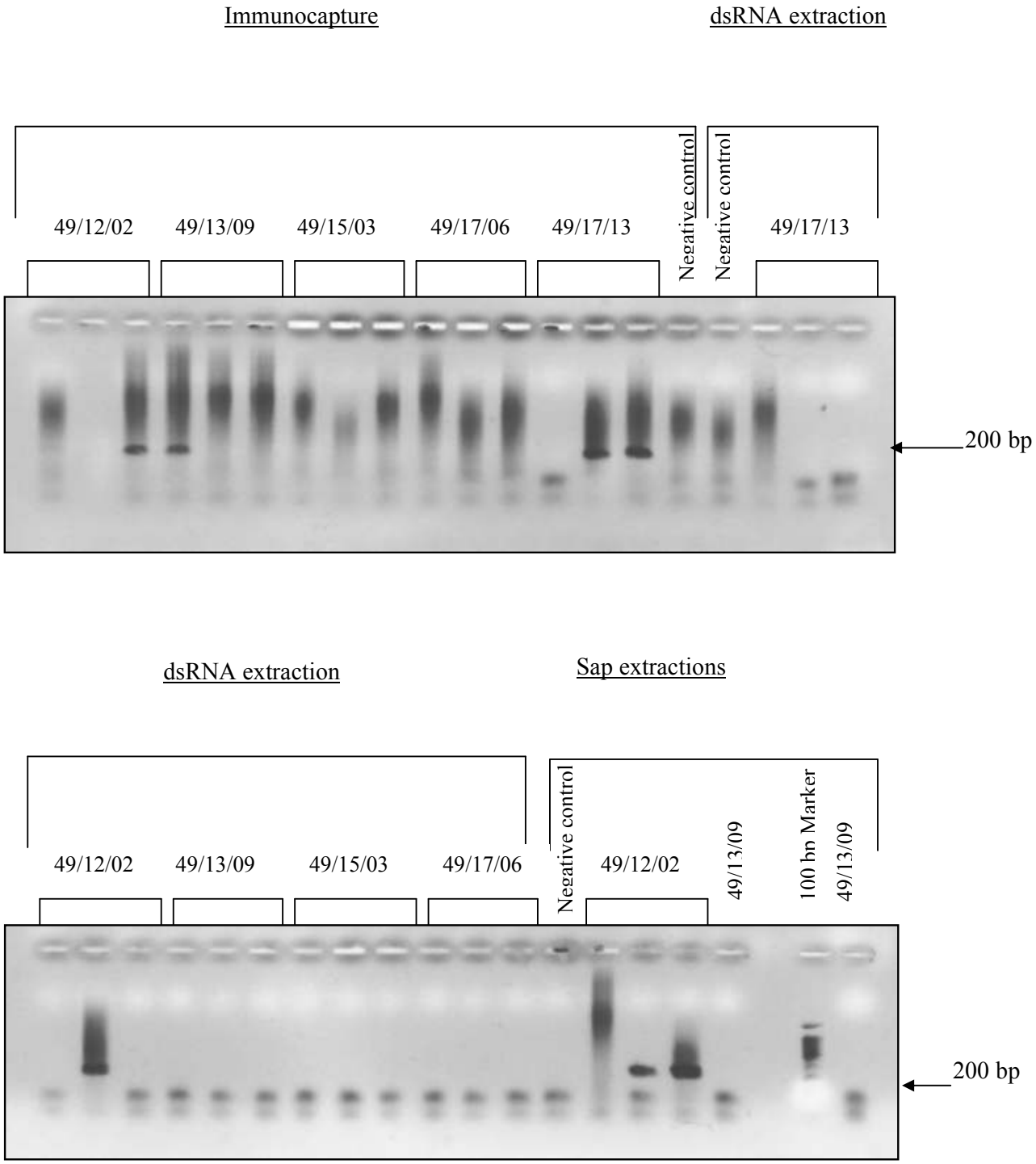

Figure 4.5: The PCR results obtained with different template preparation methods including, the immunocapture, dsRNA and sap extractions (remaining sap extraction results illustrated in Figure 4.6)

Using immunocapture PCR, only plant sample 49/12/02 and 49/13/09 (of the known positive samples) yielded amplicon of the expected size. The healthy control (49/17/13) had amplification in one replicate, probably to sample cross-contamination between PCR tubes. The healthy plant control gave no amplification in any of the other extraction methods indicating that the sample does not contain virus and that the primers do not amplify part of the plant's genome. One of the PCR's done on the dsRNA extractions (49/12/02) yielded amplicon, but showed no amplicon in the two replicates from the same extraction (49/12/02). Two PCR reactions performed on the sap extractions from plant sample 49/12/02 yielded amplicon (Figure 4.5), while one of the replicates did not show any amplification. For the healthy control (49/17/13) and plant sample 49/13/09 (macerated in ELISA extraction buffer) no amplicon were obtained (Figure 4.6) .In all the above-mentioned extraction methods plant sample 49/17/06 did not yield amplicon. Plant sample 49/17/06, was known to be infected due to the high absorbance (405 nm) obtained using ELISA test (chapter 3, section 3.3), thus amplicon was expected in all the PCR replicates performed with this sample.

4.3.4 Template preparation: Total RNA extractions, phenol-chloroform extraction and spotting

Electrophoresis gels of amplicons generated by PCR on the total RNA extractions, the phenol-chloroform extraction, spotting plant material on nitrocellulose transfer membrane and two sap extractions samples were illustrated in figure 4.6. The PCR protocol followed was exactly the same as the protocol N. Douglas used (described in section 4.3.2). The primers used were 93-110 and 92-98, followed by 93-25 and 93-40 in the nested PCR, to compare the results with the previous extraction results obtained. Performing the reaction on all plant samples in triplicate tested the consistency of the PCR. The PCR results from the plant samples prepared by spotting the sample onto a membrane are shown in figure 4.6 and figure 4.7. The results obtained from the PCR where the template was first spotted on a membrane, yielded no amplicon. The experiment was repeated, with no change in the results. The healthy control and the negative control yielded no amplification products in the PCR reactions done with both the phenol-chloroform extraction and the spotting membrane technique.

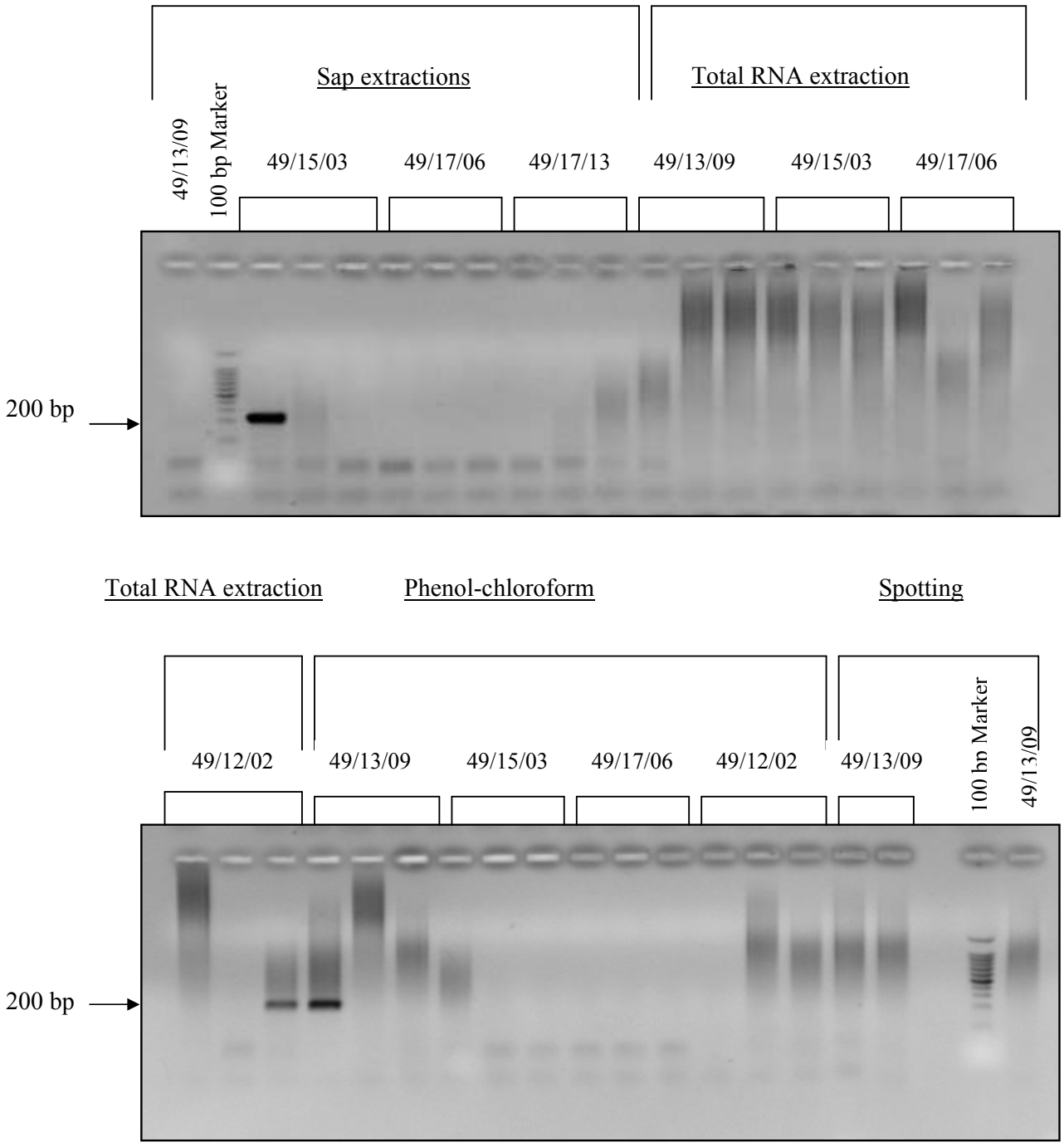

Figure 4.6: The PCR results obtained with different template preparation methods including, total RNA extraction, Phenol-chloroform and the membrane spotting

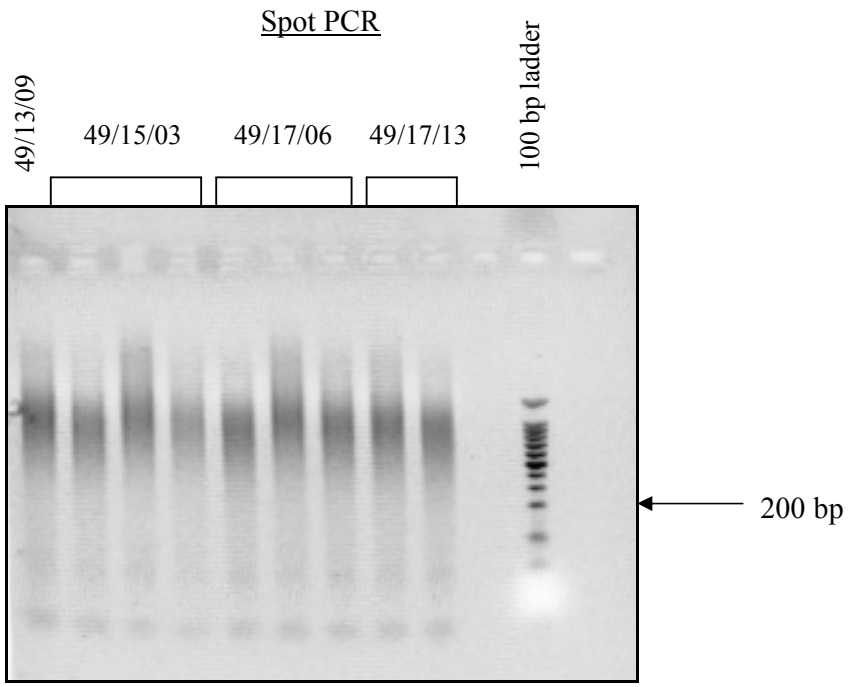

Figure 4.7: PCR results after the use of spotting template on membrane

4.3.5 Template preparation: Simple-direct-tube method

A simple-direct-tube method was also done to compare the results of this against the results of the Immunocapture step. This was done because the simple-direct-tube method utilises no antibodies, which makes it cheaper to use in large scale testing. Two buffers, the ELISA extraction buffer and PBST, were used for virus extraction in the simple-direct-tube method. The primers used were 93-110 and 92-98, followed by 93-25 and 93-40 in the nested PCR reaction in order to compare the results with the immunocapture results. The results presented in figure 4.8 showed that with the ELISA extraction buffer only plant sample 49/12/02 yielded amplicon of the expected size (200 bp), but with the PBST buffer two plants showed positive results (of expected size, 200 bp) with the PCR (49/12/02 and 46/13/09). The healthy control and the buffer control showed no amplification as expected.

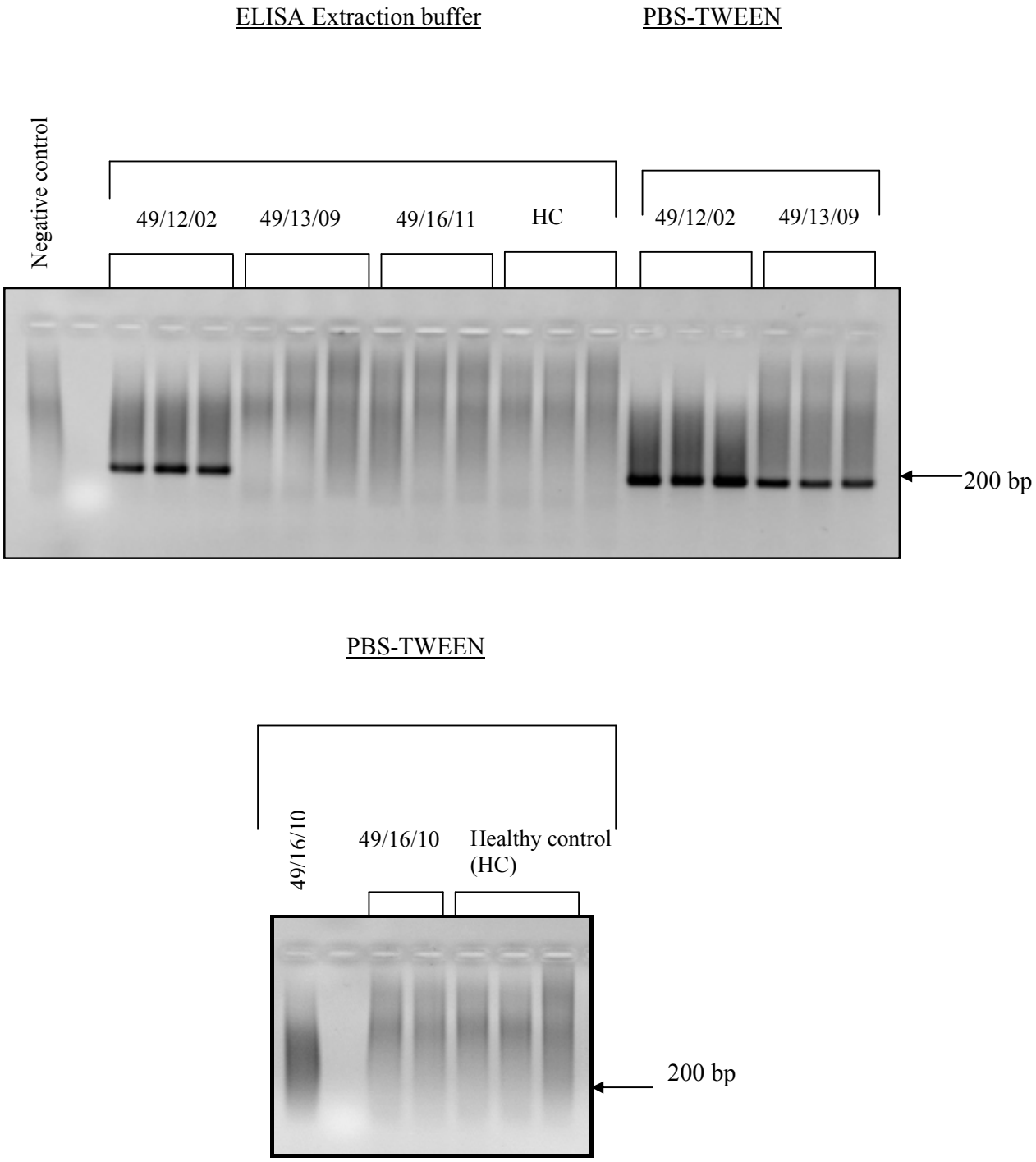

Figure 4.8: PCR products of the simple-direct-tube method

The grinding of the plant samples in PBST buffer prior to performing the simple-direct tube method gave improved amplification results when compared to samples macerated with ELISA extraction buffer. To test if the same holds true when using such preparation directly in the RT-PCR, plant material were macerated with ELISA extraction buffer and PBST buffer and the sap obtained added

directly in the RT reaction. Different quantities of the sap were added to the RT reaction to test whether the ELISA extraction buffer inhibits the PCR reactions at specific concentrations. The results are illustrated in figure 4.9. To determine whether inhibition does occur and the quantity needed before inhibition occurs, a two step RT-PCR was performed. The two step was used instead of the one step, one tube RT-PCR to be certain that any inhibition that did occur was due to the sap and not due to inhibition from the RT enzyme on the Taq polymerase enzyme.

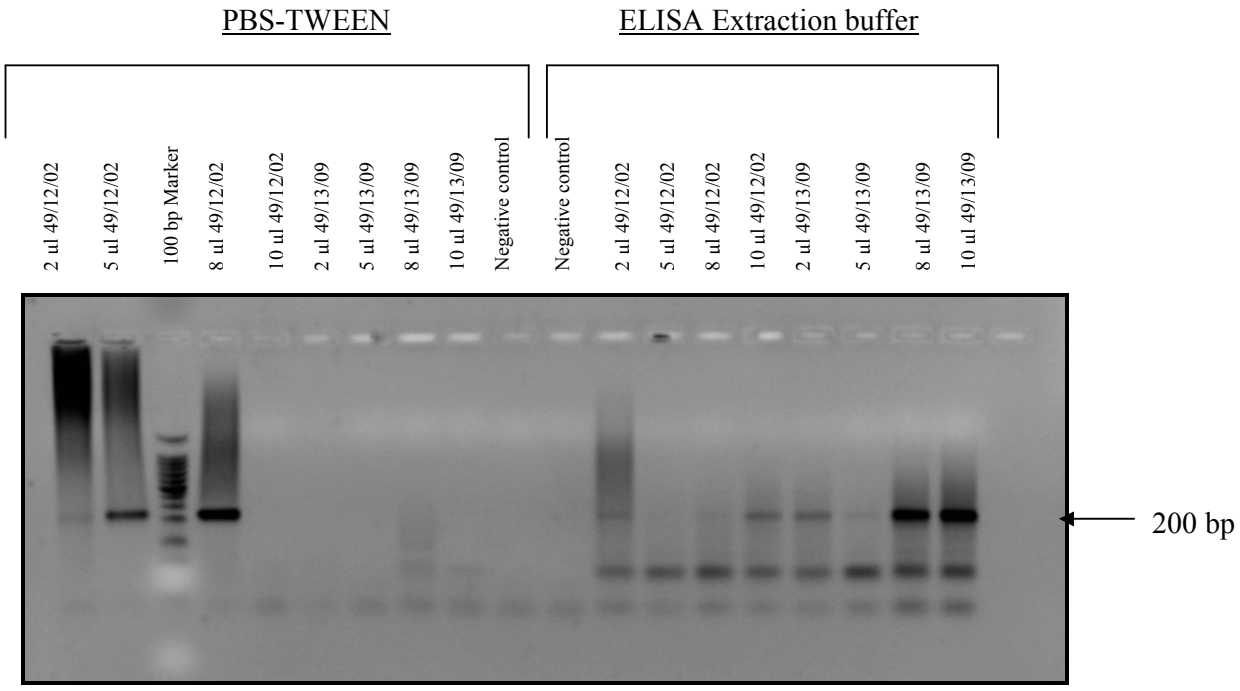

Figure 4.9: PCR results for samples ground in either extraction buffer or PBST

Extraction with PBST buffer only yielded amplicons of plant sample 49/12/02 when 5 µl and 8 µl of the extract were added to the RT reaction. No amplification occurred at higher of lower amounts (2 µl and 10 µl) of sample added or with plant sample 49/13/09 (known to be infected). The fact that only two PCR reactions (same plant different amount added to RT) amplified may be indicative of inhibition at high concentration of PBST, and sub positive/negative detection threshold at low concentration. Samples, 49/12/02 and 49/13/09 also amplified when macerated using ELISA extraction buffer. Both the samples yielded best amplification when a large amount of template was added (8 µl and 10 µl).

4.3.6 Implementation of modified PCR protocol

The following modifications were made when compared to the previous chapter (chapter 3): more sample was added to the mix, the plant material was macerated in a 1:5 ratio in the ELISA extraction buffer, and a two-step PCR was performed instead of a one tube, one step RT-PCR. The best amplification was observed when 5 µl of sap (plant material maceratedd in buffer) was added to the RT and 2 µl RT template in the subsequent PCR. The experiment was repeated with 6 plants (49/12/02, 49/13/09, 49/15/03, 49/16/09, 49/16/11 and 49/17/06) and every plant sample tested in triplicate. Plant sample 49/16/11 was used as the healthy control. All the samples yielded the expected size amplicon with the exception of the healthy control plant, which gave no amplification.

To verify that the system worked optimally, 2 additional primer sets were tested using the above-mentioned protocol. An additional 15 samples were tested with primer sets 93-110/92-98 in the RT, followed by 93-25/93-40 in the PCR reactions. Another 35 randomly chosen plant samples were tested with primer set H420/C629 (Figure 4.10). The 15 plant samples tested with primer set 93-110/92-98 were also tested in parallel with primers 1392 and 1393. All the samples were prepared and tested in duplicate. All the samples except for the healthy control amplified using primer sets 93-25/93-40, H420/C629 and 1392/1393.

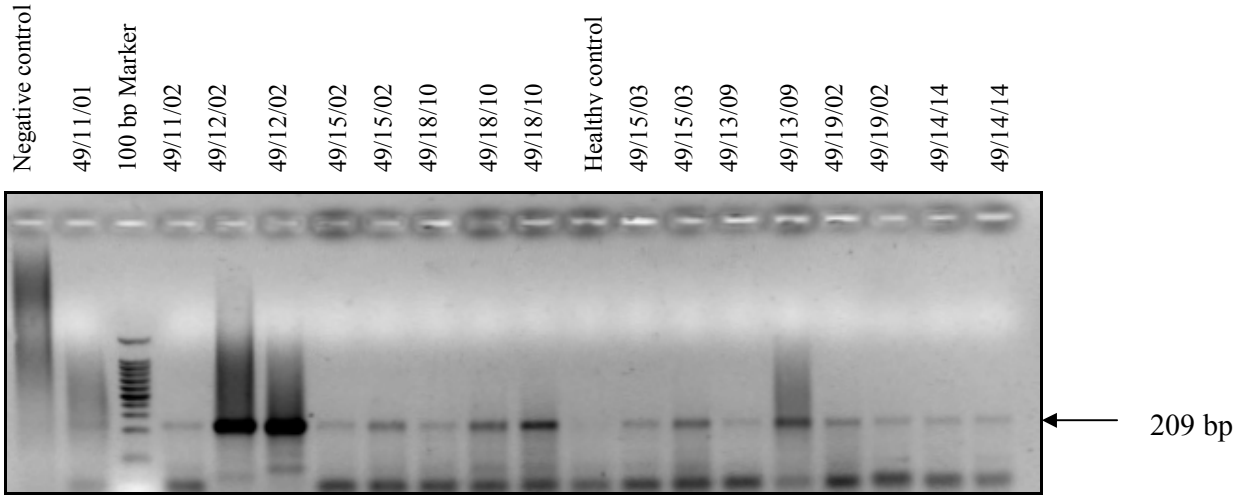

Figure 4.10: Gel electrophoresis illustrating 17 plants samples yielding amplicon using the two step RT-PCR done with primer set H420/C629 (McKenzie, 1997)

Results above suggest that some of the plant samples did not amplify in the studies conducted in Chapter 3, due to insufficient template used in the PCR reaction. Increasing template amounts clearly improved consistency of the PCR reaction. Plants in the vineyard will differ regarding their virus titre, but due to uneven distribution of the virus throughout the plant (Van der Merwe *et al*., 2007) one plant can also have petioles containing different virus titres. To determine the optimal amount of sap extract needed for positive amplification over a range of virus concentrations, a plant with low virus concentration (49/15/03) and one with high virus concentration (49/17/06) were used. ELISA results (chapter 3, section 3.3) were used to gain a measure of relative concentration in plants. RT reactions adding different quantities of template using both a plant with high viral titre and a plant with low viral titre were performed. Following the RT 2 µl of the cDNA was added to the PCR reaction. C629 and H420 were the primers chosen because 49/15/03 (known to be infected) gave negative results originally in the amplification when tested for variants (Chapter 3) and the primers have been used in previous studies (McKenzie, 1997. The plant with the low concentration (49/15/03) amplified erratically when low amounts of template were used, but results improved when more sap was added (up to 5 µl) to the RT reaction. When the amount of sap was increased beyond 5 µl, amplification no longer occurred. The plant with a higher virus titre amplified with lower amounts of sap added to the RT reaction, the amplification improved up to 5 µl of plant sample added, while amplification declined when more then 5 µl of plant sample was added.

Observations during the optimisation of the PCR with primer set 93-25/93-40 also suggested that variation in concentration was not the only factor determining the PCR quality, but that time elapsed between maceration and addition of the sample also plays a role.

Consequently, a test was done to see how long the macerated plant material could be left on ice and still yield a positive PCR result. Results, illustrated in figure 4.11, suggest that in the first 5 minutes after grinding, the sample is not influenced by RNA breakdown, however between 5 and 10 minutes on ice PCR amplification decreased. After 30 minutes on ice, samples previously positive, tested negative (data not shown). The test was done on two occasions. In the second test PCR thermocyclers (GeneAmp 2700 thermocycler (Applied biosystems, Warrington, United Kingdom)) used per treatment were chosen randomly, to make sure the same machines are not used for the same time treatment. This was done to make sure that differences in the RT amplification are not machine related; this was necessitated by the fact that different PCR machines were required in order to run the same extracts in parallel. The three plants used in this test were 49/12/02, 49/15/03 and 49/17/06. 49/12/02 was used because it amplifies consistently and the plant was macerated

freshly every time to be used as a control. The control was included to verify if the RT was working. 49/12/02 was not utilised in the time trial as 49/17/06 was used as a plant containing a high viral titre. Plant sample 49/15/03 was used as low virus titre material. Material with different titres was used to test whether the material with the lower concentration was not affected sooner by degradation with enzymes.

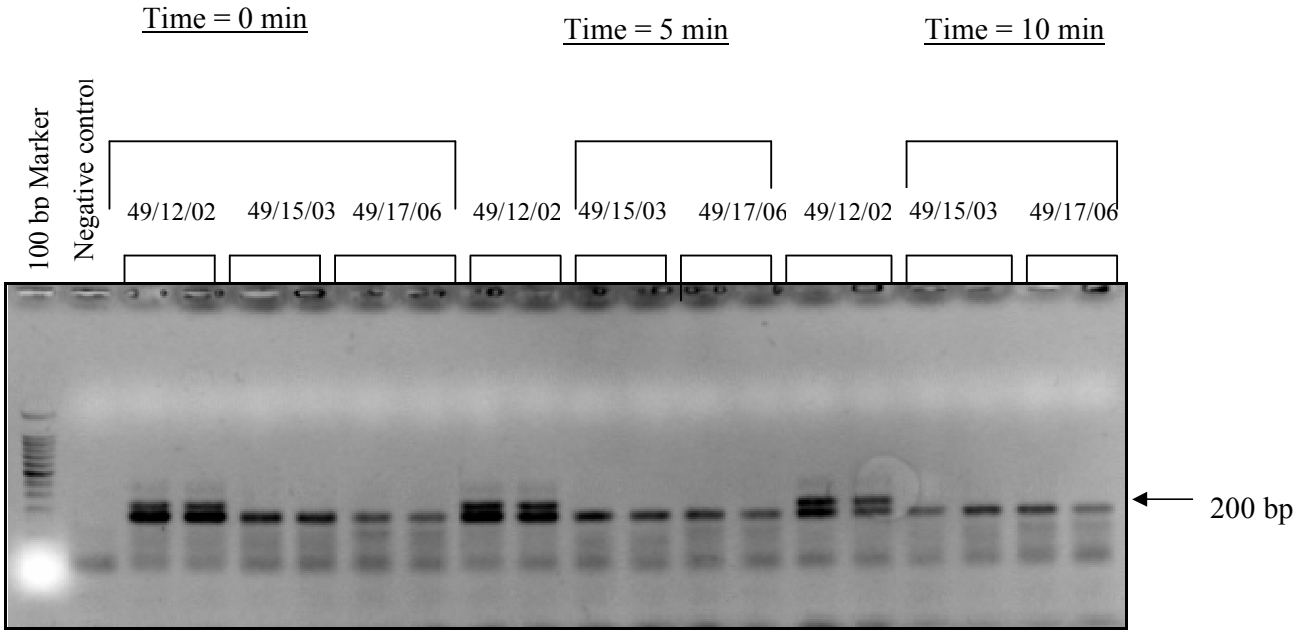

Figure 4.11: Time test to determine if virus RNA degradation does occur

4.5 DISCUSSION & CONCLUSION

In the current study plant samples often showed positive PCR results with one primer set, but negative results using another primer set (Chapter 3). Moreover these plants were often clearly positive in ELISA and showed symptoms of the disease. Anfoka *et al*. (2004) stated that GLRaV-3 is distributed unevenly throughout the plant; in their case this could have lead to a sample yielding amplicon with one primer set, but no amplicon with another primer set, since with every test new material was prepared. However this explanation can be excluded from the current study, where even a single extraction would sometimes give inconsistent amplification when PCR performed in triplicate. In a study done by Pico *et al.* (1999) erratic amplification were obtained with the fast sap extraction procedures, but in the current study the negative results were not due to erratic amplification, since there were samples (known to be infected) that yielded no amplicon

consistently with a primer set. Ling *et al*. (2001) noted that detection of GLRaV-3 in nonsymptomatic vines was erratic regardless of the detection method that was used. The erratic detection due to nonsymptomatic vines could be excluded from this study since symptomatic plants also yielded amplicon with some primer sets but no amplicon using a different primer set. Osman & Rowhani (2005) have also attempted to blot the plant tissue by pressing them onto the Nylon membrane discs, but inconsistent results were obtained possibly due to uneven distribution of the pathogens in the host tissue or the presence of higher inhibitors' concentration remaining on the membrane. The existence of different sequence variations at the primer binding sites (which could have explained the amplification scenario) can also be excluded as reason for some primers not yielding amplion since identical sequences over primer binding sites was obtained in Chapter 3.

The PCR protocol (a one step, one tube RT-PCR protocol), which gave erratic amplification in Chapter 3, was modified to determine whether the erratic results were due to sub-optimal protocol steps. The protocol was separated into a RT step and PCR reaction, to ensure that the RT enzyme did not inhibit the PCR as found to occur by Sellner *et al.* (1992) and Chandles *et al*. (1998). While this considerably improved the detection ability of the PCR, some plant samples (example 49/15/01) still did not yield any amplicon with some of the primers used. Based on this results obtained, it could be concluded that the previously negative PCR results was not the result of inhibition from the RT enzyme on the Taq polymerase in the one step, one tube RT-PCR protocol. Inhibition due to the RT:Taq ratio was excluded in further tests by separating the reverse transcriptase step from the PCR step as suggested by Sellner *et al.* (1992). Different extraction methods were tested to determine the optimal method to obtain consistent amplification with all known infected plant samples.

The RNA was first extracted using the SV Total RNA isolation System (Promega, Madison, USA). The kit was used to minimize potential PCR inhibition due to contaminants such as polyphenols present in the plant sap. Using the total RNA extract as template in the PCR, one plant sample (49/15/03, known to be infected from ELISA results (Chapter 3)) still gave no amplification. This suggests that a factor other than inhibition by the presence of contaminants plays a role. Koonjul *et al*. (1999) suggested that contaminants could be removed with the aid of resins or columns. The total RNA extraction kit from Promega is based on the column principle where the RNA binds to a membrane and the contaminants are washed away, and therefore it was expected that with all known infected samples should yield amplicons.

It is possible that the lack of detection of known positive samples could be due to inconsistency in the amplification protocol. To determine if inconsistency in the amplification step did occur the PCR reactions were performed in triplicate. Amplification results however were consistent over all the replicates. If the sample did yield amplicon, it yielded amplicon in all three replicates, but if the sample did not yield amplicon, no amplicon was observed in all three replicates. It is therefore unlikely that negative PCR results were due to inconsistency in the PCR reactions. The results from the above-mentioned experiments suggest that the lack of detection of known positive samples was not due to inhibition of any sort on the PCR protocols used, and therefore different template preparation methods were tested to obtain optimal detection in PCR.

A personal communication with N. Douglas encouraged the use of the phenol-chloroform extraction method in this study. N. Douglas tested various extraction methods including the, RNeasy Mini Kit from QIAGEN (Valencia, CA, USA), Promega SV total RNA isolation kit (Promega, Madison, USA), Gentra Purescript RNA Isolation kit (Gentra Systems Inc, Minneapolis, MN, USA), Qiagen QIAzol (Valencia, CA, USA) and Chloroform-phenol extraction (Mallory *et al.,* 2001), to determine which protocol would give optimal amplification of GLRaV-3 extracted from a vector (*Planococcus ficus*). She found that the phenol-chloroform extraction gave consistent amplification of known infected samples, however showed inconsistent amplification on plant material. N. Douglas's amplification protocol (one tube, one step RT-PCR followed by a nested PCR) was used since it gave successful results in her studies. The sample amplified in one of the replicates, but no amplicon was obtained in the remaining two replicates.

In parallel with the phenol-chloroform extraction the following extraction methods were also tested: Total RNA extraction (Promega, Madison, USA), Immunocapture and simple-direct tube method (Suehiro *et al.*, 2004), dsRNA extraction (Valverde, 1990), ELISA sap extraction (according to manufacturers, ARC-PPRI, Pretoria) and direct spotting of the sample on nitrocellulose transfer membrane (Osman & Rowhani, 2006). The above-mentioned extraction methods were tested to determine an optimal extraction method. The one step, one tube RT-PCR protocol followed by nested PCR were used since it gave optimal amplification in N. Douglas's studies and the chloroform-phenol extractions were amplified using this protocol. Using the same protocol allows for comparing the different preparation methods. All the extraction methods, except the simple-direct-tube method gave inconsistent amplification results on the plant samples performed in triplicate. For example, in the dsRNA extraction (Valverde, 1990) performed on plant 49/12/02 only one replicate yielded amplicon. Using the direct spotting of the sample on nitrocellulose transfer membrane method no amplicon was obtained from the samples, the results stayed

unchanged when the experiment was repeated. This is a pity since the advantage of the spotted membrane technique is that the membranes can be shipped overseas and processed, even after an extended period of time (La Notte *et al.*, 1997).

The simple-direct-tube method showed consistent amplication with all the samples tested. Because of this, this protocol was used for further tests to optimize the protocol. It was determined whether the samples would give consistent amplification when macerated PBS-Tween (used in the simple-direct-tube method). If the PCR gave consistent amplification with the samples ground in PBS-Tween but not with the ELISA extraction buffer, it could indicate that a chemical in the ELISA extraction buffer inhibits the PCR reaction. Common inhibitors of the PCR reaction include various compounds of body fluids and reagents encountered in forensic and clinical science (e.g. haemoglobin, urea, and heparin), food constituents (e.g. organic and phenolic compounds, glycogen, fats, and Ca^{2+}) and environmental compounds (e.g. phenolic compounds, humic acids, and heavy metals) (Wilson, 1997). Other, more widespread inhibitors include constituents of bacterial cells, non-target DNA, contaminants, and laboratory items such as pollen, glove powder, laboratory plasticware, and cellulose (Wilson, 1997). Although a wide range of inhibitors is reported, the identities and modes of action of many remain unclear. The reaction inhibition can be total or partial and manifest itself as complete reaction failure or as reduced sensitivity of detection (Wilson, 1997). To determine the amount of sample needed in the RT reaction (two step RT-PCR used for amplification) before inhibition occurs, the samples (macerated in both buffers) were added at different volumes to the RT reactions. The protocol was changed to the two step RT-PCR to ensure that if inhibition occurred, it would be due to the buffer used and not the probability of RT enzyme inhibition on the Taq. When the ELISA buffer was used to macerate plant samples (known to be infected), amplicon was obtained when a large amount (8 µl and 10 µl) of the sample was added. For this reason it was thought that some chemicals in the buffer could inhibit PCR when the target template is of low concentration. From the results obtained it was determined that the amplification was optimal when the sample was macerated in ELISA extraction buffer, but at a lower ratio (1:5) than previously used (1:10) (Chapter 3) and when the amount of sap added was increased.

The amount of sap added to the RT reaction played an important role in the results of the amplification, thus it would be necessary to determine the amount of template needed for optimal amplification results. Two scenarios were tested namely, one plant sample in which high virus titer occurs, the other were a plant sample has low virus titer. This was done to see what range of

template can be added to obtain optimal amplification. The results showed that in the plant sample containing low virus concentration, the amplification improved when more template was used, but at a certain point (more than 5µl sap) no amplification was obtained due to more inhibitors that were present in the RT reaction and the low amount of template. Thus, in a scenario where the plant has little template the amplification will not occur when an excess amount of sap is added. The result obtained indicated in the plant sample containing the high virus concentration, the amplification improved when more template was used, but at a certain point (more than 5µl sap) the amplification decreased due to more inhibitors that was added directly into the RT reaction. Amplification should occur, but not optimally, due to the high amount of template added. Thus, a plant with high virus titer will only amplify less than optimal when the quantity of the template is increased. Barlow *et al.* (1997) showed that when the copy number of the starting target was low, false negatives could be obtained. By using a lower ratio of ELISA extraction buffer for the maceration of the sample and adding more of the sample extract to the RT step, the absolute number of starting template was increased, increasing the probability of yielding amplicon.

Virus titer in the plant material tested was not the only factor that could influence PCR results, but also the interval after the material has been macerated and the sap added to the RT reaction. The sap should be added to the RT reaction within 10 minutes after the plant material was macerated. The need to do this rapidly could be due to the instability of the viral RNA and breakdown of the RNA (Valverde, 1990). This date could also explain a part of the negative PCR results (from known infected plants) obtained in Chapter 3. In the experiments all the plant material was ground and then added to the PCR reaction mix, creating a scenario where some off the samples had to be kept on the bench until it was added to the PCR reaction. This could have lead to RNA degradation and for that reason yielded no amplicon in the PCR reaction.

In this study it was therefore determined that to achieve optimal and consistent amplification of GLRaV-3 the two step RT-PCR protocol should be used. The sample should be macerated in the ELISA extraction buffer at a 1:5 ratio. From the resulting sap 5 µl should be added to the RT mix (within 10 minutes from sample grinding). The RT step should also be performed separately from the PCR and the amplification should be performed using 2 µl cDNA generated in the RT step as template.

CHAPTER 5

OVERALL CONCLUSION

This study was conducted to determine whether Grapevine leafroll type 3 variants do exist within a specific vineyard in the Western Cape. The specific vineyard was chosen as it had been used in a study to determine whether grapevine leafroll virus infected plants can be detected on a large scale with remote sensing (Pietersen, 2006b). The above-mentioned study was done to minimise the need for plant-for-plant monitoring of vines for symptoms (Pietersen, 2006b). Plant-for-plant monitoring is labour intensive and can only be performed in a short period of the year between the onset of autumn and the fall of vine leaves (Pietersen, 2006a). In order to establish a "ground-truth" an enzyme-linked immunosorbent assay (ELISA) was performed to identify infected plants (Pietersen, 2006b). In addition to ELISA, the more sensitive polymerase chain reaction (PCR) technique was performed to identify infected plants. As expected due to the greater sensitivity of PCR some ELISA negative samples, were positive by PCR (G. Pietersen, personal communication). However, sometimes the plant material tested positive for the leafroll virus in ELISA but was negative using PCR (Pietersen, 2006b). It was speculated that these apparently contradictory results may be due to the presence of grapevine leafroll type 3 variants (G. Pietersen, personal communication), and that PCR primers were possibly not binding to regions of the virus genome differing significantly form those to which the primers was designed.

Six primers sets to differing regions were designed and used in a one tube, one step PCR protocol together with ELISA to detect GLRaV-3 virus in the 135 plant samples section of the vineyard on which ELISA and PCR had originally been done (135 plants situated in the same vineyard). Comparing the ELISA and PCR results, the PCR proved to be more sensitive than the ELISA since it yielded greater number of positive plants from the 135 samples tested. The samples that yielded negative ELISA results but positive PCR results probably had a low viral titre and therefore did not yield a positive colour reaction in the ELISA test. However, some samples yielded positive results with the ELISA, but negative results with the PCR, possible due to variation. Comparing the PCR results of the different primer sets, anomalous results occurred since a plant sample will yield an amplicon with one primer set, but no amplicon with another primer set. Using the combined PCR results of all 6 primer sets, each plant was assigned a PCR 'fingerprint'. This yielded 24 different fingerprints. Mapping the spatial distribution of given fingerprints supported the possibility that strain variants exist, but after sequencing over regions including primer binding sites of selected sources of different fingerprints was performed it can be concluded that no nucleotide sequence variation at the primer binding sites exist in the 135 plant samples tested.

As these plants showed symptoms and often clearly reacted in ELISA and at least one PCR system it can be concluded that they are GLRaV-3 infected. Negative PCR results if not due to primer

failing to bind to sequence variants must be due to a sub-optimal, unreliable PCR assay. Therefore, different extraction techniques including(1) grinding in extraction buffer; 2) total RNA extraction using the Promega kit (Promega, Madison, USA); 3) Phenol-chloroform extraction (Mallory *et al.*, 2001); 4) Spotting of plant sap onto nitrocellulose transfer membrane (Osman & Rowhani, 2003); 5) double stranded RNA (dsRNA) extraction (Valverde, 1990); 6) Imumunocapture and 7) the simple-direct tube method (Suehiro *et al*., 2005) were tested. Similarly different PCR protocols (1) two step RT PCR, (2) One step, one tube RT-PCR followed by nested PCR, were tested to determine whether false negative PCR results obtained with known infected sources was a protocol error, rather than variability of the pathogen. These false negatives may be due to inhibition from sap extracts, PCR inhibition by RT enzyme or suboptimal PCR conditions. The PCR protocol initially utilised (a one step, one tube RT-PCR protocol), which gave erratic amplification was separated into a RT step and PCR reaction, to ensure that the RT enzyme did not inhibit the PCR as found to occur by Sellner *et al.* (1992) and Chandles *et al*. (1998). It was found when using the two step RT-PCR and macerating the plant sample in ELISA extraction buffer (1:5) for sample preparation, amplicon of expected size was obtained from all known infected plant samples. The protocol consisted of adding 5 µl of sap (obtained from sample preparation) to the RT step, followed by adding 2 µl of the RT product to the PCR reaction.

Therefore it can be concluded that GLRaV-3 sequence variability at the primer binding sites was not the cause of the anomalous PCR results obtained, but that it was due to sub-optimal conditions in the extraction and PCR protocol. Strangely, GLRaV-3 does seem to be a relatively homogenous virus, since there is not a great deal of literature on GLRaV-3 strains, while literature regarding different strains of CTV and GLRaV-1, belonging to the same family, is abundant. However, the current study was conducted in a small area giving a biased picture of whether strains exist or not, as plants were in close proximity to one another, probably reflecting only the spread of one strain of GLRaV-3.

If in future one should want to identify different strains for GLRaV-3, the study should be conducted over a widespread area, to increase the probability of identifying different strains. It would also be advisable to sequence the samples from the initiation of the studies, since it will be quicker to determine whether strains do occur.

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APPENDIX A

Table A1: Table showing the efficiency relative to ELISA, of primer combination 93-25 and 93-40 (Ling *et al.*, 2001) used in the one tube, one step RT-PCR in the detection of GLRaV-3 infected plants. Using the ELISA test as the standard.

	Infected plants (ELISA positive)	Healthy plants (ELISA negative)
Positive PCR resuts	57	51
Negative PCR results	11	16
Total	68	67

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \qquad \text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}}$$

$$\text{Sensitivity} = \frac{57}{57 + 11} \qquad \text{Specificity} = \frac{16}{16 + 51}$$

$$= 83.82\ \% \qquad = 23.89\ \%$$

Table A2: Table showing the efficiency relative to ELISA, of primer combination 1399 and 1400 (Ling *et al.*, 2001) used in the one tube, one step RT-PCR in the detection of GLRaV-3 infected plants. Using the ELISA test as the standard.

	Infected plants (ELISA positive)	Healthy plants (ELISA negative)
Positive PCR resuts	30	51
Negative PCR results	38	16
Total	68	67

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \qquad \text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}}$$

$$\text{Sensitivity} = \frac{30}{30 + 38} \qquad \text{Specificity} = \frac{16}{16 + 51}$$

$$= 44.12\ \% \qquad = 23.89\ \%$$

APPENDIX A (Continue)

Table A3: Table showing the efficiency relative to ELISA, of primer combination 1397 and 1398 (Ling *et al.*, 2001) used in the one tube, one step RT-PCR in the detection of GLRaV-3 infected plants. Using the ELISA test as the standard.

	Infected plants (ELISA positive)	Healthy plants (ELISA negative)
Positive PCR resuts	39	35
Negative PCR results	29	32
Total	68	67

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \qquad \text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}}$$

$$\text{Sensitivity} = \frac{39}{39 + 29} \qquad \text{Specificity} = \frac{32}{35 + 32}$$

$$= 57.35\ \% \qquad = 47.76\ \%$$

Table A4: Table showing the efficiency relative to ELISA, of primer combination 1401 and 1402 (Ling *et al.*, 2001) used in the one tube, one step RT-PCR in the detection of GLRaV-3 infected plants. Using the ELISA test as the standard.

	Infected plants (ELISA positive)	Healthy plants (ELISA negative)
Positive PCR resuts	17	11
Negative PCR results	51	56
Total	68	67

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \qquad \text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}}$$

$$\text{Sensitivity} = \frac{17}{17 + 51} \qquad \text{Specificity} = \frac{56}{11 + 56}$$

$$= 25\ \% \qquad = 83.58\ \%$$

APPENDIX A (Continue)

Table A5: Table showing the efficiency relative to ELISA, of primer combination H420 and C629 (Ling *et al.*, 2001) used in the one tube, one step RT-PCR in the detection of GLRaV-3 infected plants. Using the ELISA test as the standard.

	Infected plants (ELISA positive)	Healthy plants (ELISA negative)
Positive PCR resuts	18	23
Negative PCR results	50	44
Total	68	67

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \qquad \text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}}$$

$$\text{Sensitivity} = \frac{18}{18 + 50} \qquad \text{Specificity} = \frac{44}{23 + 44}$$

$$= 26.47\ \% \qquad = 65.67\ \%$$

Table A6: Table showing the efficiency relative to ELISA, of primer combination 1379 and 1396 (Ling *et al.*, 2001) used in the one tube, one step RT-PCR in the detection of GLRaV-3 infected plants. Using the ELISA test as the standard.

	Infected plants (ELISA positive)	Healthy plants (ELISA negative)
Positive PCR resuts	11	5
Negative PCR results	57	62
Total	68	67

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \qquad \text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}}$$

$$\text{Sensitivity} = \frac{11}{11 + 57} \qquad \text{Specificity} = \frac{62}{62 + 5}$$

$$= 16.18\ \% \qquad = 92.54\ \%$$

APPENDIX A (Continue)

Table A7: Table showing the efficiency relative to ELISA, of primer combination 1392 and 1393 (Ling *et al.*, 2001) used in the one tube, one step RT-PCR in the detection of GLRaV-3 infected plants. Using the ELISA test as the standard.

	Infected plants (ELISA positive)	Healthy plants (ELISA negative)
Positive PCR resuts	12	4
Negative PCR results	56	63
Total	68	67

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}}$$

$$\text{Sensitivity} = \frac{12}{12 + 56}$$

$$= 17.64\ \%$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}}$$

$$\text{Specificity} = \frac{63}{4 + 63}$$

$$= 94.03\ \%$$

APPENDIX B

Table B1: The 24 different PCR fingerprints obtained for the 135 plant samples after amplification with 7 primer sets using the one tube, one step RT-PCR (performed in Chapter 3).

	1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396	
49/12/03	-	-	-	-	-	-	Samples testing negative by all PCR's
49/12/06	-	-	-	-	-	-	
49/12/07	-	-	-	-	-	-	
49/13/04	-	-	-	-	-	-	
49/16/14	-	-	-	-	-	-	
49/17/01	-	-	-	-	-	-	
49/17/13	-	-	-	-	-	-	
49/14/11	-	-	-	-	-	-	
49/15/10	-	+	+	-	-	-	PCR detection pattern 1
49/15/11	-	+	+	-	-	-	
49/15/15	-	+	+	-	-	-	
49/16/10	-	+	+	-	-	-	
49/14/15	-	+	+	-	-	-	
49/11/15	+	-	-	-	-	-	PCR detection pattern 2
49/16/01	+	-	-	-	-	-	
49/16/03	+	-	-	-	-	-	
49/16/04	+	-	-	-	-	-	
49/17/11	+	-	-	-	-	-	
49/17/15	+	-	-	-	-	-	
49/14/06	+	-	-	-	-	-	
49/19/04	+	-	-	-	-	-	
49/19/05	+	-	-	-	-	-	
49/19/06	+	-	-	-	-	-	
49/19/15	+	-	-	-	-	-	
49/11/01	+	-	+	-	-	-	PCR detection pattern 3
49/11/07	+	-	+	-	-	-	
49/11/13	+	-	+	-	-	-	
49/12/01	+	-	+	-	-	-	
49/12/04	+	-	+	-	-	-	
49/12/05	+	-	+	-	-	-	
49/15/07	+	-	+	-	-	-	
49/16/02	+	-	+	-	-	-	
49/16/12	+	-	+	-	-	-	
49/17/12	+	-	+	-	-	-	
49/18/05	+	-	+	-	-	-	
49/11/03	+	+	-	-	-	-	PCR detection pattern 4
49/12/10	+	+	-	-	-	-	
49/12/14	+	+	-	-	-	-	
49/13/06	+	+	-	-	-	-	
49/15/09	+	+	-	-	-	-	
49/16/09	+	+	-	-	-	-	
49/18/08	+	+	-	-	-	-	
49/18/10	+	+	-	-	-	-	
49/18/12	+	+	-	-	-	-	
49/18/14	+	+	-	-	-	-	
49/14/09	+	+	-	-	-	-	

APPENDIX B (Continue)

	1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396	
49/14/12	+	+	-	-	-	-	PCR detection pattern 4
49/19/01	+	+	-	-	-	-	
49/19/03	+	+	-	-	-	-	
49/19/14	+	+	-	-	-	-	
49/12/08	+	+	+	-	-	-	PCR detection pattern 5
49/12/09	+	+	+	-	-	-	
49/12/11	+	+	+	-	-	-	
49/12/12	+	+	+	-	-	-	
49/12/13	+	+	+	-	-	-	
49/12/15	+	+	+	-	-	-	
49/15/01	+	+	+	-	-	-	
49/15/05	+	+	+	-	-	-	
49/15/08	+	+	+	-	-	-	
49/15/12	+	+	+	-	-	-	
49/15/13	+	+	+	-	-	-	
49/15/14	+	+	+	-	-	-	
49/16/07	+	+	+	-	-	-	
49/17/02	+	+	+	-	-	-	
49/17/14	+	+	+	-	-	-	
49/14/04	+	+	+	-	-	-	
49/15/02	+	+	+	-	-	-	
49/11/05	+-	-	+	-	-	-	
49/16/13	+	-	-	-	-	+	PCR detection pattern 5
49/17/03	+	-	-	-	-	+	
49/17/08	+	-	-	-	-	+	
49/18/11	+	-	-	-	-	+	
49/11/04	+	+	+	-	-	+	PCR detection pattern 6
49/15/03	+	+	+	-	-	+	
49/16/08	+	+	+	-	-	+	
49/18/02	-	-	-	-	+	-	PCR detection pattern 7
49/14/14	-	-	-	-	+	-	
49/11/09	-	-	+	-	+	-	PCR detection pattern 8
49/13/15	-	-	+	-	+	-	
49/13/13	-	+	-	-	+	-	PCR detection pattern 9
49/14/13	-	+	-	-	+	-	
49/13/03	-	+	+	-	+	-	PCR detection pattern 10
49/14/02	-	+	+	-	+	-	
49/13/02	+	-	-	-	+	-	PCR detection pattern 11
49/13/12	+	-	-	-	+	-	
49/17/04	+	-	-	-	+	-	
49/11/08	+	-	+	-	+	-	PCR detection pattern 12
49/11/11	+	-	+	-	+	-	
49/11/12	+	-	+	-	+	-	
49/13/01	+	-	+	-	+	-	
49/13/07	+	-	+	-	+	-	
49/13/14	+	-	+	-	+	-	
49/16/15	+	-	+	-	+	-	
49/17/06	+	-	+	-	+	-	
49/19/11	+	-	+	-	+	-	

APPENDIX B (Continue)

	1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396	
49/16/11	+	+	-	-	+	-	PCR detection pattern 13
49/14/05	+	+	-	-	+	-	
49/14/08	+	+	-	-	+	-	
49/14/10	+	+	-	-	+	-	
49/15/04	+	+	+	-	+	-	PCR detection pattern 14
49/15/06	+	+	+	-	+	-	
49/17/07	+	+	+	-	+	-	
49/14/01	+	+	+	-	+	-	PCR detection pattern 15
49/14/03	+	+	+	-	+	-	
49/14/07	+	+	+	-	+	-	
49/11/02	+	+	+	-	+	+	PCR detection pattern 16
49/12/02	+	+	+	-	+	+	
49/17/05	+	+	+	-	+	+	
49/18/09	-	-	+	+	-	-	PCR detection pattern 17
49/18/01	-	-	+	+	-	-	PCR detection pattern 18
49/18/04	-	-	+	+	-	-	
49/19/13	-	-	+	+	-	-	
49/18/06	-	+	+	+	-	-	
49/17/09	+	-	-	+	-	-	PCR detection pattern 19
49/19/08	+	-	-	+	-	-	
49/16/06	+	-	-	+	-	-	
49/11/14	+	-	+	+	-	-	PCR detection pattern 20
49/18/03	+	-	+	+	-	-	
49/19/02	+	-	+	+	-	-	
49/16/05	+	-	+	+	-	-	
49/13/10	+	+	-	+	-	-	PCR detection pattern 21
49/17/10	+	+	-	+	-	-	
49/18/13	+	+	-	+	-	-	
49/18/15	+	+	-	+	-	-	
49/19/12	+	+	-	+	-	-	
49/19/07	+	+	+	+	-	-	PCR detection pattern 22
49/19/09	+	+	+	+	-	-	
49/19/10	+	+	+	+	-	-	
49/11/10	+	-	-	+	+	-	PCR detection pattern 23
49/13/11	+	-	-	+	+	-	
49/13/09	+	-	+	+	+	-	PCR detection pattern 24
49/13/08	+	+	-	+	+	-	
49/18/07	+	+	+	+	+	-	
49/13/05	+	-	-	+	+	+	

APPENDIX C

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	+	+	-	+	+

	11	12	13	14	15	16	17	18	19
1									
2		2002							
3									
4									
5							2005		
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	10.142	7.8	
3	7.465	5.786	
4	4.773	5.449	-
5	4.773	4.093	-
6	-	-	-
7	-	-	-

Figure C1: The figure illustrates PCR fingerprint 3, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	+	-	-	-	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4									
5									
6									
7									
8								2001	
9									
10								2002	
11									
12								2004	
13									
14									2003
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.494	0.861	0.928
3	0.676	0.918	0.822
4	0.893	-	-
5	0.793	-	-
6	-	-	-
7	-	-	-

Figure C2: The figure illustrates PCR fingerprint 4, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	-	+	-	+	-

	11	12	13	14	15	16	17	18	19
1			2003						
2									
3									
4									
5									
6							2003		
7									
8									
9									
10									
11									2003
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	1.512	1.68	1.526
3	0.842	0.946	0.825
4	1.468	-	-
5	0.519	-	-
6	-	-	-
7	-	-	-

Figure C3: The figure illustrates PCR fingerprint 5, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
-	-	-	-	+	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	2	2	2
3	0.947	0.929	0.889
4	2	-	-
5	0.909	-	-
6	-	-	-
7	-	-	-

Figure C4: The figure illustrates PCR fingerprint 6, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	-	-	+	+	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.963	0.95	0.923
3	0.947	17	12
4	0.909	-	-
5	0.909	-	-
6	-	-	-
7	-	-	-

Figure C5: The figure illustrates PCR fingerprint 7, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	-	+	+	-	-

	11	12	13	14	15	16	17	18	19
1									
2									
3								2003	
4									
5						2002			
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.463	0.283	0.423
3	0.447	2.94	0.389
4	0.409	-	-
5	0.409	-	-
6	-	-	-
7	-	-	-

Figure C6: The figure illustrates PCR fingerprint 8, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	-	-	-	-	-

	11	12	13	14	15	16	17	18	19
1						2002			
2									
3						2002			
4						2001			
5									2005
6				2003					2004
7									
8									
9									
10									
11							2003		
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	3.117	1.668	1.681
3	1.117	1.782	0.585
4	2.424	-	-
5	2.569	-	-
6	-	-	-
7	-	-	-

Figure C7: The figure illustrates PCR fingerprint 9, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	+	+	-	-	-

	11	12	13	14	15	16	17	18	19
1					2001				
2					2002		2002		
3									
4									
5					2003				
6									
7						2003			
8									
9									
10									
11									
12									
13									
14							2004		
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.998	0.752	0.995
3	0.939	0.818	0.768
4	1.167	-	-
5	1.319	-	-
6	-	-	-
7	-	-	-

Figure C8: The figure illustrates PCR fingerprint 10, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

	11	12	13	14	15	16	17	18	19
1									
2			2003						
3									
4							2002		
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.463	0.45	0.423
3	0.447	0.429	0.389
4	0.409	-	-
5	0.409	-	-
6	-	-	-
7	-	-	-

Figure C9: The figure illustrates PCR fingerprint 11, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	+	-	+	-	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4									
5									
6									
7									
8									
9									
10							2001		
11									
12									2002
13								2005	
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.463	0.283	0.423
3	3.789	3.393	2.074
4	4.773	-	-
5	2.424	-	-
6	-	-	-
7	-	-	-

Figure C10: The figure illustrates PCR fingerprint 12, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
-	+	-	-	+	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.963	0.95	0.923
3	0.947	0.929	0.889
4	14	-	-
5	14	-	-
6	-	-	-
7	-	-	-

Figure C11: The figure illustrates PCR fingerprint 13, spatial distribution of plants of this specific PCR fingerprint, and Morisita index of dispersal analysis of vines of this fingerprint

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	+	+	-	+	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.463	0.45	0.423
3	7.465	5.786	4.093
4	0.909	-	-
5	4.773	-	-
6	-	-	-
7	-	-	-

Figure C12: The figure illustrates PCR fingerprint 14, spatial distribution of plants of this specific PCR fingerprint, and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	+	+	-	-	+

	11	12	13	14	15	16	17	18	19
1									
2									
3					2001				
4	2003								
5									
6									
7									
8						2004			
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.463	0.45	0.423
3	0.447	0.429	0.389
4	0.409	-	-
5	0.409	-	-
6	-	-	-
7	-	-	-

Figure C13: The figure illustrates PCR fingerprint 15, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	+	+	-	+	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4					2002				
5									
6					2005				
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.463	0.45	0.423
3	7.465	5.786	4.093
4	0.409	-	-
5	0.409	-	-
6	-	-	-
7	-	-	-

Figure C14: The figure illustrates PCR fingerprint 16, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
-	+	+	-	-	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	5.136	3.958	2.769
3	6.513	5	3.472
4	6.788	-	-
5	2.777	-	-
6	-	-	-
7	-	-	-

Figure C15: The figure illustrates PCR fingerprint 17, spatial distribution of plants of this specific PCR fingerprint, and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	-	-	+	-	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4									
5									
6						2003			
7									
8									2004
9							2003		
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.963	0.45	0.923
3	0.947	5.786	0.889
4	0.909	-	-
5	0.909	-	-
6	-	-	-
7	-	-	-

Figure C16: The figure illustrates PCR fingerprint 18, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	-	-	-	-	+

	11	12	13	14	15	16	17	18	19
1									
2									
3							2002		
4									
5									
6									
7									
8									
9									
10									
11								2003	
12									
13						2005			
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.296	0.283	0.256
3	0.281	0.262	0.222
4	0.242	-	-
5	0.242	-	-
6	-	-	-
7	-	-	-

Figure C17: The figure illustrates PCR fingerprint 19, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
-	-	-	-	-	-

	11	12	13	14	15	16	17	18	19
1							2002		
2									
3									
4			2003						
5									
6		2003							
7		2003							
8									
9									
10									
11									
12									
13							2002		
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.106	0.88	1.143
3	0.09	0.645	0.825
4	982	-	-
5	0.519	-	-
6	-	-	-
7	-	-	-

Figure C18: The figure illustrates PCR fingerprint 20, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
-	-	+	+	-	-

	11	12	13	14	15	16	17	18	19
1								2001	
2									
3									
4								2005	
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	0.106	0.88	1.143
3	0.09	0.645	0.825
4	982	-	-
5	0.519	-	-
6	-	-	-
7	-	-	-

Figure C19: The figure illustrates PCR fingerprint 21, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX C (Continue)

1392/1393	1399/1400	1397/1398	1401/1402	H420/C629	1379/1396
+	+	+	+	-	-

	11	12	13	14	15	16	17	18	19
1									
2									
3									
4									
5									
6									
7									2002
8									
9									2005
10									2002
11									
12									
13									
14									
15									

Morisita Index

p<=0.05	
p<=0.01	
p<=0.001	

	2	3	4
2	7.8	0	0
3	5.786	0	0
4	-	0	0
5	-	0	0
6	-	0	0
7	-	0	0

Figure C20: The figure illustrates PCR fingerprint 22, spatial distribution of plants of this specific PCR fingerprint, year in which symptoms were first detected (blocks without year indicate plants not showing symptoms by 2005), and Morisita index of dispersal analysis of vines of this fingerprint

APPENDIX D

Sample	Sequence	Position
Plant49/12/02	GATTGTTTTGACGTTACCGACTGAATAAGCACCGGCTAAC	40
Plant49/15/03	GatTgTTTTGACGTTACCGACtgAATAAGCACCGGCTAAC	40
Consensus	gattgttttgacgttaccgactgaataagcaccggctaac	
Plant49/12/02	TCTGTGCCANNAGCCGCGGTAATACAGAGGGTGCAAGCGT	80
Plant49/15/03	TCTGTGCCANNAGCCGCGGTAATACAgAGGGTGCAAGCGT	80
Consensus	tctgtgccannagccgcggtaatacagagggtgcaagcgt	
Plant49/12/02	TAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTT	120
Plant49/15/03	TAATCGGAATTACTGGGCGTAAAGcGCGCGTAGGTGGTTT	120
Consensus	taatcggaattactgggcgtaaagCgcgcgtaggtggttt	
Plant49/12/02	GTTAAGTTGAATGTGAAATCCCCGGGCTC..AACCTGGGA	158
Plant49/15/03	GTTAAgTTGAATGTGAAATCCCCGGGCTC..AACCTGGGA	158
Consensus	gttaagttgaatgtgaaatccccgggctc aacctggga	
Plant49/12/02	ACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGT	198
Plant49/15/03	ACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGT	198
Consensus	actgcatccaaaactggcaagctagagtatggtagagggt	
Plant49/12/02	GGTGGAATTTCCTGTGTAGCGGTGAAATGCGGAAATATAG	238
Plant49/15/03	GGTGGAATTTCCTGTGTAGCGGTGAAATGCGGAAATATAG	238
Consensus	ggtggaatttcctgtgtagcggtgaaatgcggaaatatag	
Plant49/12/02	GAAGGAACACCAGTGGNGAAGGCGACCACCTGGACTGATA	278
Plant49/15/03	GAAGGAACACCAGTGGNgAAGGCGACCACCTGGACTGATA	278
Consensus	gaaggaacaccagtggngaaggcgaccacctggactgata	
Plant49/12/02	CTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATT	318
Plant49/15/03	CTGACACTGAGGTGcGAAAGCGTGGGGAGCAAACAGGATT	318
Consensus	ctgacactgaggtgcgaaagcgtggggagcaaacaggatt	
Plant49/12/02	AGATACCCTGGTAGACCACGCCGTAAACGATGTCAACTAG	358
Plant49/15/03	AGATACCCTGGTAGACCACGCCgtAAACgATGTCAACTAG	358
Consensus	agataccctggtagaccacgccgtaaacgatgtcaactag	
Plant49/12/02	CCGTTGGGAGCCTTGAGCT.CTTAGTGGGGCANCTAACGC	397
Plant49/15/03	CCGTTGGGAGCCTTGAGCT.CTTAGTGGGGCANCTAACGc	397
Consensus	ccgttgggagccttgagct cttagtggggcanctaacgc	
Plant49/12/02	.ATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAA	436
Plant49/15/03	.ATTAAGTTGACCGCCTGGGGAGtACGGCCGCAAGGTTAA	436
Consensus	attaagttgaccgcctggggagtacggccgcaaggttaa	
Plant49/12/02	AACTCAAATGAATTGACGGGGCGGGANAANCATCT	471
Plant49/15/03	AACTCAAATGAATTGACGGGGCGGGANAAN.ATCT	470
Consensus	aactcaaatgaattgacggggcggganaan atct	

Figure D1:Multiple alignment of 2 sequences generated from 2 different samples (49/12/02; 49/15/03) for using primer 1386 to the sequencing. Consensus sequence present below the aligned sequences of the plant samples

APPENDIX D (Continue)

Sample	Sequence	Position
Plant49/12/02	GTGTGTGAAGAAGGTCTTCGGATTGT....AAAGCACTTT	36
Plant49/13/09	GTGTGTGAAGAAGGTCTTCGGATTGT....AAAGCACTTT	36
Plant49/17/06	GTGTGTGAAGAAGGTCTTCGGATTGT....AAAGCACTTT	36
Consensus	gtgtgtgaagaaggtcttcggattgt aaagcacttt	
Plant49/12/02	AAGTTGGGAGGAAGGGCAGTTNCCTAA.TACGTGATTGTT	75
Plant49/13/09	AAGTTGGGAGGAAGGGCAGTTaCCTAA.TACGTGATTGTT	75
Plant49/17/06	AAGTTGGGAGGAAGGGCAGTTNCCTAA.TACGTGATTGTT	75
Consensus	aagttgggaggaagggcagtt cctaa tacgtgattgtt	
Plant49/12/02	TTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTNC	115
Plant49/13/09	TTGACGTTACCGACaGAATAAGCACCGGCTAACTCTGTNC	115
Plant49/17/06	TTGACGTTACCGACaGAATAAGCACCGGCTAACTCTGTNC	115
Consensus	ttgacgttaccgacagaataagcaccggctaactctgtnc	
Plant49/12/02	CAGCA.GCCGCGGTAATACAGAGGGTGCAAGCGTTAATCG	154
Plant49/13/09	CAGCA.GCCGCGGTAATaCAGAGGGTGCAAGCGTTAATcG	154
Plant49/17/06	CAGCA.GCCGCGGTAATACAGAGGGTGCAAGCGTTAATCG	154
Consensus	cagca gccgcggtaatacagagggtgcaagcgttaatcg	
Plant49/12/02	GAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTG..TTA	192
Plant49/13/09	GAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTG..TTA	192
Plant49/17/06	GAATTACTGGGCGTAAAGCGcGCGTAGGTGGTTTG..TTA	192
Consensus	gaattactgggcgtaaagcgcgcgtaggtggtttg tta	
Plant49/12/02	AGTTGAATGTGAAATCCCCGGGCTCAACCTGGGAAC.TGC	231
Plant49/13/09	AGTTGAATGTGAAATCCCCGGGCTCaACCTGGGAAC.TGC	231
Plant49/17/06	AGTTGAATGTGAAATCCCCGGGCTCAACCTGGGAAC.TGC	231
Consensus	agttgaatgtgaaatccccgggctcaacctgggaac tgc	
Plant49/12/02	ATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTGGTGG	271
Plant49/13/09	ATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTGGTGG	271
Plant49/17/06	ATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTGGTGG	271
Consensus	atccaaaactggcaagctagagtatggtagagggtggtgg	
Plant49/12/02	AATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGG	311
Plant49/13/09	AATTTCctGTGTAGCGGTGAAATGCGTAGATATAGGAAGG	311
Plant49/17/06	AATTTCCtGTGTAGCGGTGAAATGCGTAGATATAGGAAGG	311
Consensus	aatttcctgtgtagcggtgaaatgcgtagatataggaagg	
Plant49/12/02	AACACCAGTGGNGAAGGCGNCCNCCTGGACTGATACTGAC	351
Plant49/13/09	AACACCAGTGGNGAAGGCgNCCNCCTGGACTGATAcTGaC	351
Plant49/17/06	AACACCAGTGGNGAAGGCgNCCNCCTGGACTGATAcTGAC	351
Consensus	aacaccagtggngaaggcgnccncctggactgataCtgac	
Plant49/12/02	ACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATN	391
Plant49/13/09	ACTGAGGTGcGAAAgCGTGGGGAGCAAACAGGATTAGATN	391
Plant49/17/06	ACTGAGGTGCGAAAgCGTGGGGAGCAAACAGGATTAGATN	391
Consensus	actgaggtgcgaaagcgtggggagcaaacaggattagatn	
Plant49/12/02	CCCTGGTAGTCCACGCCGTAAACGATGTCAAcTAGCCGTT	431
Plant49/13/09	CCCTGGTAGTCCACGCcGTAAaCGATGTCAAcTAgCCgTT	431
Plant49/17/06	CCCTGGTAGTCCACGCCGTAAACGATGTCAAcTAGCCGTT	431
Consensus	ccctggtagtccacgccgtaaacgatgtcaactagccgtt	
Plant49/12/02	GGGAGCCTTGAGCTNTTAGTGGCGCAGCTAACGCATTAAG	471
Plant49/13/09	GGGAGCCTTgAgcTNTTAGTGGCGCAGCTAACGCATTAAG	471
Plant49/17/06	GGGAGCCTTGAGCTNTTAGTGGCGCAGCTAACGCATTAAG	471
Consensus	gggagcCttgagctnttagtggcgcagctaacgcattaag	

APPENDIX D (Continue)

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Plant49/12/02          TTGACCNCCTGGGGAGTA                    488
Plant49/13/09          TTGACCNCCtGGGGAGtA                    489
Plant49/17/06          TTGACCNCCtGGGGAGTA                    489
Consensus              ttgaccncctggggagta
```

Figure D2:Multiple alignment of 3 sequences generated from 3 different samples (49/12/02; 49/13/09, 49/17/06) for using primer 1387 to the sequencing. Consensus sequence present below the aligned sequences of the plant samples

Sample	Sequence	Position
Plant49/12/02	TATCTACGCATTTCACCGCTACACAGGAAATTCCACCACC	345
Plant49/13/09	TATCTACGcATtTCaCCgctACaCAgGAAATTCCaCcaCC	360
Consensus	tatctacgcatttcaccgctacacaggaaattccaccacc	
Plant49/12/02	CTCTACCATACTCTAGCTTGCCAGTTTTGgATGCAGTTCC	385
Plant49/13/09	CTCTACCaTACTCTAgcTTGcCaGtttTGgATGCAGTTCC	400
Consensus	ctctaccatactctagcttgccagttttggatgcagttcc	
Plant49/12/02	cAGGTTGAGCCCGGGGATTTCACaTNCAACTTAACAAACC	425
Plant49/13/09	cAgGttGAGCCCgGGGATtTCACaTNCaACTTAaCAAAcC	440
Consensus	caggttgagcccggggatttcacatncaacttaacaaacc	
Plant49/12/02	ACCTACGCGCGCTTTACGCCcAGTAATTCCGATTAACGCT	465
Plant49/13/09	ACCTACgCgCGCTTTaCGCCcAGtAATTcCgAtTAACGCT	480
Consensus	acctacgcgcgctttacgcccagtaattccgattaacgct	
Plant49/12/02	TGCACCCTCTGTATTACCGCGGcTGCTGGCACAGAGTTAG	505
Plant49/13/09	TGCaCCCTCTGTATTACCGCGGcTGCTGGcACAGAGTTAG	520
Consensus	tgcaccctctgtattaccgcggctgctggcacagagttag	
Plant49/12/02	CCGGTGCTTATTCTGTCgGCTCTCANAGGCGNGNACANAA	545
Plant49/13/09	CCGGTGCTTATTCTGTCgGCTCTCANAGGCGNgNACANAA	560
Consensus	ccggtgcttattctgtcggctctcanaggcgngnacanaa	
Plant49/12/02	TAAGCACGGGCNACAACTCTGTGNCTTCGGCCNGTGCTCA	585
Plant49/13/09	TAAgCACgggc.ACAactCtgtgNCTTCGgccNgTgCTCA	598
Consensus	taagcacgggc ccacctctgtgncttcggccngtgctca	

Figure D3:Multiple alignment of 2 sequences generated from 2 different samples (49/12/02; 49/13/09) for using primer 1388 to the sequencing. Consensus sequence present below the aligned sequences of the plant samples

ABBREVIATIONS

AlkB:	Alkylate DNA repair protein
ArmV:	Arabis mosaic virus
BYV:	Beet Yellows virus
cDNA:	Complementary DNA
CP:	Coat protein
CPd:	Diverged coat protein
CPm:	Minor coat protein
CP-MR:	Coat protein-mediated resistance
CTV:	Citrus tristeza virus
CYSDV:	Cucurbit yellowing stunting disorder virus
DAS:	Double antibody sandwich
D-RNA:	Defective RNA
dNTP:	Deoxyribonucleotide triphosphate mix
DTT:	Dithiotreitol
dsRNA:	Double-stranded RNA
EDTA:	Ethylenediaminetetraacetic acid disodium salt
ELISA:	Enzyme-linked immunosorbent assay
EtBr:	Ethidium bromide
EtOH:	Ethanol
GAR-AP:	Goat anti-rabbit globulin/alkaline phosphatase
GFKV:	Grapevine fleck virus
GFLV:	Grapevine fanleaf virus
GLD:	Grapevine leafroll disease
GLRaV-1:	Grapevine-leafroll associated type 1 virus
GLRaV-2:	Grapevine-leafroll associated type 2 virus
GLRaV-3:	Grapevine-leafroll associated type 3 virus
GLRaV-4:	Grapevine-leafroll associated type 4 virus
gRNA:	Genomic RNA
GVA:	Grapevine virus A
GBV:	Grapevine virus B
HC:	Healthy control
HEL:	Helicase
HMA:	Heteroduplex mobility assay

ABBREVIATIONS (CONTINUE)

HPRI RNAse:	Human placenta ribonuclase inhibitor
HSP:	Heat-shock protein
IC:	Immunocapture
IC/RT PCR:	Immunocapture reverse transcription PCR
ICGV:	International Council for the Study of Virus and Virus-like diseases of the Grapevine
ICTV:	International Committee on Taxonomy of Viruses
ISEM:	Immunosorbent electron microscopy
Kb:	Kilobases
LChV:	Little cherry virus
LIYV:	Lettuce infectious yellow virus
miRNA:	MicroRNA
M-MLV:	Moloney-murine leukemia virus
MP:	Movement protein
mRNA:	Messenger RNA
MET:	Methyltransferase
MT:	Methyltransferase like domain
NaOAC:	Sodium acetate
Nt:	Nucleotides
OLYaV:	Olive leaf yellowing associated virus
ORF:	Open reading frame
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PDR:	Pathogen-derived resistance
PMWAV-2:	Pineapple mealybug wilt associated virus
PVP:	Polyvinylpyrrolidone
RdRp:	RNA dependent RNA polymerase
RFLP:	Restriction length polymorphism
RISC:	RNA-induced silencing complex
RNA:	Ribonucleic acid
RpRSV:	Rasberry ringspot virus
RT:	Reverse transcriptase

ABBREVIATIONS (CONTINUE)

RT-PCR:	Reverse transcriptase PCR
SA:	South Africa
SB:	Sodium boric acid
Sg:	Subgenomic
SgRNA:	Subgenomic RNA
siRNA:	Small interfering RNA
SSCP:	Single-stranded conformation polymorphism
TMV:	Tobacco mosaic virus
TRSV:	Tobacco ringspot virus
UTR:	Untranslated region