CHARACTERIZATION OF *POTATO VIRUS Y* (*PVY*) ISOLATES INFECTING SOLANACEOUS VEGETABLES IN KWAZULU-NATAL (KZN), REPUBLIC OF SOUTH AFRICA (RSA)

By

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Abstract

Potato virus Y (*PVY*) is an economically important virus worldwide. In South Africa, *PVY* has been shown to be a major limiting factor in the production of important solanaceous crops, including potato (*Solanum tuberosum* L.), pepper (*Capsicum annuum* L.), tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana* spp). The variability that *PVY* displays, wherever the virus occurs, merits the study of the isolates occurring in KwaZulu-Natal (KZN) in the Republic of South Africa (RSA). This characterization will provide a clear understanding of strains/isolates from local vegetables and how they relate to the other *PVY* strains already identified, as well as information that can be used to manage the diseases they cause. Hence, the aim of this project was to study the biological and genetic properties of *PVY* isolates infecting potato, tomato and pepper in KZN. Enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies and reverse transcription polymerase chain reaction (RT-PCR) using primers specific to all *PVY* strains were used to detect the virus in plant material showing *PVY*-like symptoms collected from various locations in KZN. A total of 39 isolates (18 isolates infecting tomato, 12 infecting potato and 9 infecting pepper) were further differentiated into strains by means of ELISA using strain specific antibodies and RT-PCR using primers specific to the different strains of *PVY* identified around the world. All *PVY* isolates infecting tomato and pepper tested positive for the ordinary PVY^{O} strain with both ELISA and RT-PCR. *PVY* isolates infecting potato were more diverse and comprised the PVY^{N}, PVY^{NTN} and PVY^{N}Wilga strains, with mixed infections noted in some cases. The biological properties were studied by mechanically inoculating *Chenopodium quinoa*, *Nicotiana tabacum* cv Xanthi, *N. tabacum* cv Samsun, *N. glutinosa*, and *N. rustica* with leaf extracts from plants infected with the different *PVY* strains detected in this study. All inoculated *C. quinoa* plants did not show symptoms. All tobacco plants showing symptoms were tested for the presence of *PVY* by means of ELISA using monoclonal antibodies targeting all strains and electron microscopy using the leaf dip technique. Not all the inoculated tobacco tested positive with ELISA. The symptoms observed were therefore divided

into *PVY*-related and *PVY* non- related. *PVY*-related symptoms included vein clearing, mosaic chlorosis, stunting, and vein necrosis. *PVY* non-related symptoms included wrinkles and leaf distortions. Potyvirus-like particles of about 700 nm were observed under the transmission electron microscope (TEM) from plants showing *PVY*-related symptoms while rod shaped viral particles of sizes varying between 70 and 400 nm were observed from plants showing non-*PVY* related symptoms. A portion of the virus genome (1067 bp) covering part of the coat protein gene and the 3’ non-translated region (NTR) of three PVY^{O} isolates infecting tomato, one PVY^{O} isolate infecting pepper and one PVY^{N}Wilga isolate infecting potato were amplified, cloned and sequenced. The 5’ NTR, P1, HC-Pro and part of P3 regions (2559 bp) of a PVY^{N} isolate infecting potato were also amplified, cloned and sequenced. Sequence data was compared with selected *PVY* sequences from different geographical locations around the world. These were available on the NCBI website and subsequently used for phylogenic analyses. The sequenced genomic regions of the PVY^{N} isolate were found to be 99% similar to the New Zealand PVY^{N} isolate (GenBank accession number: AM268435), the Swiss PVY^{N} isolate CH605 (X97895) and the American PVY^{N} isolate Mont (AY884983). Moreover, the deduced amino acid sequence comparison of the genomic regions of the PVY^{N} isolate revealed the presence of five distinct amino acids residues. The three amino acid residues (D_{205}, K_{400}, and E_{419}), which determine the vein necrosis phenotype in tobacco, were also identified. The coat protein and 3’ NTR sequences of all KZN PVY^{O} isolates infecting pepper and tomato were closely similar to each other than to KZN PVY^{N}Wilga isolate infecting potato. The phylogenic analysis clustered the KZN PVY^{N} isolate with the European sublineage N, PVY^{N}Wilga isolate infecting potato with the American PVY^{O} isolate Oz (EF026074) in the O lineage and all PVY^{O} isolates infecting tomato and pepper in a new sublineage within the O lineage. Taken together, these results point to the presence of *PVY* in solanaceous vegetables cultivated in KZN and they lay the foundation for the formulation of effective control measure against *PVY* diseases in KZN.

Keywords

Potato virus y, KwaZulu-Natal, Republic of South Africa, Tomato, Pepper, Potato, DAS-ELISA, TAS-ELISA, RT-PCR, Strain Differentiation, Host indexing, Negative Stain, Cloning, Sequencing, Phylogenic analyses.

Preface

The experimental work described in this dissertation was carried out in the School of Agricultural Science and Agribusiness, University of KwaZulu Natal, Pietermaritzburg from February 2008 to September 2009, under the supervision of Dr Augustine Gubba.

These studies represent original work by the author and have not otherwise been submitted in any form for degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Declarations

I, Jacques Davy Ibaba, declare that:

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Dedication

All those who from far or near have contributed in any way whatsoever towards the completion of this work.

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List of abbreviations

°C	:	Degree Celsius
%	:	Percent
+C	:	Positive control
-C	:	Negative control
A	:	Adenine
AAP	:	Acquisition access period
B	:	Buffer
C	:	Cytosine
cDNA	:	Complementary DNA strain
CI	:	Cytoplasmic inclusion
Chl	:	Chlorosis
cm	:	Centimetre
CMV	:	*Cucumber mosaic virus*
CP	:	Coat protein
cv	:	Cultivar
DAS	:	Double antibody sandwich
Dl	:	Dead leaves
DIBA	:	Dot immune assay
DNA	:	Deoxyribonucleic acid

dpi	:	Days post inoculation
E. coli	:	*Escherichia coli*
EIAs	:	Enzyme-based immunoassays
ELISA	:	Enzyme-linked immunosorbent assay
ELOSA	:	Enzyme-linked oligosorbent assay
FM	:	Faint mottling
G	:	Guanine
HC-Pro	:	Helper component - proteinase
hrs	:	hours
IAP	:	Inoculation access period
IPTG	:	Isopropyl-β-D-thiogalactopyranoside
ISEM	:	Immunosorbent electron microscopy
kb	:	Kilobase
KZN	:	KwaZulu-Natal
LB	:	Luria-Bertani
LD	:	Leaf distortion
M	:	Molar
Mab	:	Monoclonal antibody
ME	:	Minimum evolution
min	:	Minute
ML	:	Maximum likelihood

ml	:	Millilitre
Mo	:	Mosaic
MP	:	Maximum parsimony
NAB	:	Nucleic acid-based
NIa	:	Nuclear inclusion a
Nib-Pol	:	Nuclear inclusion b and RNA dependant RNA polymerase
NJ	:	Neighbour joining
nm	:	Nanometer
NTR	:	Non translated region
Pab	:	Polyclonal antibody
pNPP	:	4-Nitrophenyl phosphate disodium salt hexahydrate
PTGS	:	Post transcriptional genes silencing
PTNRD	:	Potato tuber necrotic ringspot disease
PVY	:	*Potato virus Y*
rpm	:	Revolution per minute
RNA	:	Ribonucleic acid
RSA	:	Republic of South Africa
RFLP	:	Restriction fragment length polymorphism
RT-PCR	:	Reverse transcription polymerase chain reaction
s	:	Second
SMo	:	Severe mosaic

T	:	Thymine
Ta	:	Annealing temperature
TAE	:	Tris-acetate-EDTA
TAS	:	Triple antibody sandwich
TBIA	:	Tissue blotting immunoassay
TEM	:	Transmission electron microscope
TMV	:	*Tobacco mosaic virus*
TSWV	:	*Tomato spotted wilt virus*
U	:	Uracil
µg	:	Microgram
UK	:	United Kingdom
UPGMA	:	Unweighted pair group method using arithmetic average
µl	:	Microliter
VC	:	Vein clearing
VC + M	:	Vein clearing and mottling
VN	:	Vein necrosis
VPg	:	Viral genome linked protein
Wr	:	Wrinkle
X-gal	:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Foreword

Accurate detection and characterization of plant pathogens are essential in formulating effective control strategies against the diseases they cause. The study of plant viruses aims at a better understanding of crop-damaging viruses in order to provide effective and durable control strategies. The discipline of plant virology started at the end of the 19th century with the discovery of organisms smaller than bacteria able to cause disease. Since then a number of techniques have been developed for the detection, characterization and control of plant viruses.

Vegetable and fruit crops grown in KwaZulu-Natal (KZN), like other plants on the surface of the planet, are not exempt from viral diseases. Plant viruses are a serious, constant threat to agricultural production due to the damages (direct and indirect) they cause. The Plant Virology Research Unit at the University of KwaZulu-Natal (UKZN), Pietermaritzburg Campus, focuses on the identification and molecular characterization of the viruses infecting important vegetable crops cultivated in the Republic of South Africa (RSA) with the ultimate aim of devising sustainable control strategies against these viruses, and the diseases they cause.

Potato virus Y (*PVY*) is an important virus of *Solanaceous* plants causing disease in potato (*Solanum tuberosum* L.), pepper (*Capsicum annuum* L.), tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana* spp). *PVY* is known for the genetic variability it displays, depending on the host, and the geographical location in which it occurs. *PVY* in RSA has been reported in areas where susceptible crops are cultivated. However, the biological and molecular properties of isolates of *PVY* are not well documented. The present study therefore seeks to fill this information gap in the literature regarding the virus genetic profile of *PVY* isolates occurring in KZN.

Chapter 1
Literature Review

1.1. Introduction

Plant pathogens are a serious concern in global food security, especially in less developed countries. The damage they cause in the field and during post-harvest varies from mild symptoms to heavy crop losses. Damage caused by plant pathogens has been estimated to reduce the global food production by 10% (Strange & Scott, 2005). Plant pathogens have been a challenge to agricultural production from the early stages of its development, when man started domesticating crop plants. The separation of crop species from their wild environment into agro-ecosystems has provided a platform for the emergence of new pathogens and the rapid evolution of the pathogen populations already existing in the wild ancestor of the cultivated crop (Stukenbrock & McDonald, 2008). Plant pathogens are grouped into viruses, bacteria, fungi, nematodes and parasitic plants (Strange & Scott, 2005).

Viruses are sub microscopic organisms made up of a set of one or more nucleic acid templates generally enclosed in a protective coat(s) of lipoprotein. They are considered to be either the vestiges of a pre-cellular world, the product of the regressive evolution of complex organisms, or genetic elements of endogenous origin (Astier *et al.*, 2007). They are obligate intracellular parasites since their replication only takes place inside a suitable living host cell and totally depends on the resources of the infected cell (Hull, 2002). A successful virus infection requires a battery of host- virus interactions that can be grouped into different stages of a replication cycle. These comprise, in consecutive order: entry into the cell, disassembly of the virus capsid(s), genome replication and transcription, encapsidation and cell to cell movement (Astier *et al.*, 2007; Nagy, 2008). Plant viruses, unlike fungi and bacteria, generally cause losses that are more insidious and frequently less conspicuous. This makes the loss assessment ambiguous and results in numerical figures that are, most of the time, below the level of actual damage. Nonetheless, many viruses are well

known for their economic importance in agriculture (Hull, 2002; Waterworth & Hadidi, 1998).

Potato virus Y (PVY) is the type-member of the genus *Potyvirus* in the *Potyviridae* family, the largest plant virus family recognized (Rigotti & Gugerli, 2007) containing some of the most damaging plant viruses. The *Potyvirus* genus represents the major genus of the six genera that compose the family and one of the two largest plant virus genera. It is characterized by a broad range of hosts that include both monocotyledonous and dicotyledonous plants. The genus also belongs to the supergroup of picorna-like viruses based on their genome expression (Astier *et al.*, 2007; Gibbs *et al.*, 2008; Shukla *et al*., 1994; Urcuqui-Inchima *et al.*, 2001). *PVY* was named by Smith (1931) from his studies on the mosaic diseases of potato. Nowadays *PVY* is among the five most economically damaging viruses (Rolland *et al*., 2008), with a host range including major crops, such as pepper (*Capsicum annuum* L.), potato (*Solanum tuberosum* L.), tobacco (*Nicotiana* spp), tomato (*Lycopersicon esculentum* Mill.), less important plants and several species of weed mainly in the *Solanaceae* family (Kerlan & Moury, 2008).

PVY particles are non-enveloped flexuous filaments (730 x 11 nm) containing a single positive single-strand positive sense ribonucleic acid (RNA) of about 9.7 Kb in length, polyadenylated at the 3'end, and covalently linked via a tyrosine residue to a genome linked protein at its 5' end. PVY encodes a single, large polyprotein which is later processed by three virus-encoded proteinase into nine polypeptides (Figure 1.1) which include the following: P1, Helper component-proteinase (HC-Pro), P3, 6K1, cytoplasmic inclusion (CI), 6K2, nuclear inclusion a (NIa), nuclear inclusion b and RNA-dependant RNA polymerase (NIb-Pol) and the capsid protein (CP) (Hu *et al*., 2009; Urcuqui-Inchima *et al*., 2001). The functions of these proteins are summarized in Table 1.1.

Table 1.1. Function of *PVY* proteins (Urcuqui-Inchima *et al.*, 2001).

Proteins	Size (KDa)	Functions
P1	32-64	Trypsin-like serine proteinase involved in C terminal autocleavage and in symptomatology.
HC-Pro	50	Multifunctional protein involved in C terminal autocleavage, local and systemic movement, gene silencing suppression, aphid transmission, synergism and symptom development.
P3	37	Involved in plant pathogenecity.
6k1	6	Function still unknown.
CI	70	The protein displays an ATPase and RNA helicase that are involved in local movement of the virus.
6k2	6	Attaches viral replication complex to endoplasmic reticulum-like membranes.
NIa	49	Trypsin-like serine proteinase that processes the polyprotein in *cis* and *trans* to produce functional proteins. It is involved in genome replication (VPg) and protein-protein interaction.
NIb-Pol	58	RNA-dependent RNA polymerase involved in genome replication.
CP	30	Multifunctional protein involved in virus assembly, local and systemic movement and aphid transmission.

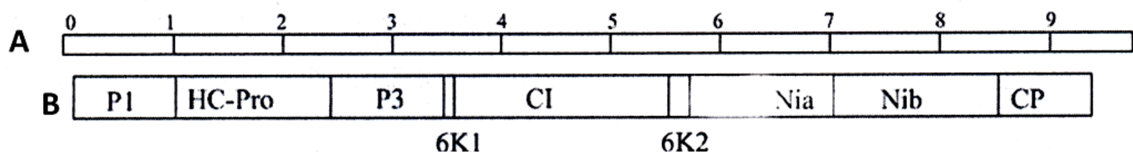


Figure 1.1. *PVY* genome organisation. **A**: Genome length in kb; **B**: Polypetides (Hu *et al.*, 2009).

PVY has been reported in almost all parts of the world where its natural hosts occur. Nowadays the virus is well known for the high variability it displays in terms of strains, pathotypes and serotypes. This variability is mainly the result of recombination processes that take place over time as the virus evolves. The following literature review will focus on the diversity of PVY, the different methods available for detection and differentiation of strains, and lastly, the epidemiology of the virus and the different ways of managing the disease.

1.2. Diversity of Potato virus Y (PVY)

The variability of *PVY* has resulted in its differentiation into strains. The primary classification of *PVY* was based on the host plant the virus was isolated from. This led to the grouping of *PVY* into potato, pepper, tobacco and tomato strains. Isolates in each group are further characterized on the basis of biological (symptoms and resistance response), serological and molecular properties (Jacquot *et al.*, 2005; Rolland *et al*., 2008; Tribodet *et al.*, 2005).

1.2.1. Potato strains

PVY infecting potato has been the group that displays the greatest variability of strains. It consists of several strains that are grouped into three main strain groups, known as O, C and N (Kerlan & Moury, 2008).

1.2.1.1. O strain (*PVY*^{O})

The O strain group is the most widely-spread group (Ogawa *et al.*, 2008; Singh *et al.*, 2008). *PVY*^{O} is identifiable by the hypersensitive resistance response it induces on potato cultivars harbouring the *Ny* genes. In term of symptoms, it generally induces mild to severe mosaic, crinkle, leaf and stem necrosis in potato, but mottling and mosaic in tobacco. The strain is present in Africa, Europe, New Zealand and South America (Lorenzen *et al.*, 2006; Rigotti & Gugerli, 2007; Rolland *et al.*, 2008).

1.2.1.2. C strain (*PVY*^{C})

Previously known as *Potato virus C* (PVC), it was renamed as *PVY*^{C} after the discovery of resistance genes based on the hypersensitive resistance response it produced on potato cultivars having the *Nc* gene. It is also known as the stipple streak group since streaking is the main symptom it induces in susceptible potato (Ogawa *et al.*, 2008; Rolland *et al.*, 2008; Singh *et al.*, 2008). *PVY*^{C} isolates were recently divided into C1 and C2 strains based on molecular studies of the coat protein sequences (Blanco-Urgoiti *et al.*, 1998; Boonham *et al.*, 2002a). This strain has been reported in America, Europe, New Zealand and South Africa (Lorenzen *et al.*, 2006).

1.2.1.3. N strain (*PVY*^{N})

The N strain is the tobacco veinal necrosis group. Early reports of this strain dates back to the 1940 – 50s from Europe and South America (Singh *et al.*, 2008). The current geographic distribution also includes Africa and New Zealand. The systemic veinal necrosis symptoms induced in *Nicotiana tabacum* is the main characteristic of the strain. It does not induce a hypersensitive resistance reaction in the presence of both *Nc* and *Ny* genes in potato, but induces mild mottling instead (Jacquot *et al.*, 2005; Kogovsek *et al.*, 2008; Lorenzen *et al.*, 2006; Ogawa *et al.*, 2008; Rigotti & Gugerli, 2007; Rolland *et al*., 2008; Singh *et al.*, 2008). This group also includes *PVY*^{NTN} and *PVY*^{N}Wilga strains (Ogawa *et al.*, 2008).

1.2.1.4. NTN strain (*PVY*^{NTN})

PVY^{NTN} strain is the tuber necrosis strain group. It was first described in Hungary in the 1980s (Ogawa *et al.*, 2008). *PVY*^{NTN} is related to *PVY*^{N} at a serological level. The ability to induce potato tuber necrotic ringspot disease (PTNRD) was the major characteristic that led to its differentiation from the other known strains. PTNRD is characterized by the appearance of external necrotic rings on tubers which may appear at harvest time but often develop under storage conditions. On tobacco, it causes veinal necrosis symptoms similar to *PVY*^{N} (Kogovsek *et al.*, 2008; Rolland *et al*., 2008).

However, the difficulty of identifying the sequence responsible for the development of PTNRD, and more especially the high variability in inducing necrotic rings on tubers recently recorded in the field and greenhouse, has brought the significance of this feature into question. The rate of necrotic ring formations on tubers ranges between 50 and 70% in the field and does not occur on all infected tubers. Moreover, the discovery of symptomless PVY^{N} tubers from fields that exhibit tuber necrosis in greenhouse experiments led to the conclusion that PTNRD is a complex phenomenon which needs further studies (Ali *et al.*, 2008; Singh *et al.*, 2008).

Studies of the genome of different NTN isolates allowed their classification into subgroups mostly based on their geographical distribution. A second factor associated with that strain is the presence or absence of recombination. PVY^{NTN} isolates with no recombination have been identified in North America, Denmark, Germany, Poland and Japan (Singh *et al.*, 2008). Most recombinant PVY^{NTN} have been identified in Europe and the term Eu-PVY^{NTN} has been used to distinguish them from the non recombinant ones. Eu-PVY^{NTN} generally consists of a genome that displays PVY^{N} and PVY^{O} like sequences, with one to three recombination junctions. P1, HC-Pro, NIa and coat protein are the regions of the genome where recombination points were found (Kogovsek *et al.*, 2008; Lorenzen *et al.*, 2006).

1.2.1.5. Wilga Strain

The name of this strain originated from the Polish cultivar on which it was first identified (Rigotti & Gugerli, 2007). PVY^{N}Wilga strain (PVY^{N}W; *PVY*N^{-Wi}) emerged and spread in the 1980s. PVY^{N}Wilga genome consists of PVY^{O} and PVY^{N} sequences that show recombinantion points in P1, HC-Pro and NIa. Thus its other name of $PVY^{N:O}$ is used in North America (Ali *et al.*, 2008; Ogawa *et al.*, 2008). As a result, PVY^{N}W is serologically related to the PVY^{O} strain but possesses the biological properties of the PVY^{N} strain (Boonham *et al.*, 2002a; Glais *et al.*, 2005; Lorenzen *et al.*, 2006). It is thought to be more infectious than O strain but its symptoms in potato are less severe than those caused by standard PVY^{N} (Kogovsek *et al.*, 2008). Wilga isolates

from Europe differ from North American isolates in that North American isolates induce a lethal necrotic reaction in *Solanum brachycarpum* (Schubert *et al.*, 2007).

1.2.1.6. Z and E strain

PVY Z strain (PVY^Z) was proposed to distinguish the isolates serologically classified as PVY^O which have overcome the resistance genes against both PVY^O and PVY^C but are unable to overcome the proposed *Nz* gene in the potato cultivar "Maris Bard" (Kerlan *et al.*, 1999). PVY^Z has been identified in Great Britain (Aramburu *et al.*, 2006). It does not induce necrosis in tobacco (Kerlan *et al.*, 1999; Singh *et al.*, 2008). A variant strain of PVY^Z known as *PVY* E strain (PVY^{ZE}; PVY^E) which overcomes the proposed *Nz* gene has been identified in Spain (Kerlan *et al.*, 1999; Singh *et al.*, 2008).

1.2.2. Pepper strains

PVY isolates infecting pepper were classified on the basis of their response against the recessive resistant genes $pvr2^1$ and $pvr2^2$ in *Capsicum annuum* L. This has led to the identification of three distinct pathotypes, namely (0), (0,1) and (0,1,2). Pathotype 0 is unable to overcome both resistant genes, therefore can only infect genotypes lacking these genes. Pathotype (0,1) infects plants having $pvr2^1$ gene. Pathotype (0,1,2) has overcome both resistance genes (Kerlan & Moury, 2008; Singh *et al.*, 2008). At a serological level, most pepper isolates were found to be closely related to PVY^O and PVY^C strains from potato, but no relationship was found between pathotypes and serotypes (Aramburu *et al.*, 2006). Studies of the coat protein sequences of these pathotypes did not show significant differences (Romero *et al.*, 2001).

Biological studies of pepper isolates definitely distinguish them from potato isolates. Pepper isolates do not infect potato mechanically and vice versa (Singh *et al.*, 2008). However some potato isolates showed limited ability to infect pepper when inoculated with aphids. Moreover, monoclonal antibodies used to detect potato strains do not detect pepper isolates (Romero *et al.*, 2001).

1.2.3. Tobacco strains

A pathotypic classification of tobacco isolates, which is elaborated on the basis of the symptoms developed in *Nicotiana tabacum* cultivars susceptible or resistant to root nematodes, distinguishes three main strain groups. M^SN^R (MsNr or MN) group includes isolates that induce necrosis in tobacco plants harbouring the dominant root-knot nematode resistant gene *Rk*. M^SM^R strain causes mosaic symptoms and N^SN^R strain causes necrotic symptoms in both susceptible and resistant cultivars (Aramburu *et al.*, 2006; Kerlan & Moury, 2008; Singh *et al.*, 2008). However, little is known on the differential interactions of these pathotypes against *Ny* and *Nc* genes in potato (Singh *et al.*, 2008). Phylogenic studies of the coat protein of the few identified M^SN^R and N^SN^R isolates revealed a close relationship with the potato PVY^C strain group (Singh *et al.*, 2008).

PVY-infecting potato strains are also able to infect tobacco. They are divided into two distinct phenotypes (mosaic and veinal necrosis) depending on the symptoms they generally produce on tobacco. The mosaic phenotype comprises PVY^O and PVY^C while PVY^N, PVY^{NTN} and PVY^NW are of the veinal necrosis phenotype (Ali *et al.*, 2008; Singh *et al.*, 2008).

1.2.4. Tomato strains

Tomato appears to be the crop lacking a defined classification of PVY isolates similar to those described for potato, pepper and tobacco. The *pot1* gene from *Lycopersicon hirsutum*, a wild relative of tomato, was found to confer resistance to *PVY* in a way similar to the *pvr2* alleles in pepper but has not yet been used for classification purposes (Moury *et al.*, 2004; Singh *et al.*, 2008). PVY^O and PVY^C induce crinkle on young leaves then necrotic mottling with sometimes veinal necrosis on the back of leaves and symptomless fruits, while PVY^N produces severe mosaic often with interveinal yellow spots and whitish spot on fruits (Aramburu *et al.*, 2006).

1.2.5. *PVY*: An ever-growing diversity

Reports of emergence of new or variant strains have become a common feature with *PVY*, especially from isolates infecting potato. Molecular biology studies and differential interactions against resistance genes are the driving factors behind this emergence of new strains. A 23 amino acid long region in the viral genome linked protein (VPg) where found to control the interactions with the *pvr2* genes. Further studies of the variability of that region on the virulence of viral variants in pepper genotypes carrying different *pvr2* alleles distinguished a total of eight pathotypes (Singh *et al.*, 2008). The NE-11 PVY isolate, previously classified as a North American NTN strain, was reclassified as a new strain variant class based on its genome sequence (Lorenzen *et al.*, 2008).

This phenomenon has started raising the question of defining an efficient system of *PVY* classification. Blanco-Urgoiti *et al.,* (1996) proposed a classification based on the restriction fragment length polymorphism assay (RFLP) pattern of the coat protein gene. According to their findings, *PVY* isolates were grouped into three main clusters which are potato PVY^{O}, potato PVY^{C} and non potato *PVY* (PVY^{NP}) that comprises pepper, tobacco and *Datura* spp isolates. Singh *et al.* (2008) suggested keeping tobacco and the differential potato cultivar assay as the standard description of potato isolates.

1.3. Methods used in detection and characterization of plant viruses

Phytopathologists nowadays have a broad spectrum of techniques available to characterize pathogenic microorganisms. Scientific discovery and technological innovation have been the main driving factors of this diversity. Method used to study plant viruses are grouped into serological assays, nucleic acid based techniques, biological indexing and electron microscopy (Boonham *et al*., 2007; Bos, 1999; Maroon-Lango, 2004; Schaad *et al*., 2003; Webster *et al.,* 2004). Specificity sensitivity, rapidity, cost effectiveness, robustness, ease of use, guarantee of

infectivity are generally the criteria taken into consideration when selecting any particular method (Bos, 1999; Maroon-Lango, 2004).

1.3.1. Serological methods

Serological detection, also known as immuno chemical techniques, involves the use of antibodies (monoclonal or polyclonal) raised against specific antigens (Albrechtsen, 2006; Bos, 1999; Maroon-Lango, 2004). It was originally developed for the detection of viruses which, unlike bacteria and fungi, cannot be cultured (Schaad *et al*., 2003). Earlier reports of these methods date back to the 1970s. Serological detection comprises several techniques that can be divided into two groups (Table 2); however Enzyme-linked immunosorbent assay (ELISA), Dot immunoblot assay (DIBA), and Immunosorbent electron microscopy (ISEM) have been the techniques frequently used in the detection of *PVY* (Ali *et al*., 2007; Aramburu *et al*., 2006; Cardin and Moury, 2008; Crescenzi *et al*., 2005; DianQiu *et al*., 2006; Fanigliulo *et al.*, 2005; Hu *et al*., 2009; Kerlan *et al*., 1999; Kogovsek *et al.*, 2008; Llave *et al*., 1999; Lorenzen *et al.*, 2006; Margaritopoulos *et al.*, 2009; Mijatović *et al.*, 2002; Schaad *et al*., 2003).

Table1. 2. Groups of serological techniques

Serological detection	
Enzyme-based immunoassays (EIAs)	Other than EIAs
ELISA	Agglutination
DIBA	Gel-diffusion
Tissue blotting immunoassay (TBIA)	Precipitin

1.3.1.1. Enzyme-linked Immunosorbent Assay (ELISA)

Clark and Adams (1977) were the pioneers of ELISA. Since its introduction, ELISA has been recognized as the most widely used serological method in plant virology (Albrechtsen, 2006; Bos, 1999; Schaad *et al*., 2003; Webster *et al.,* 2004). High sensitivity, ease of use, speed, cost effectiveness and the ability to quantify pathogen are the different characteristics that contributed to the successful use of the

technique (Clark, 1981; Maroon-Lango, 2004; Miller and Martin, 1988; Webster *et al.,* 2004). ELISA is divided into direct and indirect ELISA.

Direct ELISA, also called Double-antibody Sandwich (DAS) ELISA, is generally performed inside the well of a polystyrene microtiter plate. The principle consists of binding an antibody (either coated or uncoated) specific to an antigen to the solid phase, then adding sequentially the test sample, enzyme-labelled antibody (conjugate) and substrate enzyme (Figure 1.2). A positive test is characterized by the formation of the complex antibody, antigen, labelled antibody which is reflected through the change in colour of the substrate solution. Furthermore, spectrophotometric analysis of the intensity of the colour in a positive reaction allows the determination of the antigen concentration present in the test sample (Albrechtsen, 2006; Clark, 1981; Miller and Martin, 1988).

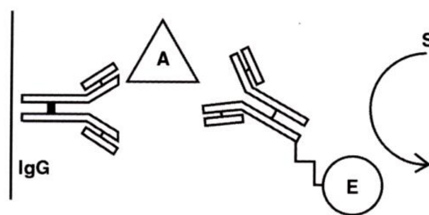


Figure 1.2. DAS-ELISA principle (Albrechtsen, 2006). IgG: antibody; A: antigen; E: enzyme; S: Substrate. The IgG bound to the solid phase reacts with the antigen in the test sample. The labelled-antibody binds to the immobilized antigen then; the enzyme on the labelled-antibody reacts with the substrate to indicate a positive reaction.

Indirect ELISA or Triple-antibody Sandwich (TAS) ELISA, as indicated by its name, differs from DAS ELISA in the number of antibodies used. In TAS-ELISA, the immobilized virus is sandwiched by an unconjugated specific antibody. The resulting complex is visualized by the successive addition of an enzyme-labelled anti-immunoglobilin antibody and the substrate enzyme (Figure1. 3). The second antibody is either from another animal species or modified. The elimination of the

conjugation induced specificity has been found to increase the antigen binding capacity of the unconjugated virus-specific antibody (Albrechtsen, 2006; Clark, 1981; Maroon-Lango, 2004; Miller and Martin, 1988).

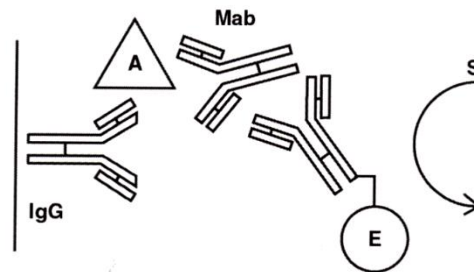


Figure 1.3. TAS-ELISA principle (Albrechtsen, 2006). IgG: antibody; A: antigen; Mab: Monoclonal antibody; E: enzyme; S: Substrate. An antibody that binds to the well of the microtiter plate is firstly added, then the test sample followed by the addition of a second antibody; lastly an enzyme-labelled anti-immunoglobilin antibody and the enzyme substrate are added.

1.3.1.2. Dot Immunoblot Assay (DIBA)

DIBA, also termed Dot-ELISA or Dot blot immunoassay (DBIA) were initiated in 1982 by Howkes *et al*. DIBA differs from ELISA in that:

i. Nitrocellulose membrane (NCM) provides the solid phase support.
ii. The test sample is applied as spots on the NCM.
iii. The subsequent reactions take place by submerging the whole support in the reagent dilutions.
iv. An insoluble coloured product that binds to the NCM at the site of dot application is the indication of a positive reaction. This unlike ELISA does not permit the quantification of the amount of antigen present in the test sample.

Similarly, DIBA follows the direct and indirect format of ELISA (Albrechtsen, 2006; Clark, 1981; Maroon-Lango, 2004; Miller and Martin, 1988).

1.3.1.3. Immunosorbent electron microscopy (ISEM)

ISEM combines electron microscopy and serology. The technique was developed by Derrick in 1973 and provides a higher sensitivity for the detection of pathogens. In the protocol, antigens are trapped on carbon-stabilized polyvinal formvar coated grids pre-treated with a specific antibody before being visualized under the transmission electron microscope (TEM) where they appear as a dark halo around the virion. ISEM enables the detection of both low and high titer virus and also permits the differentiation of viruses in mixed infection. The use of expensive and sophisticated equipment in ISEM limits its application (Albrechtsen, 2006; Clark, 1981; Maroon-Lango, 2004; Wright, 2005).

1.3.2. Nucleic acid-based (NAB) methods

NAB methods, also referred to as molecular techniques, rely on the specific complementary association of the different bases (adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U)) that compose nucleic acid molecules. Non-covalent hydrogen bonds form between A and T or U and between G and C. Molecular techniques are better alternatives in terms of sensitivity, rapidity, specificity, or in situations where suitable serological tests are not available. They can be divided into hybridization and amplification based techniques as shown in Figure 1.4 (Albrechtsen, 2006; Maroon-Lango, 2004; Webster *et al.,* 2004).

Hybridization uses probes (single RNA or DNA strands) generally labelled that anneal with target sequences in the test sample. The resulting hybrid is either visualized by autoradiography, fluorescence or enzymatic reaction. Amplification protocols on the other hand use enzymes (mainly polymerases) that amplify the copy number of the target sequence (Albrechtsen, 2006; Maroon-Lango, 2004; Webster *et al.,* 2004). Regarding *PVY* studies, amplification based reactions have been widely used (Ali *et al*., 2008; Aramburu *et al*., 2006; Boonham *et al*., 2002b; Crescenzi *et al*., 2005; Crosslin *et al*., 2006; Fomitcheva *et al*., 2009; Glais *et al.*, 2005; Hu *et al.*, 2009; Lorenzen *et al.*, 2008; Lorenzen *et al.*, 2006; Margaritopoulos *et al.*, 2009; Massumi *et al.*, 2009; Morel *et al.*, 2000; Piche *et al.*, 2004; Rigotti & Gugerli, 2007; Rosner *et*

al., 2000; Schubert *et al.*, 2007; Xianzhou *et al.*, 2004), but growing attention has been given to array technology (Agindotan and Perry, 2007; Boonham *et al*., 2003; Bystricka *et al*., 2005).

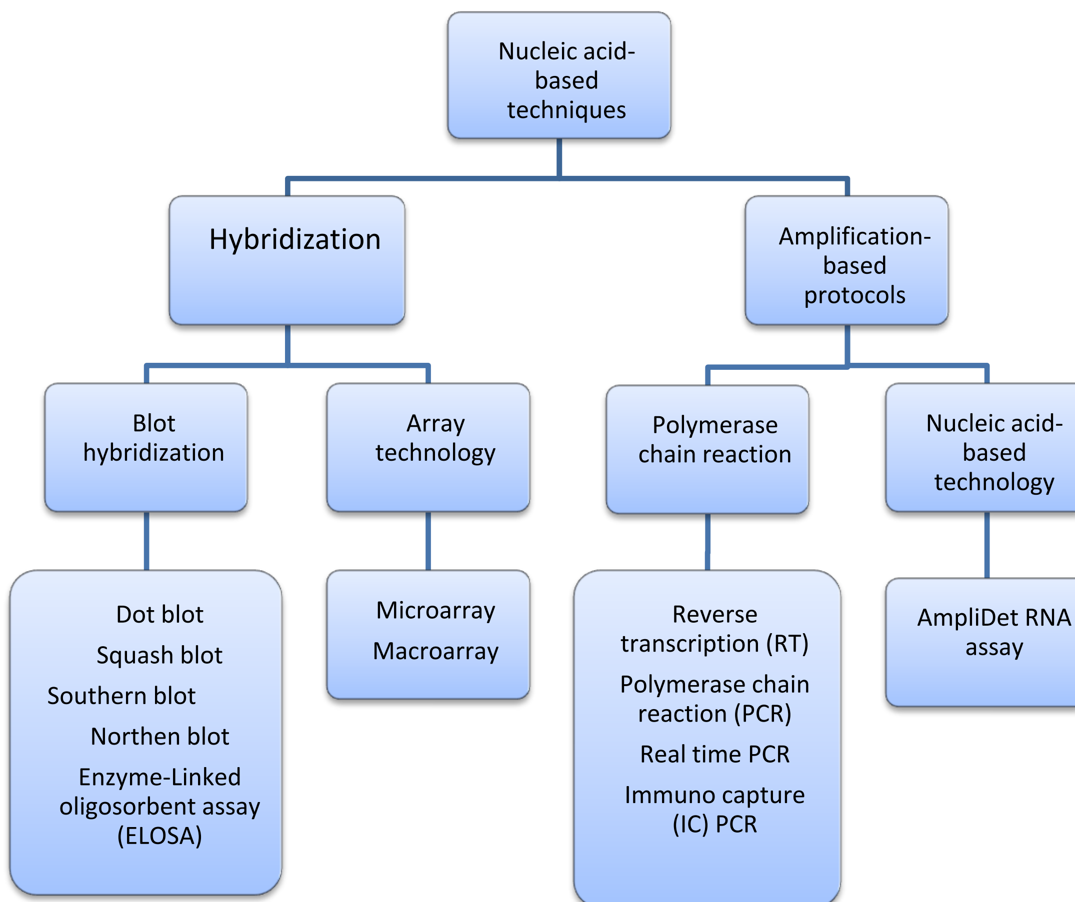


Figure 1.4. Molecular techniques used for plant pathogen detection.

1.3.2.1. Polymerase chain reaction (PCR)

The first description of PCR dates back to 1986, but the turning point occurred with the discovery and the utilization of thermostable polymerase enzyme extracted from hot water inhabiting bacteria in 1988. Henceforth, PCR has become a powerful, extremely sensitive, fairly inexpensive and simple tool in molecular biology and diagnosis (Henson & French, 1993). During PCR, deoxyribonucleic acid (DNA) is exponentially amplified by in vitro DNA synthesis through a series of repeated cycles.

A PCR cycle normally consists of denaturation, annealing and extension or elongation (Figure 1.5). The reaction occurs inside an automated thermal cycling machine (the thermocycler). Amplified DNA fragment (amplicon) can be detected, quantified or further analysed as summarized in Table 1.3. In the case of ribonucleic acid (RNA) viruses, a DNA strand complementary (cDNA) to the virus, later used as template in PCR, is previously made by reverse transcription of the viral genome (Albrechtsen, 2006; Bos 1999; Maroon-Lango, 2004; Newton & Graham, 1997; Vincelli & Tisserat, 2008; Webster *et al.,* 2004).

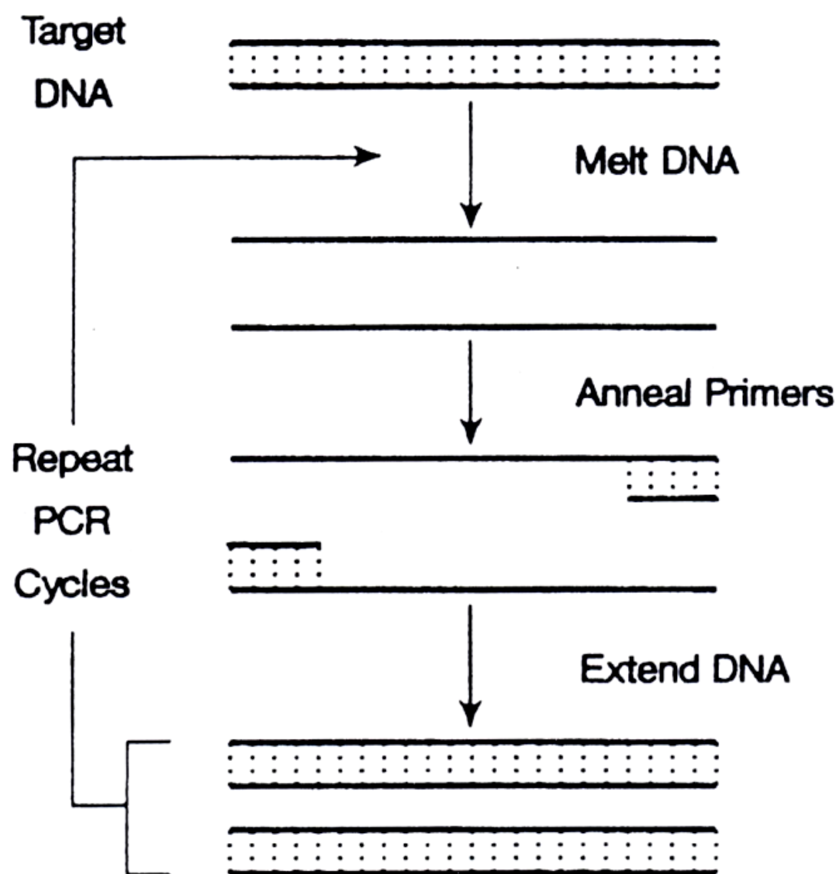


Figure 1.5. Principle of PCR. Each thermocycle consists of a denaturation step that opens the DNA. Primers then anneal at their complementary sites and direct the synthesis of a new DNA that will act as a template in the next cycle (Henson & French, 1993).

Table 1.3. Common methods used for detection, identification and quantification of PCR product (Newton & Graham, 1997).

Detection	Visualization
Agarose gel and/or polyacrylamide gel electrophoresis	▪ DNA gel stains such as ethidium bromide (EtBr), SYBR Safe, Gel red and green and EnVision (Ultra violet (UV) transilluminator, image analyser ▪ Southern blotting (hybridization with labelled probe) ▪ Incorporation of label into amplicon ▪ Addition of capture tag followed by detection ▪ Silver staining
Restriction endonuclease digestion	Agarose or polyacrylamide gel , High Performance liquid chromatography (HPLC)
Dot blots	Hybridization with label probe
HPLC	UV detection
DNA stains incorporation	UV transilluminator, image analyser
Electrochemiluminescence	Voltage-initiated chemical reaction /photon detection
Scintillation proximity assay (SPA)	Scintillation counting of captured PCR product
Direct sequencing	Radioactive or fluorescent-based DNA sequencing

Taq polymerase was the first thermostable DNA polymerase used in PCR. It was isolated from *Thermus aquaticus* (Taq) that was found in a hot spring in Yellowstone National Park. More thermostable DNA polymerase exhibiting different characteristics such as thermostability, exonuclease activity, processivity, extension rate, types of end produced, have been isolated from several different microorganisms and recombinant ones are commercially available. The choice of the enzyme is mainly dictated by the price and the type of application to be performed (Newton & Graham, 1997).

PCR draws its specificity from the uniqueness of sequences and selected probes used. Primers, also called oligonucleotides, are short nucleotides sequences that direct the DNA polymerase and define the length of the amplicon. Their design requires knowledge of the nucleotide sequence of the targeted fragment. The volume of available sequence data growing continually allows the development of primers capable of differentiating organisms at a specie, strain, group or family level. Several variants of the basic method which have been designed to meet specific requirements are available in the literature. Real time, competitive fluorescence, nested, touchdown and immocapture-PCR have been the most commonly used (Schaad *et al.*, 2003; Vincelli & Tisserat, 2008; Webster *et al.,* 2004).

Real time PCR allows the DNA amplification and its detection within the same sealed reaction vessel. This is achieved through the use of fluorescent oligonucleotides probes that emit fluorescence of defined wavelength in proportion to the amount of amplicon present after each thermocycle. PCR has been listed among the most rapid species-specific detection techniques currently available. However, the cost remains the main drawback of real time PCR besides its numerous advantages over conventional PCR. These are:

i. Data is provided in real time;
ii. It displays a much greater quantification range and greater sensitivity; and

It is time saving (Vincelli & Tisserat, 2008; Webster *et al.,* 2004).

Competitive fluorescence PCR, a variation of real time PCR, has been used for the simultaneous differentiation of virus strains and multiple virus infections. The differentiation occurs through the use of primer sets differently marked that differ only at the 3' end of a polymorphic nucleotide. Extension only occurs where the 3' nucleotide is complimentary and the amplified target is detected on the base of the wavelength of its fluorescence (Webster *et al.,* 2004).

Nested PCR is useful in very low titer virus conditions or in the presence of PCR inhibitors. The method consists of two PCR run whereby the PCR product of the first run is used as a template in the second PCR run. This reduces the level of inhibitors, concentrates the template and enhances the specificity in the second reaction. Low-specificity oligonucleotides, usually degenerate, are generally used in the first PCR run. The second run is performed with primers that anneal within the amplicon amplified in the previous run. The extreme sensitivity of this technique requires greater precautions to keep the template free of contamination (Newton & Graham, 1997; Vincelli & Tisserat, 2008; Webster *et al.,* 2004).

Touchdown PCR aims at minimizing the synthesis of non specific product and primer-dimers. In touchdown PCR the annealing temperature is incrementally lowered during cycling from an initial value above the expected melting temperature (Tm) of the primers to a value below the Tm. This enhances the annealing of primers to the target, therefore amplification of the desired amplicom (Newton & Graham, 1997).

Immunocapture-PCR is a combination of two diagnosis tools. The method exploits the high-binding affinity of antibody of serological techniques and enzyme amplification protocols (Albrechtsen, 2006). Antibodies, bound to the surface of the reaction vessel in the same way as with DAS ELISA, immobilize the antigen present in the test sample. The nucleic acid of the antigen is then released and amplified accordingly (Figure 1.6). This method has the following advantages:

i. It eliminates the problems of co-extracted PCR inhibitors;
ii. It enhances the detection sensitivity of the reaction since it does not require the extraction of the total plant nucleic acid (Albrechtsen, 2006; Mulholland, 2005; Vincelli & Tisserat, 2008; Webster *et al.,* 2004); and
iii. It is safer and time saving considering the time and toxicity level of chemicals involved in nucleic acid extraction protocols.

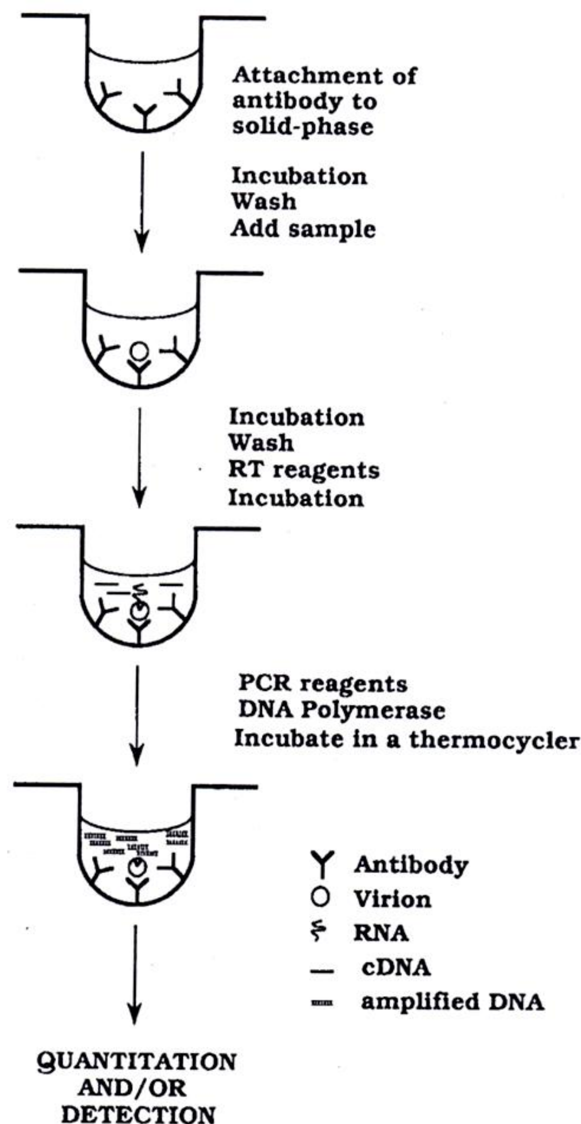
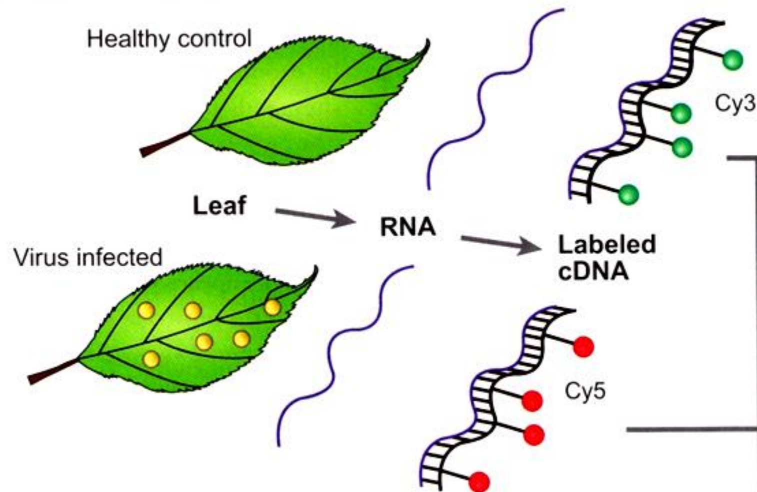


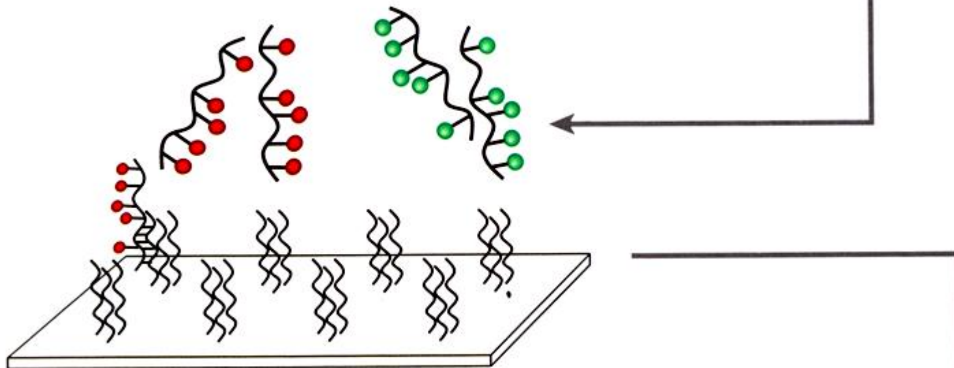
Figure 1.6. Schematic representation of the different steps of immunocapture-reverse transcription of an RNA virus (Nolasco *et al.*, 1993).

1.3.2.2. Array technology

The application of array technology in plant virology allows the detection of a whole range of viruses in a single test. It is a useful tool in screening, diagnosis and study of diseases caused by complexes of viruses. Arrays were originally designed for the study of gene expression, polymorphism and host pathogen interaction. They are based on the hybridization of fluorescently labelled sequences (targets) to their complementary sequences spotted on a solid surface acting as probes. The test requires a solid support, capture probes, marker, targets, equipment for hybridization and array analysis (Boonham *et al*., 2007; Bystricka *et al*., 2005; Webster *et al.,* 2004).

Array application in plant virology can be divided into three steps (Figure 1.7). Glass slide and nylon membrane have been used in array- based assay with oligonucleotides as capture probes and both randomly primed and virus specific amplicons as targets. Although successful results were reported with plant virus detection, the methodology still requires more attention in order to better compete with the existing recognized techniques. The use of glass support, which only happens manually, presents a drawback in the automation of the technique. The continual manual handling of glass will increase the time required to complete the protocol and will not be suitable for large scale testing. Hybridization, generally performed overnight, is another disadvantage in terms of time. Concerns were also raised about the sensitivity of the protocol. The technique has to be more sensitive while keeping its multiplex detection ability. Non specific label techniques have been reported to lower the sensitivity, while methods incorporating PCR may be more sensitive but show limitations (Boonham *et al*., 2007; Bystricka *et al*., 2005; Vincelli & Tisserat, 2008).





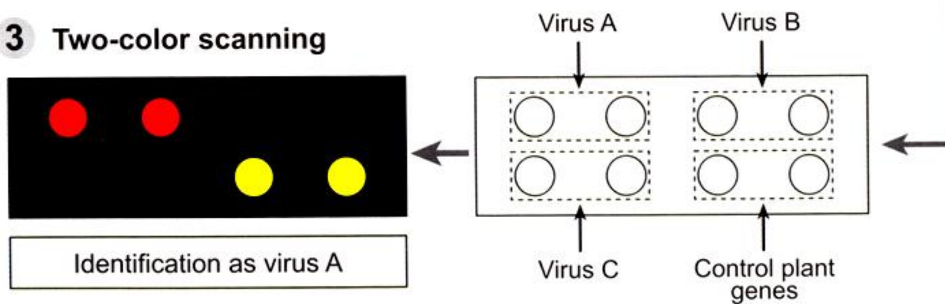


Figure 1.7. Detection of plant virus by Macro array. The technique consists of three distinct steps. **1:** RNA of diseased and health plant are both reverse-transcribed and differently marked **2:** then hybridized with probes immobilized on a solid surface and **3:** visualised for virus identification (Boonham *et al*., 2007).

1.3.3. Phylogenic analysis

Phylogeny is the discipline that studies the evolutionary history of a group of sequences or organisms by constructing evolutionary tree that describes similarities and divergences at the molecular level (Page and Holmes, 1998; Xiong, 2006). It operates on the following assumptions:

i. Sequences should be genealogically related, meaning that they are derived from a common ancestor that diverged through time.
ii. Parent branch splits into daughter branches at any point.
iii. Substitutions occur independently in a sequence.

Phylogenic construction comprises five steps. These are choice of molecular markers, alignment of markers, choice of the evolutionary model, determination of the tree building method and the assessment of the reliability of the tree (Xiong, 2006).

The choice of the marker plays an important role in the tree output. Nucleotide and protein sequences have been the markers often used. The decision to choose one to the detriment of the other depends on the properties of the sequences and the purpose of the study. Sequence alignment is the most critical step in the procedure because it provides information that determines the topology of the tree. Only correct alignment produces correct phylogenic inference. Multiple substitutions and convergence at individual positions generally gives erroneous evolutionary distances between two sequences. This is known as homoplasy (Xiong, 2006).

Substitution models or evolutionary models are statistical models used to correct homoplasy. These nucleotides substitution models infer the true evolutionary distances between sequences. Evolution distance can be corrected either with the Jukes-cantor or the Kimura model. Jukes-cantor model, which is the simplest, assumes that all the nucleotides are substituted with equal probability. The Kimura two-parameter is a more sophisticated model which assumes that mutation rates for transitions and transversions are different, with transitions occurring more frequently than transversions. Methods used to build evolutionary trees are

summarized in Figure 1.8. A constructed tree needs to be statistically evaluated to ensure its reliability, consistency and significance. Several resampling techniques have been developed for this purpose. These include bootstrapping, jackknifing, the Bayesian simulation, the Kishino-Hasegawa test and the Shimodaira-Hasegawa test. Phylogenic analysis is performed on a computer. There are several software programs with different packages and are generally freely available for phylogenic analyses (Xiong, 2006).

Phylogenic analysis has played an important role in tracking the diversification of *PVY*. One approach, which follows the standard procedure already described, has been the most frequently used by several research groups. Nucleic acid sequences in most cases served as molecular support (Ali *et al.*, 2008; Ali *et al.*, 2007; Aramburu *et al.*, 2006; Boonham *et al.*, 2002a; Comes *et al.*, 2005; Crescenzi *et al.*, 2005; Fanigliulo *et al.*, 2005 ; Glais *et al.*, 2002; Hu *et al.*, 2009). The viral genome, previously amplified by RT-PCR, is either directly sequenced (Hu *et al.*, 2009; Lorenzen *et al.*, 2008; Lorenzen *et al.*, 2006; Margaritopoulos *et al*., 2009) or ligated to a vector and later used to transform competent *Escherichia coli* before being sequenced (Ali *et al.*, 2007; Fanigliulo *et al.*, 2005; Ogawa *et al.*, 2008; Schubert *et al.*, 2007; Xianzhou *et al.*, 2004).

Sequencing of *PVY* isolates has been performed on the entire genome (Ali *et al.*, 2008; Ali *et al.*, 2007; Fanigliulo *et al.*, 2005; Hu *et al.*, 2009; Lorenzen *et al.*, 2008; Lorenzen *et al.*, 2006; Moury, 2009; Ogawa *et al.*, 2008; Schubert *et al.*, 2007; Singh and Singh, 1996; Xianzhou *et al.*, 2004), or part of it. Partial sequencing comprises any part of the genome including the 3' and 5' untranslated regions (Ali *et al.*, 2007; Aramburu *et al.*, 2006; Boonham *et al.*, 2002a; Comes *et al.*, 2005; Crescenzi *et al.*, 2005; Glais *et al.*, 2002; Llave *et al.*, 1999; Margaritopoulos *et al*., 2009; Morel *et al.*, 2000; Moury, 2009; Rosner *et al.*, 2000). Sequence alignment, distance calculation, recombination analysis and tree inference have been performed by means of the

different programs available such as Clustal, DNASIS, RDP, SISCAN, Phylip, PHYML, MEGA, DNAMAN, Simplot, MUSCLE, PAUP and Tree PUZZLE.

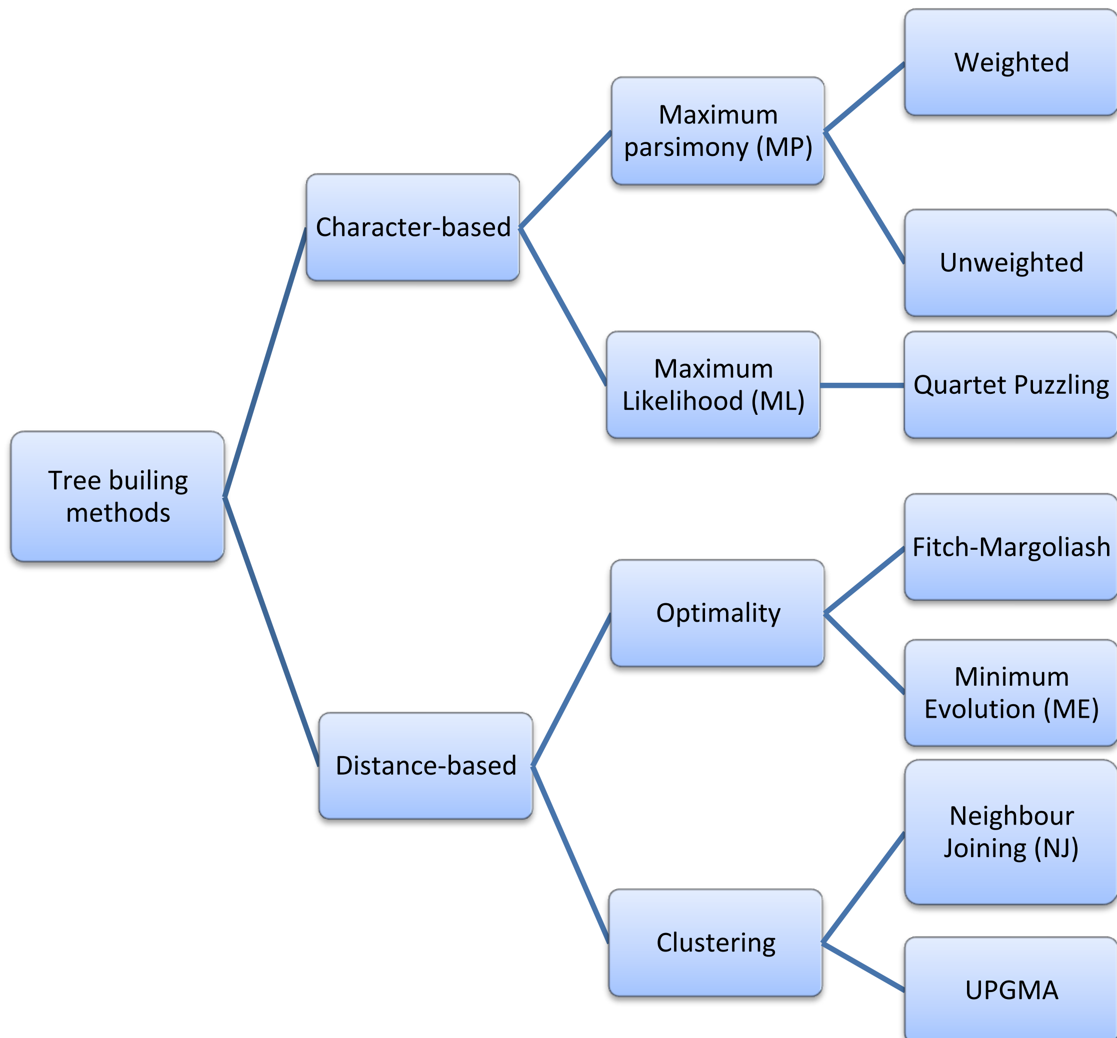


Figure 1.8. Tree building methods (Xiong, 2006).

Blanco-Urgoiti *et al.* (1996) developed the RT-PCR restriction fragment length polymorphism (RFLP) based on the restrictotype profiles displayed by *PVY* isolates after endonuclease restriction. Genome amplicon (coat protein in this case) is digested with different restriction enzymes and analysed on agarose gel. The restriction pattern on the gel is transcribed into a binary matrix (presence/absence) fragment (row) and isolates column. The presence of restriction fragment is recorded as 1 and its absence as 0 in the matrix (Figure 1.9). Restriction pattern in the matrix are then pairwise compared to generate a distance matrix that contains the distance

between all possible pairs of isolates. Phylogenic trees are computed from the distance matrix using the different algorithms methods available.

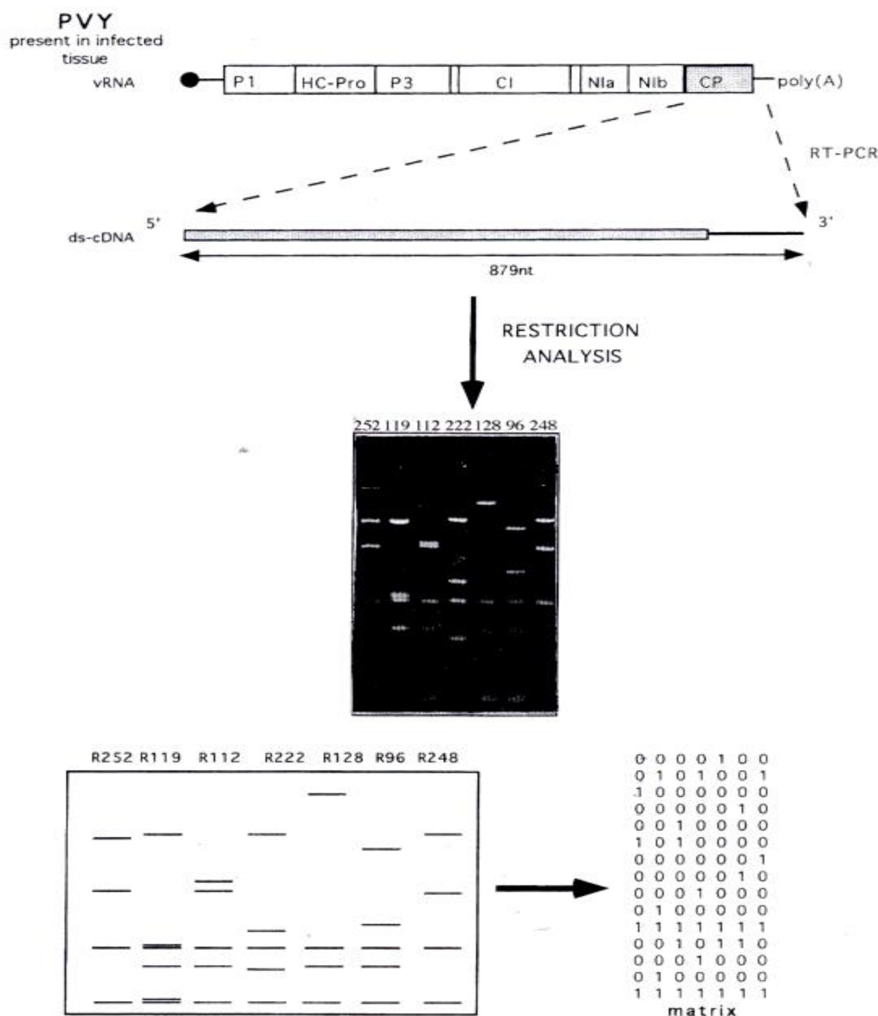


Figure 1.9. Schematic description of the RFLP method designed by Blanco-Urgoiti *et al.* (1996).

1.3.4. Biological assay

Biological assays, also called bioassay or biological indexing, are the oldest methods used in virus characterization. As the name suggests, bioassays investigate the biological properties of viruses. These include host range, symptoms and mode of transmission of viruses. Bioassays require artificial transmission of the test virus into different host or indicator plants. This is either achieved by mechanical transmission, grafting, use of dodder, vegetative propagation or by virus vector. Although they are

labour, time and space consuming compared to the other available techniques, they still have their merit in detection and diagnosis especially in differentiating pathogen strains and species. Moreover, biological assays represent the only way to propagate plant viruses (Albrechtsen, 2006; Bos, 1999; Hull, 2002). Mechanical inoculation (Ali *et al.*, 2008; Aramburu *et al*., 2006; Baldauf *et al.*, 2006; Crescenzi *et al.*, 2005; Hu *et al*., 2009; Lorenzen *et al.*, 2006; Xianzhou *et al*., 2004) and vector inoculation (Bouhachem *et al.*, 2008; Ohshima *et al.*, 2000; Romancer *et al.*, 1994) have been the two techniques used in *PVY* studies. *Chenopodium amaranticolor* (*C. amaranticolor*), *C. quinoa*, *Lycium* spp, *Physalis floridana* have been used as indicator hosts.

Mechanical or sap inoculation requires adequate vector-proof growth facilities in order to get reliable results. The method consists of transferring a virus-bearing suspension (inoculum) obtained from infected plant material onto the surface of the challenged plant in a way that permits the virus to enter the cells (Hill, 1984). Young leaves, but not the youngest, showing clear symptoms, are recommended as inoculum source since they generally contain the highest virus concentration. They are generally ground in phosphate buffer with reducing agents such as sodium sulfite, sodium diethyldithiocarbamate or 2-mercaptoethanol by means of mortar and pestle. Carborundum and celite (diatomaceous earth) are abrasive dusts used to enhance wounding and virus transmission. Carborundum is usually spread on the surface of the leaves to be inoculated, while celite is mixed with the inoculum. Inoculated plants are rinsed with water after inoculation and kept in a humid environment to prevent wilting (Albrechtsen, 2006; Bos, 1999; Hill, 1984).

1.3.5. Electron microscopy

The very small size of viruses allows their visualization under the electron microscope. The transmission electron microscope (TEM) has been used for studying plant viruses. Beside the ISEM, the TEM study of viruses includes negative staining and the ultrathin sectioning. Negative staining can be applied to both crude and purified virus sample. The technique consists of either applying a drop of the virus

suspension onto the specimen holder or by floating the formvar coated grid upside down on the virus solution and washing the excess off. The grid is then stained with uranyl acetate for about half a minute, and dried before being visualized under the TEM (Bos, 1999).

Ultrathin sectioning is used for the study of viral symptoms at a cellular level. It requires a series of treatments that can be summarized into five different stages. These comprise:

i. Fixation with glutaraldehyde and osmium tetroxide
ii. Dehydration in a graded series of ethanol dilutions
iii. Embedding with polymerizing resins
iv. Sectioning by means of an ultramicrotome
v. Staining with uranyl acetate

Pinwheel, bundle-like and non crystalline amorphous inclusions are the main structures found in *PVY* infected tissue (Bos, 1999).

1.4. Epidemiology

PVY has a host range that includes 495 species and 31 families (Kerlan & Moury, 2008). More than 50 aphid species transmit the virus in a non-persistent manner with *Myzus persicae* Sulzer (*Homoptera*: *Aphididae*), the green peach aphid, being the most efficient vector. Viruses transmitted in this way are also known as stylet-borne viruses. Acquisition access period (AAP), inoculation access period (IAP), latent period, and the feeding state of the vector are the different factors that influence the ability to transmit the virus. The virus AAP ranges between 5 seconds to 5 minutes. Longer periods increase transmission but AAP longer than 10 minutes results in very poor to no transmission at all. Starved aphids have been reported to transmit the virus more efficiently than non-starved. Transmission of the virus is more likely to happen during sampling probes of the vector when looking for a suitable host, since transmission does not require a latent period (Kanavaki *et al.*, 2006; Ng & Falk, 2006).

1.5. Control strategies

Control of *PVY* is achieved by either controlling the vector or the reproduction of the virus inside the host. Control of virus replication is managed through the replication of resistant cultivars (Garcia-Arenal and McDonald, 2003), while vector control is accomplished by cultural (Hooks and Fereres, 2006), chemical (Van Toor *et al.*, 2009) and biological methods (Cabral, *et al*., 2009; Rashki *et al.*, 2009).

Resistant cultivars are produced either by breeding or by plant transformation. Natural breeding is the oldest method used for production of resistant cultivars. This method presents two disadvantages which are the long time required to produce a resistant cultivar and the probability of the resistance to be overcome by new virus strains. Analysis of the durability of some *PVY* resistant plants in Europe showed that resistance factors (immunity, infection or accumulation) have been durable (more than 25 years) with occurrence of some breaking strains in pepper and tobacco, while some factors were overcome in potato over the same period of time (Garcia-Arenal and McDonald, 2003).

Three alternatives, which are pathogen-derived resistance; RNA-derived resistance (Zhu *et al.*, 2009) and transgenic plant expressing antibodies specific to viral components (Bouaziz *et al*., 2009), have been exploited in the production of transgenic plants. These different alternatives are based on:

i. The expression of viral component (pathogen-derived resistance) or recombinant antibody directed against viral protein.
ii. Post transcriptional genes silencing (PTGS) also referred as RNA-derived or mediated resistance.

The viral coat protein gene has been the preferred viral component used in pathogen-derived resistance. However, concerns were raised of the possibility of heterogeneous recombination with other viruses in nature that could lead to unusually dangerous viruses. RNA-derived resistance appears to be safer in this regard since it involves viral sequences only. Moreover, it allows the production of

transgenic plants with multiple virus resistance (Zhu *et al.*, 2009). Transgenic plant expressing antibodies specific to viral components have been proved efficient for large scale production. Higher levels of resistance were recorded on plants expressing antibodies against functional protein, such as proteinase and polymerase, compared to those expressing antibodies against the coat protein. However, the expression of theses recombinant antibodies in the plants needs to be improved (Bouaziz *et al*., 2009).

Chemical control includes treatment of tubers and foliar applications with insecticide classes. Insecticide classes are divided into organophosphates, dimethyl carbamates, pyrethoids, and neonicotinoids. Studies undertaken with these insecticides in New Zealand revealed that treatment of seed with imidacloprid (organophosphate) followed by foliar treatment with λ-cyhalothrin (pyrethoid), or pymetrozine (pyridine-azomethine) whenever aphid population exceeds 10/150 potato leaves was sufficient to maintain aphid populations below the action threshold without compromising or increasing virus risk in tubers. However, a control program that relies on multiple uses of the same or related insecticides leads to insecticide resistance (Van Toor *et al.*, 2009).

Biological control exploits natural enemies to control a pathogen. Biological control of aphids in *PVY* transmission can be achieved through the use of parasitoid, predator population or fungal enthomopathogen alone or in combination. A number of investigations in laboratory conditions have given satisfactory results and are waiting for field trials (Cabral, *et al*., 2009; Rashki *et al.*, 2009). Cultural control includes planting time to avoid peaks in aphid migration and the use of barrier plants. Barrier plants, generally taller than the primary crop, act as a physical barrier between the vector and the primary crop (Hooks and Fereres, 2006). Results, obtained using that approach, are summarized in Table 1.4.

Table 1.4. Effect of plant barriers in control of *PVY* (Hooks and Fereres, 2006)

Primary crop	Virus targeted	Barrier plant	Response	Mechanism
Pepper	*CMV**, *PVY*	Sorghum	Reduction of *CMV** spread; Delay of *PVY* spread	Sorghum acted as a sink for both viruses
Pepper	*CMV**, *PVY*	Maize, vetch, sorghum	Reduction in virus spread and possible yield increase	Barriers acted as a virus sink, but did not reduce aphid in crop
Pepper	*PVY*	Sunflowers	Reduction in virus spread	Blocked aphid landing rates
Potato	*PVY*	Sorghum, potato, soybean, wheat	Reduction in virus incidence along the field edge of potato	Barriers acted as a sink
Potato	*PVY*	Wheat straw mulch	Reduction in *PVY* incidence but no impact on yield	Barriers reduced optical contrast between plant and soil

**Cucumber mosaic virus*

1.6. Objectives and outlines of research project

Against this background, *PVY* isolates infecting vegetables grown in KwaZulu-Natal (KZN) were investigated using some of the methods described in this chapter. Potato, pepper and tomato are crops of economic importance in the Republic of South Africa (RSA). These vegetable crops are actively grown in KZN on commercial and small-scale farming systems. *PVY* is known to occur in KZN (Budnik *et al.,* 1996; Thompson *et al.*, 1987; Trench *et al.*, 1992; Vorster *et al.*, 1990). However, there is limited information on the existing *PVY* isolates. Knowledge of KZN *PVY* isolates will play an

important role in devising strategies to control the disease. Therefore, this research project aims at:

- Detecting *PVY* isolates in solanaceous crops in both commercial and small-scaled farms by collecting plant materials displaying *PVY*-like symptoms and test them for the presence of *PVY* by ELISA using monoclonal antibody reacting with all *PVY* strains and RT-PCR using oligonucleotides that amplify all strains of *PVY*.
- Obtaining pure isolates of *PVY* by mechanical inoculation on *C. quinoa*.
- Identifying the different strains of *PVY* isolates by performing ELISA test using strain specific antibodies and RT-PCR using primers that amplify specific strains.
- Determining the biological properties of the different strains identified by host indexing using *Nicotiana tabacum* cv Xanthi, *N. tabacum* cv Samsun, *N. glutinosa*, and *N. rustica*.
- Determining the phylogeny of the different strains identified by amplifying (RT-PCR), cloning, sequencing selected region of the *PVY* genome and comparing with available sequences of the same genomic regions on the NCBI website.

1.7. References

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Chapter 2
Detection, Differentiation and Biological Characterization of *Potato virus Y (PVY)* Isolates Infecting Selected Vegetable Crops in KwaZulu-Natal (KZN), Republic of South Africa (RSA)

Abstract

Potato virus Y (*PVY*) is a virus of economic importance with a wide host range. The virus exists as a diversity of strains at a biological, serological and molecular level. The aim of this study was to identify the strains of *PVY* infecting pepper (*Capsicum annuum* L.), potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.) in KwaZulu-Natal (KZN), Republic of South Africa (RSA) and to study their biological properties. Sampling for *PVY* isolates was done on both small-scale and commercial farms. *PVY* isolates were detected using double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). A total of 16 isolates including one isolate from pepper, three from tomato and 12 from potato were subsequently differentiated into strains using strain specific antibodies and primers. All tomato and pepper isolates of *PVY* were found to be the common PVY^{O} strain, while potato infecting isolates displayed a diversity of strains comprising PVY^{N}, PVY^{NTN} and PVY^{N}Wilga with mixed infections in some cases. Biological studies of all isolates on *Chenopodium quinoa* (*C. quinoa*), *Nicotiana glutinosa* (*N. glutinosa) N. tabacum* cv Xanthi, *N. tabacum* cv Samsun, and *N. rustica* did not to produce symptoms on *C. quinoa*. The veinal necrosis symptom, characteristic of PVY^{N}, PVY^{N}Wilga and PVY^{NTN} strains, was observed on *N. tabacum* cv Xanthi and *N. tabacum* cv Samsun but not on *N. glutinosa* nor *N. rustica*. ELISA tests, together with the leaf dip method, indicated the presence of viruses different from *PVY*, as rod-shape particles of length varying between 70 and 400 nm and *Potyvirus*-like particles were observed under the transmission electron microscope. Taken together, these results confirm the presence of *PVY* in KZN, the diversity of known potato strains and their occurrence in synergism with other viruses.

2.1. Introduction

Accurate detection of the causal agent of a disease has always been the starting point of every disease management programme. Detection of plant viruses entails the screening of plant material for a particular virus which is already known to occur in a particular host or geographic location (Bos, 1999). The identity of a pathogen provides useful information on how the disease spreads, which in turn allows the development of proper and efficient control strategies. Therefore, a control programme based on an erroneous detection will undoubtedly prove to be ineffective and a waste in terms of time, resources and energy (Grogan, 1981).

Potato virus Y (*PVY*) is the type member of the genus *Potyvirus* in the family *Potyviridae*. Virions are flexuous particles of about 730 nm long and 11 nm wide. The *PVY* genome consists of a single stranded positive sense RNA of about 10 Kb with a VPg protein covalently linked to its 5'-end and a poly-A tail at its 3'-end. It is translated into a single, large polyprotein which is subsequently processed by three virus encoded proteinase into nine gene products: P1, Helper component-proteinase (HC-Pro), P3, 6K1, cytoplasmic inclusion (CI), 6K2, nuclear inclusion a (NIa), nuclear inclusion b and RNA-dependant RNA polymerase (NIb-Pol) and the capsid protein (CP) (Urcuqui-Inchima *et al*., 2001). *PVY* infects many *solanaceous* plants including pepper (*Capsicum annuum* L.), potato (*Solanum tuberosum* L.), tobacco (*Nicotiana* spp) and tomato (*Lycopersicon esculentum* Mill.) in which it causes serious damage worldwide (Shukla *et al*., 1994). The green peach aphid, *Myzus persicae* Sulzer (*Homoptera*: *Aphididae*), is the most efficient vector of the virus among more than 50 aphid species identified to transmit the virus in a non-persistent manner (Kanavaki *et al.*, 2006).

PVY can be classified into different strains: the common or ordinary O strain (*PVY*^{O}), the stipple streak C strain (*PVY*^{C}), and the tobacco veinal necrosis strain (*PVY*^{N}). *PVY*^{N} also includes Wilga strain (*PVY*^{N}W; *PVY*N^{-Wi} or *PVY*$^{N:O}$) and NTN strain (*PVY*^{NTN}) (Margaritopoulos *et al.,* 2009; Rigotti & Gugerli, 2007). *PVY* isolates are generally

differentiated on the basis of their biological, serological and molecular characteristics. *PVY* can be separated into two serotypes, O and N. The O serotype comprises *PVY*^{O}, *PVY*^{C} and *PVY*^{N}W and the N serotype includes *PVY*^{N} and *PVY*^{NTN}. *PVY* produces two distinct symptoms in tobacco plants. *PVY*^{O} and *PVY*^{C} induce vein clearing and mosaic while *PVY*^{N}, *PVY*^{NTN} and *PVY*^{N}W cause veinal necrosis (Ali *et al.*, 2008). All *PVY* strains are able to infect potato (Singh *et al.*, 2008). *PVY*^{O}, *PVY*^{C} and *PVY*^{N} infect tomato (Aramburu *et al*., 2006; Comes *et al*., 2005). Isolates infecting pepper have been identified as *PVY*^{O} and *PVY*^{C} only (Cardin & Moury, 2008).

Serological detection of *PVY* involves the use of antibodies specific to a strain or a group of strains of the virus. Enzyme-linked immunosorbent assay (ELISA) has been the most common serological technique used in plant virus detection (Albrechtsen, 2006; Bos, 1999; Webster *et al.*, 2004). Molecular detection is achieved through reverse transcription polymerase chain reaction (RT-PCR) using primers specific to a strain or a group of strains of the virus (Baldauf *et al.*, 2006; Cardin & Moury, 2008; Crosslin *et al.*, 2006; Kogovsek *et al.*, 2008; Massumi *et al.*, 2009; Piche *et al.*, 2004; Schubert *et al.*, 2007; Webster *et al.*, 2004). Biological assays require artificial transmission of the test virus onto different host or indicator plants. This is either achieved by mechanical transmission, grafting, use of dodder, vegetative propagation or using the virus vector (Albrechtsen, 2006; Dijkstra & Khan, 2006).

Vegetable cultivation is a major farming activity in KZN. Due to favourable climatic conditions, KZN is ideal for the cultivation of various vegetables including tomato, pepper and potato at small-scale and commercial levels. Seven hundred and thirty hectares under tomato cultivation in KZN produced 36 500 tons of tomato between 2005 and 2006 (National Department of Agriculture, 2007). Although KZN is a fairly small producing region compared to the other producing regions, it annually manages to produce higher quality table and seed potatoes through the 4000 ha of planted potato and the 1600 ha of registered seed planting areas (Potato South Africa, 2009). Home-produced crops contribute to improve house nutritional status

and food security by either generating substantial monetary income or by reducing the household food expenditure (Maunder & Meaker, 2007; Van Averbeke & Khosa, 2007).

PVY has previously been reported to occur in RSA (Budnik *et al.,* 1996; Thompson *et al.*, 1987; Trench *et al.*, 1992; Vorster *et al.*, 1990). An earlier study of *PVY* isolates of tobacco occurring in the main tobacco growing areas led to the identification of four strains: PVY^{C}, PVY^{N}, $PVY^{O\text{-}chl}$ and $PVY^{N\text{-}S}$ based on their biological properties on various tobacco cultivars (Vorster *et al.*, 1990). Studies by Budnik *et al.* (1996) on viruses of pepper in KZN clearly showed *PVY* as being the predominant virus. However, differentiation of *PVY* to strain level was not done. Similar results of studies were reported with *PVY* infecting tomato and potato (Thompson *et al.*, 1987; Trench *et al.*, 1992).

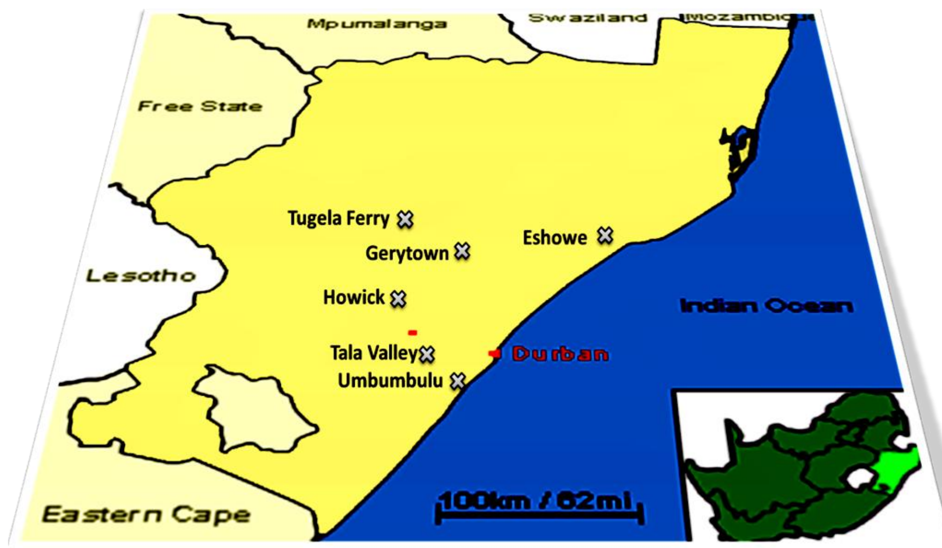
Due to the variability displayed by *PVY* around the world, an assessment of the isolates occurring locally is required. Against this background, the aim of this study was to identify, differentiate and evaluate the biological properties of *PVY* infecting tomato, pepper and potato in KZN.

2.2. Material and methods

2.2.1. Virus isolates

Sampling for *PVY* isolates was done on both small-scale and commercial farms in KZN. The locations where sampling for *PVY* isolates took place are indicated in Figure 2.1. All samples used in this study are described in Table 2.1. Isolates infecting pepper and tomato were obtained during the routine tests performed between February 2006 and May 2007. Samples were kept at -80°C in plastic bags with labels indicating the nature of the crop and the location from where it was collected. All frozen isolates were then mechanically inoculated onto *Nicotiana rustica* (*N. rustica*), a propagation host for the virus. Frozen leaves were ground using autoclaved mortars and pestles in 0.1M phosphate buffer pH 7.4 containing 0.4% sodium

sulphite. Fully expanded leaves from six week old *N. rustica* plants were dusted with carborundum before being gently rubbed with the inoculum using a pestle. Inoculated leaves were rinsed with tap water (Albrechtsen, 2006; Hill, 1984). Isolates infecting potato were obtained from volunteer potato plants showing *PVY*-like symptoms collected between 2008 and 2009 which had tested positive for *PVY*. Inoculated *N. rustica* and collected potato plants were maintained at 25°C in Jolly Roger tunnel (Discipline of Plant Pathology, UKZN-PMB).



Locations where sampling for *PVY* isolates took place

Figure 2.1. Map of KwaZulu-Natal showing the different locations where *PVY* isolates were sampled.

Table 2.1. Description of samples used in this study

Location	Crop	Sample description
Eshowe	Tomato	Leaf
Greytown	Pepper	Leaf
Howick	Potato	Full plant
Tala Valley	Tomato	Leaf
Tugela Ferry	Tomato	Leaf
Umbumbulu	Potato	Full plant

2.2.2. Detection of *PVY*

The presence of *PVY* was detected in inoculated *N. rustica* and the collected field potato plants using double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) and reverse transcription – polymerase chain reaction (RT-PCR).

2.2.2.1. Double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA)

The Monoclonal antibody (Mab) DAS ELISA reagent set for $PVY^{O/C/N}$ (Neogen Corporation, Scotland, UK) was used according to the manufacturer's instructions. Coating and conjugate antibodies were diluted at 1/100 to make working solutions in coating and conjugate buffer, respectively. High binding ELISA plates (Greiner-Bio-one, Germany) were coated with 100 µl coating antibodies and incubated for 4 hrs at 37°C. The plates were then washed three times with phosphate buffered saline containing 0,05% Tween 20 (PBS-T) and 100 µl of sample, positive and negative control were loaded in the wells before overnight incubation at 4°C. Virus free (healthy) uninoculated plants were used as negative controls. Each sample was tested in duplicate. Sample suspensions were obtained by grinding 5 discs (1 cm in diameter) in 500 µl extraction buffer in a 1.5ml eppendorf tube using an electrical drill fitted with a plastic drill bit. The suspensions were then centrifuged at 13,000 rpm for 2 min to clear the sap from plant tissues.

The plates were washed again following the overnight incubation after which 100 µl of the conjugate antibodies solution was added and incubated at 37°C for 1hr. Another wash was performed before the final addition of 100 µl of 1mg/ml of the enzyme substrate 4-Nitrophenyl phosphate disodium salt hexahydrate (SIGMA, Missouri, USA) solution (pNPP). The plates were incubated at 37°C for 30 min and results were assessed visually. A yellow colour was recorded as a positive reaction.

2.2.2.2. Reverse transcription – polymerase chain reaction (RT-PCR)

Total plant RNA was extracted from ELISA-positive *PVY* plants using the SV total RNA isolation system (Promega, Madison, USA). The degenerate primer pair, *PVY* 2F (5'-ACGTCMAAAATGAGAATGCC-3') and *PVY* 2R (CATTTGWATGTGCGCTTCC-3'), designed by Aramburu *et al.* (2006) from conserved sequences of the coat protein, was used to confirm the presence of *PVY* by RT-PCR. It yields a product of 510 bp with all *PVY* strains (Aramburu *et al.,* 2006). The first strand complementary DNA (cDNA) was synthesized using $3'_{NTR}C$ primer (5'-GTCTCCTGATTGAAGTTTAC-3') by Glais *et al.* (2005) and the Reverse Transcription System (Promega, Madison, USA) according to the manufacturer's instructions.

Go Taq PCR core systems II (Promega, Madison, USA) was used according to the manufacturer's instructions to prepare the PCR. Thermocycler conditions consisted of an initial denaturation of 2 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C followed by a final extension of 5 min at 72°C. The product was analyzed by electrophoresis on a 1% (w/v) agarose gel with Tris-acetate, EDTA (TAE) buffer containing 0,6 µg ethidium bromide (Sambrook & Russell, 2001) and photographed using the VersaDoc imaging system 4000 (Bio-Rad, California, USA).

2.2.3. Differentiation of *PVY* isolates

All *PVY* isolates identified with both ELISA and RT-PCR were differentiated into different strains using ELISA and RT-PCR respectively.

2.2.3.1. Serological differentiation

PVY isolates were tested for the serotypes O, C and N using $PVY^{O/C}$ Mab DAS ELISA, PVY^{N} Mab DAS ELISA and PVY^{O} Pab + Mab TAS ELISA reagents (Neogen Corporation, Scotland, UK). DAS ELISA was performed as described in the section 2.2.2.1. Triple-antibody sandwich ELISA was done for the PVY^{O} reagent according to the manufacturer's instructions. Coating antibodies were diluted 100 times in coating buffer, while probe antibodies and the goat anti-mouse IgG-AP were also diluted 100 times in conjugate buffer.

The steps including coating of the plates, loading of samples and overnight incubation in TAS ELISA are similar to DAS ELISA and were done as described in section 2.2.2.1. One hundred microliters of probe antibodies were added following the washing step after overnight incubation. The plate was then incubated at 37°C for 2 hrs. The plate was washed again and 100 µl of goat anti-mouse IgG-AP were added before being incubated at 37°C for 1 hr. A final wash was then performed and 100 µl of substrate solution was added. This was followed with incubation at 37°C for 30 min before observing and recording the results.

2.2.3.2. Molecular differentiation

Primers designed by Rigotti & Gugerli (2007) and by Schubert *et al.* (2007) were used to identify the strains of *PVY* isolates identified. Primers designed by Rigotti & Gugerli (2007) comprise the primer pairs *PVY*c3/f, *PVY*3+/3- and CP2+/1-. *PVY*c3/f primer pair was designed on the PVY^{O}-139 genome and are respectively located in the 5'NTR and P1 genomic regions. *PVY*3+/3- primer pair are located on the CI and 6K2 genes of the PVY^{N}-605 genome. CP2+/1- primer pair was designed on the coat protein region of the PVY^{O}-803 genome (Rigotti & Gugerli, 2007). All the primers designed by Rigotti & Gugerli (2007) are described in Table 2.2. Those designed by Schubert *et al.* (2007) are summarized in Table 2.4. *PVY* strains are differentiated with Rigotti & Gugerli (2007) primers by comparing the PCR product of each primer pair as shown in Table 2.3. Each primer pair designed by Schubert *et al.* (2007) amplifies a particular strain of *PVY* (Table 2.4).

Total plant RNA extracted with the SV total RNA isolation system (Promega, Madison, USA), reverse primers and the Enhanced Avian Reverse Transcriptase (SIGMA, Missouri, USA) was used according to the manufacturer's instructions to synthesize the first strand cDNA. PCR were run as described in section 2.2.2.2, with the amendments provided in Table 2.2 and Table 2.4. PCR products were also analysed as described in 2.2.2.2.

Table 2.2. Description of Rigotti & Gugerli (2007) primers used to differentiate *PVY* strains and PCR parameters

Primer name (F/R)	Sequence 5' – 3'	PCR annealing Temperature (°C)	PCR extension time
PVYc3 (F)	CAACGCAAAAACACTCAYAAAMGC	54	45 s
PVYf (R)	TAAGTGRACAGACCCTCTYTTCTC		
PVY3+ (F)	TGTAACGAAAGGGACTAGTGCAAAG	58	1min
PVY3- (R)	CCGCTATGAGTAAGTCCTGCACA		
CP2+ (F)	CCAGTCAAACCCGAACAAAGG	58	1 min
CP1- (R)	GGCATAGCGTGCTAAACCCA		

Each primer pair is made of a reverse (R) and a forward (F) primer.

Table 2.3. Identification of *PVY* strains using Rigotti & Gugerli (2007) primers

Strains	PCR product with *PVY*c3/f primer pair (bp)	PCR product with *PVY*3+/3- primer pair (bp)	PCR product with CP2+/1- primer pair (bp)
PVY^{N}	440	1110	-
Non recombinant *PVY*^{NTN}	440	1110	-
Recombinant *PVY*^{NTN}	440	-	-
PVY^{O}	660	-	530
PVY^{N}Wi	-	-	530
PVY^{N}Wi (*PVY*^{N}N242)	440	-	530
PVY^{C}	660	-	-

e.g. *PVY*^{N} yields 440 bp with *PVY*c3/f, 1110 with *PVY*3+/3- but no product with CP2+/1-

Table 2.4. Description of Schubert *et al.* (2007) primers for the differentiation of *PVY* strains and PCR parameters

Primer name (F/R)	Sequence 5' – 3'	strain/ Product size (bp)	PCR Ta* (°C)	PCR extension time
YO5-1005 (F)	A_{979}AATTGTACGATGCACGTTCTAGA	O/ 1553	55	1 min 35 sec
YO3-2558 (R)	A_{2556}GGCTCATCTAACAGCAACTGTC			
YN5-1780 (F)	T_{1758}CCGAATGGGACAAGAAAACTTG	N/658	56	45 sec
YN3-2438 (R)	T_{2559}GGTTCATCCAGTAGCAATTGCT			
YNA5-116 (F)	T_{95}TTGATCTTCGTCGTACAAACCG	NA/434	51	30 sec
YNA3-622 (R)	C_{645}TTGATAAGATGGTTCATTTGTTT			
YO5-5293 (F)	G_{5293}TACAGACCTCTTCGCCATCCCAA	NTN/ 3867	55	4 min
YNTN3-9160 (R)	A_{9170}AAGCATAGCGAGCCAAACTTC			
YN5-1780 (F)	T_{1758}CCGAATGGGACAAGAAAACTTG	Wilga/ 5052	54	5 min
YO3-6790 (R)	G_{6787}TTCGTGGTGTGTTTGTTGTTT			
YC5-125 (F)	A_{125}TTGAAAACCGTCTTAGTTAGTT	C/353	51	30 sec
YC3-460 (R)	G_{478}CAGCCATCTGAAAGTAGTGC			

(R): reverse primer, (F): forward primer, Ta*: Annealing temperature of primers, NA: PVY^{N} North American type. Positions of primers (lower case number in column "Sequence") are given according to the position in the genome of isolate Jakab (PVY^{N}, PVY^{N}W, PVY^{NTN}), SA110 (PVY^{O}, PVY^{N}W, PVY^{NTN}), Adgen-C (PVY^{C}) and Nicola (NA-$PVY^{N/NTN}$) (Schubert *et al.,* 2007).

2.2.4. Biological assay

All *PVY* isolates were mechanically inoculated onto *Chenopodium quinoa* (*C. quinoa*), *Nicotiana tabacum* (*N. tabacum*) cv Xanthi, *N. tabacum* cv Samsun, *N. glutinosa*, and *N. rustica* as described in section 2.2.1. Inoculated plants were maintained in a glasshouse (CERU facilities, UKZN-PMB) under natural light and ambient temperature. Symptom development was observed every second day for six weeks. DAS ELISA, using $PVY^{O/C}$ Mab and PVY^{N} Mab DAS reagents (Neogen Corporation, Scotland, UK), was performed as described in section 2.2.2.1 to confirm the presence of *PVY* in all plants showing symptoms three weeks post inoculation.

2.2.5. Electron microscopy

The leaf dip method (Bos, 1999) was used to prepare samples from different symptomatic indicator plants for electron microscope examination. The surface of a fresh cross-section of a young leaf was dipped in water and rubbed onto the surface of a carbon-coated polyvinyl formaldehyde (Formvar) grid. The grid was then negatively stained with 2% uranyl acetate for thirty seconds and viewed under the Philips CM 120 Biotwin transmission electron microscope (TEM).

2.3. Results

2.3.1. Detection of *PVY*

2.3.1.1. ELISA

The Mab specific to PVY^{O}, PVY^{C}, and PVY^{N} reacted positively with *N. rustica* inoculated with leaf extracts from all Eshowe samples and some of the samples from Tugela Ferry, Tala Valley and Greytown. Positive reactions were also observed with some of the potato plants collected from Howick and Umbumbulu. All ELISA results are summarized in Table 2.5.

2.3.1.2. RT-PCR

PCR results correlated with ELISA results. The expected 510 bp fragment was amplified in all ELISA-positive plants. A distinct band of about 1000 bp that

corresponds to the amplification product of primers 3'$_{NTR}$C and *PVY* 2F was also observed on the gels. PCR results for the detection of *PVY* in all inoculated *N. rustica* plants and potato plants tested are summarized in Table 2.5.

Table 2.5. ELISA and PCR results for the detection of *PVY* in all inoculated *N. rustica* plants and volunteer potato plants tested.

Location	Sample description	Tested samples	ELISA Positive	PCR Positive
Eshowe	*N. rustica* inoculated with leaf extracts	9	9	9
Tala Valley	*N. rustica* inoculated with leaf extracts	9	5	5
Tugela Ferry	*N. rustica* inoculated with leaf extracts	9	4	4
Greytown	*N. rustica* inoculated with leaf extracts	24	9	9
Howick	Volunteer potato	23	6	6
Umbumbulu	Volunteer potato	10	6	6

2.3.2. Differentiation of *PVY* isolates

2.3.2.1. ELISA

All *PVY* isolates infecting tomato and pepper reacted positively with Mab specific to *PVY*^{O} and *PVY*^{C} and with Pab + Mab specific to PVY^{O} but not with Mab specific to *PVY*^{N}. Three isolates (H11, H12 and H14) infecting potato reacted with Mab specific to *PVY*^{N} only. Two isolates (H17 and H23) reacted with Mab specific to *PVY*^{O} and *PVY*^{C} and with Pab + Mab specific to *PVY*^{O}. Isolate H6 reacted with all antibodies. An ELISA plate with Pab + Mab specific to *PVY*^{O} showing typical results is shown in Figure 2.2. All ELISA results for strain differentiation are summarized in Table 2.6.

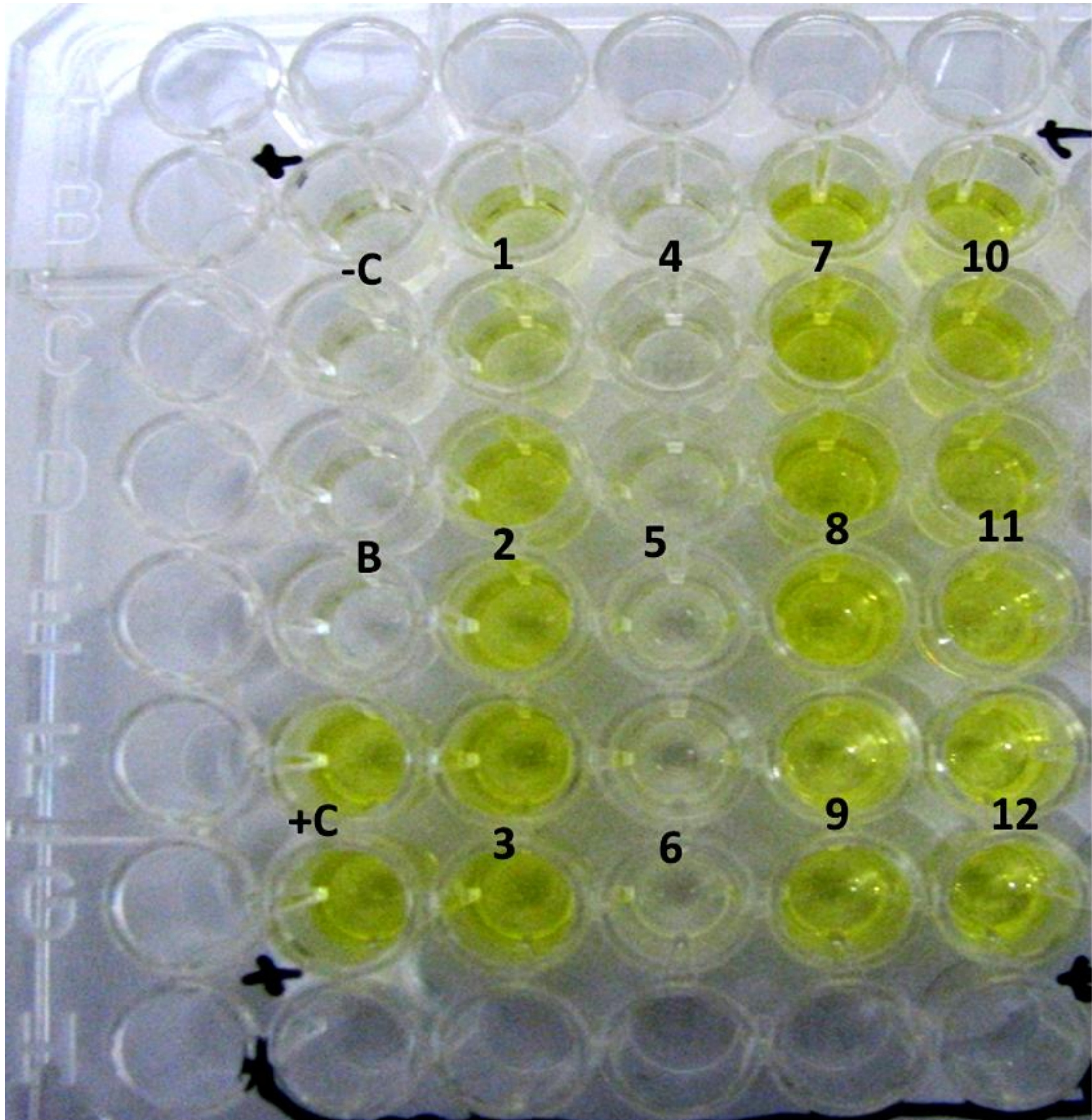


Figure 2.2. ELISA plate showing typical positive and negative reactions. Samples showing positive reaction were similar to the positive control (+C) as shown by the yellow colour they display. The extraction buffer (B) and the negative control (-C) remained colourless, indicating a negative reaction. 1- 12: *N. rustica* leaves inoculated with pepper leaf extracts collected from Greytown.

Table 2.6. ELISA results for the differentiation of *PVY* isolates infecting pepper, potato and tomato in KZN

Location	Sample description	$PVY^{O/C}$ Mab	PVY^{O} Pab + Mab	PVY^{N} Mab
Eshowe	Tomato	+	+	-
	Tomato	+	+	-
Tala Valley	Tomato	+	+	-
Tugela Ferry	Tomato	+	+	-
Greytown	Pepper	+	+	-
	Pepper	+	+	-
Howick	Potato 6 (H6)	+	+	+
	Potato 11 (H11)	-	-	+
	Potato 12 (H12)	-	-	+
	Potato 14 (H14)	-	-	+
	Potato 17 (H17)	+	+	-
	Potato 23 (H23)	+	+	-
Umbumbulu	Potato	-	-	+
	Potato	-	-	+
	Potato	-	-	+
	Potato	-	-	+
	Potato	-	-	+
	Potato	-	-	+

+: positive reaction (pNPP turned yellow); -: negative reaction (pNPP remained colourless).

2.3.2.2. RT-PCR

Amplification using Rigotti & Gugerli (2007) primers led to the conclusion that PVY^{O} is strain present in the pepper and tomato samples collected from Greytown and Eshowe. Potato infecting isolates H17 and H23 from Howick are PVY^{N}Wilga, potato infecting isolate H14 from Howick either are PVY^{N} or PVY^{NTN}, potato infecting isolate H12 from Howick is a mixture of PVY^{NTN} and PVY^{N}Wilga, and potato infecting isolates

H6 and H11 from Howick are a mixture of *PVY*^{N} and *PVY*^{N}Wilga. These conclusions were drawn by comparing the RT-PCR products (Figure 2.3) with the indications given in Table 2.3. All the results are summarized in Table 2.7. However, it was not possible to conclude on the strain of the tomato infecting *PVY* isolates from Tala Valley and Tugela Ferry because they infer multiple possible combinations. It was also noticed that the RT-PCR of tomato and pepper samples yielded more non specific amplification products (Figure 2.3A) compared to the RT-PCR of potato samples (Figure 2.3B & C).

Table 2.7. Differentiation of the *PVY* isolates infecting pepper, potato and tomato in KZN with RT-PCR using primers designed by Rigotti & Gugerli (2007)

Location	Sample	***PVY*c3/*PVY*f product (bp)**	***PVY*3+/*PVY*3- product (bp)**	**CP2+/CP1- product (bp)**	**Strain**
Greytown	Pepper	660	-	530	*PVY*^{O}
Eshowe	Tomato	660	-	530	*PVY*^{O}
Tala Valley	Tomato	660; 440	-	530	*All except PVY*^{N}
Tugela Ferry	Tomato	660; 440	1110	530	*All*
Howick	H6	440	1110	530	*PVY*^{N}, *PVY*^{N}Wilga
	H11	440	1110	530	*PVY*^{N}, *PVY*^{N}Wilga
	H12	440	-	530	*PVY*^{NTN}, *PVY*^{N}Wilga
	H14	440	1110	-	*PVY*^{NTN}, *PVY*^{N}
	H17	-	-	530	*PVY*^{N}Wilga
	H23	-	-	530	*PVY*^{N}Wilga

-: No product

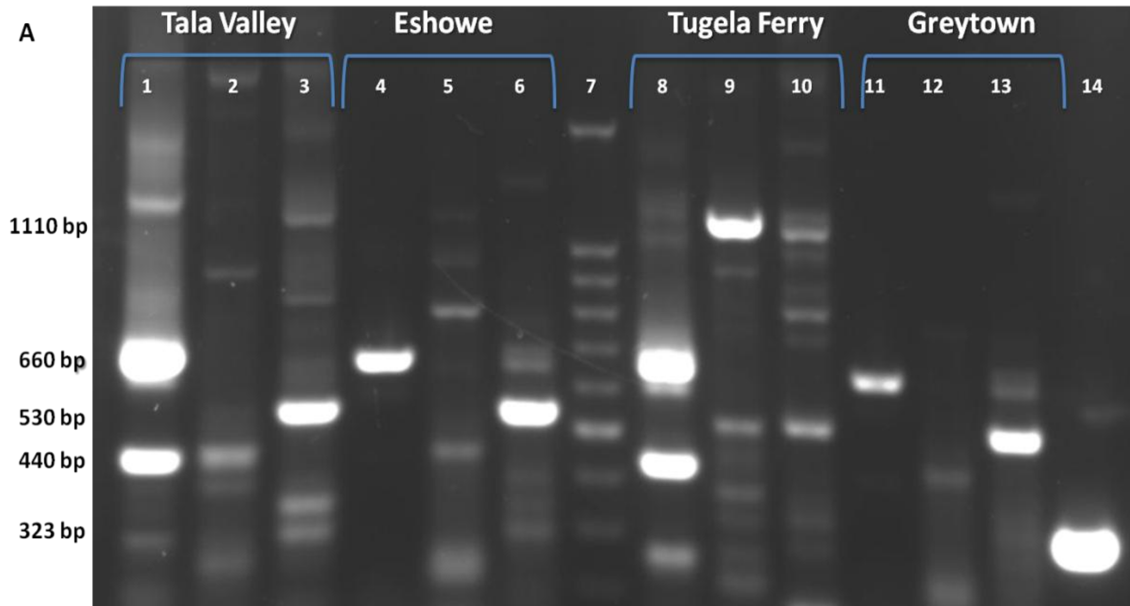


Figure 2.3A. Agarose gel of RT-PCR using primers designed by Rigotti & Gugerli (2007) with tomato infecting isolates from Tala Valley, Eshowe, Tugela Ferry and pepper infecting isolates from Greytown. Lanes 1-3: Tomato infecting isolate from Tala Valley amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lanes 4-6: Tomato infecting isolate from Eshowe amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lane 7: 100 bp DNA ladder (Promega, Madison, USA); lanes 8-10: Tomato infecting isolate from Tugela Ferry amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lanes 11-13: Pepper infecting isolate from Greytown amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lane 14: promega Go Taq PCR core systems II positive control.

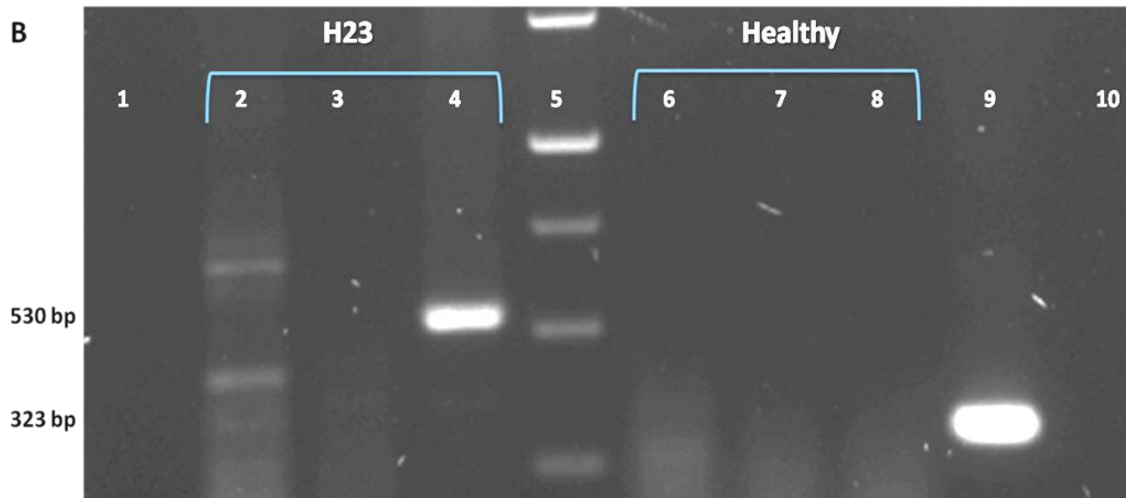


Figure 2.3B. Agarose gel of RT-PCR using primers designed by Rigotti & Gugerli (2007) with potato infecting isolates H23 from Howick. Lane 1: water control of *PVY*c3/*PVY*f; lanes 2-4: Potato infecting *PVY* isolate H23 from Howick amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lane 5: 1 kb DNA ladder (Fermentas, Canada); lanes 6-8: Healthy plant amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lane 9: promega Go Taq PCR core systems II positive control; lane 10: water control of *PVY*3+/*PVY*3-.

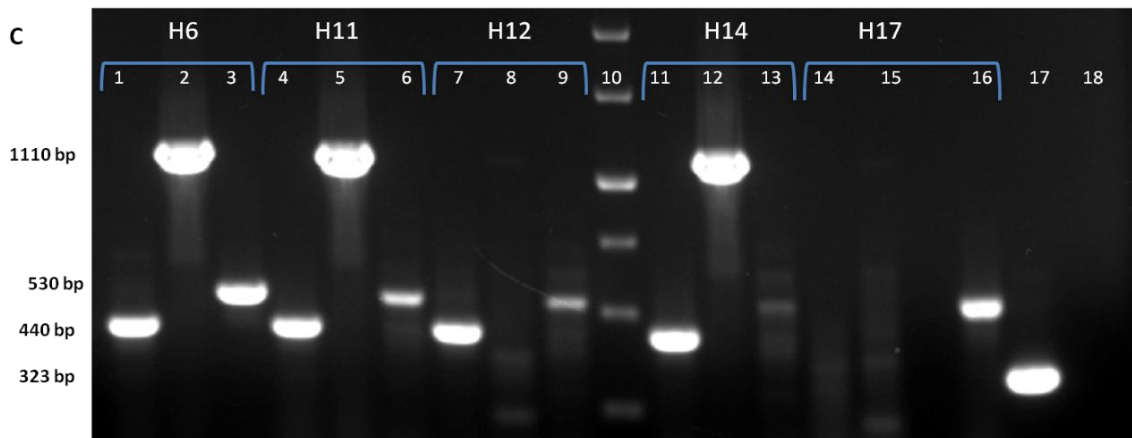


Figure 2.3C. Agarose gel of RT-PCR using primers designed by Rigotti & Gugerli (2007) with potato infecting isolates H6, H11, H12, H14 and H17 from Howick. Lanes 1-3: Potato infecting isolate H6 from Howick amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lanes 4-6: Potato infecting isolate H11 from Howick amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lanes 7-9: Potato infecting isolate H12 from Howick amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lane 10: 1 kb DNA ladder (Fermentas, Canada); lanes 11-13: Potato infecting *PVY* isolate H14 from Howick amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lanes 14-16: Potato infecting *PVY* isolate H17 from amplified with *PVY*c3/*PVY*f, *PVY*3+/*PVY*3-, and CP2+/CP1- respectively; lane 17: promega Go Taq PCR core systems II positive control; lane 18: water control of CP2+/CP1-.

RT-PCR, using Schubert *et al.* (2007) primers, provided more conclusive results compared to Rigotti & Gugerli (2007) primers. RT-PCR, using the primers N5-1780/N3-2438, yielded an 800 bp product (Figure2.4) longer than the expected fragment of 658 bp. The alignment of the primers YN5-1780 and YN3-2438 on the Jakab *PVY*^{N} (Accesion number X97895) using *MEGA* version 4 (Tamura *et al.*, 2007) hybridized in the 1758-1780 and 2537-2559 nucleotide regions of the genome respectively. This will therefore produce an 802 bp product instead of 658 as indicated by Schubert *et al.* (2007). RT-PCR, using Schubert *et al.* (2007) primers, identified potato infecting isolates H17, H23 from Howick and all tomato and pepper

infecting isolates as PVY^{O} (Figure 2.5); potato infecting isolate H12 from Howick as PVY^{NTN} (Figure 2.7); potato infecting isolates H11 and H14 from Howick as PVY^{N} (Figure 2.4) and potato infecting isolate H6 from Howick as a mixture of PVY^{O} and PVY^{N} (Figure 2.4 and 2.5). All potato infecting isolates from Umbumbulu were a mixture of PVY^{NTN} and PVY^{N} (Figure 2.4 and 2.7). RT-PCR, using primers specific to PVY^{C}, produced a non specific amplification product of about 200 bp which is about 200 bp shorter than the expected 353 bp fragment with potato infecting *PVY* isolates H11, H12, H14, H17 and H23 from Howick (Figure 2.8). PVY^{N}Wilga isolates could not be positively identified due to the multiple non specific bands observed on the gel (Figure 2.9). All the RT-PCR results using Schubert *et al.* (2007) primers are summarized in Table 2.8.

Table 2.8. Differentiation of the *PVY* isolates infecting different vegetables with RT-PCR using primers designed by Schubert *et al.* (2007)

Location	Isolate	PVY^{N}	PVY^{O}	PVY^{NTN}	NA-$PVY^{N/NTN}$	PVY^{C}	PVY^{N}Wilga
Greytown	Pepper	x	+	x	X	-	x
Eshowe	Tomato	x	+	x	X	-	x
Tala Valley	Tomato	-	+	x	X	-	-
Tugela Ferry	Tomato	-	+	x	X	-	-
Umbumbulu	Potato	+	x	+	X	x	x
Howick	H6	+	+	-	-	-	x
	H11	+	-	-	-	-	x
	H12	-	-	+	-	-	x
	H14	+	-	-	-	-	x
	H17	-	+	-	-	-	?
	H23	-	+	-	-	-	?
Refer to Figure		2.4	2.5	2.6	2.7	2.8	2.9

+: Positive result; -: Negative result; ?: Result not conclusive due to multiple non specific amplifications: x: PCR not performed.

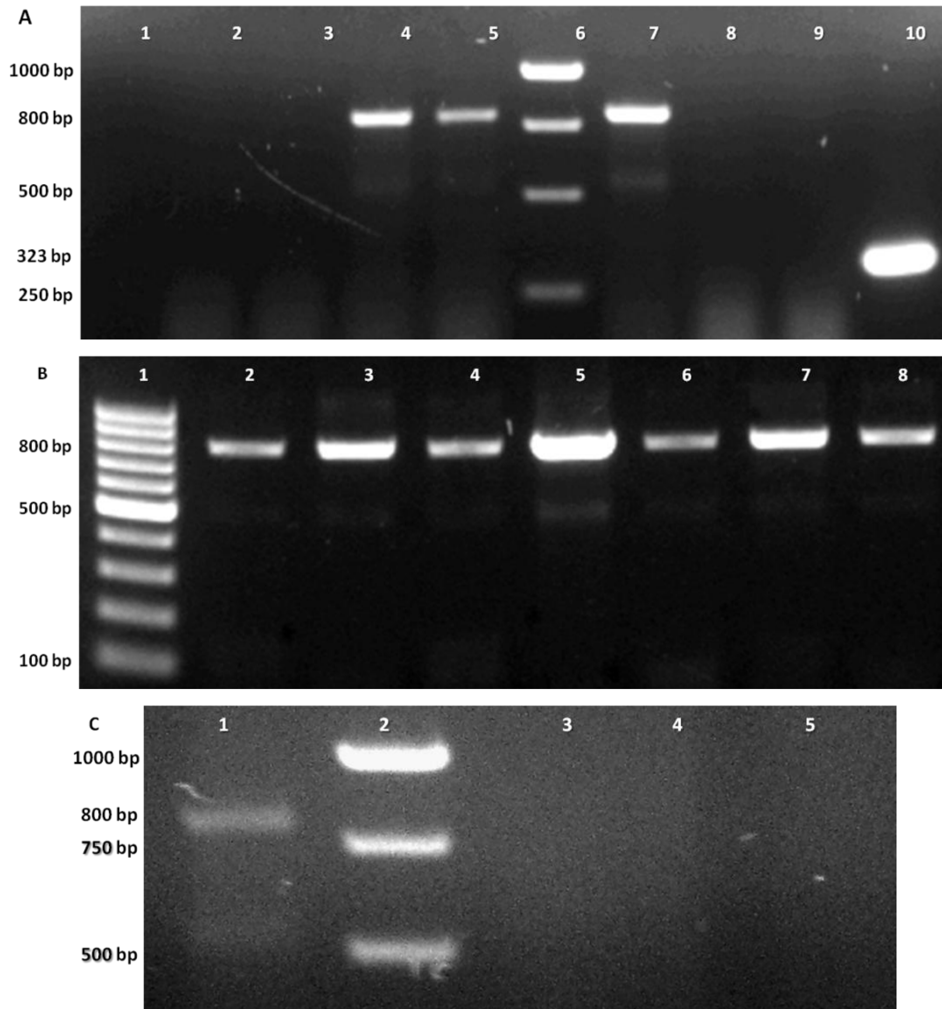


Figure 2.4. Agarose gel of RT-PCR using primers designed by Schubert *et al.* (2007) for the detection of *PVY*^{N}. **A:** Lane 1: water control; lane 2: tomato infecting isolate from Tugela Ferry; lane 3: tomato infecting isolate from Tala Valley; lane 4: potato infecting isolate H6 from Howick; lane 5: potato infecting isolate H11 from Howick; lane 6: 1 kb DNA ladder (Fermentas, Canada); lane 7: potato infecting isolate H14 from Howick; lane 8: potato infecting isolate H17 from Howick; lane 9: healthy plant; lane 10: promega Go Taq PCR core systems II positive control. **B:** Lane 1: 100 bp DNA ladder (Fermentas, Canada); lanes 2, 3, 4, 6, 7, 8: potato infecting *PVY* isolates from Umbumbulu; lane 5: potato infecting *PVY* isolate H6 from Howick. **C:** Lane 1: potato infecting *PVY* isolate H11 from Howick; lane 2: 1 kb DNA ladder (Fermentas, Canada); lane 3: potato infecting *PVY* isolate H12 from Howick; lane 4: potato infecting *PVY* isolate H23 from Howick; lane 5: healthy plant. A positive amplification is expected to produce a 658 bp fragment.

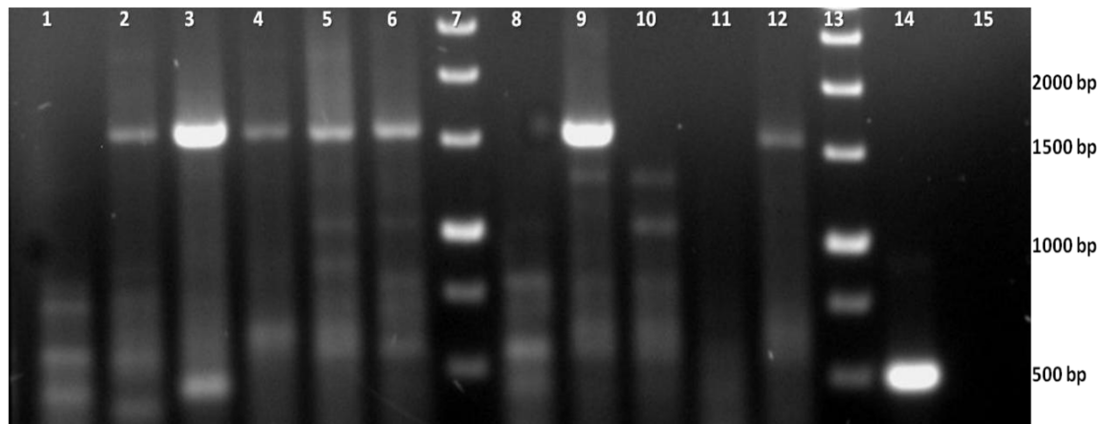


Figure 2.5. Agarose gel of RT-PCR using primers designed by Schubert *et al.* (2007) for the detection of *PVY*^{O}. Lane 1: Healthy plant; lane 2: potato infecting isolate H17 from Howick; lane 3: tomato infecting isolate from Eshowe; lane 4: potato infecting isolate H23 from Howick; lane 5: tomato infecting isolate from Tala Valley; lane 6: tomato infecting isolate from Tugela Ferry; lane 7: 1 kb DNA ladder (Fermentas, Canada); lane 8: potato infecting isolate H11 from Howick; lane 9: pepper infecting isolate from Greytown; lane 10: potato infecting isolate H12 from Howick; lane 11: potato infecting isolate H14 from Howick; Lane 12: potato infecting isolates H6 from Howick; lane 13: 1 kb DNA ladder (Fermentas, Canada); lane 14: *PVY* positive control with *PVY* 2F/2R primers; lane 15: water control. A positive amplification is expected to produce a 1553 bp fragment.

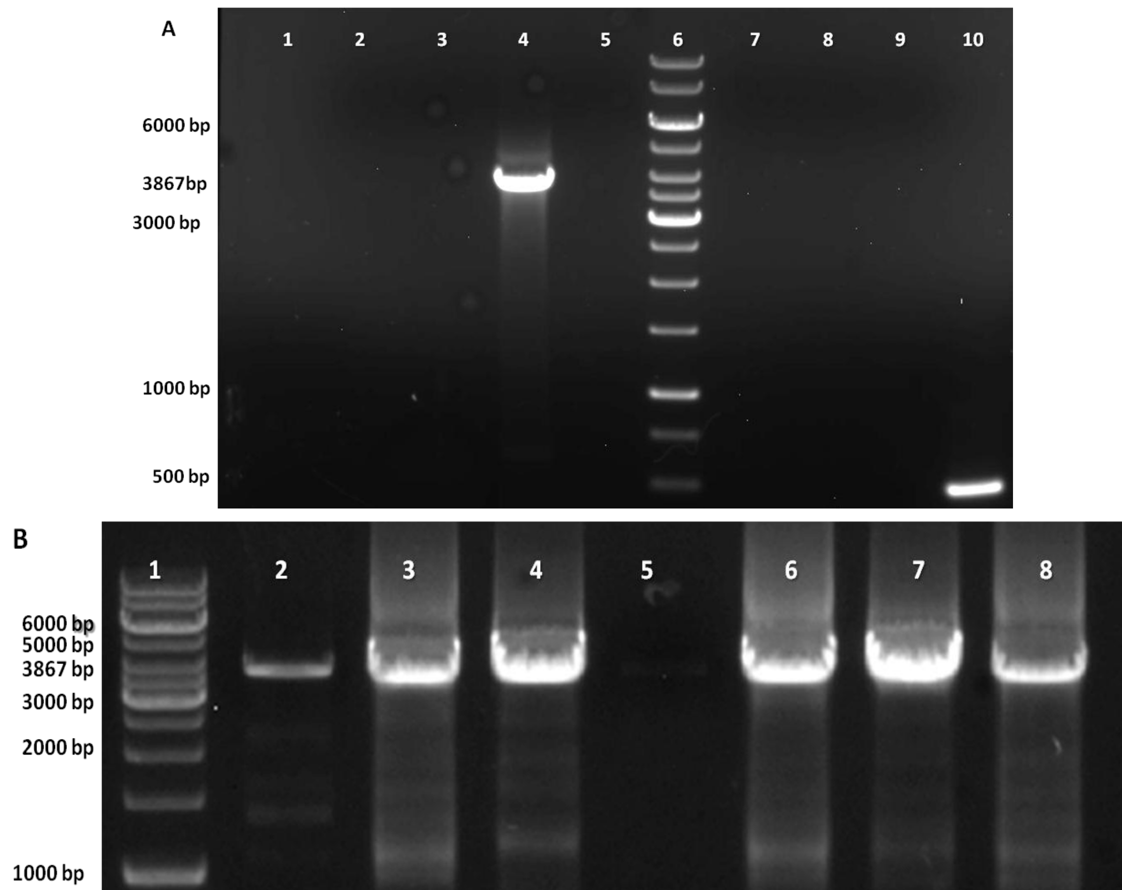


Figure 2.6. Agarose gel of RT-PCR using the primers designed by Schubert *et al. (*2007) for the detection of *PVY*^{NTN}. **A:** Lane 1: water control; lane 2: potato infecting *PVY* isolate H6 from Howick; lane 3 potato infecting isolate H11 from Howick; lane 4: potato infecting isolate H12 from Howick; lane 5: potato infecting isolate H14 from Howick; lane 6: 1 kb DNA ladder (Fermentas, Canada); lane 7: potato infecting isolate H17 from Howick; lane 8: potato infecting isolate H23 from Howick; lane 9: healthy plant; lane 10: *PVY* positive control with *PVY* 2F/2R primers. **B:** Lane 1: 1 kb DNA ladder (Fermentas, Canada); lanes 2, 3, 4, 6, 7, 8: potato infecting isolates from Umbumbulu; lane 5: potato infecting isolate H6 from Howick. A positive amplification is expected to produce a 3867 bp fragment.

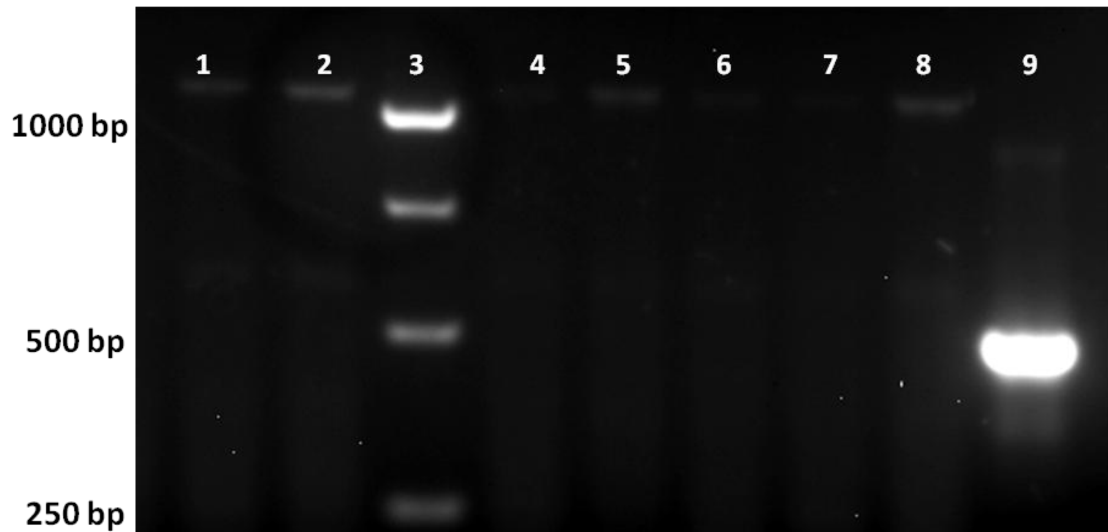


Figure 2.7. Agarose gel of RT-PCR using the primers designed by Schubert *et al.* (2007) for the detection of the North American *PVY*$^{N/NTN}$. Lane 1: potato infecting isolate H6 from Howick; lane 2: potato infecting isolate H11 from Howick; lane 3: 1 kb DNA ladder (Fermentas, Canada); lane 4: potato infecting isolate H12 from Howick; lane 5: potato infecting isolate H14 from Howick; lane 6: Healthy plant; lane 7: potato infecting isolate H17 from Howick; lane 8: potato infecting isolate H23 from Howick; lane 9: *PVY* positive control with *PVY* 2F/2R primers. A positive amplification is expected to produce a 434 bp fragment.

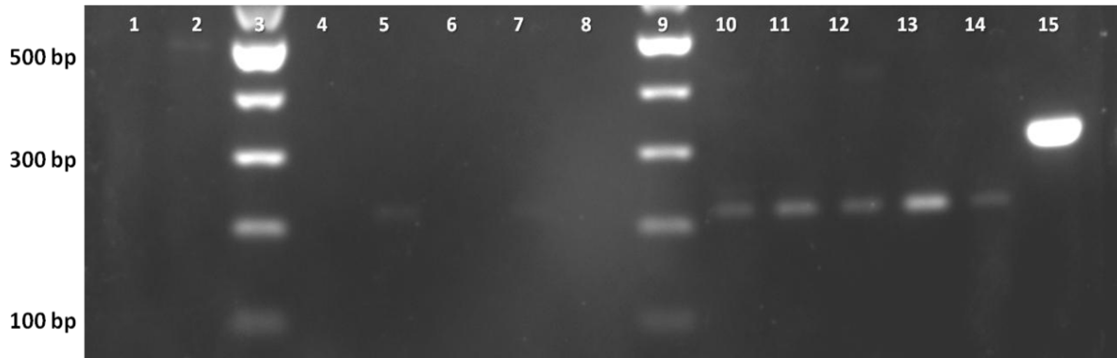


Figure 2.8. Agarose gel of RT-PCR using the primers designed by Schubert *et al.* (2007) for the detection of *PVY*^{C}. Lane 1: Healthy plant; lane 2: tomato infecting isolate from Eshowe; lane 3 and 9: 100 pb DNA ladder (Fermentas, Canada); lane 4: water control; lane 5: tomato infecting isolate from Tala Valley; lane 6: pepper infecting isolate from Greytown; lane 7: tomato infecting isolate from Tugela Ferry; lane 8: potato infecting isolate H6 from Howick; lane 10: potato infecting isolate H11 from Howick; lane 11: potato infecting isolate H12 from Howick; lane 12: potato infecting isolate H14 from Howick; lane 13: potato infecting isolate H17 from Howick; lane 14: potato infecting isolate H17 from Howick; lane 15: promega Go Taq PCR core systems II positive control. A positive amplification is expected to produce a 353 bp fragment.

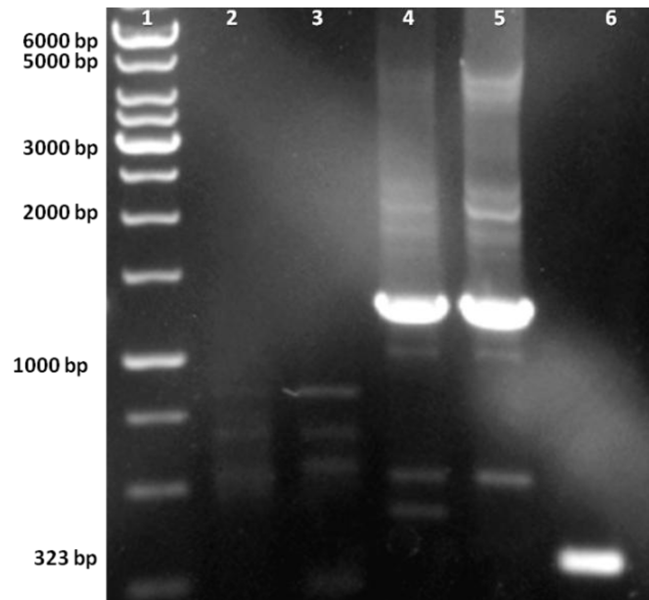


Figure 2.9. Agarose gel of RT-PCR using the primers designed by Schubert *et al.* (2007) for the detection of *PVY*^{N}Wilga. Lane 1: 1 kb DNA ladder (Fermentas, Canada); lane 2: tomato infecting isolate from Tugela Ferry; lane 3: tomato infecting *PVY* isolate from Tala Valley; lane 4: potato infecting isolate H17 from Howick; lane 5: potato infecting isolate H23 from Howick; lane 6: promega Go Taq PCR core systems II positive control. A positive amplification is expected to produce a 5052 bp fragment.

2.3.3. Biological assay

Inoculated *C. quinoa* did not show any symptoms (Figure 2.10). Symptoms on the other indicator plants started appearing 5 days post inoculation (dpi). Not all plants displaying symptoms tested positive for *PVY* using ELISA. All *PVY*-related symptoms (symptoms which tested positive for *PVY*) started with vein clearing which later turned into mosaic, mottling, wrinkle, stunting, vein necrosis, and leaf death depending on the indicator plants and *PVY* isolates. *PVY*-related symptoms observed are summarized in Table 2.9. All indicator plants infected with tomato *PVY*^{O} isolate from Eshowe displayed severe mosaic compared to the indicator plants inoculated with tomato *PVY*^{O} isolate from Tala Valley and pepper *PVY*^{O} isolate from Greytown (Figure 2.11A, B; 2.12A, B and 2.13A, B). Tomato *PVY*^{O} isolate from Tala Valley and

pepper PVY^{O} isolate from Greytown produced on *N. rustica* a very faint mottling which can go unnoticed (Figure 2.13C). Vein necrosis started appearing 21 dpi only on *N. tabacum* cv Xanthi and cv Samsun inoculated with potato PVY^{N}, PVY^{N}Wilga, PVY^{NTN} isolates from Howick and potato PVY^{N} and PVY^{NTN} isolates from Umbubulu (Figure 2.12E and F). The same potato *PVY* isolates from Howick and Umbumbulu induced mosaic and stunting on *N. glutinosa* (Figure 2.11C and D) and a very faint, almost unnoticeable mottling on *N. rustica* (Figure 2.13D). Atypical *PVY* symptoms, including necrotic spot, wrinkling and severe leaf distortion, were observed on *N. rustica*. Moderate mosaic were observed on *N. tabacum* cv Xanthi and cv Samsun (Figure 2.14).

Figure 2.10. e.g. of Symptomless *C. quinoa* inoculated with tomato PVY^{O} isolate from Eshowe.

Table 2.9. *PVY*-related symptoms observed on the different indicator plants

Locations	Isolates	Indicator plants			
		N. glutinosa	***N. tabacum* cv Xanthi**	***N. tabacum* cv Samsun**	***N. rustica***
Greytown	*PVY*^{O} infecting pepper	Vein clearing	Vein clearing, moderate mosaic	Vein clearing, moderate mosaic	Vein clearing, moderate mosaic
Eshowe	*PVY*^{O} infecting tomato	Vein clearing, mottling	Vein clearing, severe mosaic	Vein clearing, severe mosaic	Vein clearing, severe mosaic
Tala Valley	*PVY*^{O} infecting tomato	Vein clearing	Vein clearing, moderate mosaic	Vein clearing, moderate mosaic	Vein clearing, moderate mosaic
Howick	*PVY*^{N} and *PVY*^{N}Wilga infecting potato	Vein clearing, mosaic, stunting	Vein clearing, mosaic, vein necrosis	Vein clearing, mosaic, vein necrosis	Vein clearing, very faint mottling
	Potato infecting *PVY*^{NTN}	Vein clearing, mosaic, stunting	Vein clearing, mosaic, vein necrosis	Vein clearing, mosaic, vein necrosis	Vein clearing, very faint mottling
Umbumbulu	Potato infecting *PVY*^{N} and *PVY*^{NTN}	Vein clearing, mosaic, stunting	Vein clearing, mosaic, vein necrosis	Vein clearing, mosaic, vein necrosis	Vein clearing, very faint mottling
Refer to Figure		2.11	2.12	2.12	2.13

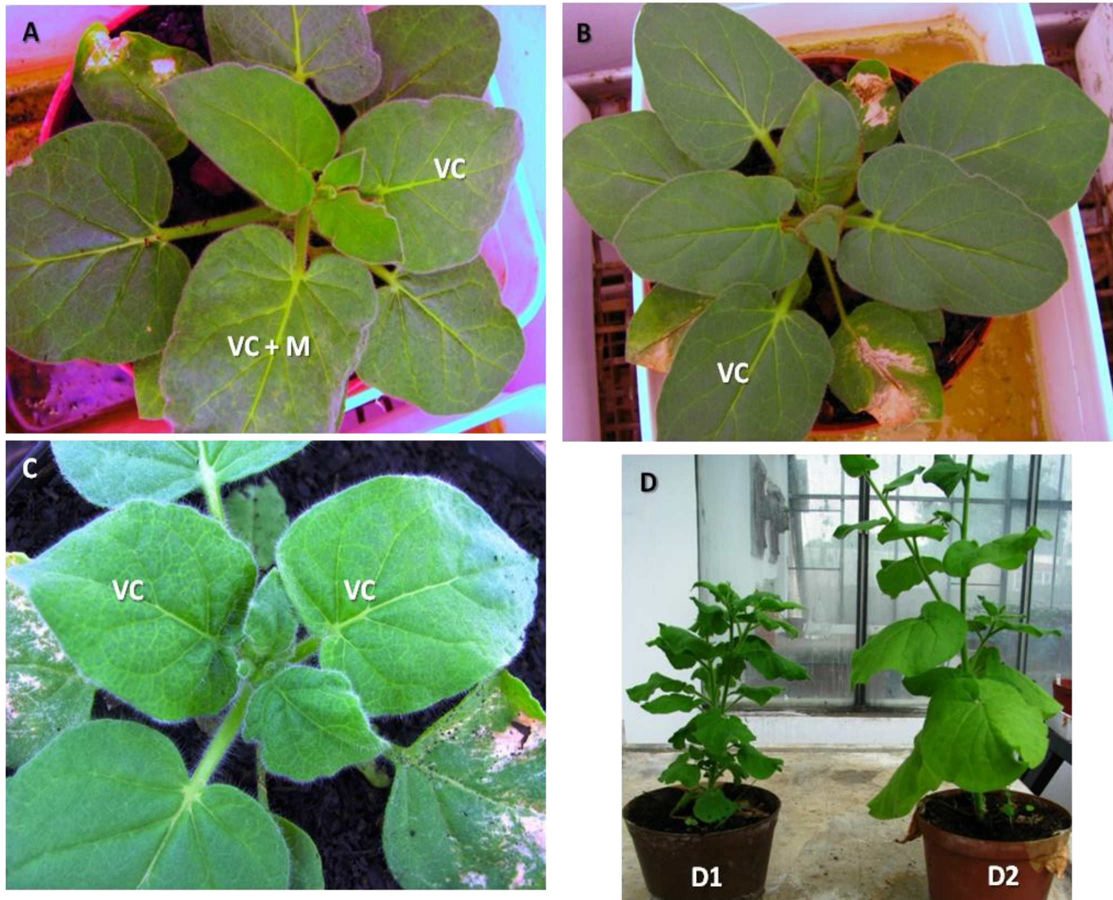


Figure 2.11. *PVY*-related symptoms on mechanically inoculated *N. glutinosa* with **A: T**omato infecting PVY^{O} isolate from Eshowe **B:** Tomato infecting PVY^{O} isolates from Tugela Ferry, Tala Valley and pepper infecting PVY^{O} isolate from Greytown separately. **C** and **D:** Potato infecting PVY^{N}, PVY^{N}Wilga, PVY^{NTN} isolates from Howick and Potato infecting PVY^{N} and PVY^{NTN} isolates from Umbumbulu separately. **D1:** infected, **D2:** Healthy (uninoculated). M: Mottling, VC: vein clearing. VC+M: Vein clearing and mottling.

Figure 2.12. *PVY*-related symptoms on mechanically inoculated *N. tabacum* cv Samsun and cv Xanthi with **A** and **B:** Tomato infecting *PVY*^{O} isolate from Eshowe. **C:** Tomato infecting *PVY*^{O} isolates from Tugela Ferry, Tala Valley and pepper infecting *PVY*^{O} isolate from Greytown separately. **D:** Tomato infecting *PVY*^{O} isolates from Tugela Ferry, Tala Valley, pepper infecting *PVY*^{O} isolate from Greytown, Potato infecting *PVY*^{N}, *PVY*^{N}Wilga, *PVY*^{NTN} isolates from Howick and Potato infecting *PVY*^{N} and *PVY*^{NTN} isolates from Umbumbulu separately. **E** and **F:** Potato infecting *PVY*^{N}, *PVY*^{N}Wilga, *PVY*^{NTN} isolates from Howick and Potato infecting *PVY*^{N} and *PVY*^{NTN} isolates from Umbumbulu separately. Chl: chlorosis, Dl: Dead leaves, FM: faint Mottling, SMo: severe mosaic, VC: vein clearing, VN: vein necrosis.

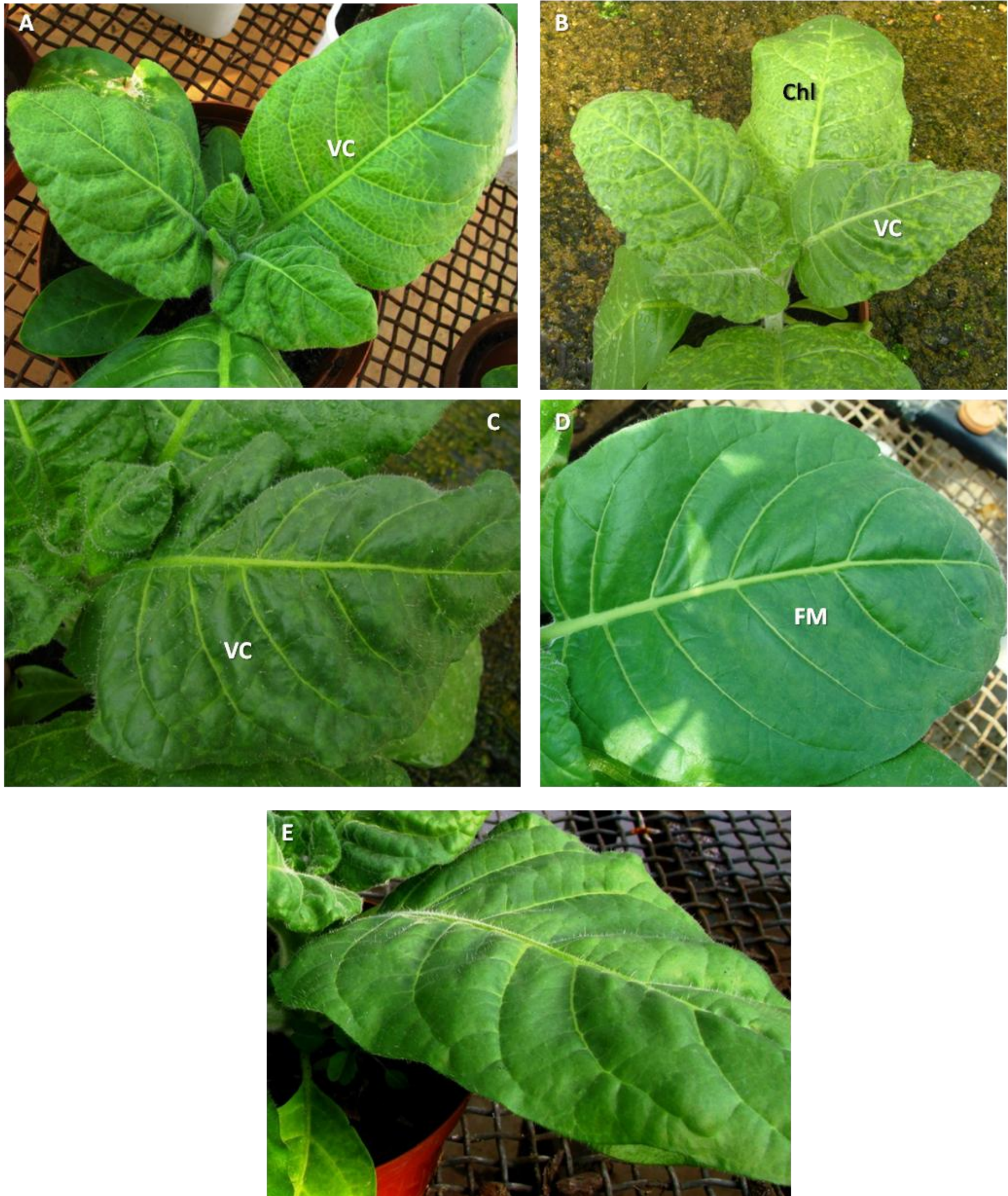


Figure 2.13. *PVY*-related symptoms on mechanically inoculated *N. rustica* with **A** and **B:** Tomato infecting *PVY*^{O} isolate from Eshowe. **C:** Tomato infecting *PVY*^{O} isolates from Tugela Ferry, Tala Valley and pepper infecting *PVY*^{O} isolate from Greytown separately. **D:** Potato infecting *PVY*^{N}, *PVY*^{N}Wilga, *PVY*^{NTN} isolates from Howick and Potato infecting *PVY*^{N} and *PVY*^{NTN} isolates from Umbumbulu separately. **E:** Healthy (uninoculated). Chl: chlorosis, FM: faint Mottling, VC: vein clearing.

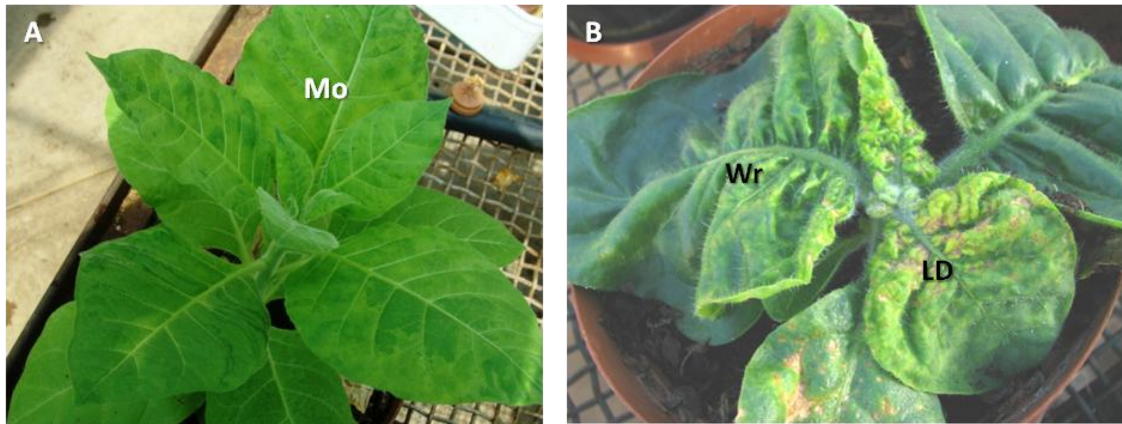


Figure 2.14. Non-related *PVY* symptoms. **A:** *N. tabacum* cv Samsun and cv Xanthi, **B:** *N. rustica*, LD: leaf distortion, Mo: mosaic, Wr: wrinkle.

2.3.4. Electron microscopy

Potyvirus-like particles (Figure 2.15A) were observed in most indicator plants showing *PVY*-related symptoms. A mixture of *potyvirus*-like particles and rod-shape particles (Figure 2.15B and C) were observed in *N. tabacum* cv Samsun and cv Xanthi showing the severe mosaic displayed in Figure 2.12D. Rod-shape particles with length varying between 70 and 400 nm (Figure 2.15D) were observed in the indicator plants showing non-related *PVY* symptoms.

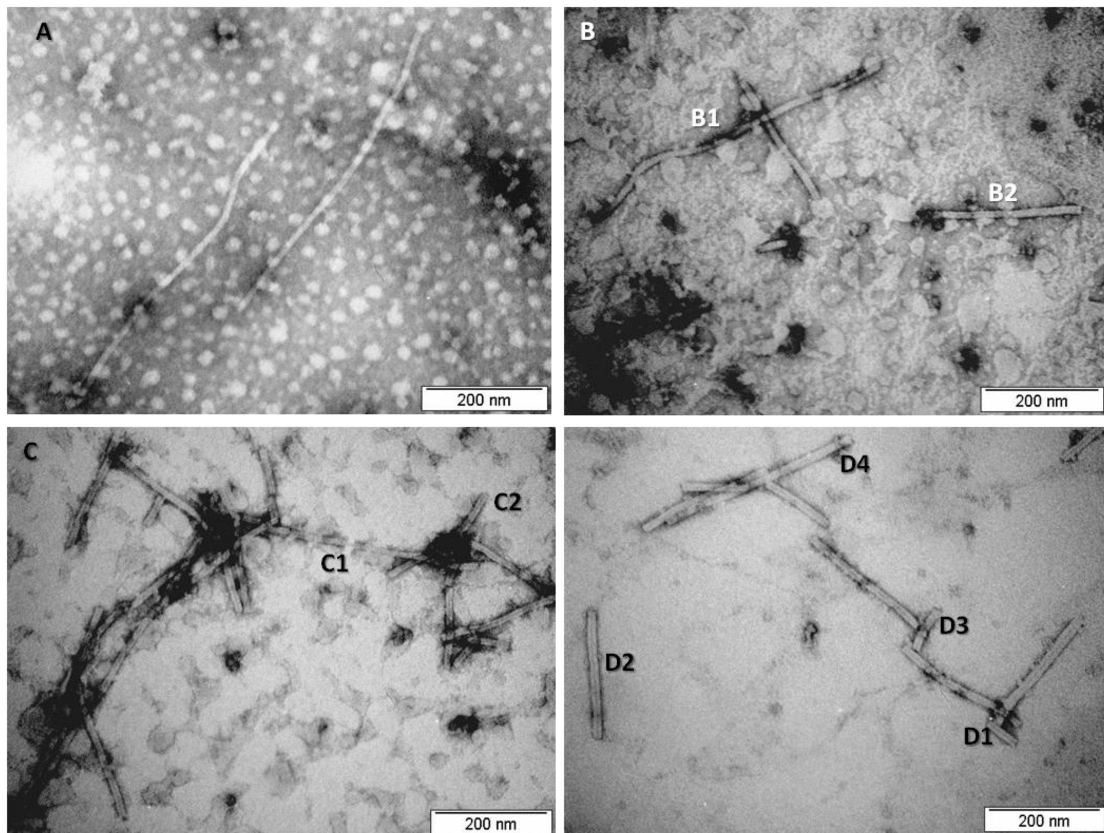


Figure 2.15. Electro-micrographs of viral particles present in infected indicator plants. **A:** *Potyvirus*-like particles (about 700 nm). **B** and **C:** mixture of *Potyvirus*-like particles (**B1**, **C1**) and rod-shape particles (**B2**, **C2**). **D:** Rod-shape particles with varying length; **D1:** 70 nm, **D2:** 250 nm, **D3:** 100 nm, **D4:** 400 nm.

2.4. Discussion

PVY was successfully detected in pepper, potato and tomato grown in KZN by ELISA using Mab and RT-PCR using primers specific to all strains of *PVY*. This report, along with similar studies (Budnik *et al.*, 1996; Thompson, 1980) confirm the prevalence of *PVY* in KZN and underline the permanent threat *PVY* presents to vegetable production in the province. *PVY* has been considered as one of the five most important viruses of vegetable crops in the world (Mijatović *et al.*, 2002). No *PVY* epidemic has been reported yet in KZN. *Nicandra physaloides* and *Solanum nigrum* were reported to be important reservoirs of the virus in KZN (Budnik *et al.* 1996). Accumulation of the virus in those plants may, in the long term, provide favourable

conditions for a widespread epidemic of the virus. *PVY* epidemics have been reported to reach 100% infection with yield losses of up to 50% (Rosner *et al.*, 2000). A break-out of such an epidemic in KZN would have negative consequences in the province.

PVY incidence always brings the effectiveness of disease management into question. Management of *PVY* is mainly achieved by controlling its vector. However, the high mobility of the vector population and the mode of transmission of *PVY* (non persistent) make its control difficult. Another alternative is the use of resistant cultivars. Resistant cultivars provide protection against a narrow range of pathogens. Genetic engineering resistance is thought to confer a wider protection range when compared to a conventional breeding programme (Zhu *et al.*, 2009). Engineered crop resistant to the major pathogens occurring locally remains the quicker way for a long term solution.

PVY^{O} is the strain present in all tomato and pepper infected samples collected in KZN since these samples reacted positively with both Mab specific to $PVY^{O/C}$ and Pab + Mab specific to PVY^{O}. RT-PCR results, especially with those using Schubert *et al.* (2007) primers, confirmed that finding as they yielded the expected product of 1553 bp (Figure 2.5). However, RT-PCR using Rigotti & Gugerli (2007) suggested mixed infections with either PVY^{N} or PVY^{N}Wilga (Figure 2.3, Table 2.7) in *PVY* infected tomato samples from Tala Valley and Tugela Ferry. Amplification of the same samples using Schubert *et al.* (2007) primers specific to PVY^{N} and PVY^{N}Wilga did not show any product (Figure2.4 and 2.9, Table 2.8). This consequently does not give us enough confidence to state the presence of PVY^{N} and PVY^{N}Wilga in those samples. Sequencing of the amplified DNA indicating the presence of PVY^{N} or PVY^{N}Wilga obtained using Rigotti & Gugerli (2007) primers with *PVY* infected tomato samples from Tala Valley and Tugela Ferry remains the only alternative to understand their identity.

The identification of PVY^{O} infecting tomato and pepper appears to be the first report of this kind in tomato and the second in pepper in KZN. *PVY* isolates infecting tomato

previously studied in different parts of the world were classified as *PVY*^{C} strain in most cases (Aramburu *et al.*, 2006; Comes *et al*., 2005; Crescenzi *et al*., 2005; Morel *et al*., 2000; Rosner *et al.*, 2000) and, *PVY*^{N} and *PVY*^{NTN} in only one case (Aramburu *et al.*, 2006). The Netherlands *PVY*^{O} isolate PK 706 was the only *PVY*^{O} isolate reported to infect pepper especially cv. Friariello di Napoli (d'Aquino *et al.,* 1995).

ELISA results obtained with isolates infecting potato were consistent with RT-PCR results using primers designed by Schubert *et al.* (2007), as well as those designed by Rigotti & Gugerli (2007). *PVY*^{N} isolates infecting potato detected with Mab specific to *PVY*^{N} (Table 2.7) were identified as *PVY*^{NTN} for H12 isolate (Table 2.7 and 2.8, Figure2.3 and 2.6), *PVY*^{N} for H6, H11 and H14 isolates (Table 2.7 and 2.8, Figure2.3 and 2.4), and a mixture of *PVY*^{NTN} and *PVY*^{N} for all Umbumbulu isolates (Table 2.8, Figure 2.4 and 2.6). Potato infecting-isolates H6, H17 and H23 that tested positive with Mab specific to *PVY*$^{O/C}$ and Pab + Mab specific to *PVY*^{O} (Table 2.6) were identified as a mixture of *PVY*^{O} (Table 2.7 and 2.8, Figure 2.3 and 2.5) and *PVY*^{N}Wilga (Table 2.7, Figure 2.3) by RT-PCR. RT-PCR results using Rigotti & Gugerli (2007) primers were used to confirm the presence of *PVY*^{N}Wilga. The faint 5000 bp (Figure 2.9) band obtained using Schubert *et al.* (2007) primers specific to *PVY*^{N}Wilga may either be the expected product or another non-specific amplification.

PVY infecting potato isolates were more diverse and mixtures of strains were observed in most cases. This reflects the diversity of *PVY* strains infecting potato reported around the world (Kerlan & Moury, 2008). *PVY* strains infecting potato comprise *PVY*^{O}, *PVY*^{C}, *PVY*^{N}, *PVY*^{NTN} and *PVY*^{N}Wilga. *PVY*^{NTN} and *PVY*^{N}Wilga isolates have been proven to be recombinant of *PVY*^{O}, and *PVY*^{N}. The genome studies of *PVY*^{NTN} especially have shown that isolates from North America, Europe and Japan differ in their recombination points (Fomitcheva *et al.*, 2009). Recombination junctions have been mainly found in the P1, HC-Pro, NIa and coat protein region of the genome (Kogovsek *et al.*, 2008; Lorenzen *et al.*, 2006). Studies of the genome of

local isolates of *PVY*^{NTN} and *PVY*^{N}Wilga will provide information on the origin of these isolates.

PVY^{C} was not detected in this study. *PVY*^{C} is known to infect potato, pepper, tomato and tobacco. Studies done on *PVY* in the main tobacco growing region of the country reported the presence of that strain (Vorster *et al.*, 1990). *PVY*^{C} is also know to have restricted distribution compared to the others strains. This is mainly due to the weak transmission of that strain by the vector (Ellis *et al.*, 1997; Kerlan *et al.*, 1999). This observation can provide a plausible explanation for the absence of *PVY*^{C} in our studies.

Biological studies of viruses require pure isolates of the viruses concerned. *C. quinoa* is known as a local lesion host of *PVY* and has routinely been used to obtain pure isolates of the virus. This could not be achieved in our study because *C. quinoa* did not show local lesions (Figure 2.10). Similar observations have been reported in different studies (Crescenzi *et al*., 2005; Lorenzen *et al*., 2006; Morel *et al.*, 2000). The fact that all tobacco plants showing symptoms did not react positively with ELISA unmistakably indicates the presence of other viruses. Pepper, potato and tomato are susceptible to several other viruses (Jones *et al.*, 1997; Pernezny *et al*., 2003; Stevenson *et al*., 2004) which can occur at the same time. Synergism is a feature very common in *potyviruses* which is mainly mediated by the *Potyvirus* HC Pro protein. Synergism in this study was confirmed with the TEM by observing flexuous *potyvirus*-like particles together with rigid rod-shape viral particles (Figure 2.15B & C). Consequently all observed *PVY* symptoms cannot be fully attributed to *PVY*.

PVY^{N}, *PVY*^{NTN} and *PVY*^{N}Wilga strains cause veinal necrosis on tobacco. The results in our studies show that *N. rustica* and *N. glutinosa* infected with *PVY*^{NTN}, *PVY*^{N} and *PVY*^{N}Wilga did not show veinal necrosis but vein clearing, mosaic and faint mottling (Figure 2.11; 2.13). This unexpected result may be an indication of a unique phenotype specific to KZN *PVY*^{N}, *PVY*^{NTN} and *PVY*^{N}Wilga isolates. An American *PVY*

isolate (L26), which displays a similar phenotype, has recently been described by Hu *et al.*, (2009).

The atypical *PVY* symptoms observed on *N. rustica* and *N. tabacum* could be attributed to the rod-shaped viruses with various lengths seen under the TEM (Figure 2.15D). The observation of the rod-shape particles (400 nm) was thought to be *Tobacco mosaic virus* (*TMV*), a highly stable infectious *Tobamovirus* infecting *solanaceous* crops and reported to occur in KZN (Trench *et al.*, 1992). A routine ELISA test with Mab specific to *TMV* (Neogen Corporation, Scotland, UK) did not produce any positive reaction (data not shown). Symptoms displayed on *N. rustica* leaves appeared to indicate infection by *Tomato spotted wilt virus* (*TSWV*), a thrips transmitted *Tospovirus* also infecting *solanaceous* crop in KZN (Sivparsad and Gubba, 2008). However, ELISA with Mab specific to *TSWV* (Bio-Rad, California, USA) was also negative (data not shown). Therefore, the hypothesis that *N. tabacum* and *N. rustica* displaying the non-related *PVY* symptoms were infected with *TMV* or *TSWV* was excluded. The presence of a newly introduced virus or virus that has been occurring but never been reported in KZN can be speculated. An attempt to identify this virus may involve full etiological diagnosis protocol.

In conclusion, this study led to the detection of *PVY* in all vegetable hosts grown in KZN. The virus is likely to occur in synergism with other viruses of vegetables. PVY^{O} is the only strain infecting pepper and tomato. Isolates infecting potato are more diverse and occur in mixed infection. They include PVY^{N}, PVY^{NTN}, PVY^{O} and PVY^{N}Wilga. Further characterization may include phylogenic analyses as they provide information on the evolution of these local strains.

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Chapter 3
Phylogenic Studies of Selected Isolates of *Potato Virus Y* (*PVY*) Infecting Selected Vegetable Crops in KwaZulu-Natal (KZN), Republic of South Africa (RSA)

Abstract

Molecular studies of plant viruses provide genetic information on biological characteristics and possible pathways of evolution. Phylogenic relationships of selected isolates of *Potato virus Y* (*PVY*) infecting different vegetables in KwaZulu-Natal (KZN) were investigated in this study. 1067 bp covering part of the coat protein gene and the 3' non-translated region (NTR) of three *PVY*^{O} isolates infecting tomato (*Lycopersicon esculentum* Mill.), one *PVY*^{O} isolate infecting pepper (*Capsicum annuum* L.) and one *PVY*^{N}Wilga isolate infecting potato (*Solanum tuberosum* L.) were amplified, cloned and sequenced. The 5' NTR, P1, HC-Pro and part of P3 regions (2559 bp) of a *PVY*^{N} isolate infecting potato were also sequenced. All genomic sequence data and related protein sequences were compared with selected sequences from *PVY* isolates from different geographical locations and subjected to phylogenic analyses. The sequence of the *PVY*^{N} isolate clustered with the European sublineage N and has five unique amino acids residues: two in the P1 and three in the HC-Pro protein. The three amino acid residues (D_{205}, K_{400}, and E_{419}), determinants of the vein necrosis phenotype in tobacco, were also identified on the HC-Pro region. The phylogenic analysis branched *PVY*^{N}Wilga isolate infecting potato with the American *PVY*^{O} isolate Oz in the O lineage. All *PVY*^{O} isolates infecting tomato and pepper were put together in a new sublineage within the O lineage. Evolutionary information described in this study will be useful in developing *PVY* management programmes of solanaceous crops in the Republic of South Africa.

3.1. Introduction

Potato virus Y (*PVY*), the type member of the *Potyvirus* genus in the family *Potyviridae*, occurs worldwide and is responsible for significant yield losses and quality degradation in agricultural production of several important *solanaceous* crops. Typical *PVY* virions are 713 nm long and 11 nm wide. *PVY* has a single 9.7 Kb single linear positive strand RNA genome which harbours a single open reading frame (Fauquet *et al.*, 2005; Shukla *et al*., 1994). Isolates of *PVY* that have been identified include the common ordinary PVY^{O}, the stipple streak PVY^{C}, the veinal necrosis PVY^{N}, the tuber necrosis PVY^{NTN}, and PVY^{N}Wilga (Fauquet *et al.*, 2005; Mijatovic *et al.*, 2002; Shukla *et al*., 1994).

Phylogenic studies are essential in the characterization of plant viruses. They are sources of valuable information on their biological characteristics and possible pathways of evolution. Molecular and phylogenic studies of *PVY* isolates have been carried out on the coding and non coding regions of the genome containing useful information (Margaritopoulos *et al.*, 2009; Ogawa *et al.*, 2008). PVY^{O}, PVY^{C} and PVY^{N} isolates have been reported to produce similar phylogenic patterns with any region of the virus genome studied (Margaritopoulos *et al.*, 2009). Phylogenic analyses of PVY^{C} led to its subdivision into *PVY*C1 and *PVY*C2 (Blanco-Urgoiti *et al.,* 1998). A point mutation found in the coat protein sequence of the Syrian *PVY*-12 isolate resulted in a double reactivity of the isolate to Mab specific to both PVY^{O} and PVY^{N} (Ali *et al.*, 2008). PVY^{N}Wilga isolates have been found to be recombinant of PVY^{O} and PVY^{N} and one or two recombinant points have been identified on their genomes. PVY^{NTN} isolates were split into European, North American and Japanese isolates depending on their recombination junctions. North American and Japanese PVY^{NTN} isolates appeared to be non-recombinant and represent a further sequence variant. Recombinant PVY^{NTN} isolates identified were found to have three to four recombinant junctions on their genomes (Formitcheva *et al.*, 2009; Hu *et al.,* 2009; Ogawa *et al.*, 2008). Two amino acid residues K_{400} and E_{419} in the C terminal part of the multifunctional HC-Pro protein have been identified as the molecular

determinants involved in the vein necrosis symptom produced by PVY^N isolates (Tribodet *et al.*, 2005) and the nucleotide change resulting in the amino acid change D_{205} to G_{205} in the central region of HC-Pro was associated with the loss of the vein necrosis phenotype in tobacco (*Nicotiana* spp) (Hu *et al.,* 2009).

Phylogenic analyses of African isolates of *PVY* are not well documented. Only one full sequence of an Egyptian isolate of PVY^N (GenBank Accession number AF522296) is available on the NCBI website. Therefore the aim of this study was to sequence and establish the phylogenic relation of selected *PVY* isolates occurring in KZN with isolates from other parts of the world.

3.2. Material and Methods

3.2.1. Viruses isolates

All KZN isolates of *PVY* used in this study have been described previously (Chapter 2) and are summarized in Table 3.1.

Table 3.1. Description of *PVY* isolates used in the present study

Crop	Strain	Location where collected
Pepper	O	Greytown
Tomato	O	Eshowe
Tomato	O	Tala Valley
Tomato	O	Tugela Ferry
Potato	N Wilga	Howick
Potato	N	Howick

3.2.2. Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR)

Immunocapture-reverse transcription-polymerase chain reaction (Albrechtsen, 2006; Nolasco *et al.*, 1993) was performed on all isolates (Table 3.1). Working solutions of (100x diluted) Mab specific to PVY^O and PVY^N (Neogen Corporation, Scotland, UK)

were used for the immunocapture of the isolates. All PVY^{O} and PVY^{N}Wilga isolates were captured using Mab specific to PVY^{O} while Mab specific to PVY^{N} were used to capture PVY^{N} isolates. 0.2 ml PCR tubes were coated with 20 µl coating antibodies and incubated at 37°C for 3 hrs. They were then washed three times with 100 ul PBS-T. 20 µl leaf sample, ground in extraction buffer, were added. The tubes were incubated overnight at 4°C. Following the overnight incubation the tubes were washed again.

RT was done using RevertAid™ Premium Reverse Transcriptase (Fermentas, Canada) according to the manufacturer's instructions. The $3'_{NTR}$C primer (5'-GTCTCCTGATTGAAGTTTAC-3') designed by Glais *et al.* (2005) was used to synthesize the cDNA of the PVY^{O} and PVY^{N}Wilga isolates and the YN3-2438 (5'-TGGTTCATCCAGTAGCAATTGCT-3') designed by Schubert *et al.* (2007) was used to synthesize the cDNA of the PVY^{N} isolates. Dream Taq Polymerase (Fermentas, Canada), which produced amplicons with 3'A overhang was used for PCR. PCR reactions were prepared according to the manufacturer's instructions (Fermentas, Canada). The forward primers CP2+ (5'- CCAGTCAAACCCGAACAAAGG-3') by Rigotti & Gugerli (2007) and Y5end (5'-AAATTAAAACAACTCAATACAACATAAGAA-3') by Schubert *et al.* (2007) were used to amplify the target region with the same reverse primers used for RT. The primer pair CP2+/$3'_{NTR}$ was expected to produce a 1067 bp amplicon covering part of the coat protein and the full 3' NTR before the poly-A tail of the PVY^{O} and PVY^{N}Wilga isolates. The primer pair Y5end/YN3-2438 was expected to produce a 2559 bp amplicon covering the 5' NTR, P1, HC-Pro and part of the P3 protein regions of the PVY^{N} isolates genome. PCR products were analysed on 1% agarose gel electrophoresis and visualized on the VersaDoc imaging system 4000 (Bio-Rad, California, USA).

3.2.3. Cloning and sequencing

Two microliters (µl) of PCR product were ligated to the pCR® 2.1 vector provided with the TA cloning® kit (Lucigen, California, USA) according to the manufacturer's

instructions. TOP10F' *Escherichia coli* (*E. coli*) competent cells were transformed by heat shock at 42°C for 30 s with 2 µl ligation reaction. 100 µl of transformed cells were plated onto Luria-Bertani (LB) plates containing 0.5 mM IPTG (Fermentas, Canada), 80 µg/ml X-Gal (Fermentas, Canada) and 50 µg /ml kanamycin. The plates were incubated overnight at 37°C. Following the incubation the plates were checked for white (transformant) colonies. About 10 white colonies were picked and grown in LB broth containing 50 µg/ml kanamycin at 37°C for eight hours in a shaker incubator. The cells were then harvested and plasmid extraction was performed using the QIAprep mini-prep System (Qiagen, Doncaster, Australia). True transformants containing the desired amplicon were checked by PCR with the primers described in section 3.2.2. A true transformant for each isolates was sent to Inqaba Biotec (Hatfield, Pretoria, RSA) for sequencing using the forward and reverse M13 primers.

3.2.4. Sequence comparisons and phylogenic analyses

The sequences of the isolates were compared with selected sequences on the NCBI website. All selected sequences from the NCBI website are summarized in Table 3.2. Genomic and amino acid sequences were aligned using the CLC main workbench version 5.5 software. *Pepper mottle virus* (*PepMoV*) was included as an outlier. Sequence similarities between isolates were evaluated using SimPlot Version 3.5.1 (Lole *et al.,* 1999). The defaults settings of RDP (Recombinant Detection Program) version 3.41 (Martin & Rybicki, 2000) was used to check for any recombinant events in the genomic regions sequenced. Phylogenic analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007). Phylogenic trees were inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Recombinant sequences were excluded from phylogenic analyses since they can create incorrect inferences (Margaritopoulos *et al.*, 2009; Schubert *et al.*, 2007).

Table 3.2. Sequences used for phylogenic analyses obtained from NCBI

Isolate	Origin	Strain	GenBank Accession number
OBR	Brazil	O	AF255659
O854	Switzerland	O	AJ223595
SON41	France	C	AJ439544
LYE84.2	Spain	C	AJ439545
SCRI-O	UK	O	AJ585196
Adgen-C	France	C	AJ890348
NZ	New Zealand	O	DQ217931
Oz	USA	O	EF026074
O-139	Canada	O	U09509
PVY-12	Syria	NTN	AB185833
NTNHO90	Japan	J-NTN	AB331517
NTNNN99	Japan	J-NTN	AB331518
Egypt	Egypt	N	AF522296
NZ	New Zealand	N	AM268435
Tu660	Canada	NA-NTN	AY166866
N-Jg	Canada	NA	AY166867
Mont	USA	N	AY884983
RRA-1	USA	NTN	AY884984
L26	USA	NTN	FJ204165
CH-605	Switzerland	N	X97895
PepMoV	USA	--	M96425

N: *PVY*^{N} European type; NA: *PVY*^{N} North American type; J-NTN: *PVY*^{NTN} Japanese type; NA-NTN: *PVY*^{NTN} North American type; O: *PVY*^{O}; C: *PVY*^{C} (Ogawa *et al.*, 2008).

3.3. Results

3.3.1. IC-RT-PCR

PCR products of the expected sizes, 2559 bp obtained using the primer pair Y5end/YN3-2438 (Figure 3.1A) and 1067 bp obtained using the primer pair CP2+/3'$_{NTR}$C (Figure 3.1B), indicated a successful amplification of the targeted region. Agarose gel of the RT-PCR products of *PVY*^{O} isolate infecting tomato from Tala Valley and Tugela Ferry also showed a non-specific band less than 750 bp (Figure 3.1B).

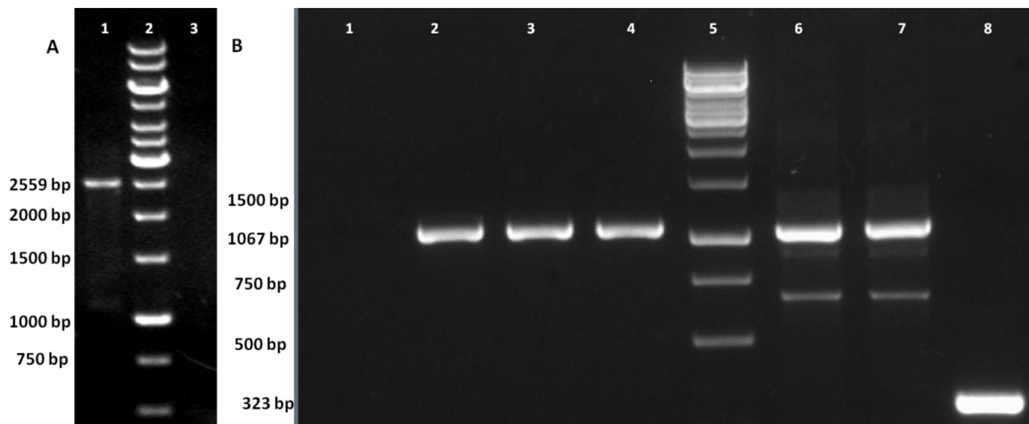

Figure 3.1. Agarose gel of the IC-RT-PCR products performed with **A:** the primer pair Y5end/YN3-2438 and with **B:** the primer pair CP2+/3'$_{NTR}$C. **A:** Lane 1: *PVY*^{N} isolate infecting potato from Howick; lane 2: 1 kb DNA ladder (Fermentas, Canada); lane 3: water control. **B**: Lane 1: water control; lane 2: *PVY*^{N}Wilga isolate H6 infecting potato from Howick; lane 3: *PVY*^{O} isolate infecting pepper from Greytown; lane 4: *PVY*^{O} isolate infecting tomato from Eshowe; lane 5: 1 kb DNA ladder (Fermentas, Canada); lane 6: *PVY*^{O} isolate infecting tomato from Tala Valley; lane 7: *PVY*^{O} isolate infecting tomato from Tugela Ferry; lane 8: promega Go Taq PCR core systems II positive control.

3.3.2. Cloning, and sequencing

Blue and white *E. coli* colonies were observed on the plates after overnight incubation as predicted by the TA cloning® kit manufacturer. An example of blue and white colonies is shown in Figure 3.2. Almost all white colonies picked were true transformants as verified by PCR (Figure 3.3 & 3.4).

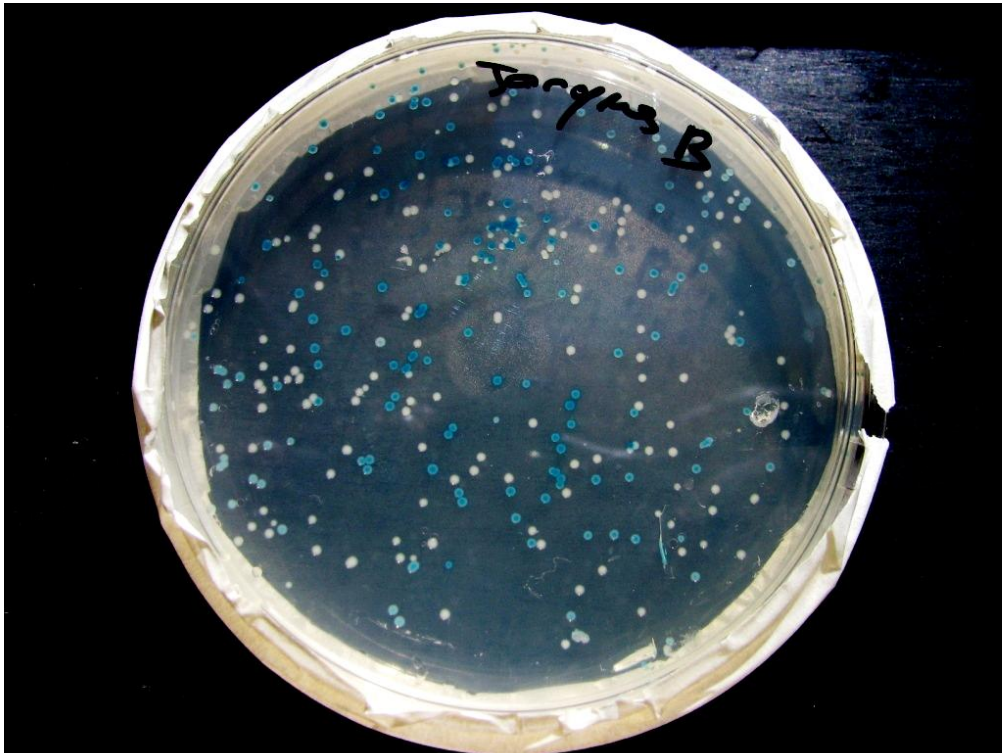

Figure 3.2. Picture of a LB plate showing blue (non-transformants) and white (transformants) colonies after overnight incubation at 37°C.

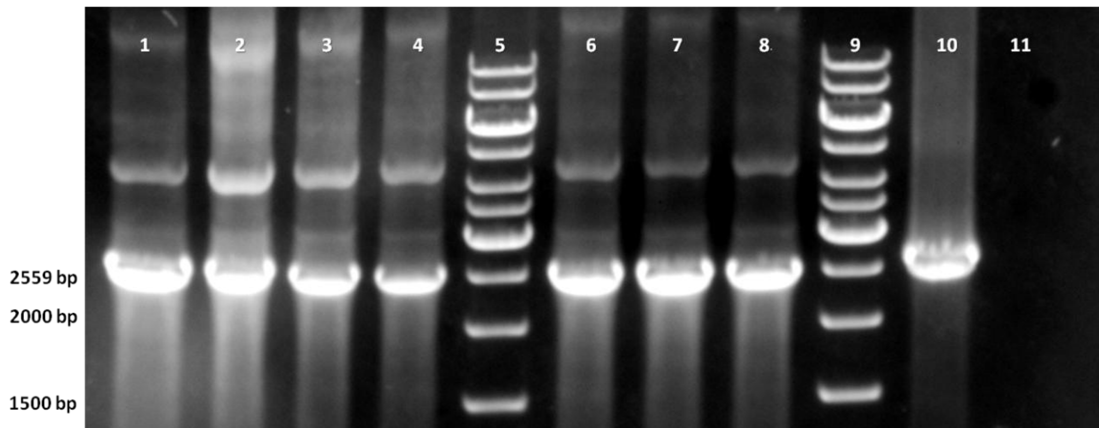

Figure 3.3. Agarose gel of PCR performed on plasmid DNA extracted from white *E. coli* colonies using the primer pair Y5end/YN3-2438 to screen for true transformants. Lanes 1-4 and 6-8: plasmid DNA extracted from white *E. coli* colonies transformed with the 2559 bp insert from PVY^{N} isolate infecting potato from Howick; lanes 5 and 9: 1 kb DNA ladder (Fermentas, Canada); lane 10: PVY^{N} isolate infecting potato from Howick; lane 11: water negative control.

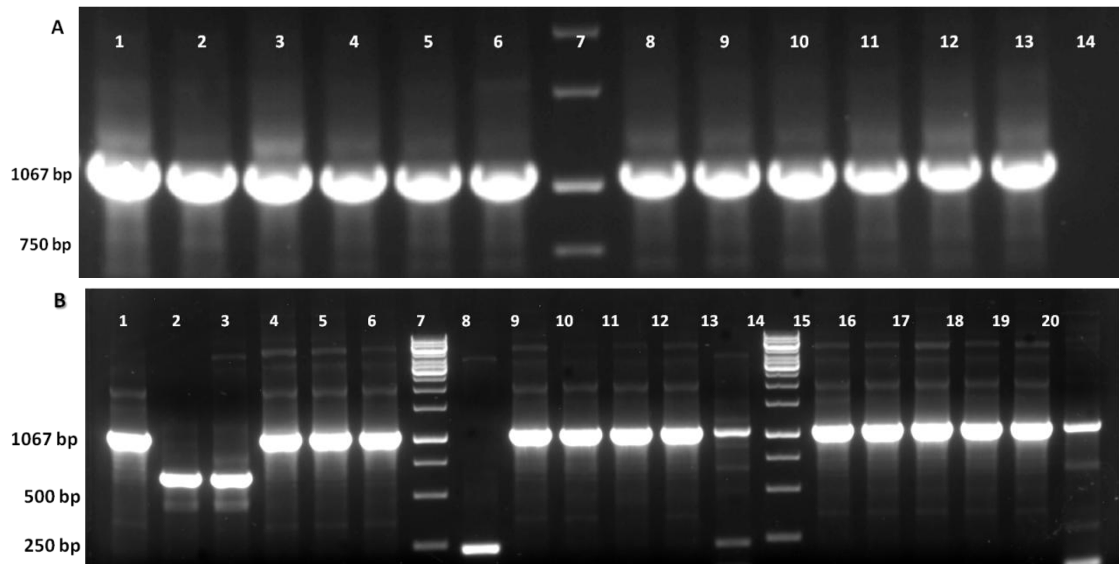

Figure 3.4. Agarose gel of PCR performed on plasmid DNA extracted from white *E. coli* colonies using the primer pair CP2+/3'$_{NTR}$C to screen for true transformants. **A:** Lanes 1-6: plasmid DNA extracted from white *E. coli* colonies transformed with the 1067 bp insert from *PVY*^{N}Wilga isolate H6 infecting potato from Howick; lane 7: 1 kb DNA ladder (Fermentas, Canada); lanes 8-13: plasmid DNA extracted from white *E. coli* colonies transformed with the 1067 bp insert from *PVY*^{O} isolate infecting pepper from Greytown; lane 14: water negative control. **B:** Lanes 1-6: plasmid DNA extracted from white *E. coli* colonies transformed with the 1067 bp insert from *PVY*^{O} isolate infecting tomato from Eshowe; lanes 7 and 14: 1 kb DNA ladder (Fermentas, Canada); lanes 8-13: plasmid DNA extracted from white *E. coli* colonies transformed with the 1067 bp insert from *PVY*^{O} isolate infecting tomato from Tala Valley, lanes 15-20: plasmid DNA extracted from white *E. coli* colonies transformed with the 1067 bp insert from *PVY*^{O} isolate infecting tomato from Tugela Ferry.

3.3.3. Sequence comparisons and phylogenic analyses

No recombination events were found in all KZN sequences studied. Analyses of these sequences with SimPlot showed that KZN *PVY*^{N} isolate infecting potato is almost identical to New Zealand *PVY*^{N} isolate (GenBank accession number: AM268435), Swiss *PVY*^{N} isolate CH605 (X97895) and American *PVY*^{N} isolate Mont (AY884983), as observed in Figure 3.5. Simplot analyses of the coat protein and 3' NTR showed that

all KZN *PVY*^{O} isolates infecting pepper and tomato were more closely related to each other than to KZN *PVY*^{N}Wilga isolate H6 infecting potato. Moreover the 5' coding region of the coat protein displayed the highest nucleotide variability while the highest nucleotide similarity between all sequences was observed within the first 100 nucleotides of the 3'NTR (Figure 3.6). KZN *PVY*^{N}Wilga isolate H6 infecting potato showed high similarity with UK *PVY*^{O} isolate SCRI-O (AJ585196), Swiss *PVY*^{O} isolate O854 (AJ223595), American *PVY*^{O} isolate Oz (EF026074), Brazil *PVY*^{O} isolate OBR (AF255659), New Zealand *PVY*^{O} isolate (DQ217931) and Canadian *PVY*^{O} isolate O-139 (U09509), as shown in Figure 3.7. The pairwise comparisons outcome with CLC main workbench version 5.5 (Appendix D) strongly supported these results.

The comparison of the protein sequences of isolates revealed that KZN *PVY*^{N} displays five unique amino acids: K_{61}, K_{194}, G_{361}, P_{393} and F_{552} (Table 3.3). K_{61} and K_{194} are located in the P1 protein while G_{361}, P_{393} and F_{552} are located in the HC-Pro protein. The residues D_{205}, K_{400}, E_{419} determinants of the vein necrosis phenotype in tobacco were identified as D_{480}, K_{675} and E_{694} on the amino acid sequences (Table 3.3). The motifs KITC and PTK involved in aphid transmission of *PVY* (Ng and Falk, 2006) were also identified within the HC-Pro protein sequence (Table 3.3). *PVY*^{N}Wilga isolate does not have any unique residues on the part of the coat protein analysed but all *PVY*^{O} isolates infecting pepper and tomato have a unique L_{34} residue in common (Table 3.4).

Ogawa *et al*. (2008) proposed an N lineage of *PVY* subdivided into the European lineage and the North American lineage. Phylogenic analyses showed that the KZN *PVY*^{N} isolate clustered within the N lineage especially within the European sublineage with a bootstrap value of 99 (Figure 3.8A) while the KZN *PVY*^{N}Wilga isolate H6 infecting potato was grouped with other *PVY* potato isolates within the O lineage with an 89 % bootstrap value and the same genetic distance as the American *PVY*^{O} isolate Oz (EF026074). All KZN *PVY*^{O} infecting tomato and pepper formed a unique clade (99 % bootstrap value) within the O lineage (Figure 3.8B).

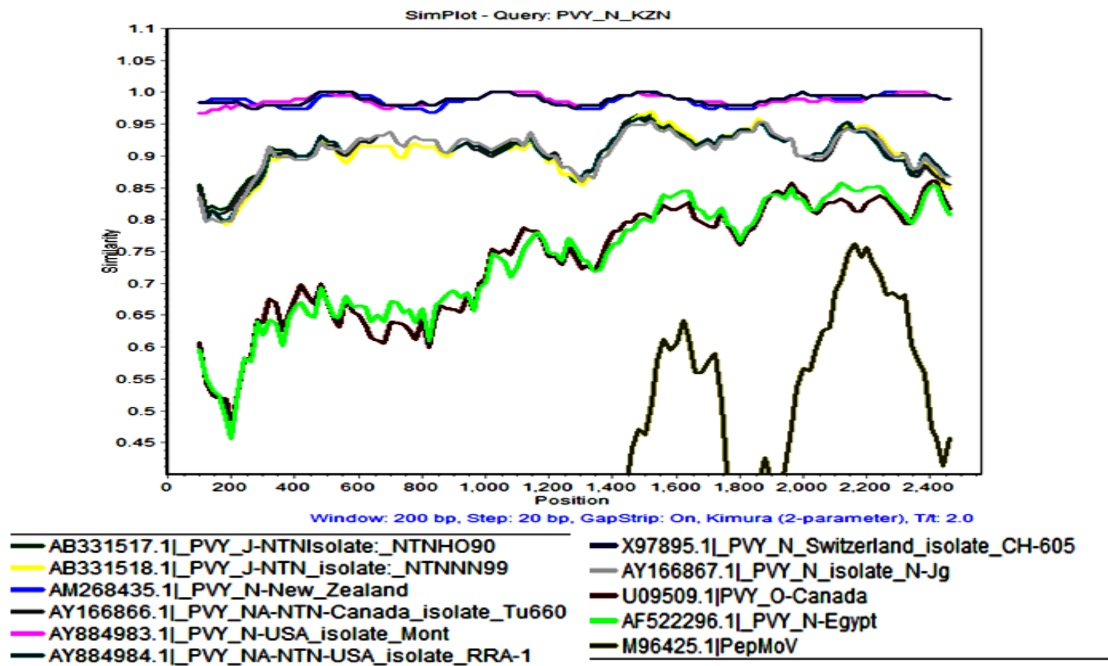

Figure 3.5. SimPlot analyses of the similarities between the 2559 nucleotides at the 5' -end of KZN PVY^N isolate and selected *PVY* isolates on the NCBI website.

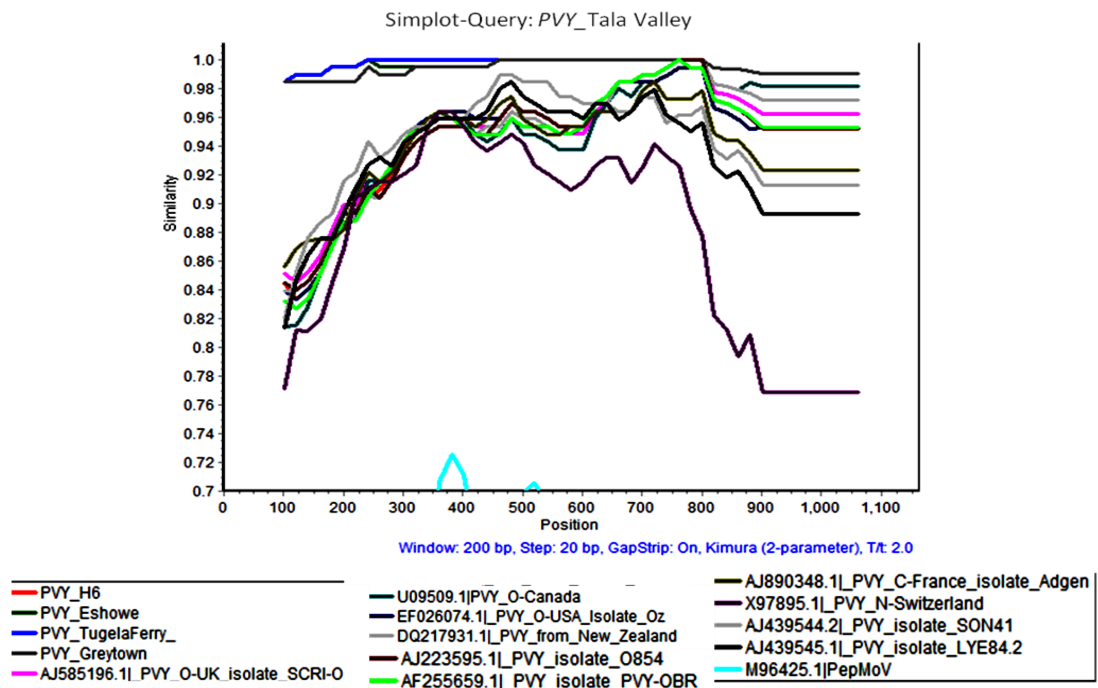

Figure 3.6. SimPlot analyses of the similarities between the 1067 nucleotides at the 3' -end before the poly-A tail of PVY^O isolate Tala Valley infecting tomato and selected *PVY* isolates on the NCBI website.

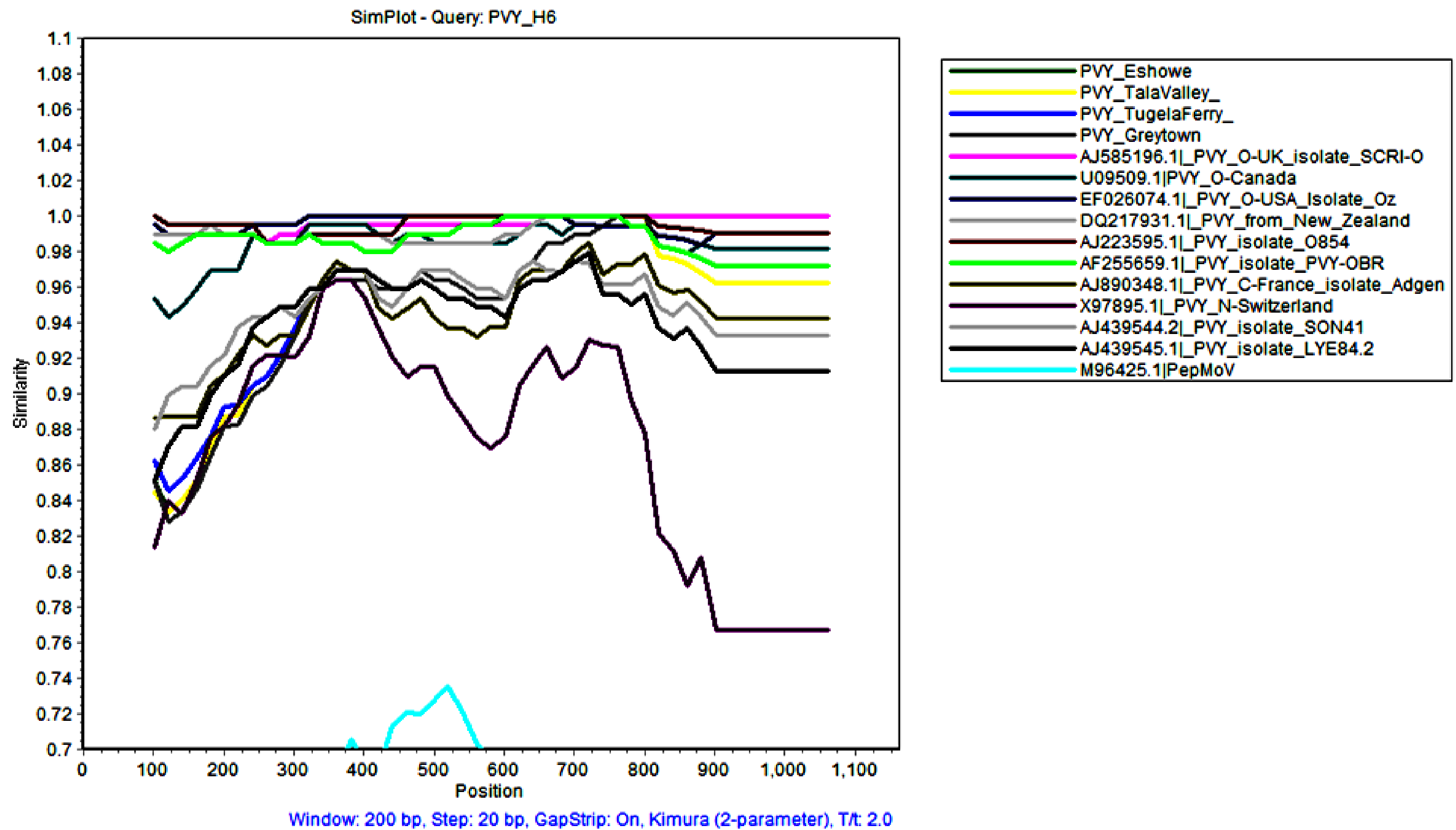

Figure 3.7. SimPlot analyses of the similarities between the 1067 nucleotides at the 3' -end before the poly-A tail of *PVY*^{N}Wilga isolate H6 infecting potato and selected *PVY* isolates on the NCBI website.

Table 3.3. Comparison of the amino acid sequence of P1, HC-Pro and part of the Part of P3 protein (Page 99-101). Motifs KITC and PTK are highlighted.

```
                                                              20                  40                  60                  80
                                                               |                   |                   |                   |
                                   PVY_N_KZN  MATYTSTIQFGSIECKLPYSPAPFGLVAGKREVSTTTDPFASLEMQLSARLRRQEFATIRKSKNGTCMYRYKTDVQIARIQKKREERE 88
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660  ....M...............E...RE.....I.....G.............L..V.T..S.I.......................... 88
  AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  ....M.................L.E...RE.....I.....G.............L..V.T..S............................ 88
    AB331517.1|_PVY_J-NTNIsolate:_NTNHO90  ....M...................E...RE.....I.....G.............L..V.T..S............................ 88
   AB331518.1|_PVY_J-NTN_isolate:_NTNNN99  ....M..................SK...RE.....I.....G.............L..V.T..S............................ 88
          AY166867.1|_PVY_N_isolate_N-Jg  ....M...................E...RE.K...I.....G................V.T..S..............H.R....... 88
          AM268435.1|_PVY_N-New_Zealand  ............................................................T.........................K. 88
       AY884983.1|_PVY_N-USA_isolate_Mont  .................................L..........................T........................... 88
X97895.1|_PVY_N_Switzerland_isolate_CH-605  ..I.........................................................T........................... 88
                    U09509.1|PVY_O-Canada  ....M...C...F.........SC..IVKE...LASVN...D..T......LK..Y..V.VL....FT......A..K.....L.RKD 88
                 AF522296.1|_PVY_N-Egypt  ....M...C...F.........SCEHIVKE...PASV....D..T......LK.KY..V.VL....FT......A..M.....L.RKD 88
                                                        100                 120                 140                 160
                                                         |                   |                   |                   |
                                   PVY_N_KZN  REEYNFQMAASSVVSKITIAGGEPPSKLESQVRRGVIHTTPRMRTAKTYHTPKLTEGQMNHLIKQVKQIMSTKGGSVQLISKKSTHVH 176
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660  ...H......P.....................Q............V.........K...............................Q 176
  AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  ...H......P.....................Q............V.........K...............................Q 176
    AB331517.1|_PVY_J-NTNIsolate:_NTNHO90  ...H......P.....................Q............V.........K...............................Q 176
   AB331518.1|_PVY_J-NTN_isolate:_NTNNN99  ...H......P.....................Q............V.........K......F........................Q 176
          AY166867.1|_PVY_N_isolate_N-Jg  ...H......P.....................Q............V.........K...............................Q 176
          AM268435.1|_PVY_N-New_Zealand  ........................................................................................ 176
       AY884983.1|_PVY_N-USA_isolate_Mont  ........................................................................................ 176
X97895.1|_PVY_N_Switzerland_isolate_CH-605  ........................................................................................ 176
                    U09509.1|PVY_O-Canada  ....H.....P.I.........D....S.P.AP..I......V.KV..RPII.........F.........E.R...H..N..T...Q 176
                 AF522296.1|_PVY_N-Egypt  ....H.....P.I.........D....S.P.AP..I........KV..RPII.............I.....E.R...H.....T...Q 176
                                               180                 200                 220                 240                 260
                                                |                   |                   |                   |                   |
                                   PVY_N_KZN  YKEVLGSHRAVVCTAHMKGLRKRVDFRCDKWTVVRLQHLARTDKWTNQVRATDLRKGDSGVILSNTNLKGNFGRSSEGLFIVRGSHEG 264
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660  ......A..........R........Q......MC.K.................................Y................. 264
  AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  ......A..........R........Q......MC.K.................................Y................. 264
    AB331517.1|_PVY_J-NTNIsolate:_NTNHO90  ......A..........R........Q......MC.K.................................Y................. 264
   AB331518.1|_PVY_J-NTN_isolate:_NTNNN99  ......A..........R....K...Q......MC.K.................................Y................. 264
          AY166867.1|_PVY_N_isolate_N-Jg  ......A..........R........Q......MC.K.................................Y...N............. 264
          AM268435.1|_PVY_N-New_Zealand  .................R...................................................................... 264
       AY884983.1|_PVY_N-USA_isolate_Mont  .................R...................................................................... 264
X97895.1|_PVY_N_Switzerland_isolate_CH-605  .................R...................................................................... 264
                    U09509.1|PVY_O-Canada  ....I.AYS.T.R....M...R.......M...GL..R.......S....TINI.R.......NTKS...H.....GD.......... 264
                 AF522296.1|_PVY_N-Egypt  ..KI..AYS.A.R....M...R.......M...GL..R.......S....TINI.R.......NTKS...H.....G........... 264
```

```
                                               280                 300                 320                 340
PVY_N_KZN                                      KIYDARSKVTQGVMDSMVQFSSAESFWKGLDGNWAQMRYPTDHTCVAGLPVEDCGRVAAIMTHSILPCYKITCPTCAQQYANLPASDL 352
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660    .............L.......N.............R.................................................... 352
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1       .............L.....................R.................................................... 352
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90          .............L.......N.................................................................. 352
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99         .............L.......N.............R.................................................... 352
AY166867.1|_PVY_N_isolate_N-Jg                 .............LY....................R.................................................... 352
AM268435.1|_PVY_N-New_Zealand                  ........................................................................................ 352
AY884983.1|_PVY_N-USA_isolate_Mont             ........................................................................................ 352
X97895.1|_PVY_N_Switzerland_isolate_CH-605     ........................................................................................ 352
U09509.1|PVY_O-Canada                          .L.....R...S.L...I...N.DN..........R...S...................L.A...................S..V... 352
AF522296.1|_PVY_N-Egypt                        .L.....R...SILN..I...N.DN..........R...S...................L.A...................S..V... 352

                                                       360                 380                 400                 420                 440
PVY_N_KZN                                      LKILHKHAGDGLNRLGADKDRFVHVKKFLTILEHLTEPVDPSLEIFNEVFKSIGEKQQSPFKNLNILNNFFLKGKENTAREWQVAQLS 440
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660    ..V..R..S...S............S....V.........LN.............................................. 440
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1       ..V..R..S...S............S....V.........LN.............................................. 440
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90          ..V..R..S...S............S....V.........LN.............................................. 440
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99         ..V..R..S...S............S....V.........LN.............................................. 440
AY166867.1|_PVY_N_isolate_N-Jg                 ..V..R..S...S............S....V.........LN.............................................. 440
AM268435.1|_PVY_N-New_Zealand                  ........S...............................L............................................... 440
AY884983.1|_PVY_N-USA_isolate_Mont             ........S...............................L............................................... 440
X97895.1|_PVY_N_Switzerland_isolate_CH-605     ........S...............................L............................................... 440
U09509.1|PVY_O-Canada                          F.L.....R.............I..N...IA.........LN..L...I.........A......V.............H........ 440
AF522296.1|_PVY_N-Egypt                        F.L.....R.............I..N...IA.........LN..L...I.........A......V.............H........ 440

                                                                 460                 480                 500                 520
PVY_N_KZN                                      LLELARFQKNRTDNIKKGDISFFRNKLSAKANWNLYLSCDNQLDKNANFLWGQREYHAKRFFSNYFEEIDPAKGYSAYENRLHPNGTR 528
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660    ................................................................F..............T......R. 528
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1       ................................................................F..............T......R. 528
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90          ................................................................F..............T......R. 528
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99         ................................................................F..............T......R. 528
AY166867.1|_PVY_N_isolate_N-Jg                 ................................................................F..............T......R. 528
AM268435.1|_PVY_N-New_Zealand                  ........................................................................................ 528
AY884983.1|_PVY_N-USA_isolate_Mont             ........................................................................................ 528
X97895.1|_PVY_N_Switzerland_isolate_CH-605     ...............................................S........................................ 528
U09509.1|PVY_O-Canada                          ...........................F....................................F..............I.K..S... 528
AF522296.1|_PVY_N-Egypt                        ................................................................F..............I.K..S... 528
```

```
                                                      540                 560                 580                 600
                                                        |                   |                   |                   |
PVY_N_KZN                                  KLAIGNLIVPLDLAEFRRKMKGDFKRQPGVSKKCTSSKDGNYVYPCCCTTLDDGSAVESTFYPPTKKHLVIGNSGDQKYVDLPKGNSE 616
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 .......................Y................................................................ 616
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1    .......................Y................................................................ 616
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90       .......................Y................................................................ 616
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99      .......................Y................................................................ 616
AY166867.1|_PVY_N_isolate_N-Jg              .......................Y................................................................ 616
AM268435.1|_PVY_N-New_Zealand               .......................Y................................................................ 616
AY884983.1|_PVY_N-USA_isolate_Mont          .......................Y................................................................ 616
X97895.1|_PVY_N_Switzerland_isolate_CH-605  .......................Y................................................................ 616
U09509.1|PVY_O-Canada                       ..S....V.........Q.....YRK.....R........................I.....................F......D.. 616
AF522296.1|_PVY_N-Egypt                     ..S....V.........Q.....YRK.....R........................I.....................F......D.. 616

                                              620                 640                 660                 680                 700
                                                |                   |                   |                   |                   |
PVY_N_KZN                                  MLYIARQGFCYINIFLAMLINISEEDAKDFTKKVRDMCVPKLGTWPTMMDLATTCAQMKIFYPDVHDAELPRILVDHETQTCHVVDSF 704
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 ........................................................................................ 704
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1    ........................................................................................ 704
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90       ........................................................................................ 704
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99      ........................................................................................ 704
AY166867.1|_PVY_N_isolate_N-Jg              .........................................................L.............................. 704
AM268435.1|_PVY_N-New_Zealand               ........................................................................................ 704
AY884983.1|_PVY_N-USA_isolate_Mont          ........................................................................................ 704
X97895.1|_PVY_N_Switzerland_isolate_CH-605  ........................................................................................ 704
U09509.1|PVY_O-Canada                       .....K..Y....V........G...................................R............SL....D.......... 704
AF522296.1|_PVY_N-Egypt                     .....K..Y....V............................................R..................D.......... 704

                                                           720                 740                 760                 780
                                                             |                   |                   |                   |
PVY_N_KZN                                  GSQTTGYHILKASSVSQLILFANDELESDIKHYRVGGIPGACPELGSTISPFREGGIIMSESAALKLLLKGIFRPKVM*QLLLDEP 790
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 ..............................................................................R....... 790
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1    ..............................................................................R....... 790
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90       ..............................................................................R....... 790
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99      ..............................................................................R....... 790
AY166867.1|_PVY_N_isolate_N-Jg              ..............................................................................K....... 790
AM268435.1|_PVY_N-New_Zealand               ..............................................................................K....... 790
AY884983.1|_PVY_N-USA_isolate_Mont          ..............................................................................K....... 790
X97895.1|_PVY_N_Switzerland_isolate_CH-605  ..............................................................................K....... 790
U09509.1|PVY_O-Canada                       .....................................V.N................V.....................R....... 790
AF522296.1|_PVY_N-Egypt                     .....................................V.N.S..............V.....................R....... 790
```

Table 3.4.Comparison of the amino acid sequence of the coat protein

```
                                                 20                  40                  60                  80
                                                  |                   |                   |                   |
PVY_Eshowe                              QSNPNKGKDKDVNVGTSGTHTVPRIKAITSKMRLPKSKGTTALNLEHLLEYAPQQIDISNTRATQSQFDTWYEAVRVAYDIG 82
PVY_Greytown                            ....................................................................N............. 82
PVY_Tala_Valley                         .................................................................................. 82
PVY_Tugela_Ferry                        .................................................................................. 82
PVY_H6                                  .............A...................M.....A.V............................*.....M..... 82
EF026074.1|_PVY_O-USA_Isolate_Oz        .............A...................M.....A.V..................................M..... 82
AJ223595.1|_PVY_isolate_O854            .............A...................M.....A.V..................................M..... 82
AJ585196.1|_PVY_O-UK_isolate_SCRI-O     .............A...................M.....A.V..................................M..... 82
DQ217931.1|_PVY_from_New_Zealand        ...L.........A...................M.....A.V..................................M..... 82
AF255659.1|_PVY_isolate_PVY-OBR         .............A...................M.....A.V..................................M..... 82
U09509.1|PVY_O-Canada                   .............A...................M.....AAV..................................M..... 82
AJ439545.1|_PVY_isolate_LYE84.2         .PT..........A...................M.....VA..........T........................M..... 82
AJ890348.1|_PVY_C-France_isolate_Adgen  .................................M.Q...A.V........................................ 82
AJ439544.2|_PVY_isolate_SON41           .R...........A...................M......AV........................................ 82
X97895.1|_PVY_N-Switzerland             .P.L..E.E.......................KM.....A.V.................................QL..... 82

                                                           100                 120                 140                 160
                                                            |                   |                   |                   |
PVY_Eshowe                              ETEMPTVMNGLMVWCIENGTSPNINGVWVMMDGDEQVEYPLKPIVENAKPTLRQIMAHFSDVAEAYIEMRNKKEPYMPRYGL 164
PVY_Greytown                            .................................................................................. 164
PVY_Tala_Valley                         .................................................................................. 164
PVY_Tugela_Ferry                        .................................................................................. 164
PVY_H6                                  .......................V.........N................................................ 164
EF026074.1|_PVY_O-USA_Isolate_Oz        .......................V.........N................................................ 164
AJ223595.1|_PVY_isolate_O854            .......................V.........N................................................ 164
AJ585196.1|_PVY_O-UK_isolate_SCRI-O     .......................V.........N................................................ 164
DQ217931.1|_PVY_from_New_Zealand        .......................V.........N................................................ 164
AF255659.1|_PVY_isolate_PVY-OBR         .......................V.......................................Q.................. 164
U09509.1|PVY_O-Canada                   .......................V.........N................................................ 164
AJ439545.1|_PVY_isolate_LYE84.2         .................................N................................................ 164
AJ890348.1|_PVY_C-France_isolate_Adgen  .................................N.....................................Q.......... 164
AJ439544.2|_PVY_isolate_SON41           .......................V.........S................................................ 164
X97895.1|_PVY_N-Switzerland             .................................N................................................ 164

                                                       180                 200                 220                 240
                                                        |                   |                   |                   |
PVY_Eshowe                              IRNLRDGSLARYAFDFYEVTSRTPVRAREAHIQMKAAALKSAQSRLFGLDGGISTQEENTERHTTEDVSPSMHTLLGVKNM 245
PVY_Greytown                            ................................................................................. 245
PVY_Tala_Valley                         ................................................................................. 245
PVY_Tugela_Ferry                        ................................................................................. 245
PVY_H6                                  ......VG...................................P..................................... 245
EF026074.1|_PVY_O-USA_Isolate_Oz        ......VG...................................P..................................... 245
AJ223595.1|_PVY_isolate_O854            ......VG...................................P..................................... 245
AJ585196.1|_PVY_O-UK_isolate_SCRI-O     ......VG.........K.........................P..................................... 245
DQ217931.1|_PVY_from_New_Zealand        V.....VG...................................P..................................... 245
AF255659.1|_PVY_isolate_PVY-OBR         ......VG...................................P..................................... 245
U09509.1|PVY_O-Canada                   ......MG......................Q............P..................................... 245
AJ439545.1|_PVY_isolate_LYE84.2         ......I....................................P..................................... 245
AJ890348.1|_PVY_C-France_isolate_Adgen  N..........................................P..................................... 245
AJ439544.2|_PVY_isolate_SON41           ....................................................V............................ 245
X97895.1|_PVY_N-Switzerland             V................................................................................ 245
```

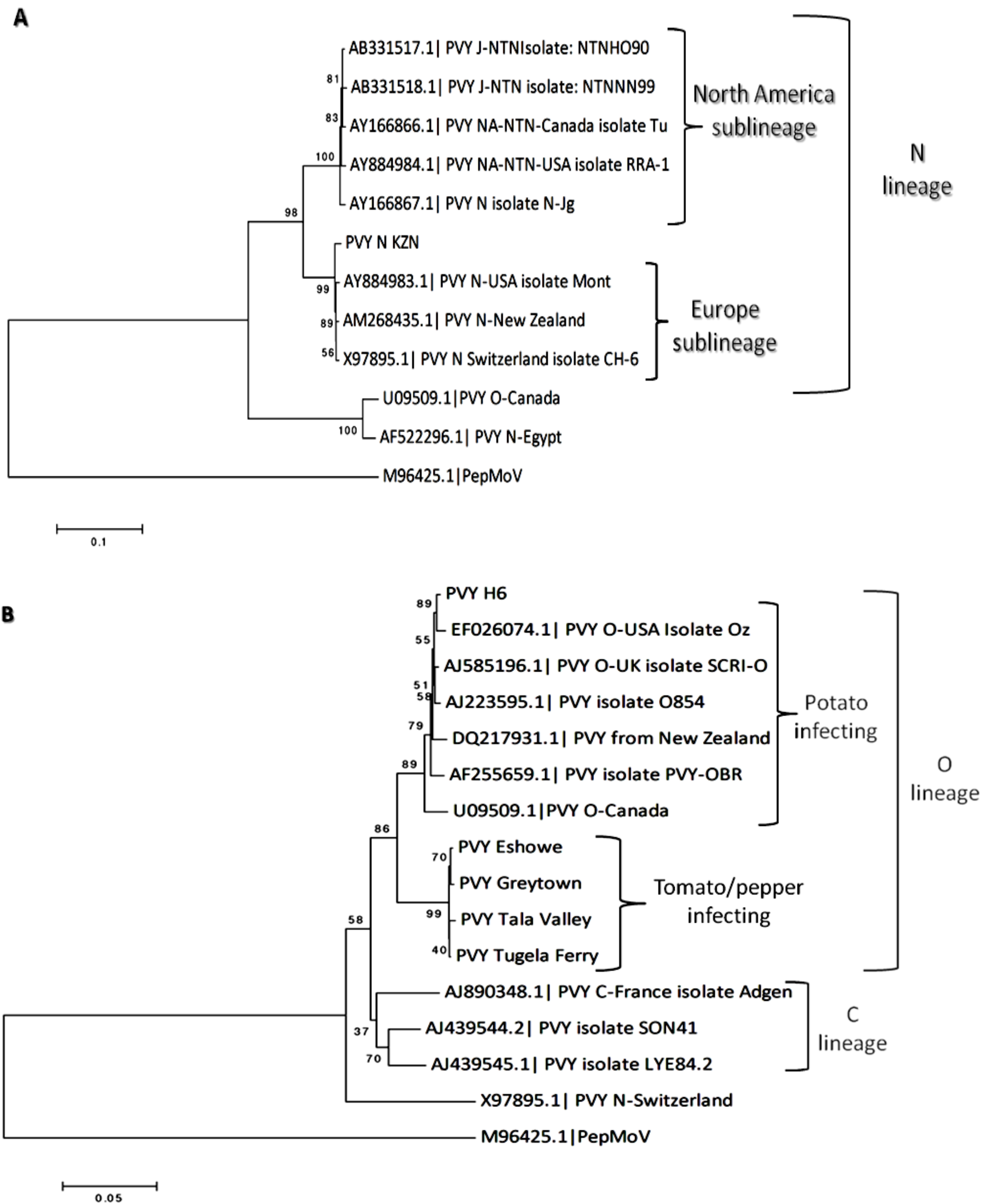

Figure 3.8. Dendrogram showing phylogenic relationships of (**A**) the 2559 nucleotides at the 5' -end of KZN *PVY*^{N} isolate infecting potato (**B**) the 1067 nucleotides at the 3' -end before the poly-A tail of KZN *PVY*^{O} isolates infecting pepper, tomato and *PVY*^{N}Wilga isolate H6 infecting potato with selected isolates on the NCBI website.

3.4. Discussion

The genomic region covering the 5'NTR, P1, HC-Pro, and part of P3 protein of KZN *PVY*^{N} infecting potato was found to be a non-recombinant. This is in accordance with the previous studies of *PVY* genome (Lorenzen *et al.*, 2006; Ogawa *et al.*, 2008; Schubert *et al.,* 2007). The high nucleotide sequence similarity observed between KZN *PVY*^{N} infecting potato and isolates of the N European sublineage compared to those from the North American sublineage with Simplot (Figure 3.5) and pairwise alignment (Appendix D) was confirmed by phylogenic analysis (Figure 3.8A). The absence of *PVY* sequences from RSA on the NCBI website implies that this is the first phylogenic study of *PVY* in RSA.

Protein sequences comparison of the HC-Pro allowed the identification of motifs important in the *PVY* infection cycle. HC-Pro is a multifunctional protein involved in self interaction, systemic movement, suppression of gene silencing, synergism and symptom development (Urcuqui-Inchima *et al*., 2001). Amino acid residues D_{205}, K_{400}, and E_{419} which are thought to be involved in the vein necrosis symptoms in tobacco and the motifs KITC and PTK, shown to be involved in virus transmission, were all identified within the HC-Pro of KZN *PVY*^{N} infecting potato. These highly conserved residues across isolates may indicate the key role they play in the infection cycle of *PVY*. Five unique amino acid residues were furthermore identified within the P1 and HC-Pro proteins of KZN *PVY*^{N} infecting potato (Table 3.3). The C- terminal half of P1 and the entire HC-Pro regions have been studied intensely in search of the determinant of the tobacco vein necrosis and the potato tuber necrotic ringspot disease (PTNRD) (Hu *et al.,* 2009; Tribodet *et al.*, 2005. Knowledge of the biological properties of KZN *PVY*^{N} infecting potato may possibly provide essential information on the effect of these unique residues on the replication cycle of *PVY*.

Phylogenic analyses divided the O lineage into two distinct clades (sublineages). Each clade is made of sequences sharing at least 99% nucleotide similarity. The nucleotide sequence similarity between clades ranges around 95% (Appendix D). It was also

remarked that both clades can also be differentiated on the basis of the hosts they infect. Therefore, they were divided into and tomato/pepper infecting sublineages. The nucleotide sequence of the KZN *PVY*^{N}Wilga isolate H6 infecting potato clustered within the potato infecting O sublineage. *PVY*^{N}Wilga, also known as *PVY*$^{N:O}$ strain in America, is a recombinant strain of *PVY*^{O} and *PVY*^{N} having serological properties of *PVY*^{O} but phenotypic properties of *PVY*^{N} (Ogawa *et al.*, 2008; Schubert *et al.,* 2007). The clustering of KZN *PVY*^{N}Wilga isolate H6 infecting potato is in accordance with the pattern recorded with *PVY*^{N}Wilga isolates around the world. Further studies of the genome of KZN *PVY*^{N}Wilga isolate H6 infecting potato need to be done in order to confirm its recombinant character.

The 3' NTR of all KZN *PVY*^{O} isolates infecting tomato and pepper share high nucleotide sequence similarity with *PVY*^{O} isolates infecting potato (Appendix D; Figure 3.6 and 3.7). Fanigliulo *et al*. (2005) reported a similar observation between the 3' NTR of an isolate of *PVY* infecting pepper and *PVY*^{O} isolates infecting potato and he suggested a *PVY*^{O} – type virus as an ancestor of *PVY* isolate infecting pepper. Phylogenic analyses of part of the coat protein and the 3'NTR region before the poly-A tail placed all KZN *PVY*^{O} isolates infecting tomato and pepper in a unique cluster closely related to *PVY*^{O} but still well separated from potato infecting *PVY*^{O} isolates (Figure 3.8B). *PVY* isolates infecting pepper and tomato were reported to cluster mainly within the C lineage (Aramburu *et al.*, 2006; Comes *et al.*, 2005; Crescenzi, 2009) even though some of these isolates were reported to react positively with Mab specific to *PVY*^{O} (Comes *et al.*, 2005).

Results obtained with KZN *PVY*^{N} isolate and *PVY*^{N}Wilga isolate H6 infecting potato raise the question of their introduction into RSA. Margaritopoulos *et al.* (2009) proposed three alternatives in an attempt to answer the same question regarding the diversity of *PVY* isolates in Greece. These are: vector migration, transportation of infested potato and the globalization of the potato trade. The long distances that separate RSA from America and Europe, combined with the mode of transmission of

the vector (non persistent), exclude the first alternative. Transportation of infested potato and the globalization of the potato trade are most likely the two alternatives that favoured the introduction of these isolates into RSA.

Studies undertaken in this chapter revealed that KZN *PVY*^{N} isolate, as is the case with all *PVY*^{N} isolates reported from potato growing regions around the world, is a non-recombinant and is closely related to the European *PVY*^{N} type. The P1/HC-Pro protein sequence of KZN *PVY*^{N} isolate possesses five unique amino acid residues (K_{61}, K_{194}, G_{361}, P_{393} and F_{552}) beside the vein necrosis determinants and the KITC and PTK motifs involved in virus transmission. KZN *PVY*^{O} isolates infecting tomato and pepper share high sequence similarity with *PVY*^{O} isolates infecting potato and form a unique cluster within the O lineage. The genome of KZN *PVY*^{N}Wilga isolate H6 infecting potato requires further study to confirm its recombinant character.

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Chapter 4
General Overview

4.1. Major Findings

The research undertaken in this study led to the identification of *PVY* strains infecting pepper, potato and tomato grown in KZN by both small-holder and commercial farmers. RSA is not the only country where *PVY* is prevalent on these vegetables. Recent studies have also reported the occurrence of *PVY* in several countries where these crops are cultivated (Aramburu *et al.*, 2006; Crescenzi, 2009; Fanigliulo *et al.*, 2005; Khalifa *et al.*, 2009; Margaritopoulos *et al.*, 2009; Massumi *et al.*, 2009; Moury, 2009). *PVY* isolates infecting potato appear to be the most studied isolates of *PVY* on the basis of the number of published documents. The variability of potato infecting *PVY* strains and the constant occurrence of new recombinant strains can explain the growing interest towards *PVY* isolates infecting potato.

The presence of *PVY* in KZN, even though not at epidemic levels, results in lower vegetable yield compared to production free of *PVY* infection. The level of damages caused by *PVY* will vary depending on the scale of farming. Consequences of *PVY* infection on the commercial farming system include loss of income and food shortage. The loss of income may result in job losses in the farming sector and in very severe cases can lead to crop loss which would have a serious sociological impact. Home produced crops improve the household nutritional status by either generating substantial monetary income or by reducing the household food expenditure (Maunder & Meaker, 2007; Van Averbeke & Khosa, 2007). However, damages caused by *PVY* will negatively affect the nutritional status of many households.

Food security is a major concern especially in developing countries. Small-scale farming is regarded as part of the solution to address the actual food shortage (Wiggins, 2009). This requires the production of good quality and consumable crops. Losses caused by plant pathogens account for 10% of global food production

(Strange and Scott, 2005). The lack of resources in small scale-farming systems amplifies plant pathogen damages. Moreover, *PVY* is listed among the five most damaging viruses worldwide (Mijatovic *et al.,* 2002). Small-scale farming systems will therefore contribute positively towards food security in environments where plant pathogen incidence and pressure is very low.

KZN *PVY* isolates infecting pepper and tomato are not diverse compared to the potato infecting isolates. PVY^{O} was the only strain identified in all the tomato and pepper samples. Neither the isolates of PVY^{C} reported to infect these crops in studies done in Italy and Spain (Aramburu *et al.*, 2006; Comes *et al.*, 2005; Fanigliulo *et al.*, 2005), nor the isolates of PVY^{N} reported in Spain (Aramburu *et al.*, 2006) were detected in this study. This useful information can contribute towards reinforcing actual control measures to keep these strains out of the province.

The occurrence of new strains of *PVY*, mostly recombinant, is a serious concern in the potato industry across the world, especially with regard to the potato tuber necrotic ringspot disease (PTNRD) which seriously affects the marketability of the crop (Hu *et al.*, 2009; Lorenzen *et al.*, 2008). The potato infecting strains PVY^{NTN} and PVY^{N}Wilga have been proven to be recombinant strains of PVY^{O} and PVY^{N} in most cases (Lorenzen *et al.*, 2006; Ogawa *et al.*, 2008; Schubert *et al.*, 2007). PVY^{NTN}, PVY^{N}Wilga and PVY^{N} were all detected in the potato infected samples studied in this study. The permanent coexistence of PVY^{O} and PVY^{N} in potato fields presents the risk of forming highly damaging recombinant strains of *PVY*. Biological properties of KZN *PVY* isolates could not be thoroughly studied because pure isolates were not obtained as *C. quinoa* did not produce typical local lesions. Therefore, no firm conclusions could be made on the role played by the five unique amino acid residues found in the P1-HC-Pro region of KZN PVY^{N} isolate.

4.2. Way forward

Information generated in this study can be used to lay the foundation for establishing sustainable control strategies of *PVY* in KZN. This work, which may be the first of its kind in the province, can constitute the beginning of a programme that can lead to the production of a database of pathogens of important crops occurring in the Southern African region. This will provide essential information for developing control strategies that will result in better production and improved food security. Presently, there is urgency to establish the severity of the disease and conduct similar studies with isolates of *PVY* occurring in the other provinces of RSA.

Results obtained in this study also raise some questions which need to be addressed. This study demonstrated that *PVY* is not the only plant virus present in vegetables grown in KZN. Pepper, potato and tomato can be infected with several viruses that belong to different families of plant viruses (Jones *et al.*, 1997; Pernezny *et al*., 2003; Stevenson *et al*., 2004). This indicates the need to undertake a comprehensive study of viruses infecting vegetable crops in KZN and in RSA as a whole.

Pure isolates of *PVY* are also needed for the biological studies of their properties. Plants such as *Chenopopdium amaticolor* and *Physalis froridana* are other indicator plants of *PVY.* They can be used as alternatives to *C. quinoa*. Pepper and potato are considered selective for *PVY* strains as host (Singh *et al.*, 2008). Pepper infecting *PVY* isolates were found to be unable to infect potato and vice versa. Biological studies of KZN isolates of *PVY* should also evaluate that property.

Part of the genome studied in this work revealed amino acid residues unique to KZN isolates of *PVY*. There is only one full sequence of an African isolate of *PVY* available on the NCBI website. Comprehensive studies of the full genome of *PVY* isolates occurring in KZN may shed light on the evolution of *PVY* and other features unique to KZN isolates. Moreover, studies will also confirm the recombinant nature of *PVY*^{NTN} and *PVY*^{N}Wilga isolates identified in this study.

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Appendix A
ELISA Buffers

- **Phosphate buffered saline (PBS) 1X for 1 l, pH 7.4**

Nacl .... 8.0 g

$Na_2HPO_4.12H_2O$ .... 2.9 g

KH_2PO_4 .... 0.2 g

KCl .... 0.2 g

NaN_3 .... 0.2 g

- **Washing buffer 1X (PBST)**

PBS .... 1 l
Tween 20 .... 0.5 ml

- **Extraction buffer 1X, pH 7.3**

PBST .... 1 l
Polyvinylpyrrolidone (PVP) .... 20 g
Ovalbumin .... 2.0 g
Sodium sulphite (anhydrous) .... 1.3 g

- **Conjugate buffer 1X pH 7.4**

PBST 1l
Ovalbumin .... 2.0 g

- **Coating buffer 1X pH 9.6**

Na_2CO_3 .... 1.59 g
$NaHCO_3$ .... 2.93 g
NaN_3 .... 0.2 g

- **Substrate buffer 1X pH 9.8 for 1l**

Diethanolamine .... 97 ml
NaN_3 .... 0.2 g

Appendix B
Mechanical Inoculation Buffers

- **Solution A (0.2 M) for 1 l**

$NaH_2PO_4.H_2O$ .... 27.6 g

- **Solution B (0.2 M) for 1 l**

$Na_2HPO_4.7H_2O$ .... 53.65 g

- **Inoculation buffer (0.1 M) pH 7.4 for 1l**

Solution A .... 95 ml

Solution B .... 405 ml

Water .... 500 ml

Sodium sulphite .... 4 g

Appendix C
Culture Media

➢ Luria-Bertani (LB) broth pH 7.0 for 1l

Tryptone .... 10 g

Yeast extract .... 5 g

NaCl .... 10 g

➢ Luria-Bertani (LB) agar pH 7.0 for 1l

Tryptone .... 10 g

Yeast extract .... 5 g

NaCl .... 10 g

Agar .... 15 g

Appendix D
Pairwise Comparisons

- **Sequence comparison of the 2559 nucleotides at the 5' -end of different isolates of *PVY***

		1	2	3	4	5	6	7	8	9	10	11
PVY_N_KZN	1		95.06	95.19	95.32	94.81	94.68	99.24	99.24	99.11	83.29	83.54
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660	2	95.06		99.62	99.75	99.37	98.73	95.44	95.44	95.32	83.80	84.30
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1	3	95.19	99.62		99.62	99.24	98.86	95.57	95.57	95.44	83.80	84.30
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90	4	95.32	99.75	99.62		99.37	98.73	95.70	95.70	95.57	83.80	84.30
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99	5	94.81	99.37	99.24	99.37		98.35	95.19	95.19	95.06	83.67	84.05
AY166867.1|_PVY_N_isolate_N-Jg	6	94.68	98.73	98.86	98.73	98.35		95.19	95.19	95.06	82.91	83.54
AM268435.1|_PVY_N-New_Zealand	7	99.24	95.44	95.57	95.70	95.19	95.19		99.75	99.62	83.54	83.80
AY884983.1|_PVY_N-USA_isolate_Mont	8	99.24	95.44	95.57	95.70	95.19	95.19	99.75		99.62	83.54	83.67
X97895.1|_PVY_N_Switzerland_isolate_CH-605	9	99.11	95.32	95.44	95.57	95.06	95.06	99.62	99.62		83.16	83.42
U09509.1|PVY_O-Canada	10	83.29	83.80	83.80	83.80	83.67	82.91	83.54	83.54	83.16		97.34
AF522296.1|_PVY_N-Egypt	11	83.54	84.30	84.30	84.30	84.05	83.54	83.80	83.67	83.42	97.34	

- **Sequence comparison of the 1067 nucleotides at the 3' -end before the poly-A tail of different isolates of *PVY***

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
PVY_H6	1		95.03	94.94	94.85	95.22	99.63	99.44	99.34	99.34	98.78	98.88	98.78	98.03	94.01	93.45	92.79	94.10	84.47	88.21	60.41
PVY_Eshowe	2	95.03		99.72	99.53	99.72	95.41	95.03	94.94	94.75	94.56	94.75	94.66	94.38	93.45	93.26	92.70	93.82	83.77	88.31	59.76
PVY_Greytown	3	94.94	99.72		99.44	99.63	95.31	94.94	94.85	94.66	94.47	94.66	94.56	94.47	93.35	93.16	92.60	93.73	83.60	88.40	59.67
PVY_Tala_Valley	4	94.85	99.53	99.44		99.63	95.22	94.85	94.75	94.56	94.38	94.56	94.47	94.19	93.26	93.07	92.70	93.63	83.51	88.31	59.85
PVY_Tugela_Ferry	5	95.22	99.72	99.63	99.63		95.60	95.22	95.13	94.94	94.75	94.94	94.85	94.56	93.45	93.26	92.70	93.82	83.86	88.31	59.57
AM236811.1|_PVY_Isolate_Henan10	6	99.63	95.41	95.31	95.22	95.60		99.44	99.34	99.34	98.78	99.06	98.78	98.03	93.82	93.45	92.79	94.10	84.29	88.03	60.59
AJ585196.1|_PVY_O-UK_isolate_SCRI-O	7	99.44	95.03	94.94	94.85	95.22	99.44		99.53	99.16	98.97	98.88	98.97	98.03	94.19	93.82	92.98	94.29	84.64	88.03	60.31
AJ223595.1|_PVY_isolate_O854	8	99.34	94.94	94.85	94.75	95.13	99.34	99.53		99.06	98.88	98.78	98.88	97.94	94.10	93.73	93.07	94.01	84.82	88.12	60.50
EF026074.1|_PVY_O-USA_Isolate_Oz	9	99.34	94.75	94.66	94.56	94.94	99.34	99.16	99.06		98.50	98.59	98.50	97.75	93.63	93.16	92.51	93.82	84.12	88.03	60.22
AJ890349.1|_PVY_strain_O_isolate_LW	10	98.78	94.56	94.47	94.38	94.75	98.78	98.97	98.88	98.50		98.41	98.50	97.75	93.73	93.63	92.70	93.82	84.12	87.84	60.68
AF255659.1|_PVY_isolate_PVY-OBR	11	98.88	94.75	94.66	94.56	94.94	99.06	98.88	98.78	98.59	98.41		98.41	97.84	93.63	93.07	92.51	93.73	84.21	87.84	60.50
DQ217931.1|_PVY_from_New_Zealand	12	98.78	94.66	94.56	94.47	94.85	98.78	98.97	98.88	98.50	98.50	98.41		97.56	93.82	93.45	92.79	94.10	84.12	88.31	60.59
U09509.1|PVY_O-Canada	13	98.03	94.38	94.47	94.19	94.56	98.03	98.03	97.94	97.75	97.75	97.84	97.56		93.16	93.16	92.13	94.01	84.12	88.03	60.78
AJ439544.2|_PVY_isolate_SON41	14	94.01	93.45	93.35	93.26	93.45	93.82	94.19	94.10	93.63	93.73	93.63	93.82	93.16		96.44	94.38	94.57	85.70	88.87	60.59
AJ439545.1|_PVY_isolate_LYE84.2	15	93.45	93.26	93.16	93.07	93.26	93.45	93.82	93.73	93.16	93.63	93.07	93.45	93.16	96.44		94.48	94.57	85.18	88.49	60.68
AJ890348.1|_PVY_C-France_isolate_Adgen	16	92.79	92.70	92.60	92.70	92.70	92.79	92.98	93.07	92.51	92.70	92.51	92.79	92.13	94.38	94.48		92.70	84.74	88.96	60.68
AF237963.2|_PVY_strain_nnp	17	94.10	93.82	93.73	93.63	93.82	94.10	94.29	94.01	93.82	93.82	93.73	94.10	94.01	94.57	94.57	92.70		83.35	88.31	60.04
FJ214726.1|_PVY_isolate_Chile3	18	84.47	83.77	83.60	83.51	83.86	84.29	84.64	84.82	84.12	84.12	84.21	84.12	84.12	85.70	85.18	84.74	83.35		81.52	56.21
X97895.1|_PVY_N-Switzerland	19	88.21	88.31	88.40	88.31	88.31	88.03	88.03	88.12	88.03	87.84	87.84	88.31	88.03	88.87	88.49	88.96	88.31	81.52		59.78
M96425.1|PepMoV	20	60.41	59.76	59.67	59.85	59.57	60.59	60.31	60.50	60.22	60.68	60.50	60.59	60.78	60.59	60.68	60.68	60.04	56.21	59.78	

Appendix E
Amino Acids and their Letter Codes

Amino acid	One-letter code
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cystein	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

Appendix F
Sequence Alignments

- **Sequence alignment of the the 2559 nucleotides at the 5' -end of different isolates of *PVY***

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                                                                             20                  40                  60                  80                 100
                                                 PVY_N_KZN  AATTAAAACAACTCAATACAACATAAGAAAATCAACGCAAAAACACTCACAAAAGCTTTCAACTCTAATTCAAACAATTTGTTAAGTTTCAATTTCGATC 100
                      AB331517.1|_PVY_J-NTNIsolate:_NTNHO90  ..............................................................T.....C....G..................C..T.... 100
                     AB331518.1|_PVY_J-NTN_isolate:_NTNNN99  ..............................................................T.....C....G..................C..T.... 100
           AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660  ..............................................................T.....C....G..................C..T.... 100
                          AY166867.1|_PVY_N_isolate_N-Jg  ..............................................................T.....C....G..................C..T.... 100
             AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  .................C.............A..............................T.....C....G..................C..T.... 100
                        AM268435.1|_PVY_N-New_Zealand  ...............................A.................................................................... 100
         X97895.1|_PVY_N_Switzerland_isolate_CH-605  .................................................................................................... 100
                  AY884983.1|_PVY_N-USA_isolate_Mont  ...............................A.............................................A...................... 100
                                   U09509.1|PVY_O-Canada  ...............................A..G..............T...C....---.T...C.C....G..TC...C.........G...AA... 97
                               AF522296.1|_PVY_N-Egypt  ...............................A..................T...C...C---.T...C.C....G...C...C.........G...AA... 97
                                       M96425.1|PepMoV  ..........TAA..-..........----------------------..G.....AA....A.T.---....G..T...TC.TGAGC..---..T...T 71
                                                                            120                 140                 160                 180                 200
                                                 PVY_N_KZN  TTCATCAAACAAACT-----CTTTCAATTTCAGTGTAAGCTAT-CGTAATCCAGTAAGTTATTTCAAACTCTCGTACATTGCAGAAGAT-CATCCATGGC 193
                      AB331517.1|_PVY_J-NTNIsolate:_NTNHO90  ...G..GT......C-----G......C...........T.C.-.....CT..A..GT.............T..A..A...AC.T.C-..G....... 193
                     AB331518.1|_PVY_J-NTN_isolate:_NTNNN99  ...G..GT......C-----G......C...........T.C.-.....CT..A..GT.............TC.A..A...AC.T.C-..G....... 193
           AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660  ...G..GT......C-----G......C...........T.C.-.....CT..A..GT.............TC.A..A...AC.T.C-..G....... 193
                          AY166867.1|_PVY_N_isolate_N-Jg  .C.G..GT......C-----G......C...........T.C.-.....CT..A..GT.............TC.A..A.T.AC.T.C-..G....... 193
             AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  ...G..GT......C-----G......C...........T.C.-.....CT..A..GT.............TC.A..A...AC.T.C-..G....... 193
                        AM268435.1|_PVY_N-New_Zealand  ...............-----......................-......T.........................A............-.......... 193
         X97895.1|_PVY_N_Switzerland_isolate_CH-605  ...............-----......................-......T.........................A............-.......... 193
                  AY884983.1|_PVY_N-USA_isolate_Mont  .C.............-----......................-......T.........................A.........G..-.......... 193
                                   U09509.1|PVY_O-Canada  A.TTC.TTG...TTC-----TC..A..CAAT.T..G..A.C..-TTC..CT..AC...CA......T-.A..TCC.ACCAATTT..A..-.C..G..... 189
                               AF522296.1|_PVY_N-Egypt  A.TTC.TTG...TTC-----TC.AG..CAAT.T..G..A.C..-TTC..CT..AC...CA......T-.A..TCC.ACCAATTTC....-.C..A..... 189
                                       M96425.1|PepMoV  ..TCCTGC.T...T.AAGCA....G.TCAA..ACA..GT..CGATT.G.ATATT.C.A..G....C.GT.T..TCTA..A...CTCC..A.TAAA..... 171
                                                                            220                 240                 260                 280                 300
                                                 PVY_N_KZN  AACTTACACATCAACAATCCAGTTTGGTTCCATTGAATGCAAACTTCCATACTCACCCGCTCCT---------TTTGGGCTAGTTGCGGGGAAACGAGAA 284
                      AB331517.1|_PVY_J-NTNIsolate:_NTNHO90  ........TG.....G..T..A..C...................................C...---------....A..........A..G........ 284
                     AB331518.1|_PVY_J-NTN_isolate:_NTNNN99  ........TG.....G..T..A..C...................................C...---------.CCAA..........A..G........ 284
           AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660  ........TG.....G..T..A..C...................................C...---------....A..........A..G........ 284
                          AY166867.1|_PVY_N_isolate_N-Jg  ........TG.....G..T..A..C...................................C...---------....A..........A..G.....A.. 284
             AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  ........TG.....G..T..A..C...................................C.T.---------....A..........A..G.G...... 284
                        AM268435.1|_PVY_N-New_Zealand  ................................................................---------........................... 284
         X97895.1|_PVY_N_Switzerland_isolate_CH-605  ..T.............................................................---------........................... 284
                  AY884983.1|_PVY_N-USA_isolate_Mont  ................................................................---------........................... 284
                                   U09509.1|PVY_O-Canada  ........TG.....G...TGT..C.....GT..........G..A..............CT..---------.GC.....TA...T.AA.G........ 280
                               AF522296.1|_PVY_N-Egypt  ........TG.........TGT........GT..........G..A...........A..CT..---------.GC.A..ATA...T.AA.G........ 280
                                       M96425.1|PepMoV  ...CAGTGTT------..T...........AT...TG.....T......A.G..-..A..CA..ATGCACAAC.G..CA..GCCCAAAACAG.G.AT..G 264
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                                                    320                 340                 360                 380                 400
                                                     |                   |                   |                   |                   |
PVY_N_KZN                                 GTTTCAACCACCAC-------TGACCCCTTCGCAAGTTTGGAGATGCAGCTTAGTGCGCGATTGCGAAGGCAAGAGTTTGCAACTATTCGAAAATCCAAG 377
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90     ..........T...-------...............G.......................C.A...........C....G...G.C..T.C....... 377
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99    ..........T...-------...............G.......................C.A...........C....G...G.C..T.C....... 377
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 ..........T...-------...............G.......................C.A...........C....G...G.C..T.C....... 377
AY166867.1|_PVY_N_isolate_N-Jg            ..........T...-------...............G.......................C.A.................G...G.C..T.C....... 377
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  ..........T...-------...............G.......................C.A...........C....G...G.C..T.C....... 377
AM268435.1|_PVY_N-New_Zealand             ..............-------..........................................A.......G...........C.......C....... 377
X97895.1|_PVY_N_Switzerland_isolate_CH-605 ..............-------.......................C..........A.......G...................C....... 377
AY884983.1|_PVY_N-USA_isolate_Mont        ....T.........-------..........................................A...........................C....... 377
U09509.1|PVY_O-Canada                     ..GCTGG.TT..GT-------.A.T..T......GA.C....A.CA..A........A......TC.A......A.A...T...G....TGTGCT.... 373
AF522296.1|_PVY_N-Egypt                   ..GC.GG.TT..GT---------.T..T......GA.C....A.CA..A........A......TC.A....A.A.A...T...G....TGTGCT...A 373
M96425.1|PepMoV                           CAC.A.TAT.GTG.GCCCAAG...T..G..T..TGAGC.C....A...T...GAAC.ATAC....AG.A.AGGAT.GA...G..A..A..CC..A.T..A 364

                                                    420                 440                 460                 480                 500
                                                     |                   |                   |                   |                   |
PVY_N_KZN                                 AATGGTACTTGCATGTATCGATACAAGACTGATGTCCAGATTGCGCGCATTCAAAAGAAGCGCGAGGAAAGAGAAAGAGAGGAATATAATTTCCAAATGG 477
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90     .G......C.........................................C...........................A...C.C..C.......... 477
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99    .G......C.........................................C...........................A...C.C..C.......... 477
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 .G.....TC.........................................C...........................A...C.C..C.......... 477
AY166867.1|_PVY_N_isolate_N-Jg            .G......C......................................A...C.G.........................A...C.C..C.......... 477
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  .G......C.........................................C...........................A...C.C..C.......... 477
AM268435.1|_PVY_N-New_Zealand             ..........................................................................A......................... 477
X97895.1|_PVY_N_Switzerland_isolate_CH-605 .................................................................................................... 477
AY884983.1|_PVY_N-USA_isolate_Mont        .................................................................................................... 477
U09509.1|PVY_O-Canada                     ..C.......TT.C...................C......AAA.........G.....A.TG...AGG.AG..T..G..A......C.C.......... 473
AF522296.1|_PVY_N-Egypt                   ..C.......TT.C...C...............C......AAT.........G.....A.TG...AGG.AG..T..G..A......C.C.......... 473
M96425.1|PepMoV                           GGC..G..GCTTG....CAA.C...T..GC..A.CTA..CGC..CA.A.AG.TT.GA...AAGC.ACGTGAG...GA.....TGCG.TTG...ATG.AC. 464

                                                    520                 540                 560                 580                 600
                                                     |                   |                   |                   |                   |
PVY_N_KZN                                 CTGCGTCAAGTGTTGTGTCGAAGATCACTATTGCTGGTGGAGAGCCACCTTCAAAACTTGAATCACAAGTGCGGAGGGGTGTCATCCACACAACTCCAAG 577
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90     ....AC............A.....T..........................G..................A........................... 577
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99    ....AC.G..........A.....T..........................G..................A........................... 577
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 ....AC.G................T..........................G..................A...A....................... 577
AY166867.1|_PVY_N_isolate_N-Jg            ....AC.G................T..........................G..................A........................... 577
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  ....AC.G................T..........................G..................A........................... 577
AM268435.1|_PVY_N-New_Zealand             .................................................................................................... 577
X97895.1|_PVY_N_Switzerland_isolate_CH-605 .................................................................................................... 577
AY884983.1|_PVY_N-USA_isolate_Mont        .................................................................................................... 577
U09509.1|PVY_O-Canada                     .C..TC.T...A.......A..A..T.....A..........T..T..A.....GTC...GC......CA.CA..A..GA....T..T........... 573
AF522296.1|_PVY_N-Egypt                   .C..TC.T...A.......A..A..T.....A.....C.....T..T..A.....GTC...GC......CA.CA..A..GA....T..T........... 573
M96425.1|PepMoV                           .A..CC..TACA.....AGT..C.....A..A.GG..A..G...GTG..C..T...A.G..GGA.GTGTCCATC.A.C.ACCGC.GA.T.A......TT- 563
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```
                                                920                 940                 960                 980                 1,000
                                                  |                   |                   |                   |                   |
PVY_N_KZN                                 TGATCTACGCAAGGGTGATAGTGGAGTTATATTGAGTAATACCAATCTCAAAGGAAACTTTGGGAGAAGCTCGGAGGGCCTATTCATAGTGCGTGGGTCG 971
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90     .........A.....C..........................T............T.............T........T.....T...........A... 971
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99    .........A.....C..........................T............T.............T........T.....T...........A... 971
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 .........A.....C..........................T............T.............T........T.....T...........A... 971
AY166867.1|_PVY_N_isolate_N-Jg            .........A.....C..........................T............T............AT..............T...........A... 971
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  .........A.....C..........................T............T.............T........T.....T...........A... 971
AM268435.1|_PVY_N-New_Zealand             ...............C..........................T......................................................... 971
X97895.1|_PVY_N_Switzerland_isolate_CH-605 ...............C..........................T......................................................... 971
AY884983.1|_PVY_N-USA_isolate_Mont        ...............C..........................T......................................................... 971
U09509.1|PVY_O-Canada                     CA.CA....A.G...............C..C....AC.CA.AA.GC........CC.......T.....T..A.GA.A.T.G...........C..A..A 967
AF522296.1|_PVY_N-Egypt                   CA.CA....A.G...............C..C....AC.CA.AA.GC........CC.......T.....T..A.GA...T.G..............A..A 967
M96425.1|PepMoV                           GTC.T..AAGCGA...........GT.G...C...A.GC..GAGCA..G.G...T..A..C..AC..T.TAGCAGA..AT.T........T..C..AAAA 958

                                                1,020               1,040               1,060               1,080               1,100
                                                  |                   |                   |                   |                   |
PVY_N_KZN                                 CACGAAGGAAAAATCTATGATGCACGTTCCAAGGTTACTCAAGGGGTTATGGATTCAATGGTTCAGTTCTCAAGCGCTGAAAGCTTTTGGAAGGGATTGG 1071
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90     ..T....................G..............A.....A...C.A..C...................A............C............. 1071
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99    ..T....................G..............A.....A...C.A..C...................A............C............. 1071
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 ..T....................G..............A.....A...C.A..C...................A............C............. 1071
AY166867.1|_PVY_N_isolate_N-Jg            ..T.........................................A...C.AT.C................................C............. 1071
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  ..T....................G..............G.....A...C.A..C................................C............. 1071
AM268435.1|_PVY_N-New_Zealand             .................................................................................................... 1071
X97895.1|_PVY_N_Switzerland_isolate_CH-605 .................................................................................................... 1071
AY884983.1|_PVY_N-USA_isolate_Mont        .................................................................................................... 1071
U09509.1|PVY_O-Canada                     ..T.....G...T.G..C...........T.GA........GA.T...T....C......A.C.....T..G.AT.....T.AT...........TC... 1067
AF522296.1|_PVY_N-Egypt                   ........G...T.G..............T.GA........GA.TA..T.AA.C......A.C.....T..G.AT..C..C.AT...........TC... 1067
M96425.1|PepMoV                           TCG..T...GTTG.ACTA......A.A......C..T..ATG.CAAC.G.AAC.CAT....AA....AT....CAC.....GCA.......GT..C..A. 1058

                                                1,120               1,140               1,160               1,180               1,200
                                                  |                   |                   |                   |                   |
PVY_N_KZN                                 ACGGCAATTGGGCACAAATGAGATATCCTACAGATCATACATGTGTGGCAGGCTTACCAGTTGAAGACTGTGGCAGAGTTGCAGCGATAATGACACACAG 1171
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90     ................G........C.................C..A................................A.....A.............. 1171
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99    ...............GG........C.................C..A................................A.....A...........T.. 1171
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 ...............GG........C.................C..A................................A.....A.............. 1171
AY166867.1|_PVY_N_isolate_N-Jg            ...............GG........C.................C..A................................A.....A.............. 1171
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1  ...............GG........C.................C..A.....T..........................A.....A.............. 1171
AM268435.1|_PVY_N-New_Zealand             .................................................................................................... 1171
X97895.1|_PVY_N_Switzerland_isolate_CH-605 .................................................................................................... 1171
AY884983.1|_PVY_N-USA_isolate_Mont        .................................................................................................... 1171
U09509.1|PVY_O-Canada                     ...............G.............T.G.....C........A..T..T.....T..C.....T.....T..G.....T..AT.G...G....... 1167
AF522296.1|_PVY_N-Egypt                   ....T..........G.............T.G.....C........A..T..T.....T..C.....T.....T..G..A..T..AT.G...G....... 1167
M96425.1|PepMoV                           .AAAG..G...AGCGTGG..C.CA.G..A..C.CA.....T...AAAC.GACG.ATT.G...TC.A....C..GGA...G..C..T..T..AG.G..AGC 1158
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                                             1,220               1,240               1,260               1,280               1,300
PVY_N_KZN                                    TATTTTACCGTGCTATAAGATAACCTGCCCTACCTGTGCCCAACAATATGCCAACTTGCCAGCCAGTGACTTACTTAAGATATTACACAAGCACGCAGGT 1271
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90        ....C.............A...........C........T.....G.....T.................T..G..C..AG.........G.......A.. 1271
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99       ....C.........................C........T.....G.......................T..G..C..AG.........G.......A.. 1271
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660  ....C.........................C........T.....G.......................T..G..C..AG.........G.......A.. 1271
AY166867.1|_PVY_N_isolate_N-Jg               ....C.........................C........T.....G.......................T..G..C..AG.........G.......A.. 1271
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1     ....C.........................C........T.....G.......................T..G..C..AG.........G.......A.. 1271
AM268435.1|_PVY_N-New_Zealand                .................................................................................................A.. 1271
X97895.1|_PVY_N_Switzerland_isolate_CH-605   .....................T...........................................................................A.. 1271
AY884983.1|_PVY_N-USA_isolate_Mont           .................................................................................................A.. 1271
U09509.1|PVY_O-Canada                        ...CC.C..A....................C........T.....G.......G......G.TT..C..TC.GT.....C.G..G..T..A..T...A.A 1267
AF522296.1|_PVY_N-Egypt                      ...CC.T.................T.....C........T.....G.......G.........TT..C..TC.GT.....C....G..T..A..T...A.A 1267
M96425.1|PepMoV                              .T.A..T......C.C...T.G..G..TGG.GAA..CT.GA..G.GATCTG.G.TC.CA.TT...A...G.GTG.GC.AGAG...T.....A.TA.---- 1254

                                             1,320               1,340               1,360               1,380               1,400
PVY_N_KZN                                    GATGGTTTAAATCGATTGGGGGCAGACAAAGATCGT------TTTGTGCATGTCAAAAAGTTCTTGACAATCTTAGAGCACTTAACTGAACCGGTTGATC 1365
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90        ..........G.........A..G..T........C--------.............GC............G..C..........G........A....... 1365
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99       ..........G......A..A..G..T........C--------.............GC............G..C..........G........A....... 1365
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660  ..........G.........A..G..T........C--------.............GC............G..C..........G........A....... 1365
AY166867.1|_PVY_N_isolate_N-Jg               ..........G.........A..G..T.....C..C--------.............GC............G.............G........A....... 1365
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1     ..........G.........A..G..T........C--------.............GC............G..C..........G........A....... 1365
AM268435.1|_PVY_N-New_Zealand                ......C............................C------.............................T............................ 1365
X97895.1|_PVY_N_Switzerland_isolate_CH-605   ......C............................C------.......................................................... 1365
AY884983.1|_PVY_N-USA_isolate_Mont           ......C............................C------.......................................................... 1365
U09509.1|PVY_O-Canada                        ........G..C........A.....T.....C..G------...A.A.....T..T..........T.GCG........TC.............G...T 1361
AF522296.1|_PVY_N-Egypt                      ........G...........A..G..T.....C..G------...A.A.....T..T..........T.GCG........TC.............G..C. 1361
M96425.1|PepMoV                              --.TCC..GGCA.TGGAAA..ATGA....TCTA.ACCCTGAA...CAA..CA.TGTT...G.G....GTG.TG.TAG...GC.C......G.ATCCA... 1352

                                             1,420               1,440               1,460               1,480               1,500
PVY_N_KZN                                    CGAGTCTAGAAATTTTCAATGAAGTATTCAAGTCTATAGGGGAGAAGCAACAATCACCTTTCAAAAACCTGAATATTCTGAATAATTTCTTTTTGAAAGG 1465
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90        TA.A.........C..........................A........................................................... 1465
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99       TA.A.........C...........G..............A........................................................... 1465
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660  TA.A.........C..........................A........................................................... 1465
AY166867.1|_PVY_N_isolate_N-Jg               TA.A.........C...........G..............A.............................A............................. 1465
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1     TA.A.........C..........................A........................................................... 1465
AM268435.1|_PVY_N-New_Zealand                T................................................................................................... 1465
X97895.1|_PVY_N_Switzerland_isolate_CH-605   T................................................................................................... 1465
AY884983.1|_PVY_N-USA_isolate_Mont           T................................................................................................... 1465
U09509.1|PVY_O-Canada                        T..A...C..GC..........GA....T..A..C..............G...G....G.....G..TT.A...G.CT.............CC....... 1461
AF522296.1|_PVY_N-Egypt                      T..A...C..GC..........GA....T..A..C...........A..G...G....G.....G..TT.A...G.CT.A...........CC....... 1461
M96425.1|PepMoV                              ATG.GACG....CA...G.....A.C.....AATG..T..ATCC..AAC....AGT.......CTC.TT.A....AA..C...G.A..TA.G........ 1452
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                                                          1,520               1,540               1,560               1,580               1,600
                                                              |                   |                   |                   |                   |
PVY_N_KZN                                  AAAGGAAAATACAGCTCGTGAATGGCAGGTGGCTCAATTAAGCTTACTTGAATTGGCAAGATTCCAAAAGAACAGAACGGATAATATCAAGAAAGGAGAC 1565
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90      ......G....................A...............................................G....................T... 1565
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99     ......G....................A...............................................G....................T... 1565
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 ......G....................A...............................................G..A.................T... 1565
AY166867.1|_PVY_N_isolate_N-Jg             ......G....................A...............................................G....................T... 1565
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1   ......G....................A............................................T..G....................T... 1565
AM268435.1|_PVY_N-New_Zealand              .................................................................................................... 1565
X97895.1|_PVY_N_Switzerland_isolate_CH-605 .................................................................................................... 1565
AY884983.1|_PVY_N-USA_isolate_Mont         .................................................................................................... 1565
U09509.1|PVY_O-Canada                      ...A.............A.....................G..T..G..C.....A.....G.....G.....T.....T.....C...........T..T 1561
AF522296.1|_PVY_N-Egypt                    ...A.............A............A........G..T..G..C.....A.....G.....G...........T.....C...........T..T 1561
M96425.1|PepMoV                            G..T.........AG.G.G..G...TT.ACT..G.G.CA.CAT...AGG..G....TG.....T..............T........A.....G..T... 1552

                                                          1,620               1,640               1,660               1,680               1,700
                                                              |                   |                   |                   |                   |
PVY_N_KZN                                  ATCTCGTTCTTTAGGAATAAACTATCTGCCAAAGCAAATTGGAACTTGTATCTGTCATGTGATAACCAGCTGGATAAGAATGCAAACTTCCTGTGGGGAC 1665
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90      ...........C........G.....C................................C.....T...T..........C................... 1665
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99     ....................G.....C................................C.....T...T..........C................... 1665
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 ...........C........G.....C................................C.....T...T..........C................... 1665
AY166867.1|_PVY_N_isolate_N-Jg             ..T........C........G.....C................................C.....T...T..........C................... 1665
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1   ...........C........G.....C................................C.....T...T..........C................... 1665
AM268435.1|_PVY_N-New_Zealand              ........................................................G........................................... 1665
X97895.1|_PVY_N_Switzerland_isolate_CH-605 .....................................................................................G.............. 1665
AY884983.1|_PVY_N-USA_isolate_Mont         .............................A...................................................................... 1665
U09509.1|PVY_O-Canada                      ..A..C.....C..A......T...T......G...........TC.....T....G..C..C.....AT....C..A........T............. 1661
AF522296.1|_PVY_N-Egypt                    ..A..T.....C..A......T..........G.....C.....TC.....T....G..C..C..............A..............C....... 1661
M96425.1|PepMoV                            T.AG.A.CA..C..A.....G..T.....TCGT...C.G.AC..T......T.A.....C...........T..C........T.GT..T........T. 1652

                                                          1,720               1,740               1,760               1,780               1,800
                                                              |                   |                   |                   |                   |
PVY_N_KZN                                  AGAGGGAATATCATGCTAAGCGATTTTTCTCAAACTATTTCGAGGAAATTGATCCAGCGAAGGGCTATTCAGCATACGAAAATCGTCTGCATCCGAATGG 1765
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90      ................C.....G........G....T..............................C.....G.......C....T............. 1765
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99     ................C.....G........G....T..............................C.....G.......C....T............. 1765
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660 ................C.....G........G....T..............................C.....G.......C....T............. 1765
AY166867.1|_PVY_N_isolate_N-Jg             ................C.....G........G....T..............................C.....G.......C....T............. 1765
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1   ................C.....G........G....T..............................C.....G.......C....T............. 1765
AM268435.1|_PVY_N-New_Zealand              ...............................G............................................T.........T............. 1765
X97895.1|_PVY_N_Switzerland_isolate_CH-605 ...............................G......................................................T............. 1765
AY884983.1|_PVY_N-USA_isolate_Mont         ...............................G......................................................T............. 1765
U09509.1|PVY_O-Canada                      .A.....G..............G.............TC..T.................A.....A..C........T....TC..CAA......A.G... 1761
AF522296.1|_PVY_N-Egypt                    .A.....G..............G.............TC..T.................A.....A..C........T....TC..CAA......A.G... 1761
M96425.1|PepMoV                            ..C.A.....C..C..ACGT..G......CTG....TC...C.AC....A..C...T.A..A..T....TG.....T...G....GAC.ATA..A..... 1752
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                                                 1,820               1,840               1,860               1,880               1,900
                                                     |                   |                   |                   |                   |
PVY_N_KZN                                     GACAAGAAAACTTGCAATTGGAAACCTAATCGTACCACTTGATCTGGCTGAGTTTAGGCGGAAGATGAAAGGTGATTTTAAAAGACAGCCAGGGGTGAGT 1865
AB331517.1|_PVY_J-NTNIisolate:_NTNHO90        ..G...........................T..T.....C.....................................A...................... 1865
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99        ..G...........................T..T.....C.....................................A...................... 1865
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660   ..GG..........................T..T.....C.....................................A...................... 1865
AY166867.1|_PVY_N_isolate_N-Jg                ..GG..........................T..T.....C.....................................A...................... 1865
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1      ..G...........................T..T.....A.....................................A...................... 1865
AM268435.1|_PVY_N-New_Zealand                 ..............................T..............................................A...................... 1865
X97895.1|_PVY_N_Switzerland_isolate_CH-605    ..............................T..............................................A...................... 1865
AY884983.1|_PVY_N-USA_isolate_Mont            ..............................T..............................................A...................... 1865
U09509.1|PVY_O-Canada                         A.....G..G..CT.......T...T..G.T..C.........T.A.............A...............C.A..GG.A...A........C..C 1861
AF522296.1|_PVY_N-Egypt                       A.....G..G..CT.......T...T..G.T..C.........T.A.............A...............C.A..GG.A...A........C..C 1861
M96425.1|PepMoV                               TT.TC.G..GT.A..T..A..C...T....T..T.....C...T.A.....A..CC.AAAACGC.....T..CATCGAC.CTCAG..A...CCAA.TG.. 1852

                                                 1,920               1,940               1,960               1,980               2,000
                                                     |                   |                   |                   |                   |
PVY_N_KZN                                     AAGAAGTGCACGAGTTCGAAGGATGGAAACTACGTGTATCCCTGTTGTTGCACTACACTTGATGATGGCTCAGCTGTTGAATCAACATTTTACCCGCCAA 1965
AB331517.1|_PVY_J-NTNIisolate:_NTNHO90        ..............C...................................T........C........................................ 1965
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99        ..............C...................................T........C........................................ 1965
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660   ..............C...................................T........C........................................ 1965
AY166867.1|_PVY_N_isolate_N-Jg                ..............C...................................T........C........................................ 1965
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1      ..............C...................................T........C........T............................... 1965
AM268435.1|_PVY_N-New_Zealand                 ..............C..................................................................................... 1965
X97895.1|_PVY_N_Switzerland_isolate_CH-605    ..............C..................................................................................... 1965
AY884983.1|_PVY_N-USA_isolate_Mont            ..............C.................................................................G................... 1965
U09509.1|PVY_O-Canada                         .GA.................A.....T..T..T....................A..............T.....CA.............C..T..A.... 1961
AF522296.1|_PVY_N-Egypt                       ..A.................A.....T..T..T....................A..............T.....CA.............C..T..A.... 1961
M96425.1|PepMoV                               ...T.C..T..A..CCAATT......G..T.TT........G..C..C..T..A..G...........TCA.C.AA..CG...GG.TG.....G.A.... 1952

                                                 2,020               2,040               2,060               2,080               2,100
                                                     |                   |                   |                   |                   |
PVY_N_KZN                                     CTAAGAAGCACCTCGTAATAGGTAATAGTGGCGACCAAAAGTATGTTGACTTACCAAAAGGGAATTCTGAGATGTTATATATTGCCAGGCAAGGCTTCTG 2065
AB331517.1|_PVY_J-NTNIisolate:_NTNHO90        ....A.....T..T...........C..C..T..T..............TC...................A........C..............G..T.. 2065
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99        ....A.....T..T...........C..C..T..T..............TC...................A........C..............G..T.. 2065
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660   ....A.....T..T...........C..C..T..T..............TC...................A........C..............G..T.. 2065
AY166867.1|_PVY_N_isolate_N-Jg                ....A.....T..T...........C..C..T..T..............TC...................A........C..............G..T.. 2065
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1      ....A.....T..T...........C..C..T..T..............TC...................A.......................G..T.. 2065
AM268435.1|_PVY_N-New_Zealand                 .................................................................................................... 2065
X97895.1|_PVY_N_Switzerland_isolate_CH-605    .................................................................................................... 2065
AY884983.1|_PVY_N-USA_isolate_Mont            .................................................................................................... 2065
U09509.1|PVY_O-Canada                         ....A........T........C........T..........T......T............G....A...........C.......A...G..T.AT.. 2061
AF522296.1|_PVY_N-Egypt                       ....A........T.....T..C........T........A.T......T............G....G...........C.......A...G..T.AT.. 2061
M96425.1|PepMoV                               .C.....A..TT.A..TG.T.....C.CA..A...AC......CA.CA....G..T.....AG.CA.A..A...C....C.....ACTTG.T....AT.. 2052
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                                                            2,120               2,140               2,160               2,180               2,200
                                                                |                   |                   |                   |                   |
PVY_N_KZN                                       TTACATTAACATTTTCCTCGCGATGTTGATTAACATTAGTGAGGAAGACGCAAAGGATTTCACTAAGAAGGTTCGTGACATGTGTGTGCCAAAGCTTGGA 2165
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90           ...T.....T..A.....T..........................G..T..............G.................................... 2165
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99          ...T.....T..A.....T.............................T..............G.................................... 2165
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660     ...T.....T..A.....T..............T..............T..............G.................................... 2165
AY166867.1|_PVY_N_isolate_N-Jg                  ...T.....T..A.....T.............................T..............G.................................... 2165
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1        ...T.....T..A.....T.............................T..............G.................................... 2165
AM268435.1|_PVY_N-New_Zealand                   ................................................T..........................C........................ 2165
X97895.1|_PVY_N_Switzerland_isolate_CH-605      ................................................T................................................... 2165
AY884983.1|_PVY_N-USA_isolate_Mont              ...............T................................T................................................... 2165
U09509.1|PVY_O-Canada                           ...T......G.G.T...T..A...C.A.........G.C.....G..T..............A.....A..C..C..............G......... 2161
AF522296.1|_PVY_N-Egypt                         ...T......G.G.T...T..A...C.............C.....G..T..............A.....A.....C........................ 2161
M96425.1|PepMoV                                 ..............AT..G..A.....AG.C.....A..C........G..T...........A.....A.....G.....C.TCA.............G 2152
                                                            2,220               2,240               2,260               2,280               2,300
                                                                |                   |                   |                   |                   |
PVY_N_KZN                                       ACCTGGCCAACCATGATGGATCTGGCTACAACTTGTGCTCAAATGAAAATATTCTACCCTGATGTTCATGATGCAGAACTGCCTAGAATACTAGTCGATC 2265
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90           .............................T...............................................G.....C...........T..C. 2265
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99          .............................T...............................................G.....C...........T..C. 2265
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660     .............................T...............................................G.....C...........T..C. 2265
AY166867.1|_PVY_N_isolate_N-Jg                  .............................T............T..................................G.....C...........T..C. 2265
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1        .............................T...............................................G.....C..G........T..C. 2265
AM268435.1|_PVY_N-New_Zealand                   .................................................................................................... 2265
X97895.1|_PVY_N_Switzerland_isolate_CH-605      .................................................................................................... 2265
AY884983.1|_PVY_N-USA_isolate_Mont              .....................T.............................................................................. 2265
U09509.1|PVY_O-Canada                           ...........T.........T....G..C................G.........T.....C..G...........G........TT..T.G..T..C. 2261
AF522296.1|_PVY_N-Egypt                         ...........T.........T....G..C................G.........T.....C..A............T....C......T.G..T..C. 2261
M96425.1|PepMoV                                 .AG........GT........T..........A.........C.TCG.......C..........G...........G......C.T..TT.G..G.... 2252
                                                            2,320               2,340               2,360               2,380               2,400
                                                                |                   |                   |                   |                   |
PVY_N_KZN                                       ACGAAACGCAGACATGCCATGTGGTTGACTCGTTTGGCTCACAAACAACTGGGTATCATATTTTGAAAGCATCTAGCGTGTCCCAACTTATTTTGTTTGC 2365
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90           .T.....T.................C...........T..G...........T...........A..G..............T..G.....C........ 2365
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99          .T.....T.................C...........T..G...........T...........A..G..............T..G.....C........ 2365
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660     .T.....T.................C...........T..G...........C........CC.A..G..............T..G.....C........ 2365
AY166867.1|_PVY_N_isolate_N-Jg                  .T.....T.................C...........T..G...........T...........A..G..............T..G.....C........ 2365
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1        .T.....T..A..............C...........T..G...........T...........A..G..G...........T..G.....C........ 2365
AM268435.1|_PVY_N-New_Zealand                   .................................................................................................... 2365
X97895.1|_PVY_N_Switzerland_isolate_CH-605      ......................A............................................................................. 2365
AY884983.1|_PVY_N-USA_isolate_Mont              .................................................................................................... 2365
U09509.1|PVY_O-Canada                           .T..C..T..A..G..T..............A........G..G........A.........C.A........C........T........C........ 2361
AF522296.1|_PVY_N-Egypt                         .T..C..T..A..G..T..............A........G..G........A.........C.A........C........T........C........ 2361
M96425.1|PepMoV                                 ..A.C..A..A..G..T........C..T..A.A...A...ATT.GT...........C...C.......TG.A.CT..T..A...T.AG.G........ 2352
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                                                               2,420               2,440               2,460               2,480               2,500
                                                                   |                   |                   |                   |                   |
PVY_N_KZN                                      TAATGATGAGTTGGAGTCTGACATTAAGCACTATAGAGTTGGTGGTATTCCTGGAGCATGCCCTGAGCTTGGGTCCACAATATCACCTTTTAGAGAAGGA 2465
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90          A..C...........A.......................................G.....T........C..A.....G...........C......... 2465
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99         A..C...........A....................G..................G.....T........C..A.....G...........C......... 2465
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660    A..C...........A.......................................G.....T........C..A.....G...........C......... 2465
AY166867.1|_PVY_N_isolate_N-Jg                 A..C...........A.......................................G.....T........C..A.....G..T........C......... 2465
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1       A..............A.......................................G.....T........C..A.................C......... 2465
AM268435.1|_PVY_N-New_Zealand                  .................................................................................................... 2465
X97895.1|_PVY_N_Switzerland_isolate_CH-605     .................................................................................................... 2465
AY884983.1|_PVY_N-USA_isolate_Mont             .................................................................................................... 2465
U09509.1|PVY_O-Canada                          A........A..A..A.....T..A..A..T..............CG.....AAT...........A...........G..................... 2461
AF522296.1|_PVY_N-Egypt                        A........A..A..A.....T..A..A..T...............G.....AAT..TA.......A.......................C......... 2461
M96425.1|PepMoV                                .G.C..CA.C.....A.....G..A....................A...G-.A...A..-.ATAA..TGCAAA.AG.T..C-CA....AG....TGT... 2449

                                                               2,520               2,540               2,560               2,580
                                                                   |                   |                   |                   |
PVY_N_KZN                                      GGAATCATAATGTCTGAGTCGGCAGCGCTAAAACTGCTCCTAAAGGGAATTTTTAGGCCCAAAGTGATGTAGCAATTGCTACTGGATGAACCA 2558
AB331517.1|_PVY_J-NTNIsolate:_NTNHO90          ....................A.....A..G........TT.......T..C.....A.....G......AGA..G..A...T.A......... 2558
AB331518.1|_PVY_J-NTN_isolate:_NTNNN99         ....................A.....A..G........TT.......T..C.....A.....G......AGA..G..A...T.A......... 2558
AY166866.1|_PVY_NA-NTN-Canada_isolate_Tu660    ....................A.....A..G........TT.......T..C.....A.....G......AGA..G..A...T.A......... 2558
AY166867.1|_PVY_N_isolate_N-Jg                 ....................A.....A..G........TT.......T..C.....A.....G......A.A..G..A...T........... 2558
AY884984.1|_PVY_NA-NTN-USA_isolate_RRA-1       ....................A.....A..G........TT.......T..C.....A.....G......AGA..G..A...T.A......... 2558
AM268435.1|_PVY_N-New_Zealand                  ....................A................................................A....................... 2558
X97895.1|_PVY_N_Switzerland_isolate_CH-605     ....................A................................................A....................... 2558
AY884983.1|_PVY_N-USA_isolate_Mont             ....................A................................................A....................... 2558
U09509.1|PVY_O-Canada                          ...G.T.......................G........TT.G..............A..T..G......AGA..G.....GT.A.....G..T 2554
AF522296.1|_PVY_N-Egypt                        ...G.T.......................G........TT.G..............A..T..G......AGA..G.....GT.A.....G..T 2554
M96425.1|PepMoV                                .---------..AG.....TCCAT..CA..CGTA..T.AA.T..A..G..C.AC.....A.GT..C.....TG....A..CTCT..A...... 2533
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- **Sequence alignment of the 1067 nucleotides at the 3’ -end before the poly-A tail of different isolates of *PVY***

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                                                          20                  40                  60                  80
                       PVY_TalaValley_ CCAGTCAAACCCGAATAAAGGAAAAGACAAGGATGTAAATGTTGGTACATCAGGAACACATACTGTACCAAGAATAAAGGCCATTACATCCAAAATGAG 99
                            PVY_Eshowe ...............C...............................................C................................... 99
                          PVY_Greytown ...............C................................................................................... 99
                      PVY_TugelaFerry_ ...............C.....................................................G............................. 99
                                PVY_H6 ...............C...........T........G....C.........T..G...........G..G.....C.....T..C..G........... 99
   AJ585196.1|_PVY_O-UK_isolate_SCRI-O ...............C...........T........G....C.........T..G...........G..G.....C.....T..C..G........... 99
          AJ223595.1|_PVY_isolate_O854 ...............C...........T........G....C.........T..G...........G..G.....C.....T..C..G........... 99
      EF026074.1|_PVY_O-USA_Isolate_Oz ...............C...........T........G....C.........T..G...........G..G.....C.....T..C..G........... 99
       AF255659.1|_PVY_isolate_PVY-OBR ...............C...........T........T....C...C.....T..G...........G..G.....C.....T..C..G........... 99
      DQ217931.1|_PVY_from_New_Zealand ...........T...C...........T........G....C.........T..G...........G..G.....T.....T..C..G........... 99
                 U09509.1|PVY_O-Canada ...............C...........T..A.....G....CC..C.....T..G.....C........G.....C.....T..C..G........... 99
         AJ439544.2|_PVY_isolate_SON41 ....CG...T..T..C..G.....G..T.....C..G....CC........T..............G...............T..C..G..A........ 99
       AJ439545.1|_PVY_isolate_LYE84.2 T...C...CT..T..C..G.....G..T.....C..G....CC........T.............................T.....G..A........ 99
AJ890348.1|_PVY_C-France_isolate_Adgen .........T..T..C..G.....G..T.....C.................T.............................G.....G........... 99
           X97895.1|_PVY_N-Switzerland T..AC....T.TC..C..G.A...G..A.....C..G........A.....T.....T........G...C....T..A..T..C..G..........A 99
                       M96425.1|PepMoV AAGAAGG.GGTT....C..C-.CGC..TTCT.....G....CG..A..TGTT......TTC..CA.T........C..AT.A..C..TGAA..G...C. 98
                                     100                 120                 140                 160                 180
                       PVY_TalaValley_ ATTGCCCAAAAGCAAGGGAACAACCGCACTAAATTTGGAACACTTGCTCGAATATGCTCCGCAGCAGATAGATATCTCAAACACTCGAGCAACGCAATC 198
                            PVY_Eshowe ....................................A.............................................................. 198
                          PVY_Greytown ........................T...........A.............................................................. 198
                      PVY_TugelaFerry_ ....................................A.............................................................. 198
                                PVY_H6 .A.................G......TG.....C..A...........T..G........A..A..A..T.....T.....T.....G.....T..... 198
   AJ585196.1|_PVY_O-UK_isolate_SCRI-O .A.................G......TG.....C..A...........T..G........A..A.....T.....T.....T.....G.....T..... 198
          AJ223595.1|_PVY_isolate_O854 .A.................G......TG.....C..A...........T..G........A..A..A..T.....T.....T.....G.....T..... 198
      EF026074.1|_PVY_O-USA_Isolate_Oz .A.................G......TG.....CC.A...........T..G........A..A..A..T.....T.....T.....G.....T..... 198
       AF255659.1|_PVY_isolate_PVY-OBR .A.................G......TG.....C..A.....T.....T..G........A..A..A..T.....T.....T.....G.....T..... 198
      DQ217931.1|_PVY_from_New_Zealand .A.................G......TG.....C..A...........T..G........A..A..A..T.....T.....T.....G.....T..... 198
                 U09509.1|PVY_O-Canada .A.................G..G.T.TG..G.....A...........T..G........A..A..A..T.....T.....T.....G.....T..... 198
         AJ439544.2|_PVY_isolate_SON41 .A....T........A......G...TG.....C..A.....T.................A..A.................T.....G.....T..... 198
       AJ439545.1|_PVY_isolate_LYE84.2 .A....T........A...GTGG..........C..A..............G..CA....A..A.................T.....G.....T..... 198
AJ890348.1|_PVY_C-France_isolate_Adgen .A....TC..........GG.G....TG.....C.................G........A.....A..T...........T.....G.....T..... 198
           X97895.1|_PVY_N-Switzerland .A.......G..T..A..TG....T.T..................A.....G........A.....A..T..C........T...........T..... 198
                       M96425.1|PepMoV TA....T...CAG...A...AGGGT.TT..C..C....CT..T..A..T.....CAAA..AAGC..AG.T..C..A..G........TT....C..GG. 197
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                                    200                 220                 240                 260                 280
PVY_TalaValley_                    ACAGTTTGACACGTGGTATGAAGCAGTGCGGGTGGCATACGACATAGGGGAAACTGAGATGCCAACTGTGATGAATGGGCTTATGGTTTGGTGCATTGA 297
PVY_Eshowe                         ................................................................................................... 297
PVY_Greytown                       .......A........................................................................................... 297
PVY_TugelaFerry_                   ................................................................................................... 297
PVY_H6                             .........T....G......G.........A................A.................................................. 297
AJ585196.1|_PVY_O-UK_isolate_SCRI-O .........T...........G.........A................A.................................................. 297
AJ223595.1|_PVY_isolate_O854       .........T...........G.........A................A.................................................. 297
EF026074.1|_PVY_O-USA_Isolate_Oz   .........T...........G.........A................A.................................................. 297
AF255659.1|_PVY_isolate_PVY-OBR    .........T...........G.........A................A.................................................. 297
DQ217931.1|_PVY_from_New_Zealand   .........T...........G.........A................A...............................................C.. 297
U09509.1|PVY_O-Canada              .........T.....................A................A.................................................. 297
AJ439544.2|_PVY_isolate_SON41      .........T...............................................A......................................... 297
AJ439545.1|_PVY_isolate_LYE84.2    .........T.....................A.........................A......................................... 297
AJ890348.1|_PVY_C-France_isolate_Adgen .........T..A..............A........G....................A......................................... 297
X97895.1|_PVY_N-Switzerland        .........T.................A.AAC.T..............A........A......................................... 297
M96425.1|PepMoV                    ...A........AT......TGT.A...TAT.AA.........TC..CAA..GGAG.CA...GGT..A...........CT.A................. 296

                                     300                 320                 340                 360                 380
PVY_TalaValley_                    AAATGGAACCTCGCCAAACATCAACGGAGTCTGGGTTATGATGGATGGCGATGAACAAGTCGAATATCCGTTGAAACCAATCGTTGAGAATGCAAAACC 396
PVY_Eshowe                         ................................................T.................................................. 396
PVY_Greytown                       ................................................T.................................................. 396
PVY_TugelaFerry_                   ................................................................................................... 396
PVY_H6                             ..................TG..........T.................GA.............G..C................................ 396
AJ585196.1|_PVY_O-UK_isolate_SCRI-O ..................TG..........T.................GA..........T..G..C................................ 396
AJ223595.1|_PVY_isolate_O854       ..................TG..........T.................GA.C........T..G..C................................ 396
EF026074.1|_PVY_O-USA_Isolate_Oz   ..................TG..........T.................GA.............G..C................................ 396
AF255659.1|_PVY_isolate_PVY-OBR    ..................TG..........T.................G...........T..G..C................................ 396
DQ217931.1|_PVY_from_New_Zealand   ..................TG..........T.................GA..........T..G..C................................ 396
U09509.1|PVY_O-Canada              ..................TG..........T.................GA..........T..G..C................................ 396
AJ439544.2|_PVY_isolate_SON41      ..................TG..........T.................AAG.........T...................................... 396
AJ439545.1|_PVY_isolate_LYE84.2    ......G...........T...........T.................AA..........T..G.................T................. 396
AJ890348.1|_PVY_C-France_isolate_Adgen G.................T...........T.................AA..........T.....C................................ 396
X97895.1|_PVY_N-Switzerland        ..............................T.................AA................C..AC............................. 396
M96425.1|PepMoV                    ......C..G..C........T.GT...ACA...ACC...........A..C........G....TC..A..A..G..CG.GA.A........C..G.. 395

                                      400                 420                 440                 460                 480
PVY_TalaValley_                    AACCCTTAGGCAAATCATGGCACATTTCTCAGATGTTGCAGAAGCGTATATAGAAATGCGCAACAAAAAGGAACCATATATGCCACGATATGGTTTAAT 495
PVY_Eshowe                         ................................................................................................... 495
PVY_Greytown                       ................................................................................................... 495
PVY_TugelaFerry_                   ................................................................................................... 495
PVY_H6                             ................................................................................................... 495
AJ585196.1|_PVY_O-UK_isolate_SCRI-O ................................................................................................... 495
AJ223595.1|_PVY_isolate_O854       ................................................................................................... 495
EF026074.1|_PVY_O-USA_Isolate_Oz   ................................................................................................... 495
AF255659.1|_PVY_isolate_PVY-OBR    ........................................C.......................................................... 495
DQ217931.1|_PVY_from_New_Zealand   .................................................................................................G. 495
U09509.1|PVY_O-Canada              ................................................................................................... 495
AJ439544.2|_PVY_isolate_SON41      G.................................................................................................. 495
AJ439545.1|_PVY_isolate_LYE84.2    ................................................................................................... 495
AJ890348.1|_PVY_C-France_isolate_Adgen ................................................................C.................................A 495
X97895.1|_PVY_N-Switzerland        ...A.............................................................................................G. 495
M96425.1|PepMoV                    G..TT..C.......A.....G..C..T..T.....G..T..G..A.................T..GC.A........C................GG. 494
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                                         500                 520                 540                 560                 580
                                         |                   |                   |                   |                   |
PVY_TalaValley_                      TCGAAATCTGCGGGATGGAAGTTTAGCGCGCTATGCCTTTGACTTTTATGAAGTTACATCACGAACACCAGTGAGGGCTAGGGAAGCGCACATTCAGAT 594
PVY_Eshowe                           ................................................................................................... 594
PVY_Greytown                         ................................................................................................... 594
PVY_TugelaFerry_                     ................................................................................................... 594
PVY_H6                               .................TGG..........T....................G..C.........................................A.. 594
AJ585196.1|_PVY_O-UK_isolate_SCRI-O  .................TGG..........T..................A.G..C.........................................A.. 594
AJ223595.1|_PVY_isolate_O854         .................TGG..........T....................G..C.........................................A.. 594
EF026074.1|_PVY_O-USA_Isolate_Oz     .................TGG..........T....................G..C.........................................A.. 594
AF255659.1|_PVY_isolate_PVY-OBR      ...G.............TGG..........T....................G..C.........................................A.. 594
DQ217931.1|_PVY_from_New_Zealand     .................TGG............................C..G..C.........................................A.. 594
U09509.1|PVY_O-Canada                ................ATGG..........T.....T..............G..C...................................A.....A.. 594
AJ439544.2|_PVY_isolate_SON41        ......................................................C......................................A..A.. 594
AJ439545.1|_PVY_isolate_LYE84.2      ................AT................................................G..........................A..A.. 594
AJ890348.1|_PVY_C-France_isolate_Adgen .......T................G.............................C......................................A..A.. 594
X97895.1|_PVY_N-Switzerland          ...T........C...........G..T........T..........................G.................A..G..A........A.. 593
M96425.1|PepMoV                      .......T.A..A..CATGG..C.G..T..A..C..A........C........C........T..GT..ACAC.T...C.C.....C..T..C..A.. 593
                                          600                 620                 640                 660                 680
                                          |                   |                   |                   |                   |
PVY_TalaValley_                      GAAGGCCGCAGCATTGAAATCAGCCCAATCTCGACTTTTCGGGTTGGATGGTGGCATCAGTACACAAGAGGAGAACACAGAGAGGCACACCACCGAGGA 693
PVY_Eshowe                           ................................................................................................... 693
PVY_Greytown                         ................................................................................................... 693
PVY_TugelaFerry_                     ................................................................................................... 693
PVY_H6                               ............................C...................C.................................................. 693
AJ585196.1|_PVY_O-UK_isolate_SCRI-O  ............................C...................C.................................................. 693
AJ223595.1|_PVY_isolate_O854         ............................C...................C.................................................. 693
EF026074.1|_PVY_O-USA_Isolate_Oz     ............................C...................C.................................................. 693
AF255659.1|_PVY_isolate_PVY-OBR      ............................C...................C.................................................. 693
DQ217931.1|_PVY_from_New_Zealand     ............................C...................C.................................................. 693
U09509.1|PVY_O-Canada                ............................C...................C.................................................. 693
AJ439544.2|_PVY_isolate_SON41        ................................................C......G........................................... 693
AJ439545.1|_PVY_isolate_LYE84.2      ........................T...C...................................................................... 693
AJ890348.1|_PVY_C-France_isolate_Adgen ...A....................T...C...................................................................... 693
X97895.1|_PVY_N-Switzerland          ............T..A........T.................A..............T..............A.......................... 693
M96425.1|PepMoV                      ...A..A..............T..T...A.AA.GT.A..T..A..............AG..A.......GA..A.........C.C........T..A.. 692
                                             700                 720                 740                 760                 780
                                             |                   |                   |                   |                   |
PVY_TalaValley_                      TGTCTCTCCAAGTATGCATACTCTACTTGGAGTCAAGAACATGTGAT-GTAGTGTCTCTCCGGACGATATATAAGTATTTACA-TATGCAGTAAGTATT 790
PVY_Eshowe                           ...............................................-...................................-............... 790
PVY_Greytown                         ...............................................-...................................-............... 790
PVY_TugelaFerry_                     .............................................R.-...................................-............... 790
PVY_H6                               ...............................................-...................................-............... 790
AJ585196.1|_PVY_O-UK_isolate_SCRI-O  ...............................................-...................................-............... 790
AJ223595.1|_PVY_isolate_O854         ...............................................-...................................-............... 790
EF026074.1|_PVY_O-USA_Isolate_Oz     ...............................................-...................................-............... 790
AF255659.1|_PVY_isolate_PVY-OBR      ...............................................-...................................-............... 790
DQ217931.1|_PVY_from_New_Zealand     ...............................................-...................................-............... 790
U09509.1|PVY_O-Canada                ...............................................-..........................A........-............... 790
AJ439544.2|_PVY_isolate_SON41        ...T...........................................T..GA...............................-............... 791
AJ439545.1|_PVY_isolate_LYE84.2      ...T...........................................T..GA...............................-............... 791
AJ890348.1|_PVY_C-France_isolate_Adgen ...T...........................................T...A...............................-............... 791
X97895.1|_PVY_N-Switzerland          ...T.............................G.............T.........T...............GA......TG-.T............. 791
M96425.1|PepMoV                      ...GAGC..CGAC.........CT.G.....G....G.G.A......CT.AT...GTCTCTG...T..A....T.T...A.GT.G......A..TA..G. 791
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                                         800                 820                 840                 860                 880
PVY_TalaValley_                          TTGGCTTTTCCTGTACTACTTTTATCATAATTAA-------------TAATCAGTTTGAATATTACTAATAGATAGAGGTGGCAGGGTGATTTCGTCATT 877
PVY_Eshowe                               ..................................-------------..................................................... 877
PVY_Greytown                             ..................................-------------..................................................... 877
PVY_TugelaFerry_                         ..................................-------------..................................................... 877
PVY_H6                                   ..................................-------------..................................................... 877
AJ585196.1|_PVY_O-UK_isolate_SCRI-O      ..................................-------------..................................................... 877
AJ223595.1|_PVY_isolate_O854             ..................................-------------..................................................... 877
EF026074.1|_PVY_O-USA_Isolate_Oz         ....A.............................-------------..................................................... 877
AF255659.1|_PVY_isolate_PVY-OBR          ..................................-------------..........................G.......................... 877
DQ217931.1|_PVY_from_New_Zealand         ..................................-------------..................................................... 877
U09509.1|PVY_O-Canada                    ..................................-------------..................................................... 877
AJ439544.2|_PVY_isolate_SON41            .........................TG...C....------------.................T................................... 878
AJ439545.1|_PVY_isolate_LYE84.2          .........................TGC..C....------------.................T....................A.............. 878
AJ890348.1|_PVY_C-France_isolate_Adgen   .........................TG...C....------------..................................................... 878
X97895.1|_PVY_N-Switzerland              ..........................GC......------------.....-..............GGC......G.....T.TA.C....C....G... 877
M96425.1|PepMoV                          A.........TC..T.C.G.C..TAT..T.A.G.GAGTAACTTAAG...GT.A....T.CT.C.AGG..TA..CA.....A.TCTC...CACTC...G.. 890

                                         900                 920                 940                 960                 980
PVY_TalaValley_                          GTGGTGACTCTATCTGTTGACTTCGCATTATTAAGTTTAGATAAAAGTGCCGGGTTGTCGTTGTTGTGGATGATTCATCGATTAGGTGATGTTGCGAT 976
PVY_Eshowe                               ....................T.........W.................................................................... 976
PVY_Greytown                             ....................T.............................................................................. 976
PVY_TugelaFerry_                         ....................T.............................................................................. 976
PVY_H6                                   ...............T..A.T.C.............C.............................................................. 976
AJ585196.1|_PVY_O-UK_isolate_SCRI-O      ...............T..A.T.C.............C.............................................................. 976
AJ223595.1|_PVY_isolate_O854             ...............T..A.TAC............................................................................ 976
EF026074.1|_PVY_O-USA_Isolate_Oz         ...............T.CA.T.C.............C........................................................C..... 976
AF255659.1|_PVY_isolate_PVY-OBR          ..................A.T.C..T..........C.............................................................. 976
DQ217931.1|_PVY_from_New_Zealand         ..................A.T.C.............C....G......................................................... 976
U09509.1|PVY_O-Canada                    ..................A.T...............C.............................................................. 976
AJ439544.2|_PVY_isolate_SON41            ..................ATT.CT................T..................T........A.G...AC....................... 977
AJ439545.1|_PVY_isolate_LYE84.2          ..................ATT.CT..............A.T...........................A.....AC....................... 977
AJ890348.1|_PVY_C-France_isolate_Adgen   ..................ATT.CT...............GT.....................C.....A....G.CT............G....AT... 977
X97895.1|_PVY_N-Switzerland              ..A.....CT..G....C.TT.CT.T.......T.-...GT..................T..........C....CT...................... 975
M96425.1|PepMoV                          .A....--------------------...G..T...C.G..T.T.C----------.A.T..GAG.A.AA.GA.CTC..AGAA.--------------- 947

                                         1,000               1,020               1,040               1,060               1,080
PVY_TalaValley_                          TCTGTCGTAGCAGTGACTATGTCTGGATCTATCTGCTTGGGTGGTGTTGTGATTTCGTCATAACAGTGACTGTAAACTTCAATCAGGAGAC 1067
PVY_Eshowe                               .......................................................M...M............................... 1067
PVY_Greytown                             ........................................................................................... 1067
PVY_TugelaFerry_                         ........................................................................................... 1067
PVY_H6                                   ......................................................C.................................... 1067
AJ585196.1|_PVY_O-UK_isolate_SCRI-O      ..................................A........................................................ 1067
AJ223595.1|_PVY_isolate_O854             ..................................A........................................................ 1067
EF026074.1|_PVY_O-USA_Isolate_Oz         ..............................................................C............................ 1067
AF255659.1|_PVY_isolate_PVY-OBR          ........................................................................................... 1067
DQ217931.1|_PVY_from_New_Zealand         ..................................A..........................G............................. 1067
U09509.1|PVY_O-Canada                    ...........................................A...........T................................... 1067
AJ439544.2|_PVY_isolate_SON41            .T.......A................C.......A...................C.......G............................ 1068
AJ439545.1|_PVY_isolate_LYE84.2          .T................................A........................................................ 1068
AJ890348.1|_PVY_C-France_isolate_Adgen   .T....A.G.................GCT.....A........A..............................G................ 1068
X97895.1|_PVY_N-Switzerland              .-..........................T..GT.A........A..C.......CT......G............................ 1065
M96425.1|PepMoV                          ------ACGAG......-------A..CAC.C-----------------------....TAGGAG....TC...GTTGG..TGAGA..... 1004
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Appendix G
Fermentas DNA Ladders Used